A Fast, Accurate, and Sensitive Quantitiation of Vitamin C in Plasma by Impact™ Protein Precipitation and Kinetex® 5 µm Core-Shell XB-C18 HPLC Columns

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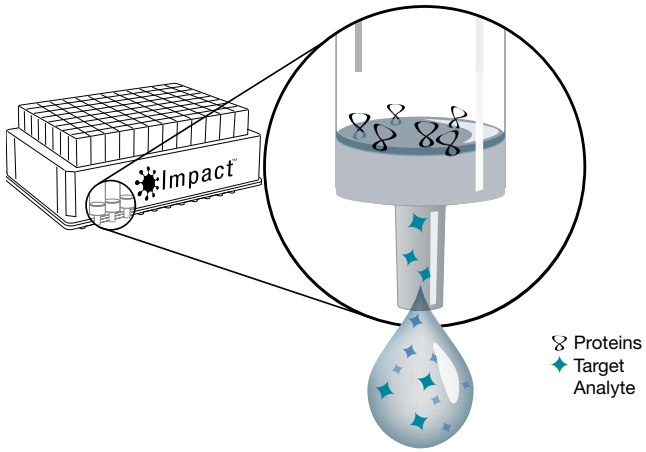
Vitamin C was extracted from human plasma by performing a rapid protein precipitation using Impact Protein Precipitation Plates followed by HPLC analysis using a Kinetex 5 µm core-shell XB-C18 150 x 4.6 mm HPLC column and UV detection at wavelength 245 nm. Impact technology offers easy, fast protein removal while providing maximized recovery of the target analytes. The Kinetex 5 µm core-shell XB-C18 HPLC column produced excellent chromatographic resolution, sensitivity, and high peak capacities, even at a low sample volume of only 100 µL, as well as a fast run time of under 3 minutes.

Introduction

Vitamin C is a very polar compound and is difficult to retain on reversed phase HPLC/UHPLC columns. The instability of Vitamin C at room temperature makes sample preparation and further analysis challenging. Our goal was to generate a stable HPLC method for accurate analysis of human plasma Vitamin C with an easy and fast sample preparation method and efficient HPLC columns.

Figure 1.
Protein Precipitation Using Impact Protein Precipitation Plates



Materials and Methods

Sample Preparation
Protein precipitation was performed using an Impact Protein Precipitation Plate.

Protein Precipitation	
96-Well Plate:	Impact Protein Precipitation Plate (Part No.: CE0-7565)
Part No.:	CE0-7565
Dispense:	300 µL of cold 5% Meta Phosphoric acid into each well
Add:	100 µL of plasma or serum sample
Mix:	5x by aspirating with the same pipette tip
Centrifuge:	the plate at 500 g for 5 minutes at 4°C. Collect purified filtrate in the 96-well collection plate.

* A vacuum manifold may be used however 25" Hg vacuum pressure must be applied for up to 10 minutes or until sample is completely pulled through the Impact plate.

After filtrate is collected, the collection plate containing the purified samples should be covered using a sealing mat. It is important that a polypropylene collection plate be used because polypropylene displays minimal degradation as compared to other plastics. The sample is now ready to be injected onto the LC/UV.

Because Vitamin C is light sensitive, the filtrate was transferred to Verex™ 9 mm Screw-top, µVial i3 (Qsert) amber autosampler vials (part no. AR0-3921-12) to protect from light. The vials were placed on a cooled autosampler (4 °C) then 30 µL was injected onto the HPLC system.

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HPLC Conditions
An Agilent® 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for LC/UV analysis.

Column: Kinetex® 5 µm XB-C18, 100Å
Dimensions: 150 x 4.6 mm
Part No.: 00F-4605-E0
Guard: SecurityGuard™ ULTRA UHPLC C18 Cartridges
Guard Part No.: AJ0-8768
Mobile Phase: A: 0.1 % Formic acid in water
B: Acetonitrile

Gradient:

Time (min)	% B
0	0
3.5	0
3.6	100
5.0	100
5.1	0
7.0	0

Flow Rate: 800 µL/min
Temperature: Ambient
Detection: UV wavelength @ 245 nm
Injection: 30 µL

Standard Preparation
Standard curves were prepared using defibrinated human plasma which was first prepared by following the same Impact™ protein precipitation procedure outlined on pg. 1. Different concentrations of Vitamin C standard solutions were then spiked in the Impact extracted defibrinated plasma.

