Determination of Impurities and Related Substances for Acetylsalicylic Acid (Ph. Eur. Monograph 0309): Increased Sensitivity and Resolution, Faster Analysis, and Reduced Solvent Usage Using Kinetex™ 2.6 µm Core-Shell LC Columns

Ellie Abbasi, Jeff Layne, Heiko Behr, and Philip J. Koerner
Phenomenex, Inc., 411 Madrid Ave, Torrance, CA 90501 USA

- *Methods can be improved for ultra-high performance without the need for higher-pressure capable instrumentation*
- *Within allowable modifications for system suitability, this method was improved for better resolution, higher sensitivity, and an 80 % reduction in analysis time.*

Introduction

Presently, HPLC methods for the determination of impurities and related substances of drug products specified in monographs by the various Pharmacopoeia agencies typically employ LC columns packed with fully porous 3 and 5 micron (µm) spherical silica chromatographic media. Due to the performance limitations of fully porous 3 and 5 µm spherical silica chromatographic media, these analytical methods commonly require long analysis times to provide the required chromatographic resolution for the impurities present. Additionally, accurate quantitation of low-level impurities in routine LC-UV applications may be challenging due to the low intensity peaks generated by these columns.

In recent years, smaller fully porous LC particles (sub-2 µm diameter) have been introduced that offer faster analysis times and generate higher intensity peaks for better sensitivity. Unfortunately, since the smaller particle columns generate system backpressures that require specialized ultra-high pressure capable LC instrumentation, widespread adoption of this sub-2 µm HPLC column technology has been slow.

Recently, a newly developed Kinetex 2.6 µm core-shell chromatographic particle has been commercialized that offers the performance benefits of fully porous sub-2 µm particles but at substantially lower operating pressures. To demonstrate the performance benefits of this new core-shell technology, a Kinetex 2.6 µm core-shell C18 column was compared with a fully-porous 5 µm C18 column referenced in European Pharmacopoeia [Ph. Eur.] Monograph 0309 for Acetylsalicylic acid and related substances on a conventional HPLC instrument with an upper pressure limit of 400 bar.

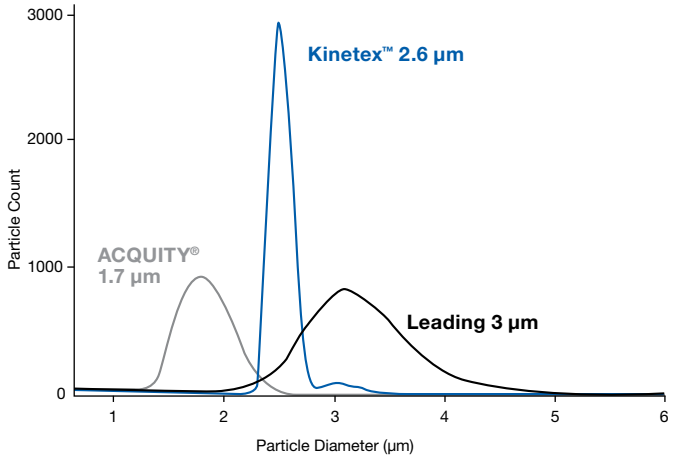
First, to demonstrate equivalency, a Kinetex column of the closest available dimension to the column referenced was operated under the conditions specified in the monograph. Then, in order to illustrate the extent of the performance benefits of the Kinetex column, a shorter Kinetex column (by 60 %) was operated at a 50 % faster flow rate; with both column length and flow rate maintained within the adjustments allowed by the Ph. Eur. for meeting system suitability. The Kinetex column achieved 80 % shorter analysis time (greater than 5x productivity improvement) and significantly improved resolution and sensitivity versus the Ph. Eur. referenced fully porous 5 µm column, while meeting the system suitability requirements.

