

APPLICATIONS

European Pharmacopoeia Paracetamol Monograph Draft Method: Achieving Improved Sensitivity, Resolution, and Separation for Paracetamol and All 14 Related Impurities using Kinetex[®] 5 μ m C18 Core-Shell Columns

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Introduction

N-(4-hydroxyphenyl) acetamide, commonly referred to as paracetamol, is one of the most familiar analgesics and antipyretic therapeutics in today's drug market. As a result of its wide usage and applicability, there is significant interest in the development of fast and efficient analysis of paracetamol and related impurities. For this report, we focused on the complex related substances profile of paracetamol as identified in the Pharmedica draft monograph (PA/PH/Exp. 10A/T (19) 136 ANP – 32.1). This draft is currently under review to possibly replace the existing monograph. By leveraging column selectivity, we were able to provide improved resolution for paracetamol and the principal degradation product, 4-aminophenol (impurity K), an impurity that can be formed by the hydrolysis of paracetamol¹. The Pharmedica draft monograph requires that resolution between impurity K and paracetamol be a minimum of 5.0 to meet system suitability; resolution significantly greater than this was achieved here. In addition, resolution of all 14 related impurities was achieved. An HPLC column packed with core-shell, or superficially porous, silica particles containing a C18 bonded phase, was utilized to maximize the performance of the analytical method. It should be noted that the proposed draft method for paracetamol utilizes a superficially porous 5 μ m column (HALO[®] 5 μ m C18 150 x 4.6 mm) in the same dimension as the Kinetex C18 5 μ m core-shell column used here. The performance of the Kinetex 5 μ m core-shell column was compared with the performance of the superficially porous HALO C18 column and all method parameters (column dimension, injection volume, and gradient mobile phase conditions) were consistent with the Pharmedica draft monograph for paracetamol.

Experimental

Analytical reference standards for paracetamol and paracetamol impurity K (4-Aminophenol) were obtained from Sigma-Aldrich[®] (St. Louis, Missouri, USA) and evaluated with the Kinetex 5 μ m C18 (Phenomenex, Torrance, California, USA). In addition, we compared the Kinetex results with the superficially porous (aka core-shell) HALO 5 μ m C18 column referenced by the Pharmedica draft method for Paracetamol related substances. Additional paracetamol impurities (A, B, C, D, E, F, G, H, I, L, M, and N) were obtained from Sigma-Aldrich and TLC Pharmaceutical Standards (Ontario, Canada).

To ensure similar and comparative results, the columns were individually tested using the same isocratic performance test conditions to confirm both columns were operating within expected efficiency/performance levels. The Kinetex C18 and HALO columns were both of core-shell solid support particle design with unique performance characteristics associated with this type of particle morphology. The system used for this method was an Agilent[®] 1290 binary UHPLC system equipped with a UV-VIS detection set at 254 nm (no reference wavelength was utilized).

A mixture of analytical reference standards [15 ppm; dissolved in methanol, water (15:85 v/v)], was used to investigate and identify relative analyte retention and assess selectivity suitability, per Ph.Eur. guidelines as indicated in the paracetamol draft monograph. The system suitability solution (Reference solution e) was prepared per the Pharmedica draft monograph by mixing 1 mL of reference solution (a) and 1 mL of reference solution (c) and diluting to 10 mL with the solvent mixture. The gradient mobile phase A = phosphate buffer (prepared by dissolving 1.7 g of potassium dihydrogen phosphate and 1.8 g of dipotassium hydrogen phosphate in HPLC grade water and diluting to 1000 mL with water); and mobile phase B = methanol. The flow rate was 1.5 mL/min, column oven was set to 30 °C and a 50 μ L injection volume was used.

Solution	Step 1	Step 2	Step 3	Step 4	Final Conc.
Test Solution	50.0 mg paracetamol	dissolve with 0.75 mL methanol	dilute to 5.0 mL with water		10 mg/mL paracetamol
Ref a	dilute 1.0 mL Test Solution to 100.0 mL with solvent mixture	dilute 1.0 mL of that solution to 20.0 mL with solvent mixture			5 μ g/mL paracetamol
Ref b	15.0 mg of imp J	dissolve with 25 mL of methanol	dilute to 250.0 mL with solvent mixture	dilute 1.0 mL of that solution to 200.0 mL with solvent mixture	0.1 μ g/mL imp J
Ref c	5.0 mg imp K	dissolve with solvent mixture	dilute to 100.0 mL with solvent mixture	dilute 1.0 mL of that solution to 10.0 mL with solvent mixture	5 μ g/mL imp K
Ref d	dilute 1.0 mL Ref c to 10.0 mL with solvent mixture				0.5 μ g/mL imp K
Ref e	1 mL Ref a	+ 1 mL Ref c	dilute to 10 mL with solvent mixture		0.5 μ g/mL paracetamol 0.5 μ g/mL imp K

APPLICATIONS

Gradient table:

(original used for HPLC with 1.3 mL dwell volume)

Time (min)	Mobile Phase A (% v/v)	Mobile Phase B (% v/v)
0	95	5
1.5	95	5
14.4	90	10
28.8	90	10
57.6	66	34
60	66	34

Gradient table:

(used for Agilent® 1290 binary UHPLC with 150 µL dwell volume)
(adjusted isocratic hold according to Ph. Eur. Chapter 2.2.46)

Time (min)	Mobile Phase A (% v/v)	Mobile Phase B (% v/v)
0	95	5
2.3	95	5
15.2	90	10
29.6	90	10
58.4	66	34
60.8	66	34

Flowrate: 1.5 mL/min

Temperature: 30 °C

Detection: UV @ 254 nm

Injection Volume: 50 µL

Autosampler: 5 °C

The system suitability requirement pertains to the critical resolution of impurity 4-aminophenol (impurity K) and paracetamol (minimum 5.0). The analytical challenges with the separations of 4-aminophenol and paracetamol are associated with reversed phase retention challenges and the stability of 4-aminophenol. It has been observed that when 4-aminophenol is exposed to environmental heat and light it undergoes accelerated degradation.² Additionally, 4-aminophenol is also the principal degradation product related to the hydrolysis of paracetamol.¹

After system suitability was demonstrated, (resolution >5.0 between 4-aminophenol and paracetamol), a more complex mixture containing 14 impurities (in addition to paracetamol) was used to demonstrate the complete separation of all impurities.

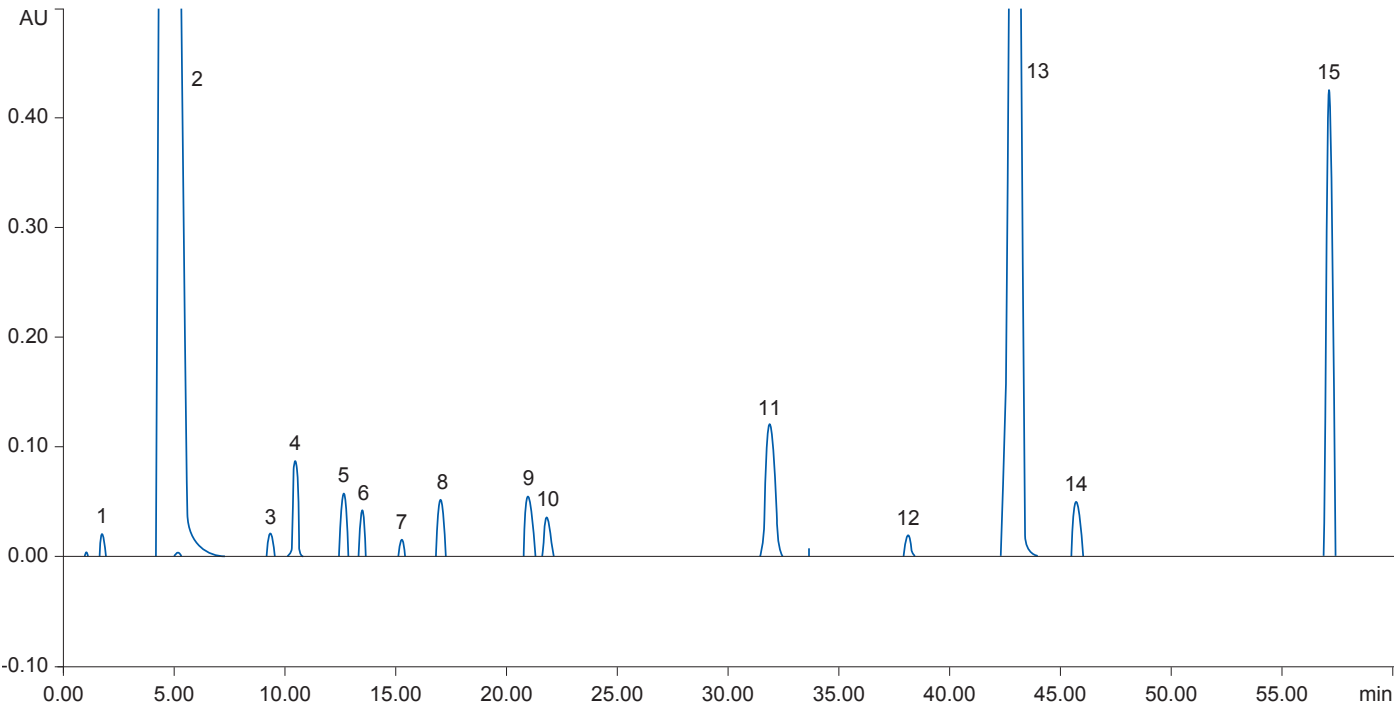
Results

Figure 1a shows the original EDQM published chromatogram (also published in Ph. Eur. 32.1) for paracetamol and 14 related impurities as obtained using the HALO® 5 µm C18 150 x 4.6 mm column. This shows the expected elution order for paracetamol and the 14 related impurities using the conditions as published in the Ph. Eur. method for paracetamol. **Figure 1b** shows the chromatogram obtained in our laboratory running a solution containing paracetamol and all 14 impurities on the recommended HALO column. As noted in the chromatogram, we observed a separation of all impurities, but an elution order reversal for impurities I, J and L, when compared with the EDQM published chromatogram. A new HALO column was used to generate the chromatograms shown here. **(It should be noted that retention times and elution orders were confirmed by injecting the individual standards, which is seen in Figure 1d.)** The Kinetex® 5 µm C18 150 x 4.6 mm column gave the same elution order (**Figure 1c**) as was obtained on the HALO column; however, we also observed a peak order reversal for impurities I, J and L as was observed on the HALO column compared to the peak order in Ph. Eur. 32.1 (**Figure 1a**). In addition, the elution order for impurities G and M was reversed on the Kinetex column. **(It should be noted that retention times and elution orders were confirmed by injecting the individual standards, which is seen in Figure 1e.)**

Comparative separations may not be representative of all applications.

