How Extra-Column Volume Affects Cannabinoids Analysis and LC Column Choice

By Jamie York, PhD

When developing or transferring cannabis and hemp potency methods, extra is not always better—in fact extra-column volume in your LC system can be detrimental to peak width, resolution, and efficiency. Since these factors affect method performance and analytical results, it is important to understand the impact of extra-column volume on your analysis. In this article, we will explore the effects that extra-column volume has on the chromatographic separations in cannabinoids analysis and also provide guidance on how to choose a column that will best suit your laboratory's needs.

What is Extra-Column Volume?

Extra-column volume is defined as the volume from the injector to the detector, excluding the volume of the column itself. It is different from dwell volume, which is the volume that the LC pumps need to move for a change in the gradient to reach the column head. The adverse effects of extra-column volume are dependent in part on column dimensions and particle size. The larger the column, the less impact extra-column volume will have on chromatographic separations. This is because the ratio of column volume to extra-column volume is low, meaning that extra-column volume contributes a smaller proportion to the overall band broadening than it does when smaller columns are used.

How Do Column Size and Extra-Column Volume Affect Results?

The effects of extra-column volume are usually more detrimental for isocratic methods than for gradient methods. Restek has published several isocratic methods that are suitable for analyzing potency in cannabis and hemp samples. Each method offers particular advantages, but they use different size columns so the extra-column volume effects on cannabinoid separations should also be considered before selecting a method. The similarities and differences among these methods are presented in Tables I and II.

Table I: Universal Conditions for Evaluated Methods

Phase	Raptor ARC-18
Column Oven	30 °C
MP Conditions	Isocratic (25% mobile phase A; 75% mobile phase B)
Mobile Phase	A. Water, 5 mM ammonium formate, 0.1% formic acid B. Acetonitrile, 0.1 % formic acid
Detector	UV/Vis @ 228 nm

Table II: Method-Specific Conditions for Cannabinoid Analysis

	Method 1	Method 2	Method 3	Method 4
Column dimensions	150 x 4.6 mm, 2.7 µm	150 x 3 mm, 2.7 µm	150 x 2.1 mm, 2.7 µm	100 x 3 mm, 1.8 µm
Flow	1.5 mL/min	1 mL/min	0.4 mL/min	1 mL/min
Injection volume	5 µL	2 µL	2 µL	1 µL
Run time*	9 min	6 min	10 min	4 min
Use/benefits	Standard HPLC	Fast HPLC	Solvent saver	Fast UHPLC

* Run times shown are for optimized conditions. When extra-column volume is increased, as in the experiments described here, run times may be extended to elute all compounds.