Overview of Kinetex 2.6 µm Core-Shell Technology

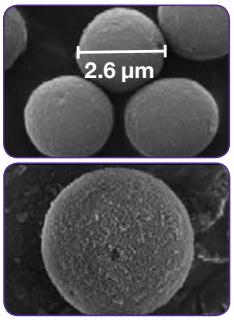
Precision Core-Shell Manufacturing

The Kinetex technology is comprised of a nearly monodispersed 1.9 µm solid silica core and a 0.35 µm porous silica shell. This particle design results in a very stable and homogeneous packed column bed that significantly reduces peak dispersion due to eddy diffusion (the "A" term of the van Deemter equation). Additionally, the short diffusion path of the 0.35 µm porous silica shell allows for faster kinetics of diffusion, thereby minimizing peak dispersion due to resistance to mass transfer (the "C" term in the van Deemter equation) **(Figure 1 & 2).**

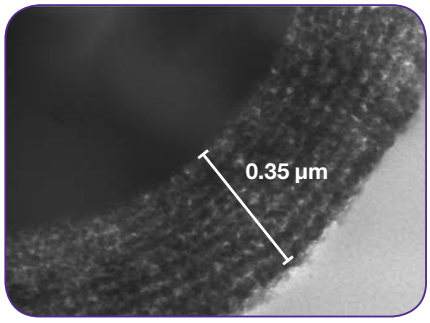
Figure 1.
Uniform Particle Size Distribution



Kinetex 2.6 µm Particle with 0.35 µm Porous Shell



Cross-section Image of Kinetex 2.6 µm Core-Shell Particle



APPLICATIONS

Ultra-High Efficiency Particle

Columns packed with Kinetex 2.6 µm core-shell silica particles are capable of maintaining ultra-high efficiencies across an extended range of mobile phase linear velocity. In **Figure 2**, van Deemter plots of plate height versus mobile phase linear velocity are presented for the Kinetex 2.6 µm column and a leading sub-2 µm column. Data was generated on an Agilent 1200SL instrument with an upper pressure limit of 600 bar. Note that the Kinetex 2.6 µm column achieved plate heights equivalent to the sub-2 µm column and was able to be operated at a higher flow rate before the upper system pressure limit was reached. Also note that there is no significant increase in plate height as mobile phase velocity is increased. This is due to the very low resistance to mass transfer of analytes into and out of the porous shell containing the stationary phase that surrounds the solid silica core (minimizing the contribution of the "C" term to plate height).

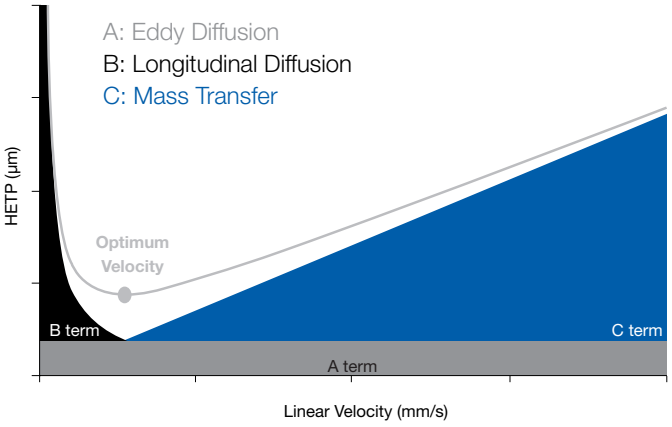
Reasonable LC System Operating Pressures

The comparison results in **Figure 3** demonstrate the ability of the Kinetex 2.6 µm core-shell technology to achieve chromatographic efficiencies comparable to those of fully-porous sub-2 µm columns at substantially lower system backpressures. The lower pressures generated by columns packed with Kinetex 2.6 µm particles allow them to be used on conventional LC instruments for routine analysis under 400 bar whereas traditional fully-porous sub-2 µm particles have limited utility below 400 bar and therefore require specialized ultra-high pressure capable LC instrumentation. This capability eliminates the challenges associated with the transfer of ultra-high performance methods across various LC system platforms, and makes ultra-high performance LC accessible to more scientists and laboratories.

Figure 2.

van Deemter Equation

$$H = 2\lambda d_p + 2GD_m/\mu + w(d_e)^2\mu/D_m + Rd^2_e\mu/D_s \;^*$$



* d_e refers to the effective particle size. For Kinetex 1.7 µm particles, d_e = 1.5 µm and for Kinetex 2.6 µm particles, d_e = 1.7 µm. For fully porous particles, $d_e = d_p$.