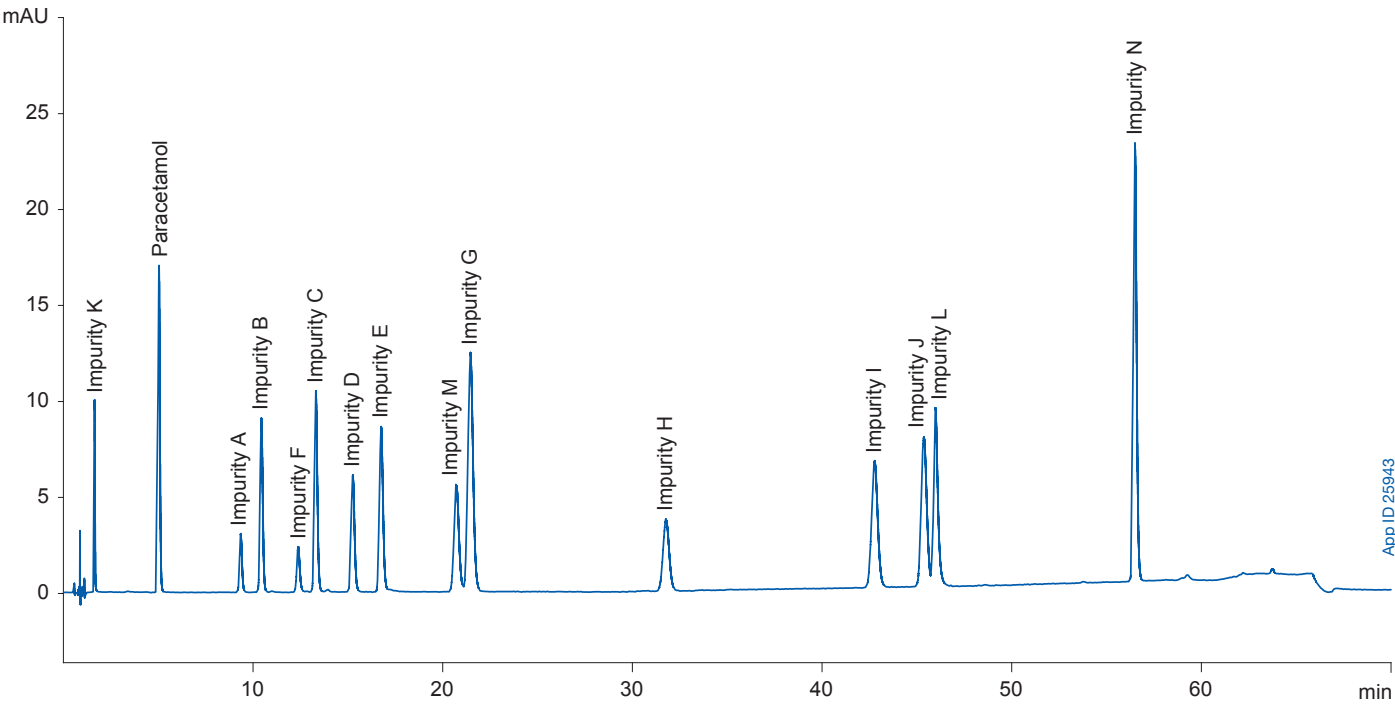
APPLICATIONS

Figure 1a.
HALO[®] 5 μ m C18; 14 impurities plus paracetamol, as published in Pharmeuropa (solution of paracetamol spiked with impurities A to N).



1	Impurity K	5	Impurity F	9	Impurity M	13	Impurity I
2	Paracetamol	6	Impurity C	10	Impurity G	14	Impurity J
3	Impurity A	7	Impurity D	11	Impurity H	15	Impurity N
4	Impurity B	8	Impurity E	12	Impurity L		

Figure 1b.
HALO 5 μ m C18; 14 impurities plus paracetamol, as obtained using solution of paracetamol with impurities A to N added on Agilent[®] 1290.

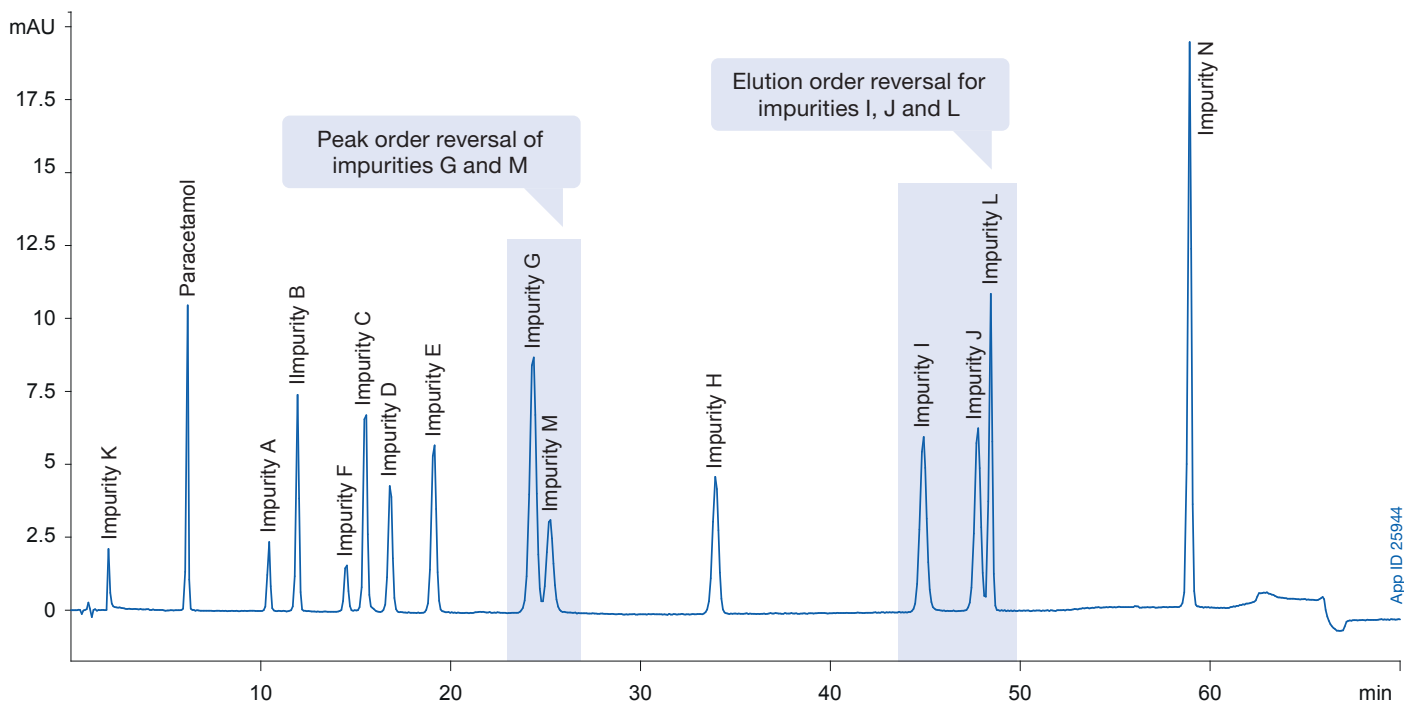


App ID 25943

APPLICATIONS

Figure 1c.

Kinetex[®] 5 μ m C18; 14 impurities plus paracetamol, as obtained using solution of paracetamol with impurities A to N added on Agilent[®] 1290.