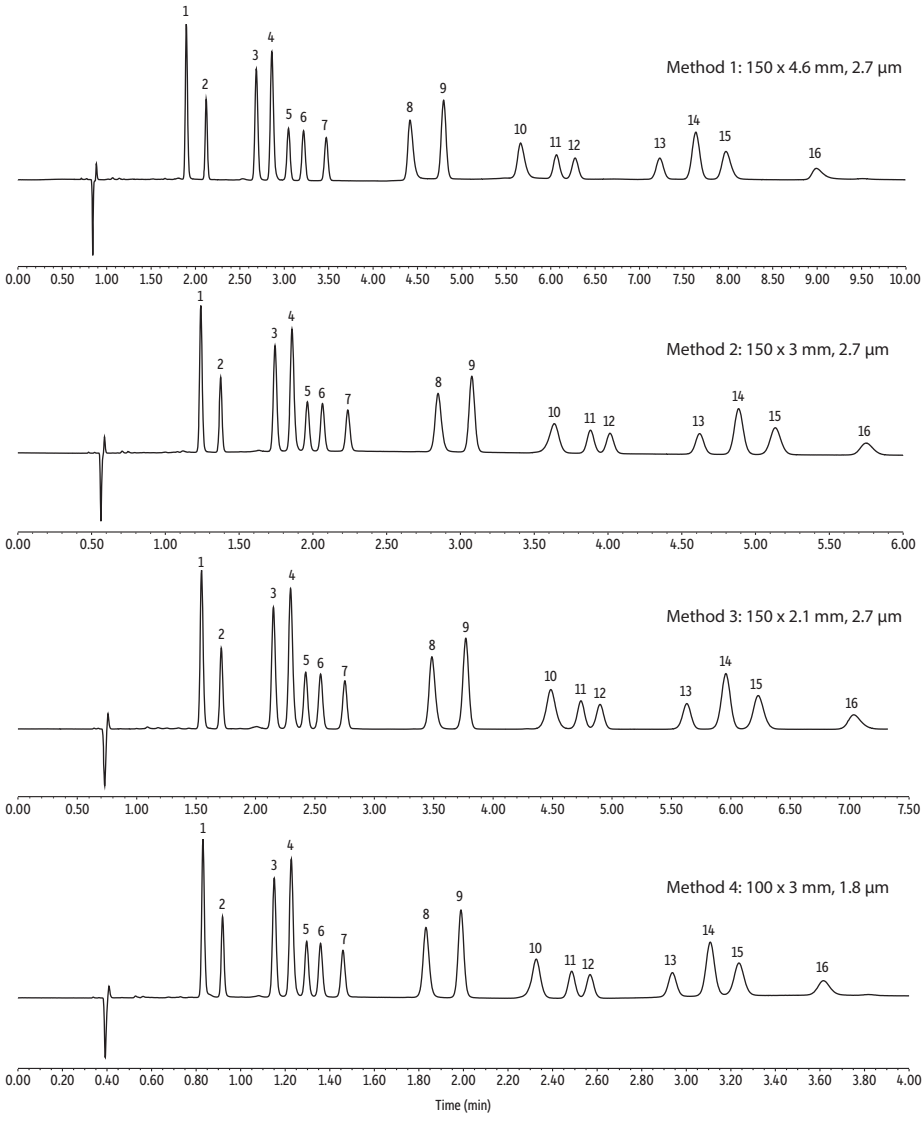
Using these cannabinoid analysis methods, let's delve into some data-driven examples to see how extra-column volume from different sources and instrument setups can affect the separation of 16 cannabinoids on the different column dimensions used in the methods.

Example 1: Using Excess Tubing

Regardless of the instrument you are using, best practices dictate plumbing it with short sections of tubing and making good, properly seated connections. This is because extra tubing increases extra-column volume, which can cause peak dispersion (band broadening) and poor chromatographic results. In this example, we used an LC with a 500 nL flow cell and then varied the post-column tubing volume and evaluated its effect.

In the first comparison of how extra-column volume affects the methods, a short segment of post-column tubing with a volume of 4 µL was used, and chromatographic results from all four columns are shown in Figure 1. With an optimized system such as this one, which uses a low volume flow cell and a minimal amount of post-column tubing, all four potency methods produced good results, and the analyst would be able to choose which method best suits their lab's needs based on other factors, such as desired speed or solvent use.

Figure 1: When extra-column volume is minimized using a low volume flow cell and short section of tubing, all four potency methods produce excellent results.

