Fast and Robust Analysis of Organic Acids from Wine using HPLC-UV

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Introduction

Organic acids are a major contributor to the stability, flavor, color, and balance of wine. Organic acids are found in both wine and the grapes from which they are derived. Of particular importance are Tartaric and Malic acids. Tartaric acid contributes largely to a wine's acidity and can form crystalline deposits, which affect red wines more than white wines. This is the preferred acid addition to wine because it is not as easily degraded by microbes[1]. Another critical acid is Malic acid, which is metabolized through the ripening stage and drops in concentration after harvest. Acetic, Citric, and Succinic acids are also critical acids in wine and contribute to the overall acidity and flavor profile for both red and white wines[2].

Previous studies have shown that HPLC can be used as a method for the quantitation of organic acids, which can be used for both fermentation monitoring and quality control [3,4]. Typical separation modes by HPLC include ion-exclusion and reversed phase. Ion-exclusion, though simple and straightforward, is limited in experimental design space and can result in run times exceeding 30 minutes. Reversed phase allows for more flexibility in method development and relatively faster analysis times, but can present challenges since organic acids are relatively polar compounds.

As such, column selectivity plays a critical role in development of a quantitative reversed phase method for organic acids. In this study, we present two orthogonal methods for the testing of organic acids in red and white wines. The first method uses a Kinetex® F5 core-shell pentafluorophenyl phase HPLC column and is an appropriate screening method for the identification of some critical acids, such as Tartaric and Lactic acid. However, for full separation of all relevant organic acids, a Synergi™ Hydro-RP column with enhanced polar selectivity is needed. Reasonable run times are below 10 minutes and the following organic acids can be accurately quantitated: Tartaric acid, Malic acid, Lactic acid, Citric acid, Acetic acid and Succinic acid. Additionally, Shikimic acid, a known interference, can also be separated.

Materials and Methods

Standards were purchased from Sigma Aldrich. Red and white wines were purchased from a local grocer.

Standards were prepared in water, then diluted 1:10 in mobile phase A prior to injection. Wine samples were also diluted 1:10 in mobile phase, then filtered with a Phenex™ 0.2 µm syringe filter (P/N AF0-3203-12) prior to injection onto the HPLC system.

HPLC analysis was performed on an Agilent® 1100 system with a DAD detector and data was collected using ChemStation software (Agilent, Santa Clara, CA. USA).

Results and Discussion

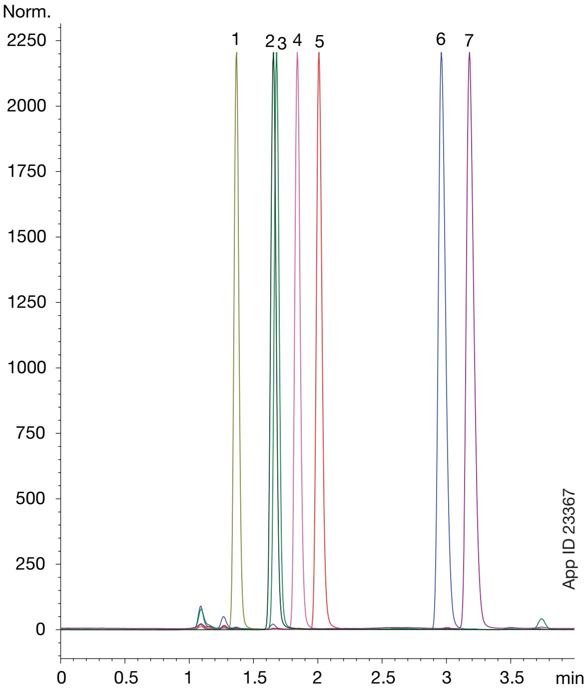
The initial screening method used a Kinetex 2.6 µm F5 core-shell column. Although the column performed well with neat standards, co-elution of interference peaks in red and white wine samples complex indicated this column might not be optimal for quantitative organic acid sample analysis. Specifically, the known interferent Shikimic acid coeluted with Malic acid **(Figure 1)**.

Nonetheless, the column did show a good proof of concept for a simple chromatographic screen that could be used for the quantitation of certain organic acids, such as Tartaric acid, or if the analyst is not particularly interested in quantitation of Malic acid.