Peak No.	Peak ID	HALO [®] Retention Time, min	Kinetex [®] Retention Time, min
1	Impurity K	1.65	2.01
2	Paracetamol	5.04	6.13
3	Impurity A	9.35	10.43
4	Impurity B	10.44	11.94
5	Impurity F	12.38	14.49
6	Impurity C	13.31	15.51
7	Impurity D	15.26	16.83
8	Impurity E	16.76	19.12
9	Impurity M	20.72	25.22
10	Impurity G	21.46	24.34
11	Impurity H	31.77	33.96
12	Impurity I	42.78	44.89
13	Impurity J	45.37	47.75
14	Impurity L	45.98	48.43
15	Impurity N	54.50	58.91

Peak order reversal of impurities G and M on Kinetex

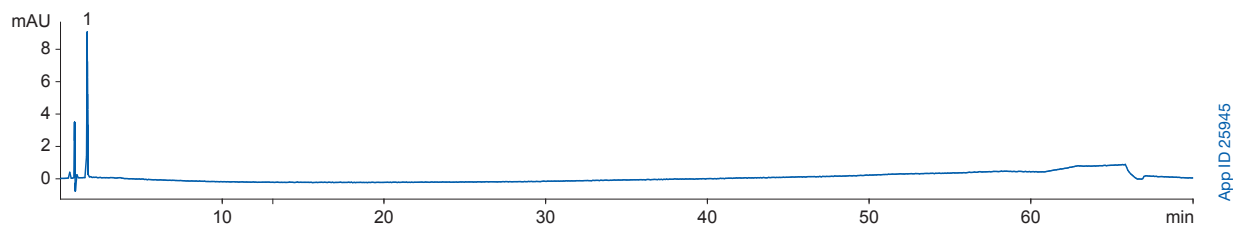
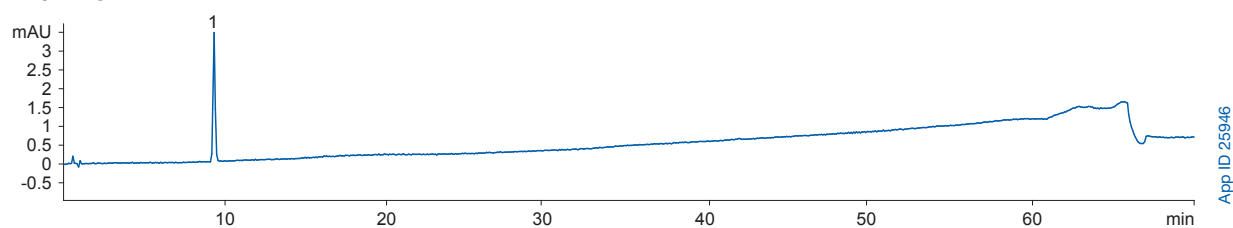
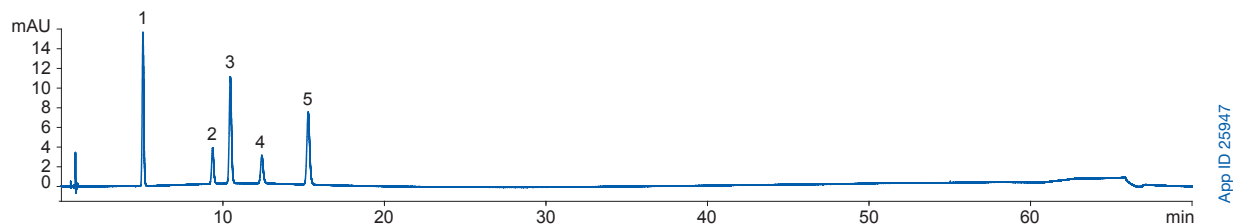
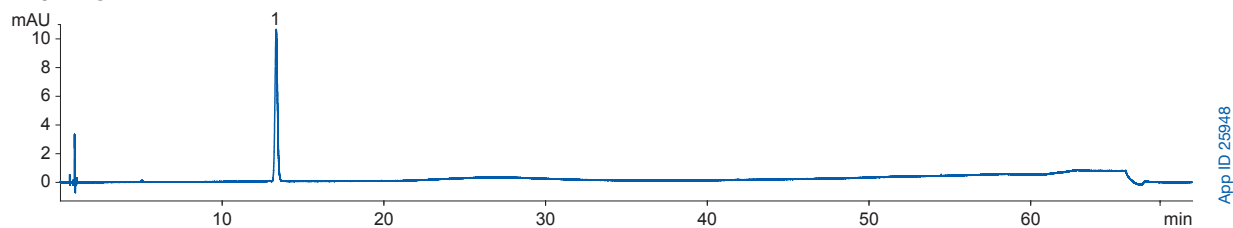
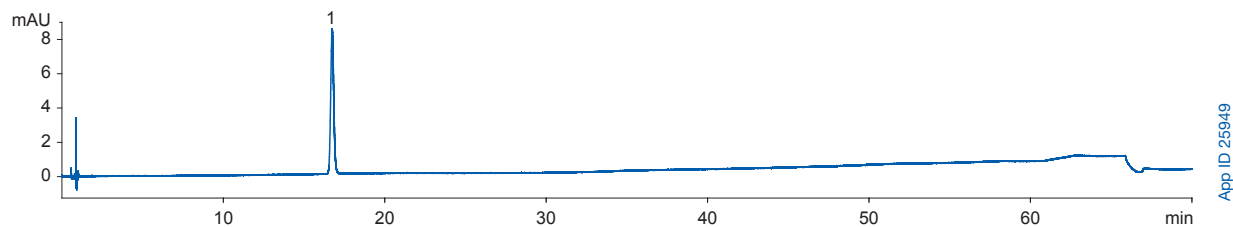
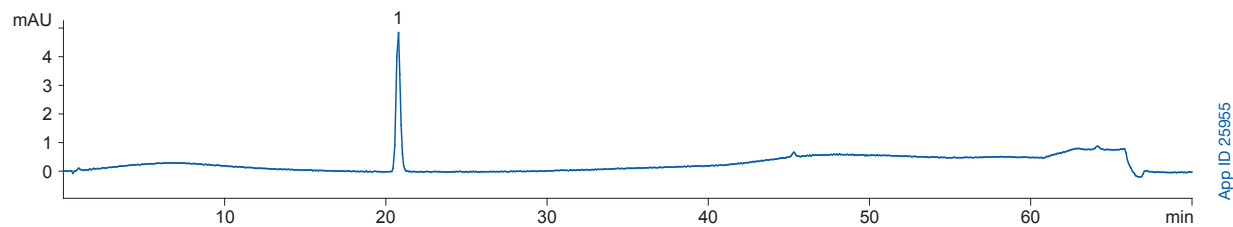
NOTE: Different elution order observed for impurities on both columns versus Pharmeuropa chromatogram

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Comparative separations may not be representative of all applications.

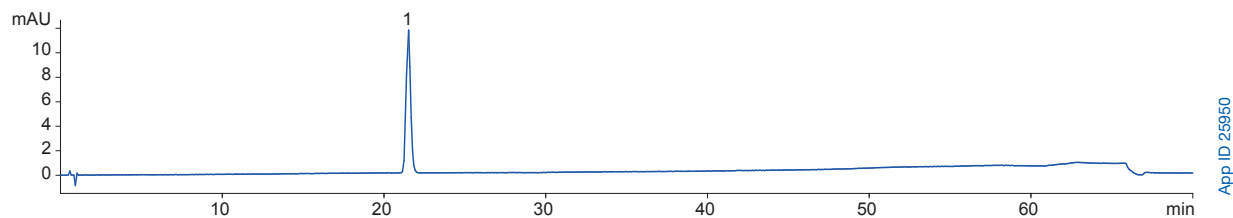
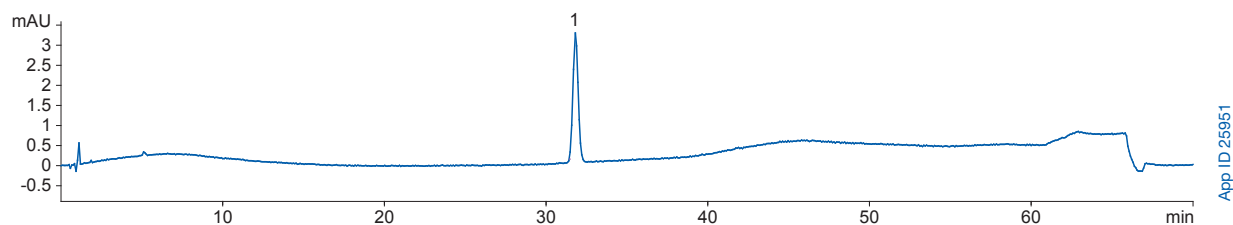
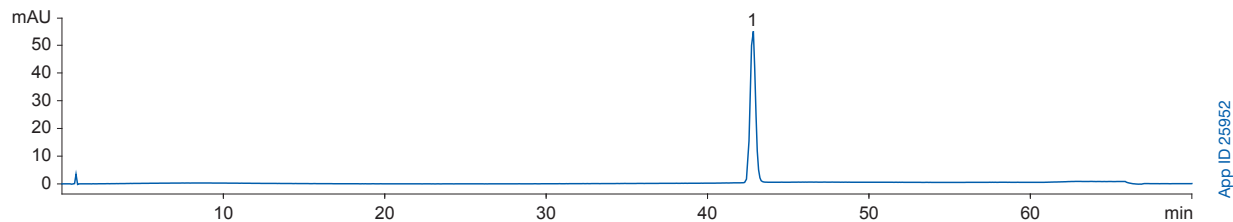
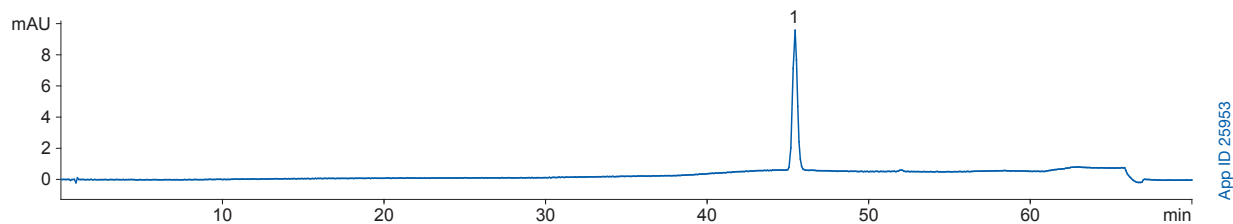
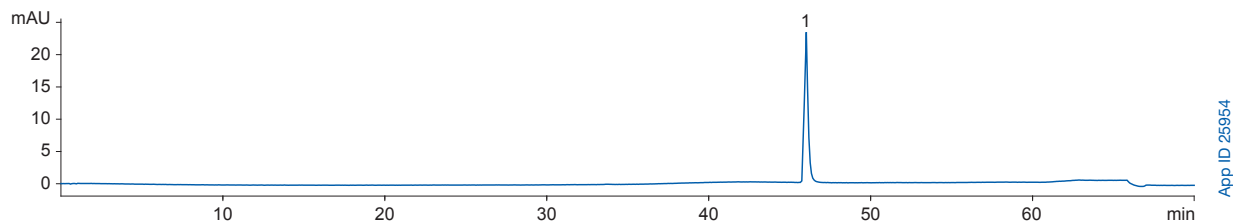
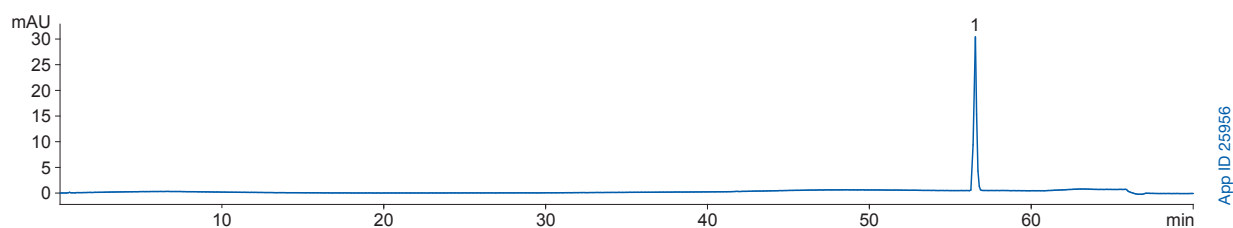
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Figure 1d.
HALO[®] 5 μ m C18; individual impurities A to N on Agilent[®] 1290.