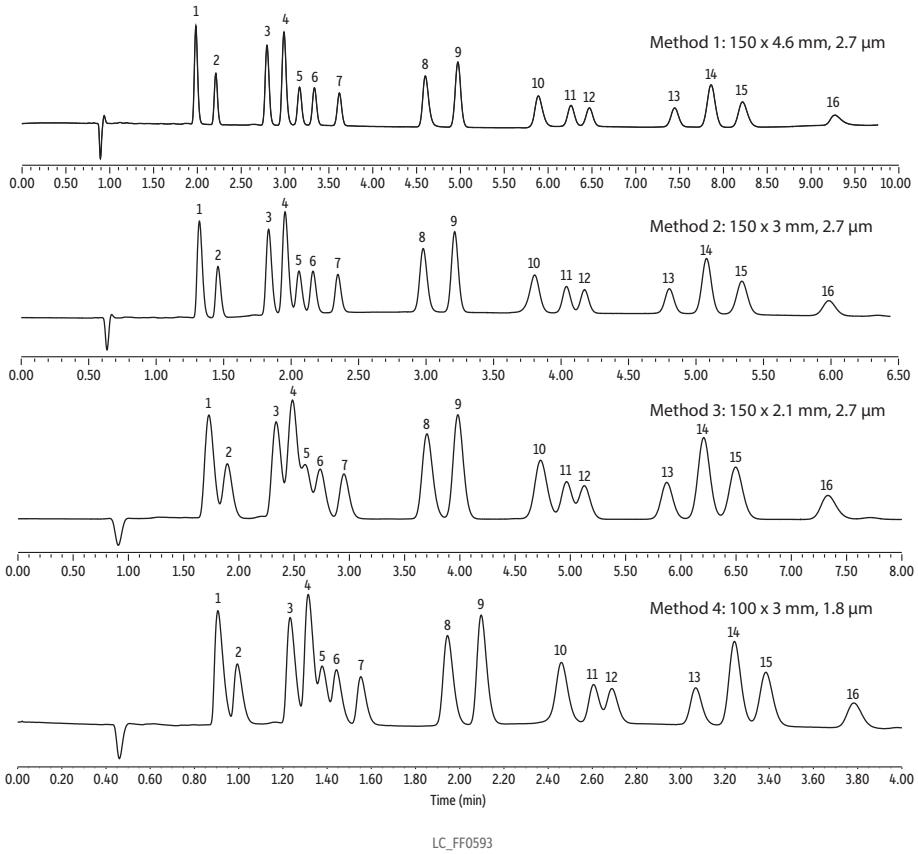


Peaks	tR (min)	Conc. (µg/mL)
1. Cannabidivarinic acid (CBDVA)	1.897	50
2. Cannabidivarin (CBDV)	2.121	50
3. Cannabidiolic acid (CBDA)	2.685	50
4. Cannabigerolic acid (CBGA)	2.860	50
5. Cannabigerol (CBG)	3.047	50
6. Cannabidiol (CBD)	3.217	50
7. Tetrahydrocannabivarin (THCV)	3.472	50
8. Tetrahydrocannabivarinic acid (THCVA)	4.416	50
9. Cannabinol (CBN)	4.794	50
10. Cannabinolic acid (CBNA)	5.661	50
11. Δ9-Tetrahydrocannabinol (Δ9-THC)	6.064	50
12. Δ8-Tetrahydrocannabinol (Δ8-THC)	6.275	50
13. Cannabicyclol (CBL)	7.228	50
14. Cannabichromene (CBC)	7.634	50
15. Tetrahydrocannabinolic acid A (THCA-A)	7.973	50
16. Cannabichromenic acid (CBCA)	8.992	50

Retention times are for top chromatogram (Method 1).

In the next comparison, we increase the extra-column volume by using a 75 µL section of post-column tubing, still using the same 500 nL flow cell. As shown in Figure 2, differences in method performance are now apparent. The larger column dimensions used in Methods 1 and 2 still show excellent chromatographic performance, and all 16 cannabinoids are well separated. However, for the smaller columns used in Methods 3 and 4, significant band broadening is observed, and even this qualitative assessment makes it clear that adequate separation and accurate results could not be obtained for all compounds.

Figure 2: When extra-column volume is increased by increasing tubing volume, larger columns are relatively unaffected, but smaller columns exhibit band broadening and poor separations.



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Peaks	t_R (min)	Conc. (µg/mL)
1. Cannabidivarinic acid (CBDVA)	1.982	50
2. Cannabidivarin (CBDV)	2.21	50
3. Cannabidiolic acid (CBDA)	2.794	50
4. Cannabigerolic acid (CBGA)	2.987	50
5. Cannabigerol (CBG)	3.165	50
6. Cannabidiol (CBD)	3.335	50
7. Tetrahydrocannabivarin (THCV)	3.619	50
8. Tetrahydrocannabivarinic acid (THCVA)	4.599	50
9. Cannabinol (CBN)	4.971	50
10. Cannabinolic acid (CBNA)	5.888	50
11. Δ9-Tetrahydrocannabinol (Δ9-THC)	6.26	50
12. Δ8-Tetrahydrocannabinol (Δ8-THC)	6.471	50
13. Cannabicyclol (CBL)	7.444	50
14. Cannabichromene (CBC)	7.859	50
15. Tetrahydrocannabinolic acid A (THCA-A)	8.218	50
16. Cannabichromenic acid (CBCA)	9.267	50

Retention times are for top chromatogram (Method 1).