Results and Discussion

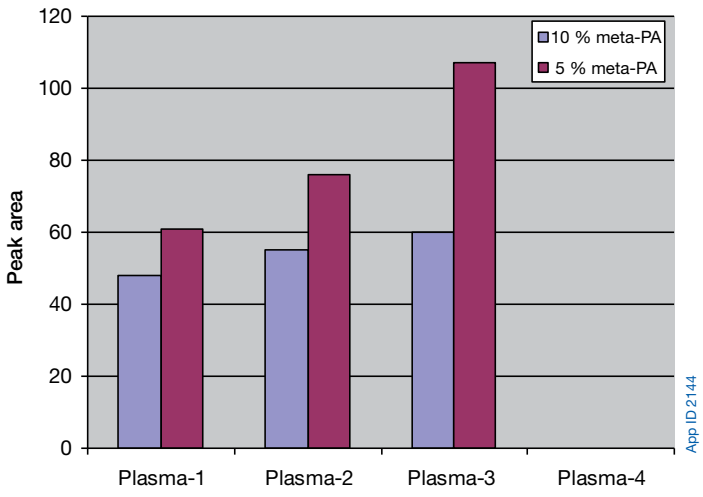
When developing a method for the analysis of Vitamin C in human plasma, it was important that the method be rapid, sensitive, and accurate. Traditionally, a protein precipitation step is used for fast cleanup of plasma samples. Protein precipitation is normally performed using a centrifuge tube or a 96-well collection plate; however, this process requires that supernatant be collected while being careful not to disrupt pelleted protein in the bottom of the tube or collection plate. This step was greatly simplified by using Impact Protein Precipitation Plates. The Impact plate allows for the analysis of 96 samples at once, eliminates the transfer steps that are commonly associated with protein precipitation, and can also be automated. Protein precipitation was performed within the wells of the Impact plate and sample was not allowed to pass through the filter of the plate until centrifugal force was applied. This ensured that the precipitated protein was left within the wells of the Impact plate while protein free sample was allowed to pass through the filter and into a collection plate (**Figure 1**).

Because Vitamin C is unstable at room temperature, we performed stability studies using several different precipitation agents when developing our precipitation method. Recent studies suggested that Vitamin C samples extracted with 10 % Meta-phosphoric acid were stable on a cooled autosampler at 4 °C for at least 10 hours with good precision. We tested several precipitation reagents including:

- 10 % Meta-phosphoric acid
- 5 % Meta-phosphoric acid
- 1.0 M/L Perchloric acid
- Acetonitrile (ACN) with 0.1 M/L Hydrochloric acid (HCl)
- ACN with 0.1 M/L Formic acid
- ACN with 0.2 M/L Formic acid
- Methanol with 0.1 M/L Formic acid
- Methanol with 0.2 M/L Formic acid

It was determined that Vitamin C was not stable under most of precipitation reagents tested. The amount of Vitamin C present in our extracts declined dramatically within a few hours. However, promising results were seen when the plasma was extracted using 5 % Meta-phosphoric acid which yielded higher plasma Vitamin C levels as compared to the 10 % Meta-phosphoric acid extraction. The increased levels varied as we tested different individual's plasma (plasma 1 to 3, **Figure 2**). Although most of our plasma samples resulted in increased levels of plasma Vitamin C in the 5 % Meta-phosphoric acid extraction, plasma 4 (defibrinated plasma) did not show an increase (**Figure 2**).

Figure 2.
Comparison of human plasma Vitamin C levels from extractions using 10 % Meta-phosphoric acid vs. 5 % Meta-phosphoric acid as a precipitation solvent.