van Deemter Data
Agilent 1200 SL - 50 x 2.1 mm columns

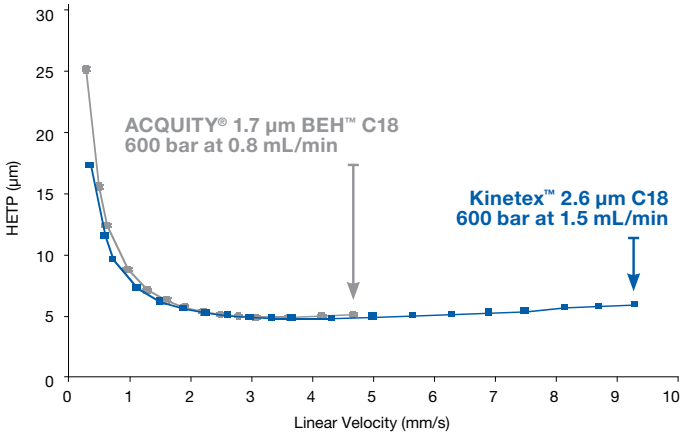
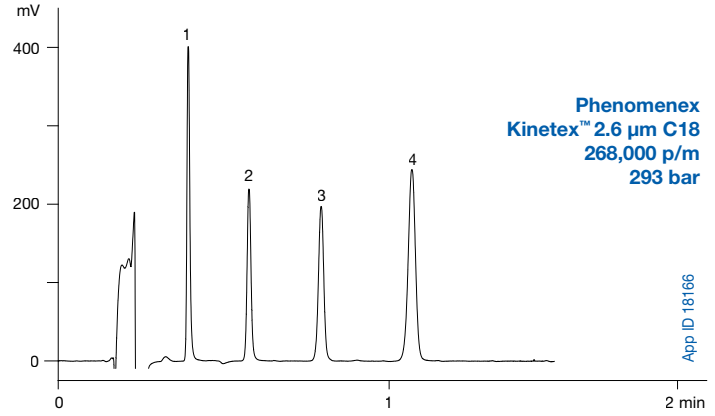
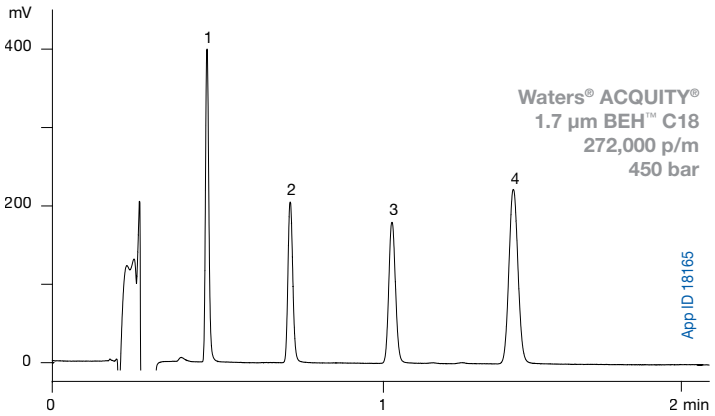


Figure 3.





Conditions for both columns:

Dimensions: 50 x 2.1 mm
Mobile Phase: Acetonitrile / Water (50:50)
Flow Rate: 0.6 mL/min
Temperature: 25 °C
Detection: UV @ 254 nm
Instrument: Waters® ACQUITY® UPLC®
Sample: 1. Acetophenone
2. Benzene
3. Toluene
4. Naphthalene

Experimental

Acetylsalicylic Acid and Related Substances:
European Monograph 0309

Columns Used:
A fully porous 5 µm C18 250 x 4.6 mm column (as specified by the monograph) was compared with a Kinetex 2.6 µm C18 150 x 4.6 mm column (The closest available dimension).

Instrumentation:
Agilent 1100 LC System (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a Quaternary gradient pump, autosampler, column oven, and variable wavelength detector.

Mobile Phase Preparation:
Phosphoric acid (H_3PO_4)/Acetonitrile/Water (2:400:600, v/v/v) was prepared by adding 2.0 mL of concentrated phosphoric acid to a solution containing 400 mL of acetonitrile and 600 mL of HPLC grade water.

Sample Preparation:
Acetylsalicylic Acid Certified Reference Standard (CRS) for system suitability was obtained from the Ph. Eur. Reference Solution (a) was prepared by dissolving 50.0 mg of Acetylsalicylic Acid CRS in mobile phase then diluted to 50 mL with mobile phase. 1.0 mL of this solution was diluted to 100 mL with mobile phase.

Acetylsalicylic Acid Analysis Method:
The monograph calls for 10 µL of sample to be injected with isocratic chromatographic separation using 100 % of mobile phase as prepared above at 1.0 mL/min. Column temperature maintained at ambient and UV detection wavelength set at 237 nm.