Screening Method using Kinetex 2.6 µm F5

Column:	Kinetex 2.6 µm F5, 100Å
Dimensions:	150 x 4.6 mm
Part No.:	00F-4723-E0
Mobile Phase:	20 mM Potassium Phosphate, pH 1.6
Flow Rate:	1.25 mL/min
Temperature:	Ambient
Detection:	UV @ 210 nm
Injection:	10 µL
Sample:	1. Tartaric Acid 2. Shikimic Acid 3. Malic Acid 4. Lactic Acid 5. Acetic Acid 6. Citric Acid 7. Succinic Acid

Figure 1.
Acid Standards Screening Method using Kinetex 2.6 µm F5 Core-Shell Column



Further method development was then performed on a Synergi™ 4 µm Hydro-RP column. This column was selected due to its stationary phase hydrogen bond donating capabilities. To prevent carryover of late eluting matrix interferences, a gradient clean up step was implemented. Although the total run time was extended to 10 minutes, the analysis was still a shorter run time than previously reported methods.

An acidic mobile phase with a pH of 1.5 was required to ensure protonation of the organic acids, thus helping in retention by reversed phase mechanisms. Again, this is primarily based upon the hydrogen bond donating capabilities of the polar endcapped Synergi Hydro-RP column.

Finally, temperature was another parameter that was investigated during method development and helped in the improved resolution of Shikimic acid, and thus the higher range of the column stability (60°C) was used.

Final Optimized HPLC-UV Method using Synergi 4 µm Hydro-RP Column

Column: Synergi 4 µm Hydro-RP 80Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4375-E0
Mobile Phase: A: 20 mM Potassium Phosphate, pH 1.5
B: Methanol
Gradient:

Time (min)	% B
0	0
2.5	0
2.6	30
2.9	30
3.0	0
10	0

Flow Rate: 1 mL/min
Temperature: 60°C
Detection: UV @ 210 nm (ambient)
Injection: 20 µL
Sample: 1. Tartaric Acid
2. Malic Acid
3. Shikimic Acid
4. Lactic Acid
5. Acetic Acid
6. Citric Acid
7. Succinic Acid

After method optimization, a five point calibration curve was generated and a linear regression was used to determine the organic acid content for red and white wines. **Figures 2 and 3** show calibrator levels at lowest and highest concentration. All sample components are baseline resolved, allowing for the easy quantitation of organic acids.

Figure 2.
Organic Acid Standard (Calibrator Level 1 - Low)

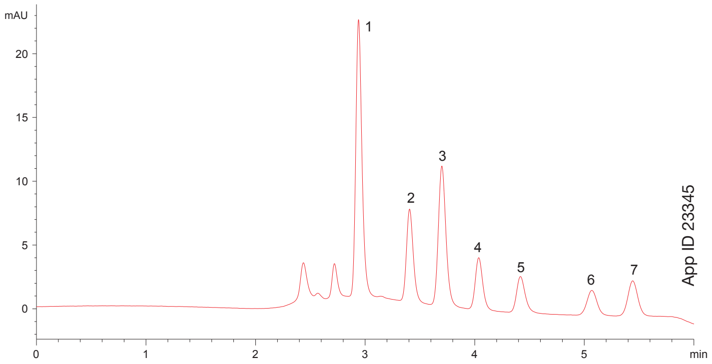


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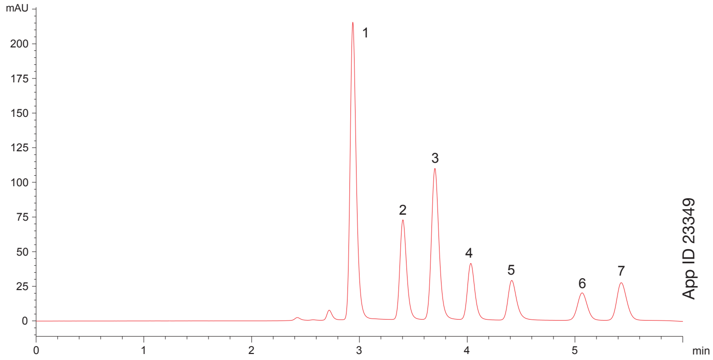
Figure 3.
Organic Acid Standard (Calibrator Level 5 - High)



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Data for organic acid standards are summarized in **Table 1**. Using peak height of the standards run in triplicate, linearity was determined for each organic acid. A correlation coefficient of >0.99 for all standards was observed and % RSD for all standards were below 5% with the exception of one data point for Citric acid (7.63%). Succinic acid was included in the standard mix. Although linearity for the standards was determined to be 0.25 to 2.5 mg/mL, matrix interferences did not allow for quantitative measurements when samples were evaluated.