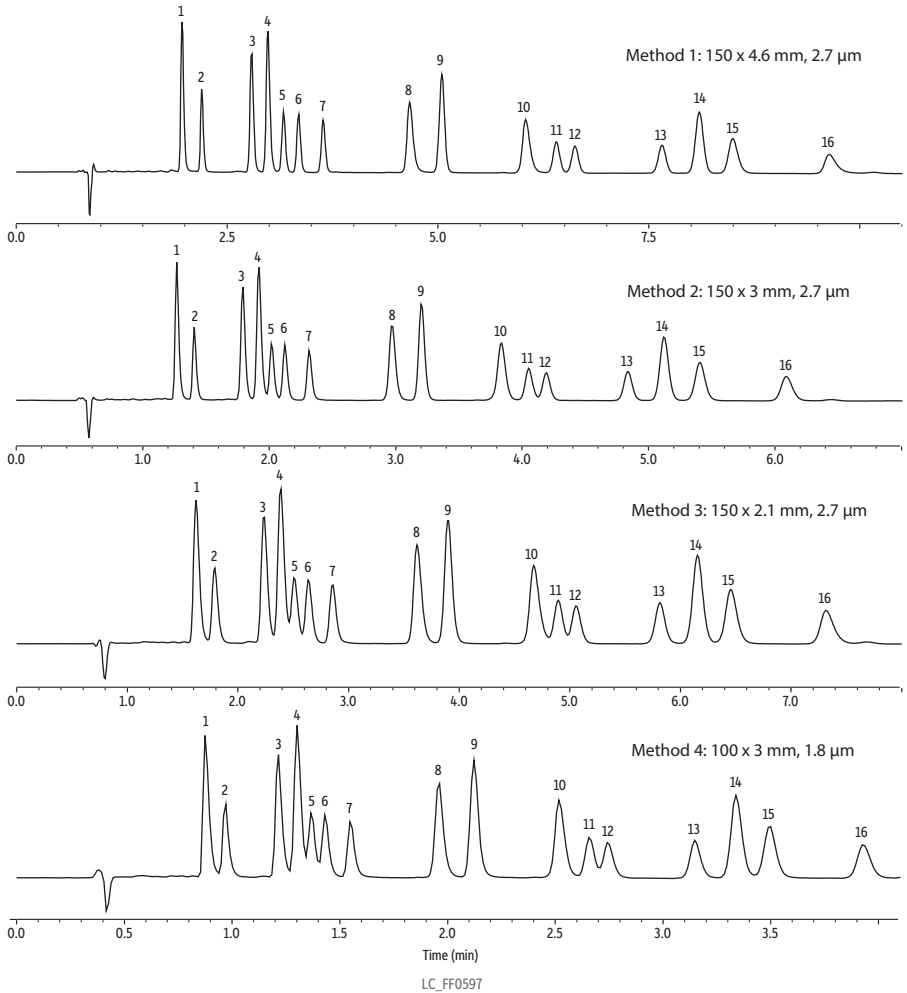
Example 2: Changing Flow Cell Volume

Flow cell size is generally not specified during method development, but flow cells range widely in volume, which means they can cause significant differences in extra-column volume and chromatographic performance between instruments.

The instrument used in Example 1 had a 500 nL flow cell and post-column tubing that could be accurately measured. However, in Example 2, we experimented using a different instrument that did not allow exact measurement of the post-column tubing. To evaluate the effect of extra-column volume contributed by the flow cell on the same four methods for cannabinoids analysis, we tested larger flow cells that differed substantially in volume.

Figure 3 shows the effect of extra-column volume on method performance using a 2.5 µL flow cell. Broader peaks were observed on the smaller columns for several commonly analyzed cannabinoids, and it is clear even just from visual inspection that compound resolution is very poor on the smaller columns, especially in congested areas of the chromatogram. Less band broadening and better separations are seen on the larger columns, which produced better chromatographic results overall.

Figure 3: Using a 2.5 µL flow cell, band broadening is observed for the smaller columns used in cannabinoids analysis Methods 3 and 4 due to extra-column volume effects, whereas method performance using the larger columns is relatively unaffected.