Results and Discussion

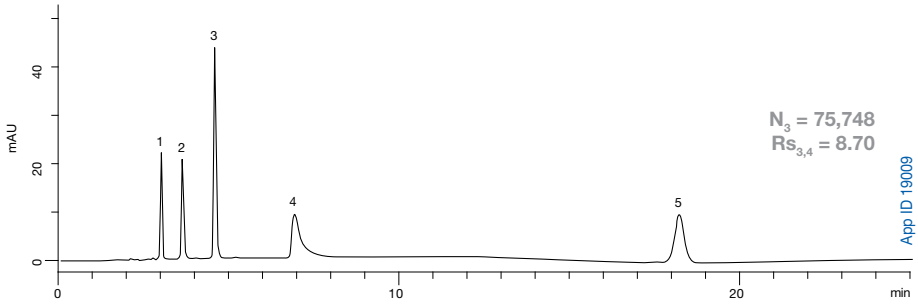
Equivalency Study:
Following the methodology described in Ph. Eur. Monograph 0309 and using a fully porous 5 µm C18 250 x 4.6 mm column as referenced in the method, a chromatogram similar to that of the specimen chromatogram provided with the Acetylsalicylic Acid CRS was obtained **(Figure 4)**.

A Kinetex 2.6 µm C18 150 x 4.6 mm column (the closest available dimension) was used according to the conditions specified in the monograph. The resulting chromatogram demonstrated equivalent selectivity (e.g. no change in elution order) and significantly improved sensitivity in less than one-half the analysis time **(Figure 5)**.

Table 1 summarizes the data comparing the Kinetex column to the fully porous 5 µm column at the specified flow rate of 1.0 mL/min. The monograph requires resolution between Acetylsalicylic acid CRS and Salicylic acid (Impurity C) of at least 6.0. Due to the significantly narrower peaks generated by the higher efficiency Kinetex column, a substantial improvement in resolution between acetylsalicylic acid and salicylic acid was achieved.

Sensitivity was also significantly improved for all impurities. Signal-to-noise ratios for the early eluting impurities were increased by roughly a factor of 2 and by roughly a factor of 7 for the later eluting salicylic acid impurity.

Figure 4.
Acetylsalicylic acid CRS: 10 µL injection on fully porous 5 µm C18 250 x 4.6 mm at 1.0 mL/min; column and conditions as specified in monograph.



Column: Fully Porous 5 µm C18
Dimensions: 250 x 4.6 mm
Mobile Phase: Phosphoric acid / Acetonitrile / Water (2:400:600)
Flow Rate: 1 mL/min
Temperature: 25° C
Detection: UV @ 237 nm
Sample: 1. Impurity A
2. Impurity B
3. Acetylsalicylic CRS
4. Impurity C (salicylic acid)
5. Impurity D

APPLICATIONS

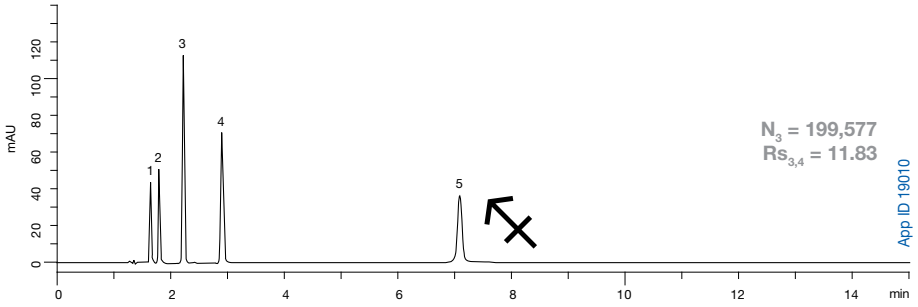
With 10 µL of the Acetylsalicylic acid CRS injected, the fully porous 5 µm C18 column generated a signal-to-noise ratio of 150 for Impurity C. Multiple 10 µL injections were performed on the fully porous 5 µm C18 column and the resulting % RSD value for the peak area of Impurity C was 2.77. By comparison, with 10 µL of the Acetylsalicylic acid CRS sample injected on the Kinetex 2.6 µm core-shell C18 column, a signal-to-noise ratio of 979 was observed for Impurity C. Multiple 10 µL injections were performed on the Kinetex column and the resulting % RSD value for peak area of Impurity C was 1.61.