Impurity K**Impurity A****Mix of paracetamol and 4 impurities (A,B,F,D)****Impurity C****Impurity E****Impurity M**

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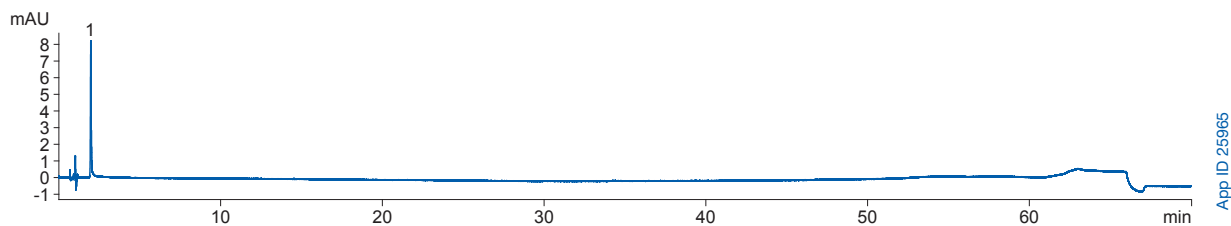
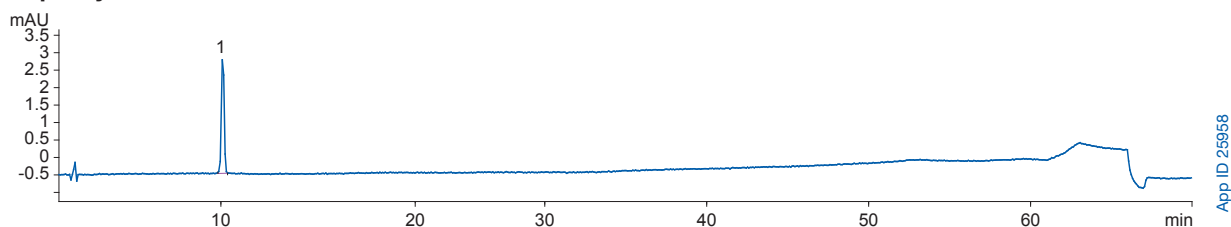
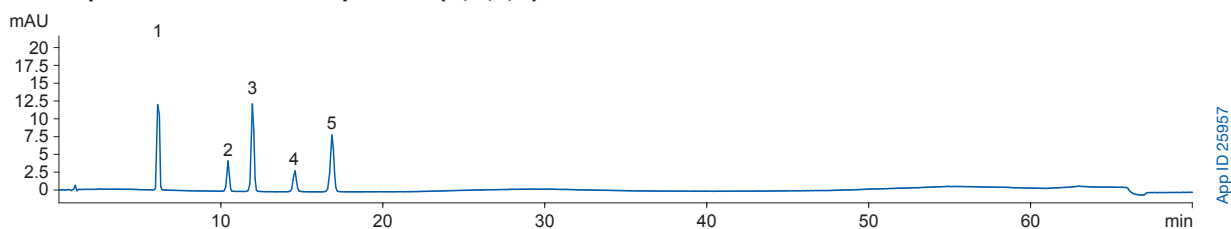
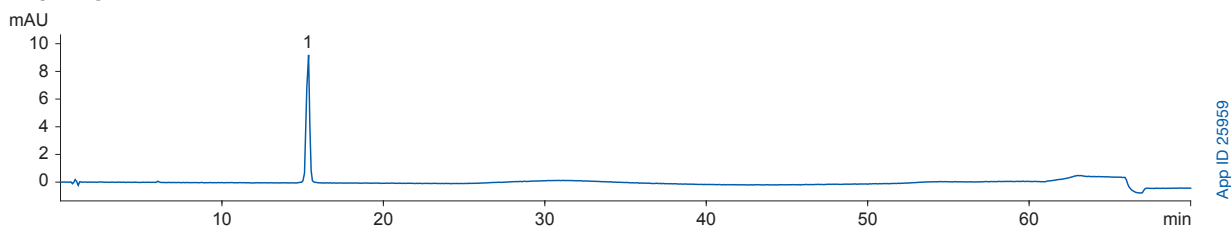
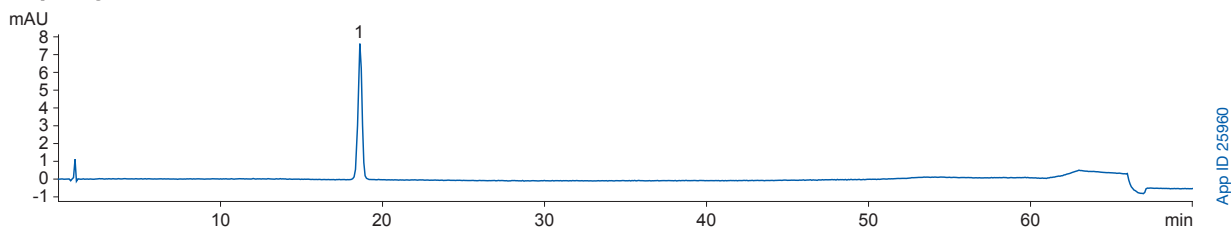
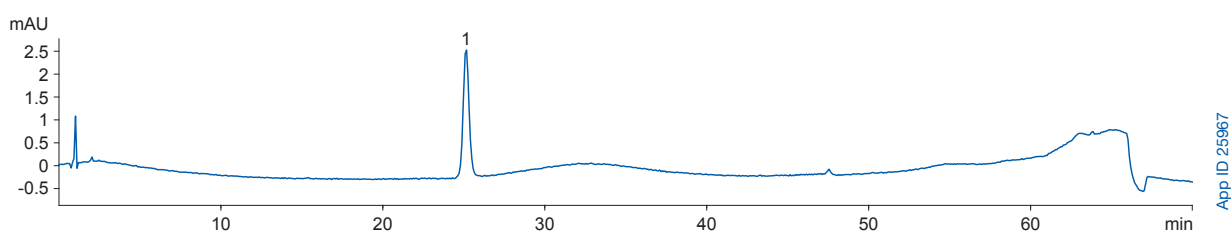
Figure 1d. (continued)
HALO® 5 µm C18; individual impurities A to N on Agilent® 1290.