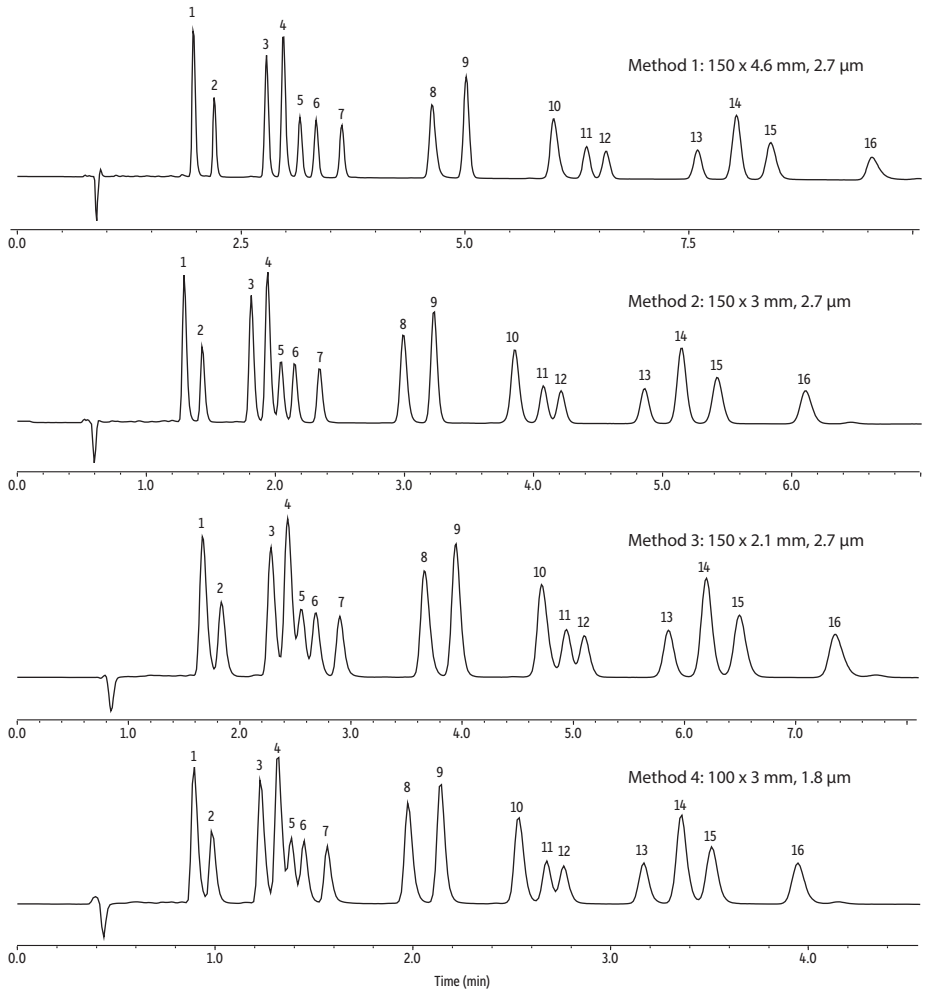


	Peaks	t_R (min)	Conc. (µg/mL)
1.	Cannabidivarinic acid (CBDVA)	1.966	50
2.	Cannabidivarin (CBDV)	2.199	50
3.	Cannabidiolic acid (CBDA)	2.79	50
4.	Cannabigerolic acid (CBGA)	2.984	50
5.	Cannabigerol (CBG)	3.169	50
6.	Cannabidiol (CBD)	3.348	50
7.	Tetrahydrocannabivarin (THCV)	3.639	50
8.	Tetrahydrocannabivarinic acid (THCVA)	4.664	50
9.	Cannabinol (CBN)	5.046	50
10.	Cannabinolic acid (CBNA)	6.039	50
11.	Δ9-Tetrahydrocannabinol (Δ9-THC)	6.404	50
12.	Δ8-Tetrahydrocannabinol (Δ8-THC)	6.624	50
13.	Cannabicyclol (CBL)	7.656	50
14.	Cannabichromene (CBC)	8.098	50
15.	Tetrahydrocannabinolic acid A (THCA-A)	8.494	50
16.	Cannabichromenic acid (CBCA)	9.639	50

Retention times are for top chromatogram (Method 1).

Next, we installed a larger 10 µL volume flow cell in the instrument and tested the result. The 150 x 4.6 mm column still has baseline resolution, demonstrating that Method 1 is the most robust method and is easily transferrable from system to system. As in the first experiment, resolution on the smaller columns suffered significantly due to band broadening. However, flow cells are simple to replace, so when transferring a method to a different instrument, it can be beneficial to consider installing a smaller flow cell if extra-column volume needs to be reduced.