Table 1.
Organic Acid Standards (Collected data)

Sample Number	Standard		Rep 1	Rep 2	Rep 3	Mean	% RSD	Calculated Conc. (mg/mL)	Expected Conc. (mg/mL)	% Error for Standards
1	Tartaric Acid	Standard 1	84.8	84.3	84.7	84.60	0.31	0.51	0.5	1.75
		Standard 2	170.3	170	174.4	171.57	1.43	1.01	1	1.18
		Standard 3	419.9	421.5	422.5	421.30	0.31	2.46	2.5	-1.74
		Standard 4	645.1	644.6	654.3	648.00	0.84	3.77	3.75	0.48
		Standard 5	865	855.5	865.2	861.90	0.64	5.01	5	0.10
2	Malic Acid	Standard 1	31.4	31.8	31.4	31.53	0.73	0.51	0.5	2.59
		Standard 2	63.1	63.4	65.4	63.97	1.95	1.02	1	1.63
		Standard 3	155	156	154.6	155.20	0.46	2.43	2.5	-2.71
		Standard 4	241.5	240.9	245	242.47	0.91	3.79	3.75	0.98
		Standard 5	323.3	315.6	323.4	320.77	1.40	5.00	5	0.04
3	Shikimic Acid	Standard 1	53.1	53.3	53.2	53.2	0.19	0.0051	0.005	2.79
		Standard 2	107.1	107	107	107.0333333	0.05	0.0101	0.01	1.16
		Standard 3	263.1	262.9	262.7	262.9	0.08	0.0245	0.025	-1.90
		Standard 4	403.5	402.8	406	404.1	0.42	0.0376	0.0375	0.21
		Standard 5	541.9	536	542.1	540	0.64	0.0501	0.05	0.28
4	Lactic Acid	Standard 1	19.3	19.3	19.4	19.33	0.30	0.26	0.25	3.74
		Standard 2	38.9	39	39.1	39.00	0.26	0.51	0.5	1.22
		Standard 3	95.3	96.5	96.3	96.03	0.67	1.22	1.25	-2.26
		Standard 4	148.2	147.8	149.1	148.37	0.45	1.88	1.875	0.18
		Standard 5	199.3	197.2	199.5	198.67	0.64	2.51	2.5	0.38
5	Acetic Acid	Standard 1	15.8	15.7	15.9	15.80	0.63	0.26	0.25	2.30
		Standard 2	32	32.3	32.1	32.13	0.48	0.51	0.5	1.43
		Standard 3	79.6	79.5	79.3	79.47	0.19	1.24	1.25	-1.14
		Standard 4	120.2	119.9	119.9	120.00	0.14	1.86	1.875	-0.81
		Standard 5	162.7	162.7	162.6	162.67	0.04	2.52	2.5	0.66
6	Citric Acid	Standard 1	13.4	12.7	12.6	12.90	3.38	0.0269	0.025	7.63
		Standard 2	25.7	25.8	25.5	25.67	0.60	0.0508	0.05	1.62
		Standard 3	61.5	63	62.6	62.37	1.25	0.1195	0.125	-4.38
		Standard 4	99	99.4	100	99.47	0.51	0.1890	0.1875	0.79
		Standard 5	132.9	132.6	132.7	132.73	0.12	0.2513	0.25	0.51
7	Succinic Acid	Standard 1	17.2	17.5	17.1	17.27	1.21	0.25	0.25	0.70
		Standard 2	35.1	34.9	35.3	35.10	0.57	0.51	0.5	1.40
		Standard 3	86.1	86.4	87	86.50	0.53	1.24	1.25	-0.59
		Standard 4	128.7	129.7	130.7	129.70	0.77	1.86	1.875	-0.75
		Standard 5	174.2	175.7	175.8	175.23	0.51	2.51	2.5	0.51

The eight samples of red and white wines were run in triplicate. Organic acids were identified based upon retention time. Concentrations for organic acids were then determined for each organic acid of interest. **Table 2** indicates the calculated organic acid concentration in each tested wine. Organic acid concentrations calculated as below their respective standard 1 concentration is indicated with a "0†". Matrix interferences also contributed to the inability to quantitate some organic acids. This is especially true with the late eluting succinic acid. **Figures 3 - 10** illustrate the chromatogram for each of the wine samples. All confirmed peaks are labeled, based on known retention times.