Impurity G**Impurity H****Impurity I****Impurity J****Impurity L****Impurity N**

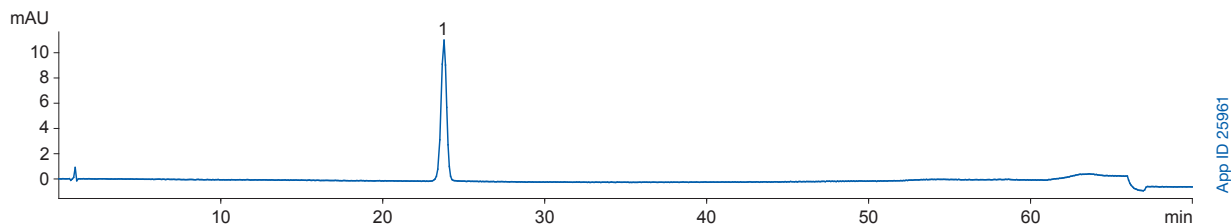
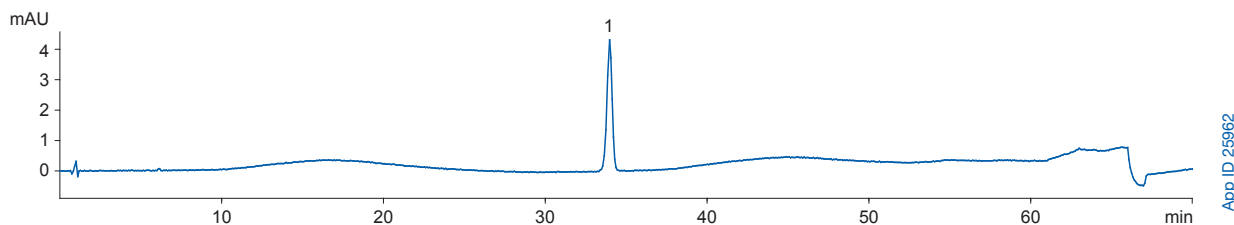
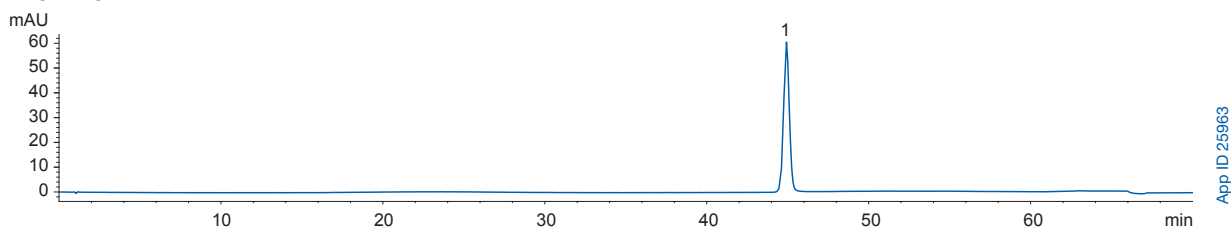
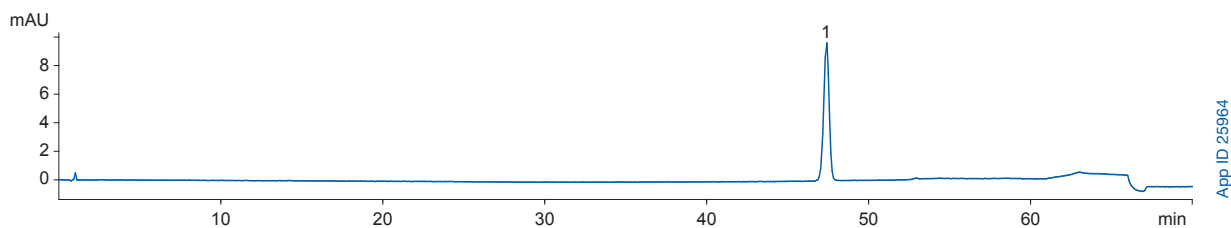
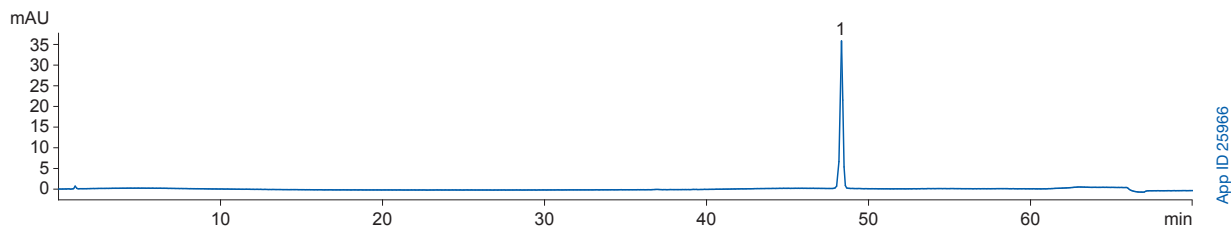
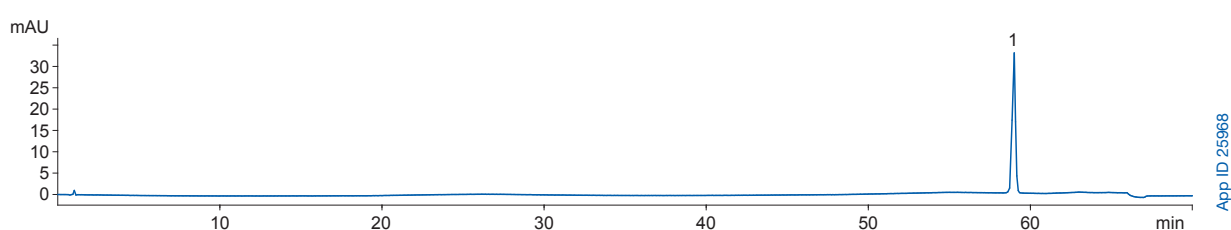
APPLICATIONS

Figure 1e.

Kinetex® 5 µm C18; individual impurities A to N on Agilent® 1290.

Impurity K**Impurity A****Mix of paracetamol and 4 impurities (A,B,F,D)****Impurity C****Impurity E****Impurity M**

APPLICATIONS

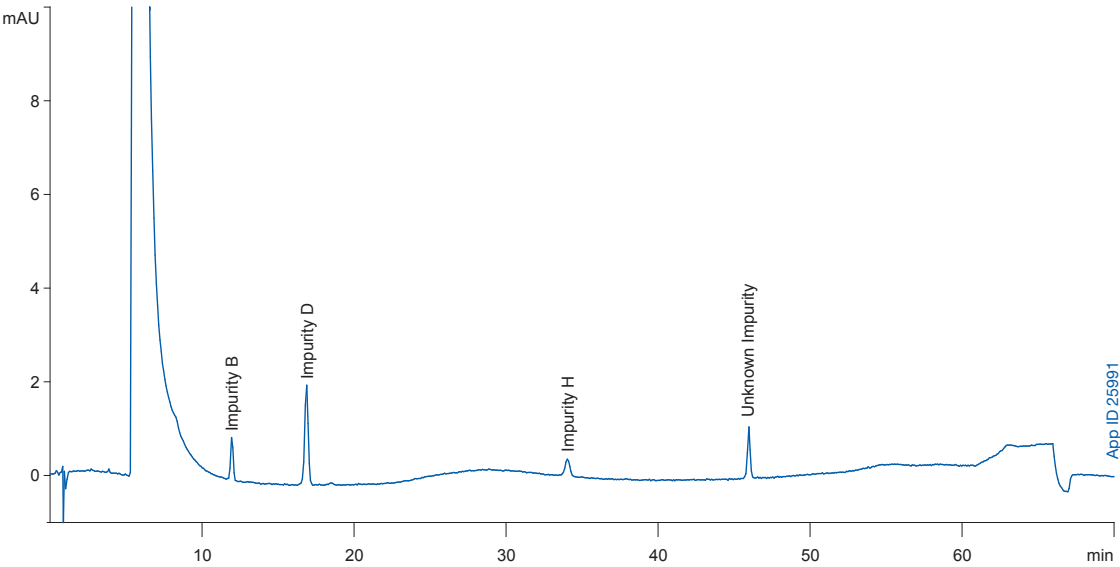
Figure 1e. (continued)Kinetex[®] 5 μ m C18; individual impurities A to N on Agilent[®] 1290.**Impurity G****Impurity H****Impurity I****Impurity J****Impurity L****Impurity N**

APPLICATIONS

Test and Reference solutions:

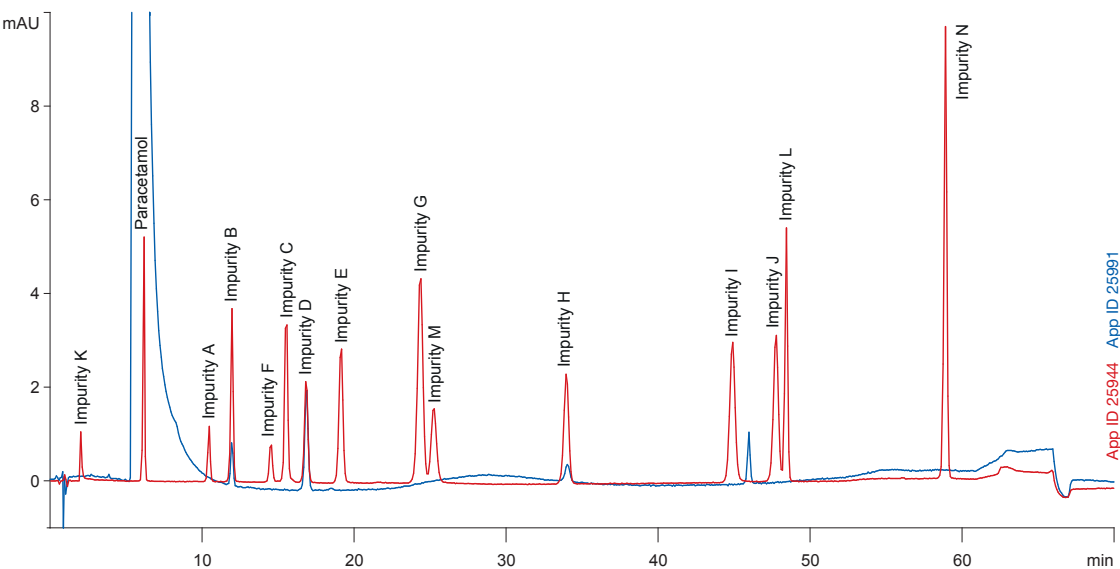
Test solution:
50 mg of paracetamol was dissolved in 0.75 ml of Methanol and diluted to 5 mL with Water – Injection: 50 µL.

Figure 2a.
Test solution on Kinetex[®] 5 µm C18 on Agilent[®] 1290.