Figure 4: Band broadening is also observed for the smaller columns when using a 10 µL flow cell, meaning the larger column methods are more rugged when transferred between instruments. Matching the extra-column volume on the original instrument (if known) may allow smaller column methods to be successfully transferred.



Peaks	t_R (min)	Conc. (µg/mL)
1. Cannabidivarinic acid (CBDVA)	1.967	50
2. Cannabidivarin (CBDV)	2.2	50
3. Cannabidiolic acid (CBDA)	2.782	50
4. Cannabigerolic acid (CBGA)	2.971	50
5. Cannabigerol (CBG)	3.157	50
6. Cannabidiol (CBD)	3.337	50
7. Tetrahydrocannabivarin (THCV)	3.624	50
8. Tetrahydrocannabivarinic acid (THCVA)	4.633	50
9. Cannabinol (CBN)	5.013	50
10. Cannabinolic acid (CBNA)	5.99	50
11. Δ9-Tetrahydrocannabinol (Δ9-THC)	6.358	50
12. Δ8-Tetrahydrocannabinol (Δ8-THC)	6.576	50
13. Cannabicyclol (CBL)	7.598	50
14. Cannabichromene (CBC)	8.033	50
15. Tetrahydrocannabinolic acid A (THCA-A)	8.416	50
16. Cannabichromenic acid (CBCA)	9.546	50

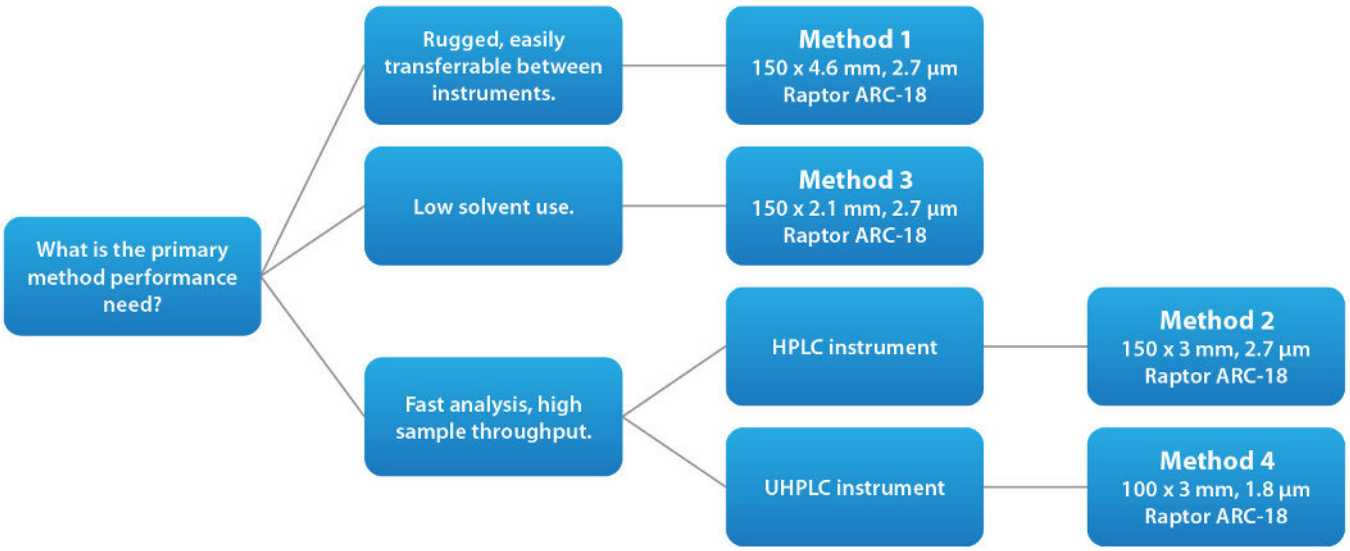
Retention times are for top chromatogram (Method 1).

Conclusions and Recommendations for Cannabinoids Analysis

To summarize, these experiments confirmed that the effects of extra-column volume are more pronounced on smaller columns. This occurs because the contribution of extra-column volume to total volume (and thus to band broadening) is relatively greater on smaller, lower volume columns than on larger, higher volume columns.