Meeting the LOQ (defined by the European Pharmacopoeia as corresponding to a signal-to-noise ratio of 10 for a chromatographic peak) requirement can be one of the most challenging parameters in routine operation; however, the higher signal-to-noise ratios and lower % RSD values observed with the Kinetex core-shell technology column represents a significant performance advantage – allowing one to easily achieve the required LOQ with improved precision.

Table 1.
Equivalency Study

	Fully porous 5 µm C18	Kinetex 2.6 µm C18	Comment
Column Dimensions	250 x 4.6 mm	150 x 4.6 mm	
Particle Size	5 µm fully porous	2.6 µm core-shell	
Flow Rate	1.0 mL/min	1.0 mL/min	
Backpressure	150 bar	294 bar	
Resolution of Acetylsalicylic acid and Salicylic acid	8.70	11.83	36 % increase
S/N ratio for Salicylic acid	150 (% RSD 2.77)	979 (% RSD 1.61)	553 % increase
N for Acetylsalicylic acid (plates/m)	75,748	199,577	163 % increase
Elution time of last peak	18.16 min	7.09 min	61 % faster
Solvent Usage	~ 20 mL	~ 8 mL	> 50 % decrease

Figure 5.
Acetylsalicylic acid CRS: 10 µL injection on Kinetex 2.6 µm C18
150 x 4.6 mm at 1.0 mL/min; backpressure = 294 bar.



Column: Kinetex 2.6 µm C18
Dimensions: 150 x 4.6 mm
Mobile Phase: Phosphoric acid / Acetonitrile / Water (2:400:600)
Flow Rate: 1 mL/min
Temperature: 25° C
Detection: UV @ 237 nm
Sample: 1. Impurity A
2. Impurity B
3. Acetylsalicylic CRS
4. Impurity C (salicylic acid)
5. Impurity D

Fast Method

As demonstrated in **Figure 2**, columns packed with Kinetex 2.6 µm core-shell particles are capable of maintaining high efficiencies (low plate heights) with increasing mobile phase flow rates. This is due to favorable physical, kinetic, and thermodynamic properties attributed to core-shell particles. Shorter analysis times may be achieved with Kinetex core-shell technology either by reducing the length of the column or increasing the mobile phase flow rate (or a combination of both) without significantly compromising chromatographic performance.

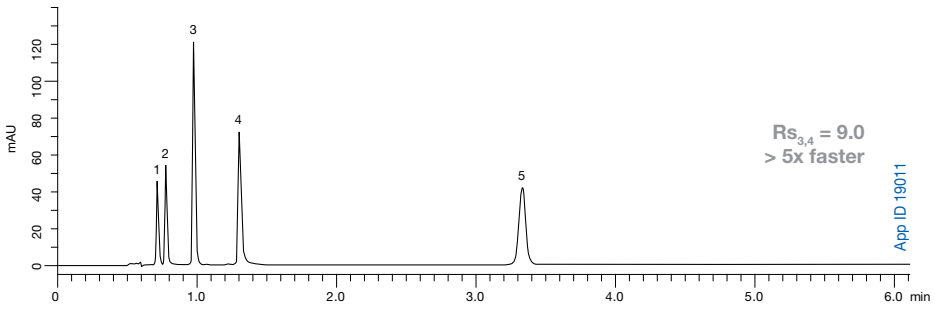
Following European Pharmacopoeia guidelines, the extent to which the various parameters of a chromatographic test may be adjusted to satisfy system suitability (when replacing one column with another of the same type, for example) is summarized in **Table 2**. Staying within these guidelines, a shorter Kinetex 2.6 µm core-shell C18 column (100 x 4.6 mm, representing a decrease in column length of 60 % relative to the monograph) was run according to the conditions specified in the monograph, but with 50 % increase in the flow rate (from 1.0 mL/min to 1.5 mL/min).

With a flow rate of 1.5 mL/min, the total analysis time was shortened from over 20 minutes (using the fully porous 5 µm 250 x 4.6 mm column specified in the monograph) to less than 4 minutes **(Figure 6)**. Resolution, efficiency and sensitivity remained substantially higher with the Kinetex column at the higher flow rate **(Table 3)**. The reduction in analysis time achieved in this example represents a greater than 5-fold increase in sample throughput capability. Note that this performance was achieved using an Agilent 1100 LC system with an upper pressure capability of 400 bar. A flow rate of 1.5 mL/min generated a system pressure of 293 bar.