After it was determined that extraction with 5 % Meta-phosphoric acid resulted in higher levels of plasma Vitamin C, further stability studies were performed. It was found that Vitamin C extracted using this reagent produced samples that were stable for up to 48 hours. Vitamin C levels in all 3 plasma samples studies (plasma 1 to 3) declined less than 5 % within 48 hours (**Figure 3a**). However, significant declines in plasma Vitamin C levels were observed from 48 to 82 hours (**Figure 3a**). In contrast, plasma Vitamin C levels in the 10 % Meta-phosphoric acid extracted plasma samples were stable for up to 24 hours for plasma samples 1 and 2 while plasma sample 3 showed a 20 % increase in Vitamin C levels within the first 8 hours after extraction and continued to increase up to 48 hours (**Figure 3b**). The defibrinated plasma sample, plasma 4, displayed no Vitamin C under both extraction reagents from 0 to 82 hours, indicating that there were no non-specific interferences to the Vitamin C levels. After stability studies, it was determined that 5 % Meta-phosphoric acid was the optimum precipitation reagent because it produced high levels of plasma Vitamin C across several plasma samples and showed the most stability during our 82 hour study.

Figure 3a.
Vitamin C levels after plasma extracted with 5 % Meta-phosphoric acid

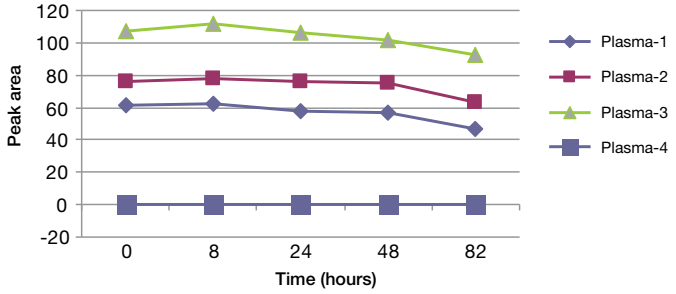
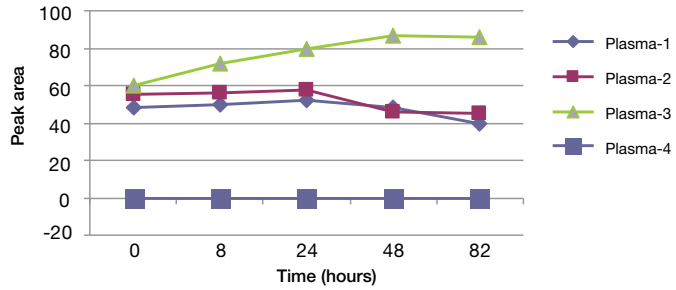
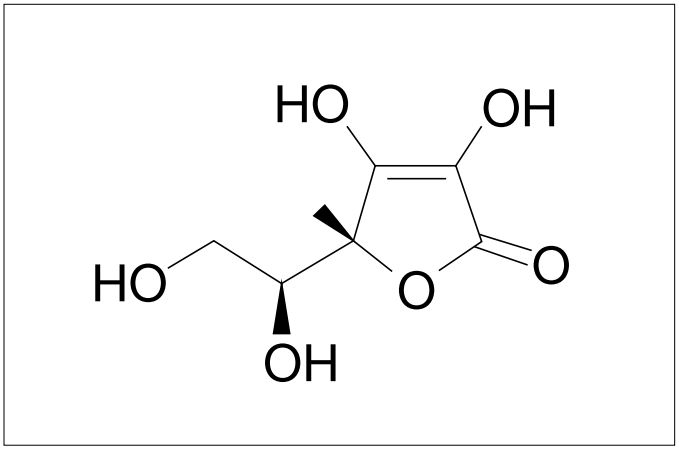


Figure 3b.
Vitamin C levels after plasma extracted with 10 % Meta-phosphoric acid



After the plasma samples were cleaned up, Vitamin C was analyzed by HPLC using a Kinetex® 5 µm core-shell XB-C18 HPLC column equipped with a UV detector at 245 nm. Vitamin C is a very polar compound (**Figure 4**) and is rather difficult to retain on a reversed phase HPLC/UHPLC column however, the Kinetex core-shell XB-C18 particle has an increased affinity for hydrogen bonding interactions, making it an ideal column for retention of Vitamin C. The Kinetex particle is based on core-shell technology which has a homogenous porous shell grown onto a solid silica core, providing the 5 µm core-shell particle with efficiencies similar to a 3 µm fully porous particle. The core-shell particle also provides faster run times as compared to traditional, fully porous 5 µm particles with a total run time of under 3 minutes, with Vitamin C eluting at 2.731 minutes (**Figures 5** and **6**).