#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Paracetamol	5.89	156222.4	3892.5	0.6689	99.966	1.683
2	Impurity B	11.972	10.7	9.6E-1	0.1852	0.007	0.947
3	Impurity D	16.872	33.2	2.2	0.1808	0.021	0.957
4	Impurity H	34.05	8.8	3.9E-1	0.3783	0.006	1.158

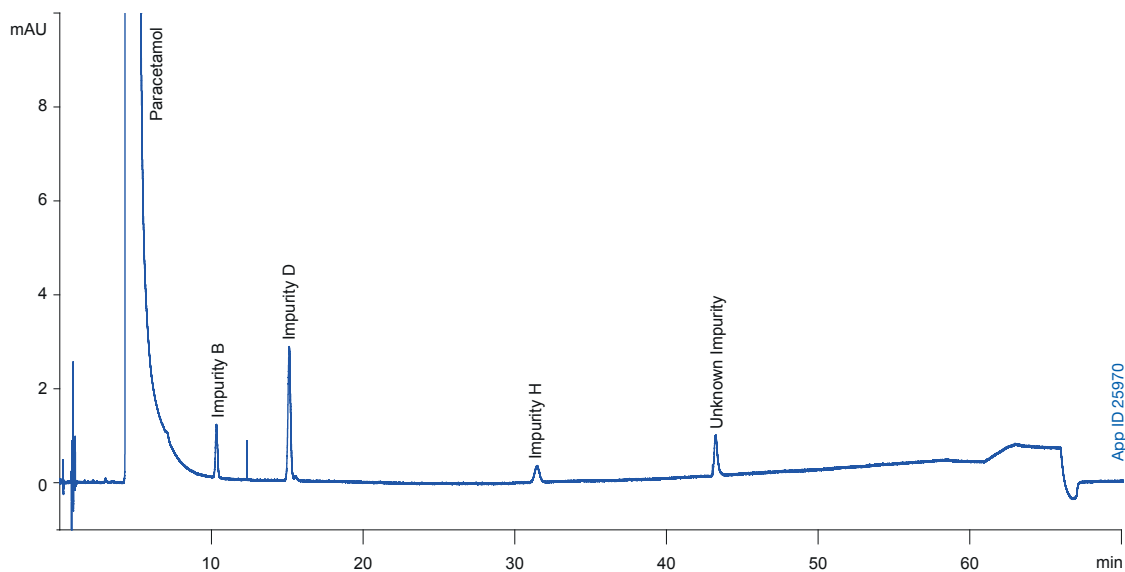
Figure 2b.
Test solution on Kinetex 5 µm C18 overlaid with 14 impurities standard Agilent 1290.



APPLICATIONS

Figure 2c.

Test solution on HALO[®] 5 µm C18 on Agilent[®] 1290.



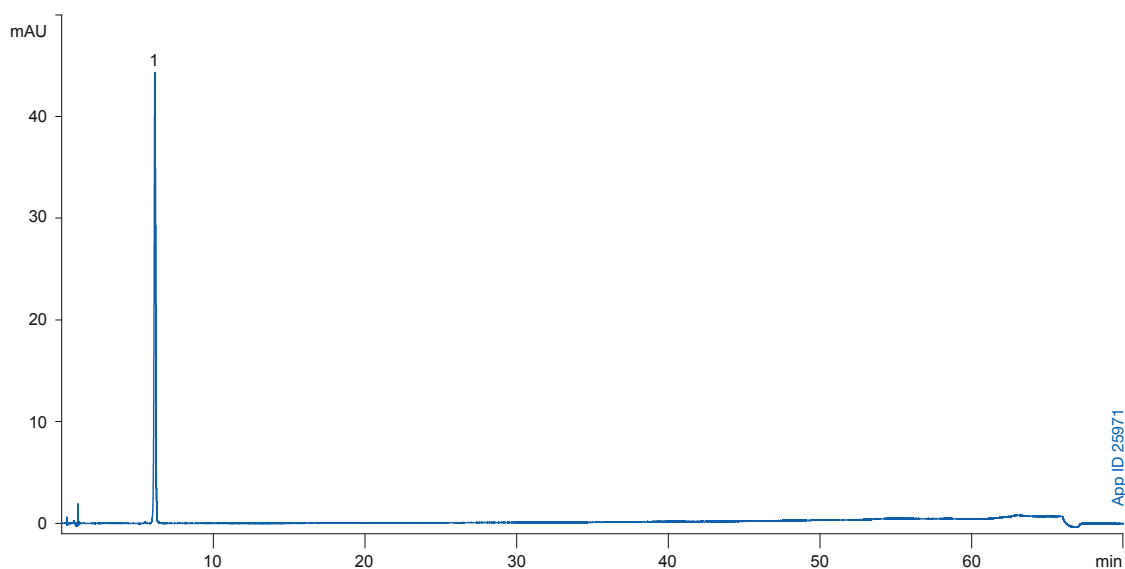
#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Paracetamol	4.743	149678.5	3858.6	0.6465	99.964	1.723
2	Impurity B	10.313	10.8	1.1	0.1139	0.007	0.886
3	Impurity D	15.101	33.8	2.8	0.1412	0.023	0.881
4	Impurity H	31.418	8.8	3.7E-1	0.4009	0.006	0.89

Reference solution (a):

1 mL of the test solution was diluted to 100 mL with the solvent mixture [methanol, water (15:85 v/v)]. 1 mL of this solution was then diluted to 20 mL with the solvent mixture – Injection: 50 µL.

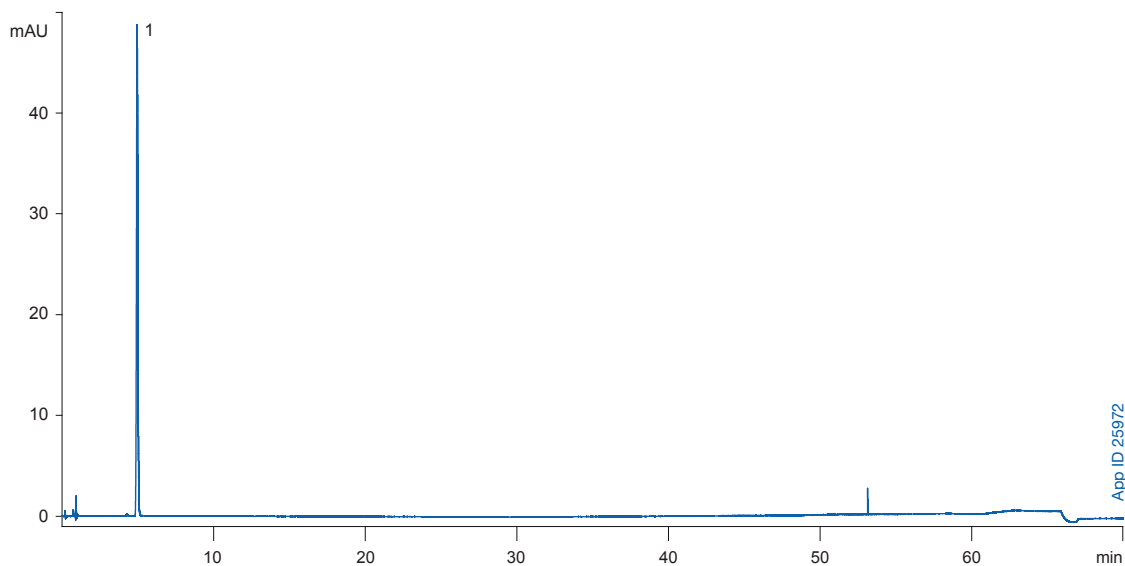
Figure 3a.

Reference solution (a) on Kinetex[®] 5 µm C18 on Agilent 1290.



#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Paracetamol	6.148	303.9	44.2	0.1057	100.000	1.023

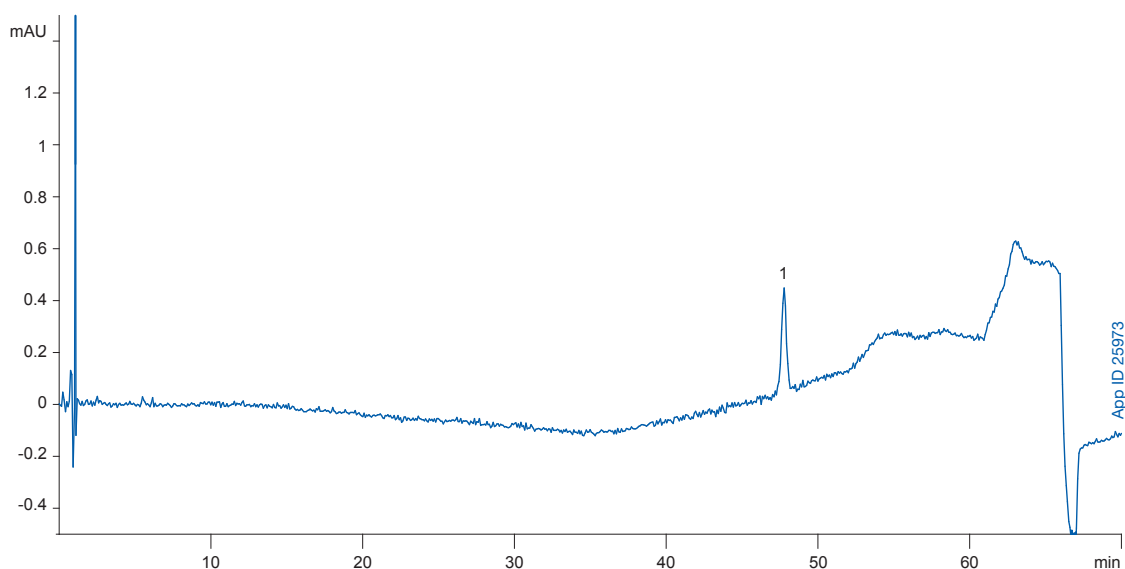
APPLICATIONS

Figure 3b.Reference solution (a) on HALO[®] 5 μ m C18 on Agilent[®] 1290.