Table 2:
Calculated Concentrations for Organic Acid Standards (mg/mL)

Wine	Tartaric Acid	Malic Acid	Shikimic Acid	Lactic Acid	Acetic Acid	Citric Acid	Succinic Acid
Mixed Red	1.51	0†	0.0234	1.83	0.37	0.0708	0†
Cabernet Sauvignon C	1.67	0†	0.0280	1.6	0.53	0†	0†
Cabernet Sauvignon P	1.84	0†	0.0408	1.39	0.73	0†	0†
Bordeaux	1.19	0†	0.0257	1.34	0†	0.0408	0†
Pinot Noir	1.85	0†	0.0177	2.07	0.61	0†	0†
Chardonnay	2.17	0†	0.0182	2.47	0†	0†	0†
Sauvignon Blanc	2.23	1.25	0.0079	0.52	0.36	0.0443	0†
Chablis	1.58	0.91	0.0288	2.79	0†	0.0405	0†

† excluded because concentration is below standard 1 level.

Figure 3.
Mixed Red

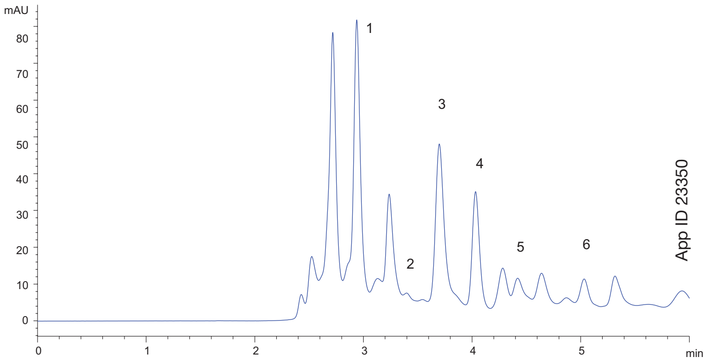


Figure 4.
Cabernet Sauvignon C

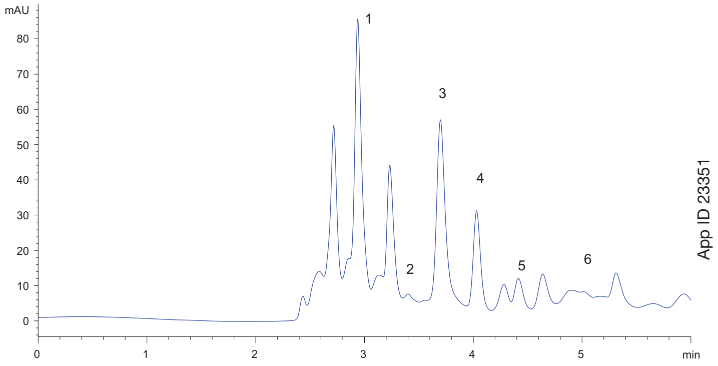


Figure 5.
Cabernet Sauvignon P

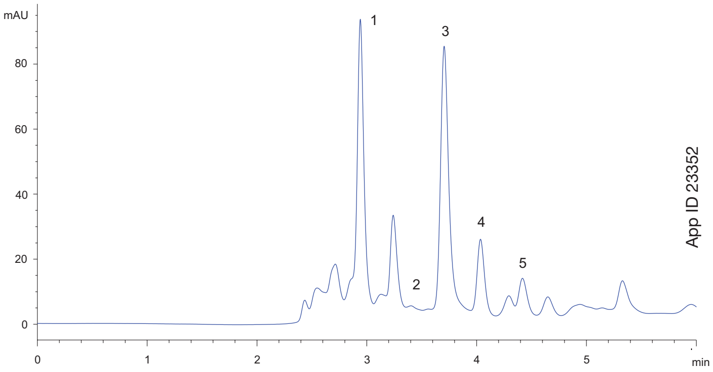


Figure 6.
Bordeaux

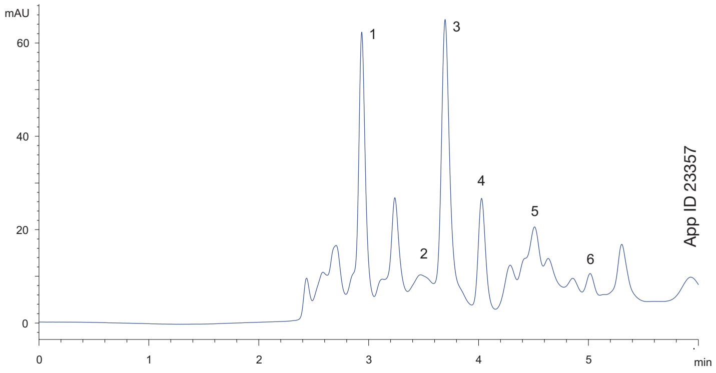


Figure 7.
Pinot Noir

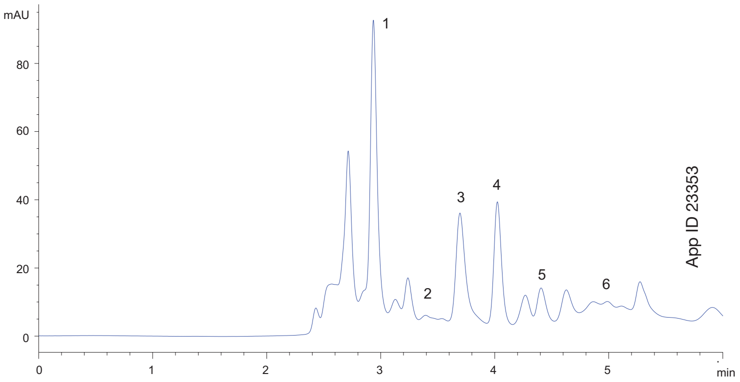


Figure 8.
Chardonnay

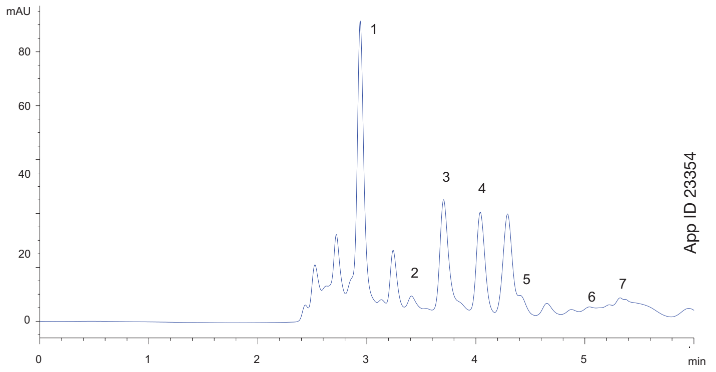


Figure 9.
Sauvignon Blanc

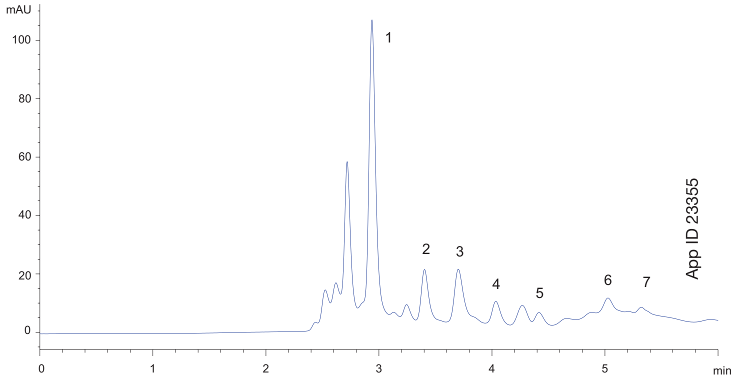
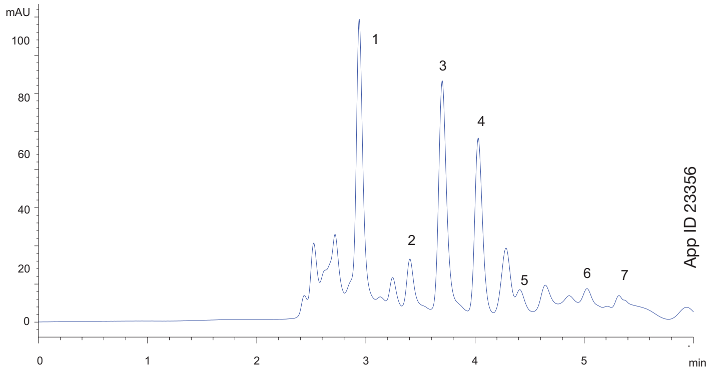