In practice, this means that when selecting a method, it is important to understand how extra-column volume is likely to affect the chromatographic results produced by different column sizes. Figure 5 provides a generalized framework for selecting which of the four methods discussed here would best suit a laboratory's needs.

Figure 5: Decision tree for cannabis and hemp potency testing. When setting up a new method, minimize extra-column volume for best chromatographic performance, especially when using smaller columns.



As originally shown in Figure 1, all four methods will provide excellent chromatographic results for cannabis and hemp potency testing when extra-column volume is reduced by using small flow cells and minimal tubing. However, our testing demonstrated that the methods using smaller columns are more susceptible to the negative impacts of excessive extra-column volume. Therefore, for best results when establishing a method on a new instrument, be sure to minimize extra-column volume, especially when adopting a method that uses a smaller column.

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- Part of Restek's Raptor LC column line featuring 1.8, 2.7, and 5 µm SPP core-shell silica.

ID	Length	qty.	cat.#
1.8 µm Particles			
3.0 mm	100 mm	ea.	931421E
2.7 µm Particles			
2.1 mm	150 mm	ea.	9314A62
3.0 mm	150 mm	ea.	9314A6E
4.6 mm	150 mm	ea.	9314A65

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- Guard column cartridges require EXP direct connect holder (cat.# 25808).
- Pair with EXP hand-tight fitting (cat.# 25937–25938) for tool-free installation.

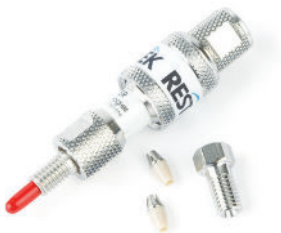
Description	Particle Size	Size	qty.	cat.#
Raptor ARC-18 EXP Guard Column Cartridge	UHPLC	5 x 2.1 mm	3-pk.	9314U0252
	UHPLC	5 x 3.0 mm	3-pk.	9314U0253
	2.7 µm	5 x 2.1 mm	3-pk.	9314A0252
	2.7 µm	5 x 3.0 mm	3-pk.	9314A0253
	2.7 µm	5 x 4.6 mm	3-pk.	9314A0250
	5 µm	5 x 2.1 mm	3-pk.	931450252
	5 µm	5 x 3.0 mm	3-pk.	931450253
	5 µm	5 x 4.6 mm	3-pk.	931450250

Maximum cartridge pressure: 1034 bar/15,000 psi* (UHPLC), 600 bar/8700 psi (2.7 µm); 400 bar/5800 psi (5 µm)
* For maximum lifetime, recommended maximum pressure for UHPLC particles is 830 bar/12,000 psi.
Intellectual Property: optimizetech.com/patents

EXP Direct Connect Holder

Description	qty.	cat.#
EXP Direct Connect Holder for EXP Guard Cartridges (includes hex-head fitting & 2 ferrules)	ea.	25808

Maximum holder pressure: 20,000 psi (1400 bar)
Intellectual Property: optimizetech.com/patents



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- Fit most standard 12 x 32 mm autosamplers, including UHPLC instruments.

Description	Color	Porosity	qty.	cat.#
Nylon				
Thomson SINGLE StEP Standard Filter Vial	black preslit cap	0.2 µm	100-pk.	25891
	pink preslit cap	0.45 µm	100-pk.	25892
PES (polyethersulfone)				
Thomson SINGLE StEP Standard Filter Vial	grey preslit cap	0.2 µm	100-pk.	25897
PTFE (polytetrafluoroethylene)				
Thomson SINGLE StEP Standard Filter Vial	green preslit cap	0.2 µm	100-pk.	25893
	blue preslit cap	0.45 µm	100-pk.	25894
PVDF (polyvinyldifluoride)				
Thomson SINGLE StEP Standard Filter Vial	red preslit cap	0.2 µm	100-pk.	25895
	yellow preslit cap	0.45 µm	100-pk.	25896

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26431

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Description	qty.	Similar to Part #	cat.#
Frit Adaptor, PTFE	4-pk.	Agilent 5062-8517	26392
Glass Solvent Filter, 15 µm frit	ea.	Agilent 5041-2168	26431

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