Figure 4.
Chemical Structure of Vitamin C (ascorbic acid)



A wavelength of 245 nm was selected as the optimal wavelength for measuring Vitamin C because the signal generated using this wavelength resulted in a signal that was 4 times higher than the signal from UV 265 nm which had previously been published as the maximum UV response for Vitamin C (**Figure 5**). A wavelength of 235 nm was also tested and the resulting signal was lower than the signal produced using 245 nm (**Figure 6**).

Figure 5.
Optimized HPLC chromatogram using wavelength 245 nm for measuring Vitamin C in human plasma (defribinated) by protein precipitation using the Impact™ Protein Precipitation Plate

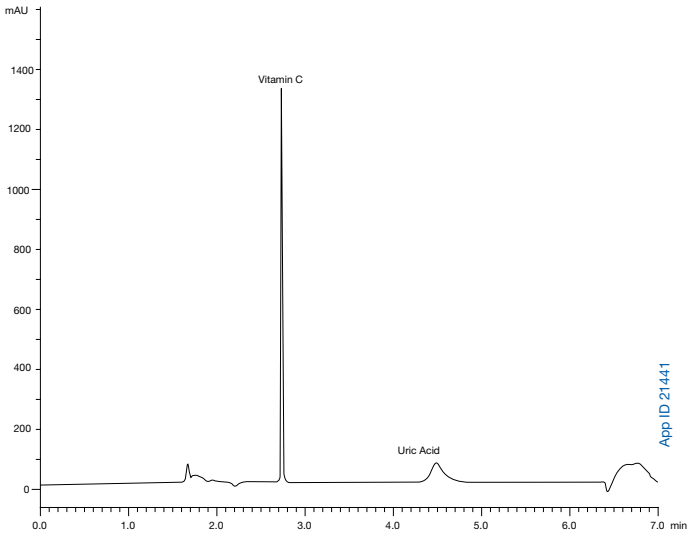
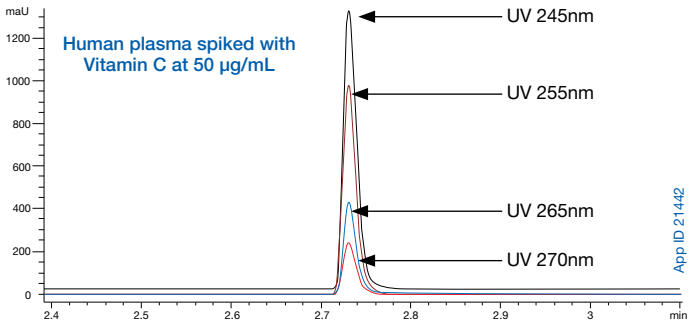


Figure 6.
HPLC chromatogram of human plasma (defribinated) spiked with Vitamin C at 50 µg/mL by protein precipitation using the Impact plate at various wavelengths



After determining the ideal wavelength at which to perform our analysis, endogenous levels of Vitamin C were studied alongside spiked samples to ensure that the sample preparation and analysis were fit for purpose (**Figures 7a** and **7b**).