Figure 10.
Chablis



Conclusion

Previously reported HPLC methods for the quantitation of organic acids from wine are either limited in selectivity or time prohibitive. In this study, we developed a fast and robust method using the Synergi™ 4 µm Hydro-RP column to resolve the organic acids of interest in 6 minutes. Including a gradient cleanup step, the total runtime is 10 minutes. If small matrix interferences are not a concern, the method could easily be shortened by simply increasing the flow rate. Finally, should a fast, simple screen be needed, the screening method developed on a Kinetex® F5 core-shell column might be of particular interest, especially if Malic acid is not a concern.

Further studies could include identification and quantitation of additional interferences in the complex wine sample matrices. Specificity could then be better assessed. Additionally, other sample preparation methods such as solid phase extraction might be investigated to further improve quantitation and robustness of the assay across other wine samples.

References

1. Kliewer, W. M., L. Howarth, and M. Omori. 1967. Concentrations of Tartaric Acid and Malic Acids and Their Salts in Vitis Vinifera Grapes. American Journal of Enology and Viticulture 18:42-54.
2. Jackson, Ron S. *Wine Science Principles and Applications*. 3rd ed. Amsterdam: Elsevier/Academic, 2008. 357-359.
3. Lopez, E. F., and E. F. Gomez. “Simultaneous Determination of the Major Organic Acids, Sugars, Glycerol, and Ethanol by HPLC in Grape Musts and White Wines.” *Journal of Chromatographic Science* (1996): 254-57.
4. Kordis-Krapez, M., Abram, V., Kac, M. et al., Determination of organic acids in white wines by RP-HPLC. Food Tech. Biotechnol. 2001, 39(2), 93–99.

Ordering Information
Kinetex

2.6 µm Analytical Columns (mm)						SecurityGuard™ ULTRA Cartridges‡
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**3/pk**
EVO C18	—	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	AJ0-9296
F5	—	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	AJ0-9320
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	AJ0-8774

for 4.6 mm ID

5 µm Analytical Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00B-4633-E0	00D-4633-E0	00F-4633-E0	00G-4633-E0	AJ0-9296
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207
XB-C18	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJ0-8768
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
C8	00B-4608-E0	00D-4608-E0	00F-4608-E0	00G-4608-E0	AJ0-8770
Phenyl-Hexyl	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJ0-8774

for 4.6 mm ID

If Kinetex core-shell columns do not provide at least an equivalent separation as compared to a competing column of the same phase, return the column with the comparative data within 45 days for a FULL REFUND.

Ordering Information
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4 µm MidBore™ Columns (mm)					SecurityGuard™ Cartridges (mm)
Phases	**30 x 3.0**	**50 x 3.0**	**150 x 3.0**	**250 x 3.0**	**4 x 2.0***
Max-RP	—	00B-4337-Y0	00F-4337-Y0	00G-4337-Y0	AJ0-6073
Hydro-RP	—	00B-4375-Y0	00F-4375-Y0	00G-4375-Y0	AJ0-7510
Polar-RP	00A-4336-Y0	00B-4336-Y0	00F-4336-Y0	00G-4336-Y0	AJ0-6075
Fusion-RP	—	00B-4424-Y0	00F-4424-Y0	00G-4424-Y0	AJ0-7556

for ID: 2.0-3.0 mm

4 µm Analytical Columns (mm)						SecurityGuard™ Cartridges (mm)
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**150 x 4.6**	**250 x 4.6**	**4 x 3.0***
Max-RP	00A-4337-E0	00B-4337-E0	00C-4337-E0	00F-4337-E0	00G-4337-E0	AJ0-6074
Hydro-RP	00A-4375-E0	00B-4375-E0	00C-4375-E0	00F-4375-E0	00G-4375-E0	AJ0-7511
Polar-RP	00A-4336-E0	00B-4336-E0	00C-4336-E0	00F-4336-E0	00G-4336-E0	AJ0-6076
Fusion-RP	—	00B-4424-E0	00C-4424-E0	00F-4424-E0	00G-4424-E0	AJ0-7557

for ID: 3.2-8.0 mm

* SecurityGuard™ Analytical Cartridges require holder, Part No.: KJ0-4282

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SecurityGuard is patented by Phenomenex. U.S. Patent No. 6,162,362

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