Table 2.
Acceptable Modifications for Meeting System Suitability

Method Parameter	Acceptable Modification	Monograph 0309 Acetylsalicylic Acid	Kinetex 2.6 µm Fast Method	Modification
Mobile phase pH	± 0.2 units	as specified	No Change	--
Concentration of salts in buffer	± 10 %	as specified	No Change	--
Ratio of components in mobile phase	± 30 % relative of the minor component(s), or 2 % absolute of that component, whichever is greater, but a change in any component cannot exceed ± 10 % absolute.	as specified	No Change	--
Wavelength of UV-Detector	no deviations permitted	237 nm	No Change	--
Injection volume	May be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory.	10 µL	No Change	--
Column temperature	± 10 %, to a maximum of 60 °C	ambient	No Change	--
Column length	± 70 %	250 mm	100 mm	**- 60 %**
Column inner diameter	± 25 %	4.6 mm	4.6 mm	--
Particle size	- 50 %	5 µm	2.6 µm	**- 48 %**
Flow rate	± 50 %	1.0 mL/min	1.5 mL/min	**+ 50 %**

Figure 6.
Acetylsalicylic acid CRS: 10 µL injection on Kinetex 2.6 µm C18 100 x 4.6 mm at 1.5 mL/min; backpressure = 293 bar



Column: Kinetex 2.6 µm C18
Dimensions: 100 x 4.6 mm
Mobile Phase: Phosphoric acid / Acetonitrile / Water (2:400:600)
Flow Rate: 1.5 mL/min
Temperature: 25° C
Detection: UV @ 237 nm
Sample: 1. Impurity A
2. Impurity B
3. Acetylsalicylic CRS
4. Impurity C (salicylic acid)
5. Impurity D

APPLICATIONS

Operating outside of the allowed adjustments and using a Kinetex 2.6 µm C18 50 x 4.6 mm column at a flow rate of 1.5 mL/min resulted in a further reduction in analysis time to less than 3 minutes with substantially higher efficiency and sensitivity as compared to the fully porous 5 µm 250 x 4.6 mm column **(Figure 7)**. However, resolution between acetylsalicylic acid and salicylic acid decreased to 5.3, which is below the system suitability requirement of 6.0. While the shorter column represents a greater than 7-fold increase in productivity for this analysis, the system suitability requirements of the Ph. Eur. method are not met. The use of the shorter 50 x 4.6 mm Kinetex column might be possible if modifications to reduce system dead volume, including the use of a smaller volume UV detector flow cell, could be made to reduce band broadening with a resulting increase in efficiency and resolution.

Figure 7.
Acetylsalicylic acid CRS: 10 µL injection on Kinetex 2.6 µm C18 50 x 4.6 mm at 1.5 mL/min; backpressure = 115 bar