Figure 7a.
Comparisons of HPLC chromatograms of endogenous levels of Vitamin C at 5 µg/mL from a random human plasma sample (plasma 3) alongside the same sample spiked with an additional 3 µg/mL of Vitamin C standard, each prepared by protein precipitation using the Impact plate

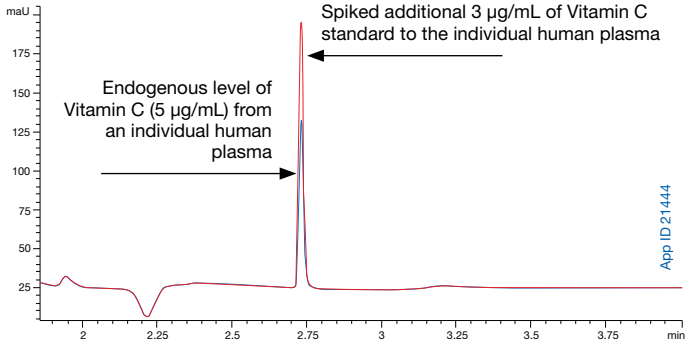
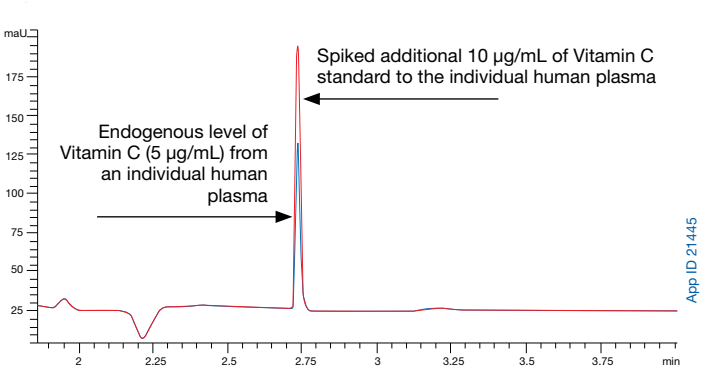


Figure 7b.
Comparisons of HPLC chromatograms of endogenous levels of Vitamin C at 5 µg/mL from a random human plasma sample (plasma 3) alongside the same sample spiked with an additional 10 µg/mL of Vitamin C standard, each prepared by protein precipitation using the Impact plate



The reproducibility of our analysis was then determined by producing a standard curve of Vitamin C at a concentration range of 0 to 100 µg/mL (**Figure 8**). Not only was our method reproducible, it also resulted in high recoveries of Vitamin C across several Vitamin C concentration levels (**Table 1**), resulting in an average recovery of 98 % with a CV of 4.8 % (n = 7). The intra-assy imprecision was 1.74 to 2.90 % and inter-assay imprecision was 1.80 to 4.82 % (**Table 2**). A lower limit of detection (LOD) of 0.78 µg/mL and lower limit of quantitation (LOQ) of 1.56 µg/mL showed a signal-to-noise (S/N) ratio of 14.6 (**Table 3** and **Figure 9**) and a mean S/N ratio of 14.21 (CV = 2.85 %). Therefore, human plasma Vitamin C can be accurately quantitated.

Figure 8.
Representative standard curve of Vitamin C at a concentration range of 0 to 100 µg/mL

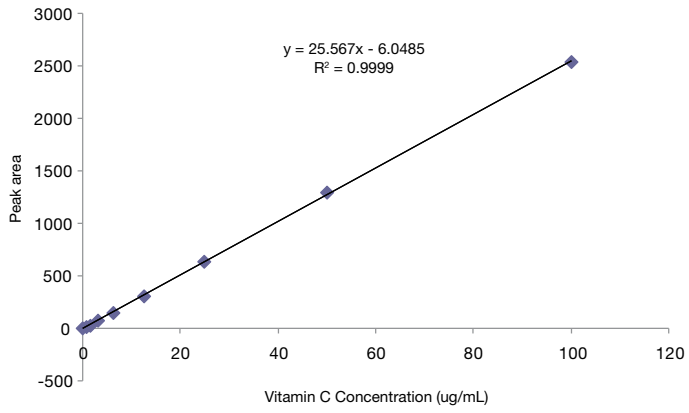


Table 1.
Recovery of Vitamin C from spiked human plasma

Added Vitamin C (µg/mL)	Observed (µg/mL)	Recovery (%)
0	5.0	-
3	7.9	98.8
5	9.0	90.0
10	14.1	94.0
20	24.8	99.2
30	35.1	100.3
40	46.8	104.0
60	65.5	100.8
	Mean	**98.2**
	CV	**4.8**