#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Paracetamol	4.956	305.6	48.7	0.0972	100.000	0.86

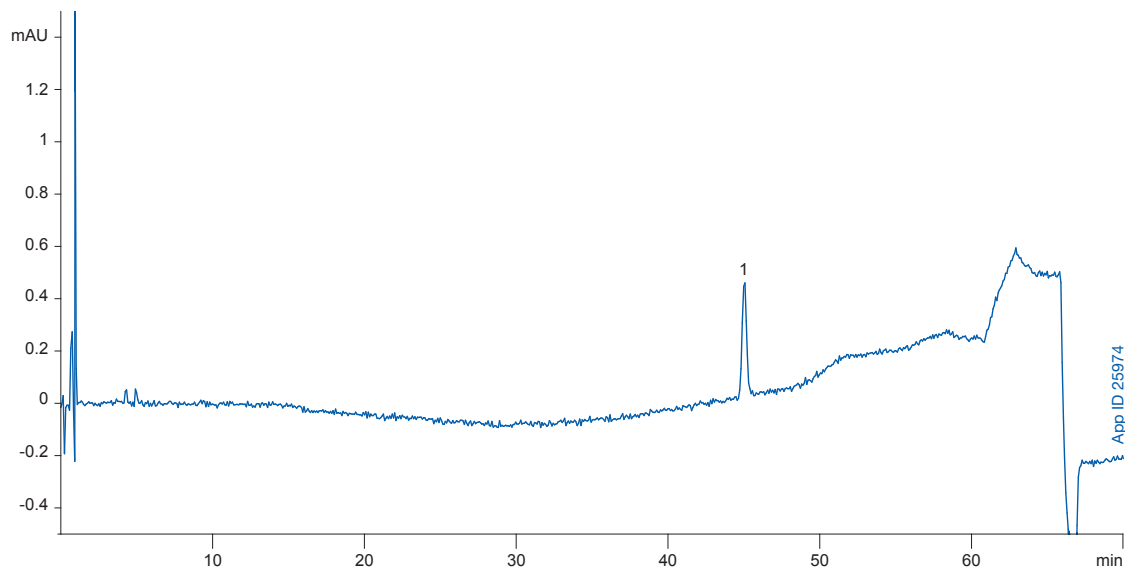
Reference solution (b):

5 mg of paracetamol impurity J was dissolved in 25 mL methanol and diluted to 250 mL with the solvent mixture. 1 mL of this solution was then diluted to 200 mL with the solvent mixture – Injection: 50 μ L

Figure 4a.Reference solution (b) on Kinetex[®] 5 μ m C18 on Agilent 1290.

#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Impurity J	47.755	9.6	4.2E-1	0.3834	100.000	0.864

APPLICATIONS

Figure 4b.Reference solution (b) on HALO[®] 5 μ m C18 on Agilent[®] 1290.

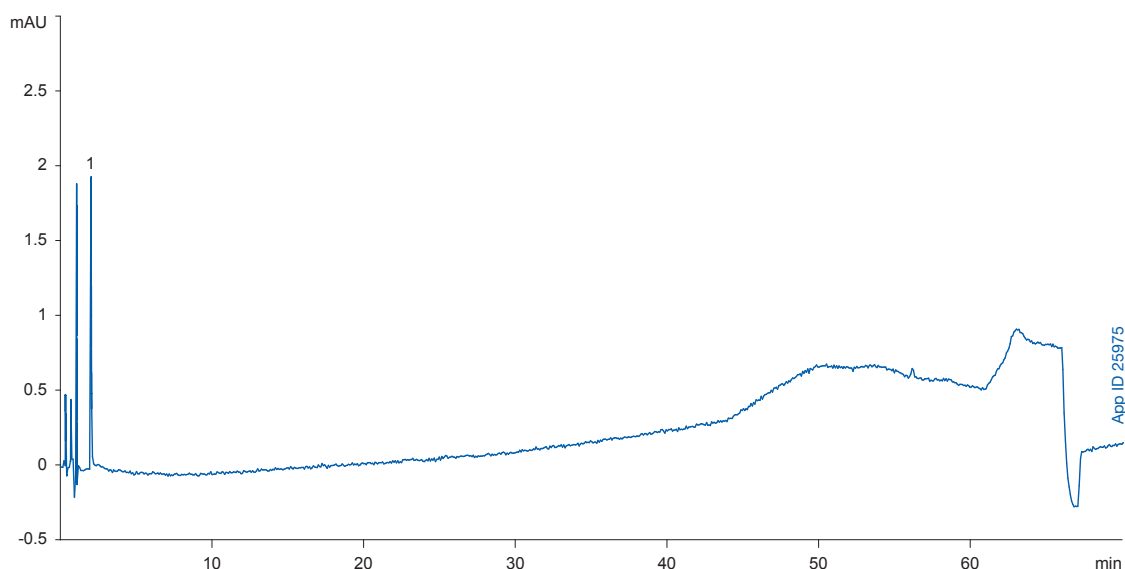
#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Impurity J	45.045	9	4.5E-1	0.3315	100.000	1.058

Reference solution (c):

5 mg of paracetamol impurity K was dissolved in the solvent mixture and then diluted to 100 mL with the solvent mixture. 1 mL from this solution was then diluted to 10 mL with the solvent mixture.

Reference solution (d):

1 mL of the reference solution (c) was diluted to 10 mL with the solvent mixture – Injection: 50 μ L.

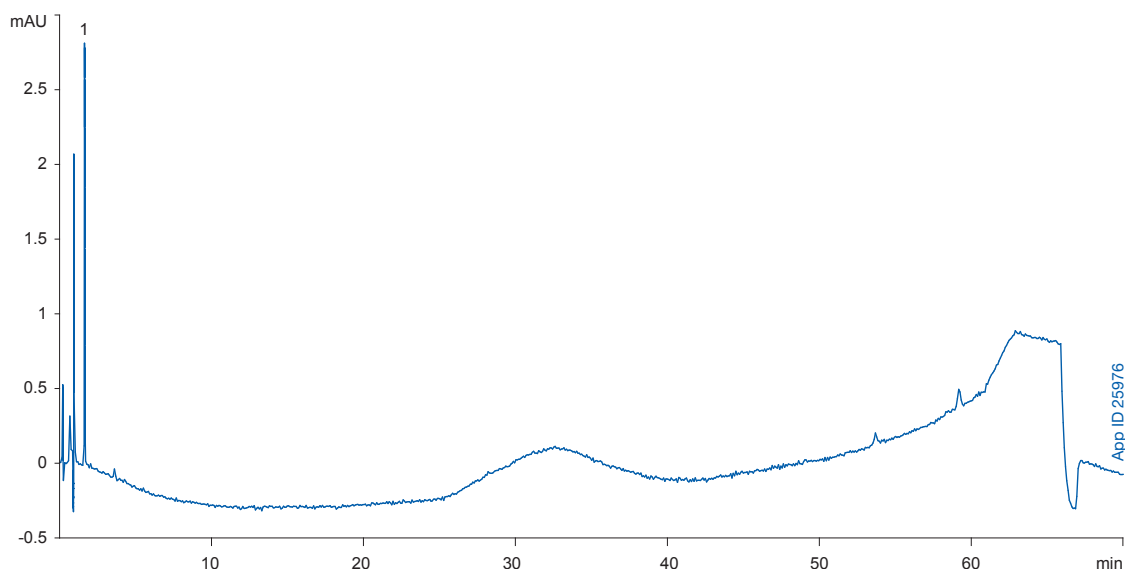
Figure 5a.Reference solution (d) on Kinetex[®] 5 μ m C18 on Agilent 1290.

#	Analyte	Time	Area	Height	Width	Area %	Symmetry
1	Impurity K	2.026	5.4	2	0.0409	100.000	1.023

APPLICATIONS

Figure 5b.

Reference solution (d) on HALO[®] 5 μ m C18 on Agilent[®] 1290.



#	Analyte	Time	Area	Height	Width	Area %	Symmetry
Impurity K	1.647	5.6	2.8	0.0299	100.000	0.785	1.023

Reference solution (e) (System suitability):

A mixture of 1 mL of reference solution (a) and 1 mL of reference solution (c) were diluted to 10 mL with the solvent mixture. Injection: 50 μ L.

The Kinetex[®] 5 μ m C18 column was able to provide a resolution factor between paracetamol and impurity K of 33.55 (**Table 1**), which is well above the system suitability requirement of 5.0, and ~22% more than the resolution achieved on the HALO column (27.5, **Table 2**).

Figure 6a.

Reference solution (e) on Kinetex 5 μ m C18 150 x 4.6 mm on Agilent 1290.