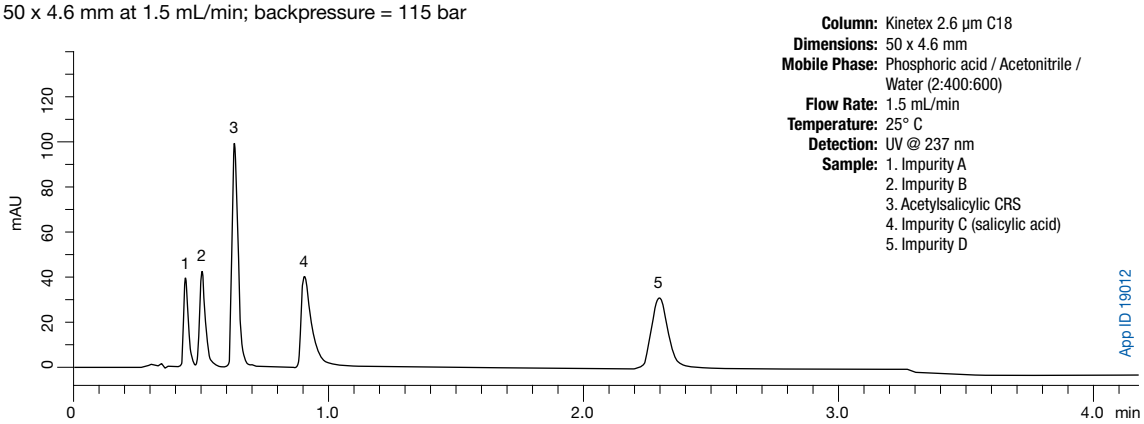


Table 3.
Improvements To The Monograph

	Fully-porous 5 µm	Kinetex 2.6 µm C18	Kinetex 2.6 µm C18
Column Dimensions	250 x 4.6 mm	100 x 4.6 mm	50 x 4.6 mm
Particle Size	5 µm fully porous	2.6 µm core-shell	2.6 µm core-shell
Flow Rate	1.0 mL/min	1.5 mL/min	1.5 mL/min
Backpressure	150 bar	293 bar	115 bar
Resolution of Acetylsalicylic acid and Salicylic acid	8.70	9.0	5.3
S/N ratio for Salicylic acid	150	509	1008
N for Acetylsalicylic acid	75,748	171,900	90,740
Elution time of last peak	18.16 min	3.33 min	2.30 min
Solvent Usage	~ 20 mL	< 6 mL	< 4.5 mL

Conclusion

Newly developed Kinetex 2.6 µm core-shell particles are capable of achieving chromatographic performance equivalent to columns packed with traditional fully porous sub-2 µm particles at substantially lower operating pressures that are compatible with conventional HPLC instrumentation.

Laboratories performing routine API and related substance analysis with traditional fully porous LC columns can benefit from the increased speed, resolution and sensitivity that Kinetex 2.6 µm columns provide without having to replace existing instrumentation with ultra-high pressure capable LC systems. Faster analysis times resulting in higher throughput and productivity can be achieved with Kinetex columns with minimal changes to validated methods by employing shorter length columns and/or higher mobile phase flow rates without sacrificing performance. Improved resolution and higher sensitivity resulting from narrower and taller chromatographic peaks generated by Kinetex columns allow for more precise detection and quantitation of low level impurities in routine operation.

For this monograph two options are illustrated for providing significant improvement in sample throughput while meeting the system suitability requirement, and operating within the allowable adjustments specified by the Ph. Eur. Reducing column length by 40 % to 150 mm while maintaining the flow rate specified in the monograph provides greater than 2x improvement in sample throughput, while further reducing column length to 100 mm (60 % reduction) with a 50 % increase in flow rate (to 1.5 mL/min) results in greater than 5x improvement in sample throughput.

An additional, and not insignificant, benefit resulting from these two options is a greater than 50 % reduction in solvent usage.

APPLICATIONS

Kinetex™ Ordering Information

1.7 µm Minibore Columns (mm)

Phases	50 x 2.1	100 x 2.1	150 x 2.1
C18	00B-4475-AN	00D-4475-AN	00F-4475-AN
PFP	00B-4476-AN	00D-4476-AN	00F-4476-AN
HILIC	00B-4474-AN	—	—

2.6 µm Minibore Columns (mm)

Phases	50 x 2.1	100 x 2.1	150 x 2.1
C18	00B-4462-AN	00D-4462-AN	00F-4462-AN
PFP	00B-4477-AN	00D-4477-AN	00F-4477-AN
HILIC	00B-4461-AN	00D-4461-AN	00F-4461-AN

2.6 µm MidBore™ Columns (mm)

Phases	50 x 3.0	100 x 3.0	150 x 3.0
C18	00B-4462-Y0	00D-4462-Y0	00F-4462-Y0
PFP	00B-4477-Y0	00D-4477-Y0	00F-4477-Y0
HILIC	—	—	00F-4461-Y0

2.6 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6
C18	00B-4462-E0	00D-4462-E0	00F-4462-E0
PFP	00B-4477-E0	00D-4477-E0	00F-4477-E0
HILIC	00B-4461-E0	00D-4461-E0	00F-4461-E0

KrudKatcher™ Ultra In-line Filter

The KrudKatcher Ultra filter body houses an integrated 0.5 µm 316 stainless steel filter element that efficiently removes microparticulates from the flow stream without contributing to system backpressure or dead volume (<0.2 µL).

KrudKatcher™ Ultra In-Line Filter Ordering Information

Part No.	Description	Unit
AF0-8497	KrudKatcher Ultra In-Line Filter, 0.5 µm Porosity x 0.004 in. ID	3/pk

KrudKatcher Ultra requires $^{5}/_{16}$ in. wrench. Installation wrench not provided.

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: +31 (0)30-2418700
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09-4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: (55) 5018 3791
f: (310) 328-7768
tecnicomx@phenomenex.com

Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 81 00 20 05
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

All other countries:
Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com

TN72351209_L