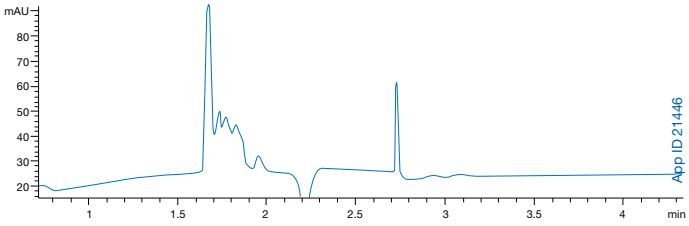
Table 2.
Intra- and inter-day imprecision of plasma Vitamin C analysis

	Intra Imprecision (n=12)			Inter Imprecision (n=6)		
	Mean (µg/mL)	SD	% CV	Mean	SD	% CV
QC 1	1.1	0.0309	**2.90**	1.1	0.0532	**4.82**
QC 2	11.2	0.3552	**3.16**	11.1	0.3857	**3.47**
QC 3	34.1	0.5910	**1.74**	34.9	0.6290	**1.80**

Table 3.
LOD, signal-to-noise ratio, and % CV of Vitamin C

	LOD			LOQ		
	Concentration (µg/mL)	S/N Ratio	% CV (n=3)	Concentration (µg/mL)	S/N Ratio	% CV (n=3)
Vitamin C	0.78	5.79	4.05	1.56	14.21	2.85

Figure 9.
Chromatogram of LOQ levels of plasma Vitamin C (1.56 µg/mL) with a signal-to-noise ratio of 14.6



Conclusion

High-throughput laboratories must adopt a rapid and robust method to accurately analyze and quantitate vitamins and their derivatives. With these goals in mind, we developed a method that can be easily automated, can be used in high-throughput labs, and provides sensitivity, speed, and reproducibility. The sample preparation step using Impact™ Protein Precipitation Plates is simple, requires no method development, and can process 96 samples at once. The downstream HPLC analysis using a Kinetex® 5 µm XB-C18 HPLC column provides excellent peak shape, in under 3 minutes.

Ordering Information

Impact™ Precipitation Plates

Part No.	Description	Unit
Impact Precipitation Plates		
CE0-7565	Impact Protein Precipitation, Square Well, Filter Plate, 2 mL	2/box
Impact Starter Kit for Protein Precipitation		
CE0-8201	Impact Protein Precipitation Plate (2 ea) Collection Plate 2 mL (2 ea) Sealing Mat, Santoprene™ (AH0-8199) (2 ea)	ea

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

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Ordering Infomation

Kinetex® HPLC Columns

5 µm Columns (mm)		SecurityGuard™ ULTRA Cartridges‡					SecurityGuard ULTRA Cartridges‡
Phases	50 x 2.1	3/pk	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
XB-C18	00B-4605-AN	AJ0-8782 for 2.1 mm ID	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJ0-8768 for 4.6 mm ID

2.6 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	3/pk
XB-C18	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768 for 4.6 mm ID

2.6 µm MidBore™ Columns (mm)						SecurityGuard ULTRA Cartridges
Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775 for 3.0 mm ID

2.6 µm Minibore Columns (mm)					SecurityGuard ULTRA Cartridges
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
XB-C18	00A-4496-AN	00B-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782 for 2.1 mm ID

1.7 µm MidBore Columns (mm)				SecurityGuard ULTRA Cartridges
Phases	30 x 3.0	50 x 3.0	100 x 3.0	3/pk
XB-C18	00A-4498-Y0	00B-4498-Y0	00D-4498-Y0	AJ0-8775 for 3.0 mm ID

1.7 µm Minibore Columns (mm)					SecurityGuard ULTRA Cartridges
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
XB-C18	00A-4498-AN	00B-4498-AN	00D-4498-AN	00F-4498-AN	AJ0-8782 for 2.1 mm ID

‡SecurityGuard ULTRA cartidges require holder, Part No.: AJ0-9000

guarantee

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