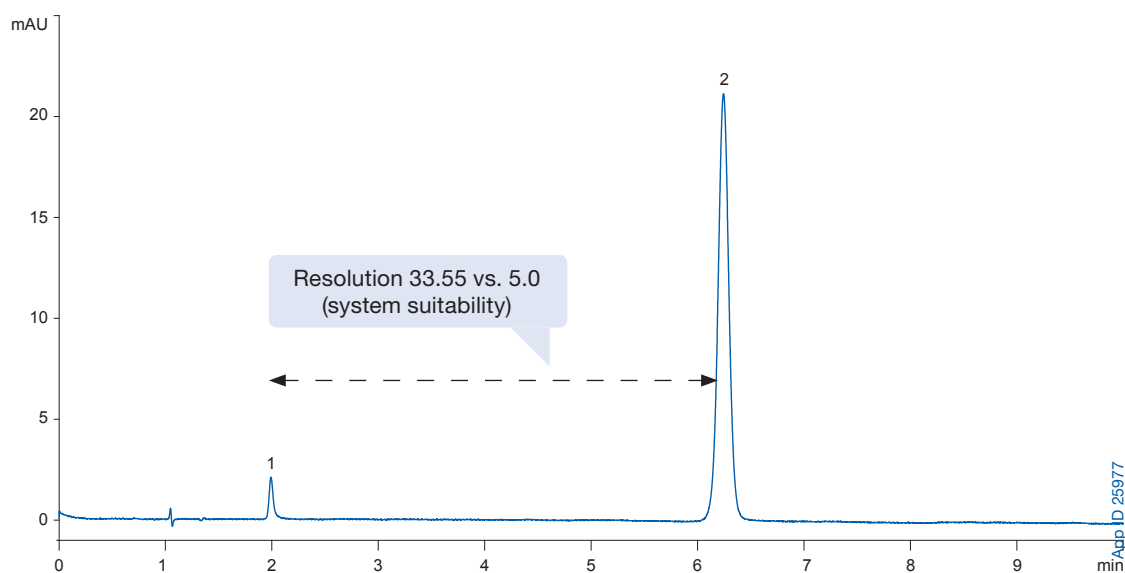
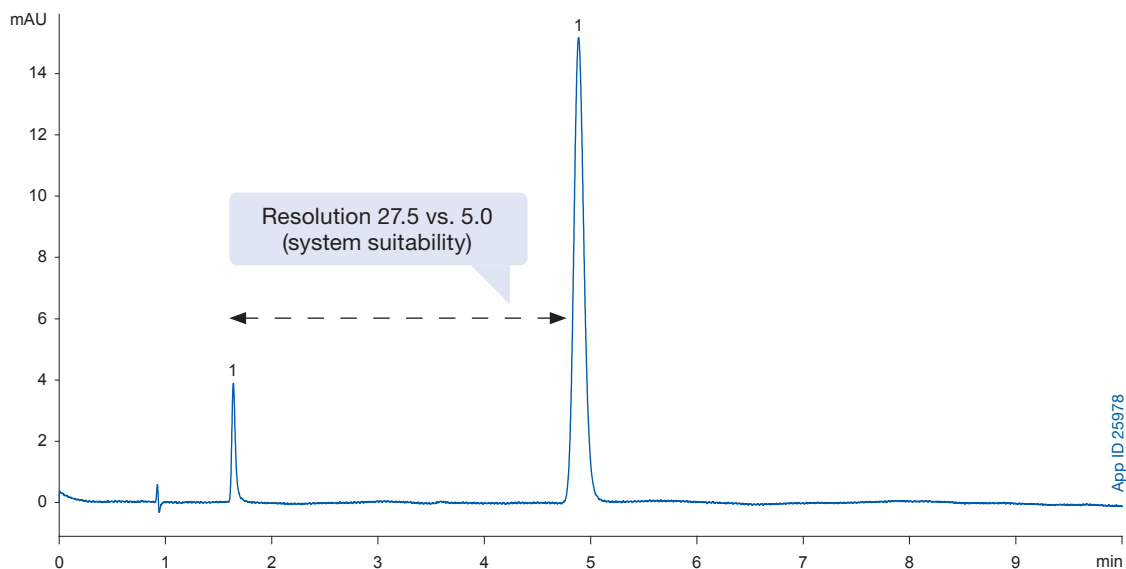


Table 1.

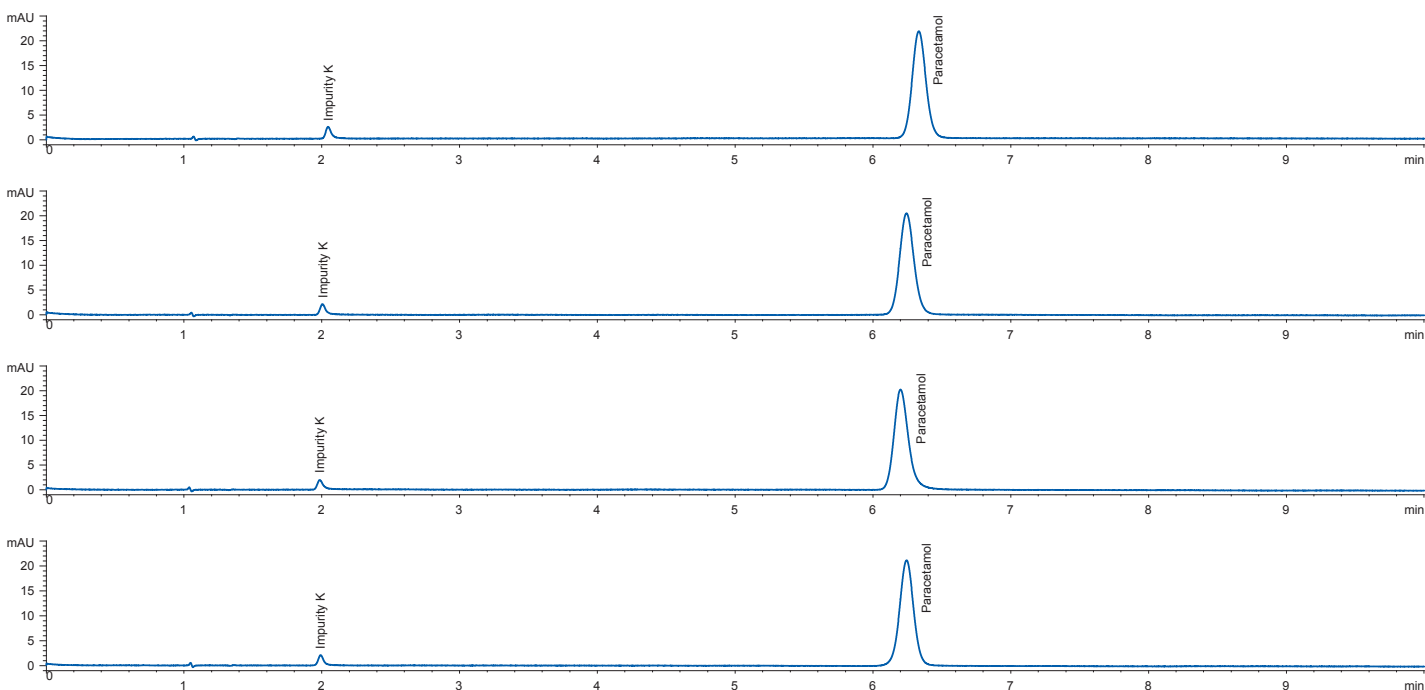
Kinetex 5 μ m C18 Results Summary.

Analyte	Retention Time	Resolution (USP)
Impurity K	2.01	
Paracetamol	6.24	33.55

APPLICATIONS

Figure 6b.Reference solution on HALO[®] 5 μ m C18 150 x 4.6 mm on Agilent[®] 1290.**Table 2.**HALO 5 μ m C18 Results Summary.

Analyte	Retention Time	Resolution (USP)
Impurity K	1.64	
Paracetamol	4.89	27.5

Figure 6c.The chromatograms obtained on Kinetex[®] 5 μ m C18 columns from 4 different manufacturing batches (lots) and **Table 3** shows the retention times for Impurity K and Paracetamol, and the resolution results for each column.

APPLICATIONS

Table 3.Kinetex[®] 5 µm C18 Results Summary.

Batch	RT Impurity K	RT Paracetamol	Resolution (USP)
0070	2.05	6.33	33.23
0075	2.01	6.24	32.20
0077	1.99	6.20	32.27
0079	1.99	6.24	33.55

Conclusion

The above experiments show Kinetex 5 µm C18 is suitable under the conditions outlined in the draft monograph for paracetamol and even gives increased resolution for the system suitability solution, reference solution (e). With the Kinetex 5 µm C18 column we also demonstrated batch to batch reproducibility (retention times and resolution) across multiple (4) batches. Therefore, Kinetex 5 µm C18 is a reliable solution for the analysis of paracetamol in routine laboratories following the Ph. Eur. regulations. Please also refer to our technical notes covering the current monograph for paracetamol^{5,6}.

Sources of Standards

Standard	Source
Paracetamol	Sigma-Aldrich
Impurity E (1-(4-hydroxyphenyl)ethan-1-one)	Sigma-Aldrich, PHR1971-100MG
4-Aminophenol (Acetaminophen Related Compound K)	Sigma-Aldrich, PHR1148-1G
Acetaminophen Related Compound J (4'-Chloroacetanilide)	Sigma-Aldrich, PHR1149-1G
Impurity A (2-Acetamidophenol)	Sigma-Aldrich, A7000-25G-A
Acetaminophen Impurity G (1-(4-hydroxyphenyl)ethanone oxime)	Sigma-Aldrich, PHR1972-100MG
Impurity H (4'-Acetoxyacetanilide)	Sigma-Aldrich, A-135-1ML
Impurity C (N-(3-Chloro-4-hydroxyphenyl)acetamide)	Ark Pharm, AK-3964-54-3-1G
Paracetamol EP Impurity L (N-[4-(4-acetamido-2-hydroxyphenoxy)phenyl]acetamide)	TLC Pharmaceutical Standards, A-0232
Paracetamol Impurity M (4,4-Iminodiphenol HCl)	TLC Pharmaceutical Standards, A-0234
Paracetamol EP Impurity N (N,N-(oxybis(4,1-phenylene))diacetamide)	TLC Pharmaceutical Standards, A-0227
Impurity I (2'-Hydroxyacetophenone)	Sigma-Aldrich, H18607-5G
Paracetamol Impurity Mixture Paracetamol plus impurities B, C, D, and F	Cerilliant, A-134-1ML

References

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3. United States Pharmacopeia and National Formulary (USP 39-NF 34). Rockville, MD: United States Pharmacopeial Convention; 2016. http://127.0.0.1:39341/uspnf/pub/data/v39341/usp39nf34s1_c227.xml#usp39nf34s1_c227. Accessed September 25, 2018.
4. Hanyšová, L & Kastner, P & Klimeš, J. (2004). Study of stability of 16-aminophenol as dominant decomposition product of paracetamol. Chemické Listy. 1998. 152-156.
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6. Zeshan Aqeel, Heiko Behr, Ryan Splitstone, and Philip J. Koerner (2020) TN-1273 Analysis Paracetamol and Critical Impurities Under European Pharmacopoeia Conditions and Utilizing the Kinetex 2.7 µm C18 Core-Shell LC Column.

APPLICATIONS

Kinetex[®] Ordering Information

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
F5	00B-4724-E0	00D-4724-E0	00F-4724-E0	00G-4724-E0	AJ0-9320
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

[†]SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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