UHPLC²

Guidelines for high throughput separations

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chromatography@merckgroup.com

Introduction

Increase sample throughput by speeding up separation by 10 times

Fast and ultra-fast separations have become particularly important due to the need for high sample throughput and higher productivity in daily lab work.
Using UHPLC methods with short columns, narrow inner diameters and small particles sizes, it is possible to speed up analyses up to ten-fold.
The choice of UHPLC columns depends on the sample being analyzed.
To ensure that you have the most appropriate solution for your application, Merck Millipore offers two different column materials for UHPLC separations:
Relatively clean samples are optimally separated on columns with narrow inner diameters and small particle sizes like Purospher® STAR UHPLC columns.
Samples with more complex matrices usually require intensive sample preparation to avoid column contamination or clogging.
In such cases, Chromolith® columns are the best choice, since they enable fast separations with minimum sample preparation – saving time and costs.

Purospher® STAR UHPLC columns are the best choice for relatively clean samples.
They deliver excellent peak symmetry, outstanding performance and pH stability, combined with the highest reliability.

Chromolith® 2 mm i.d. columns are the optimal solution for more complex and matrix-rich samples, which require sample preparation when particulate columns are used.
These columns are unbeatable in terms of the lifetime and low back-pressure.

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Two choices for high throughput separations

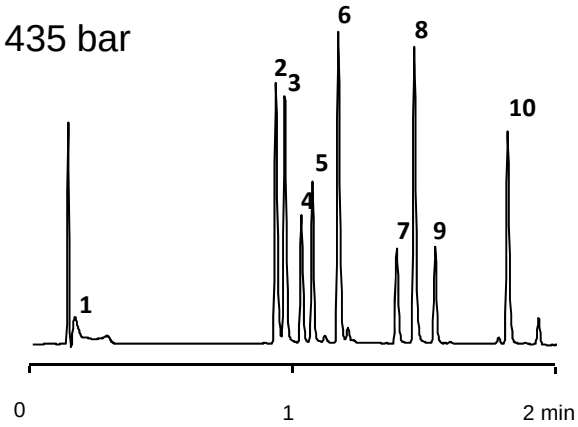
- 10 compounds in 2 minutes

UHPLC enables very fast separations of even complex samples with high resolution in very short time. This is mainly possible by using short column length.
Ultra fast separations can be achieved with Purospher® STAR UHPLC columns packed with small particles or Chromolith® 2 mm i.d. monolithic silica columns using any UHPLC instrument.

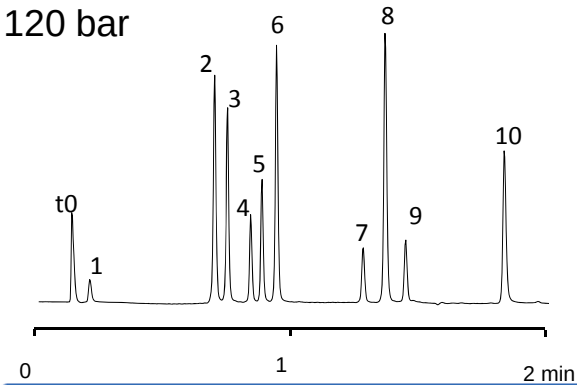
The separation of 10 Antihistamines shown below is achieved on both columns – but efficiency, lifetime and column backpressures are different.

Purospher® STAR = higher performance and extended pH stability
Chromolith® = lower backpressure and longer column lifetime

Purospher® STAR RP-18 endcapped, (2 µm)
Hibar® HR 50-2.1 mm

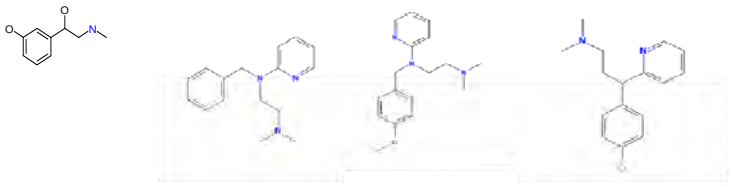


Chromolith® FastGradient RP-18 endcapped, 50-2 mm

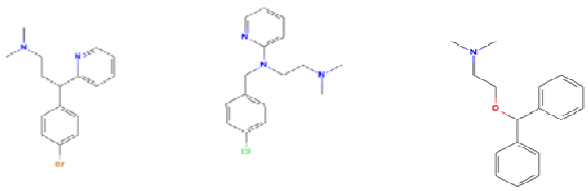


Antihistamine

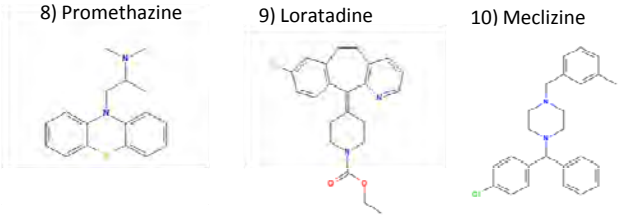
1) Phenylephrine 2) Tripelennamine 3) Pyrilamine 4) Chloropheniramine



5) Brompheniramine 6) Chloropyramine 7) Diphenhydramine



8) Promethazine 9) Loratadine 10) Meclizine



Mobile Phase:	A: 0,1% TFA in water B: 0,1% TFA in ACN Gradient
Flow rate	1 mL/min (Chromolith®); 1.103 mL/min (Purospher®)
Detection	UV 230 nm
Temp.	Ambient (Chromolith®); 60°C (Purospher®)
Inj.Volume	200 nL

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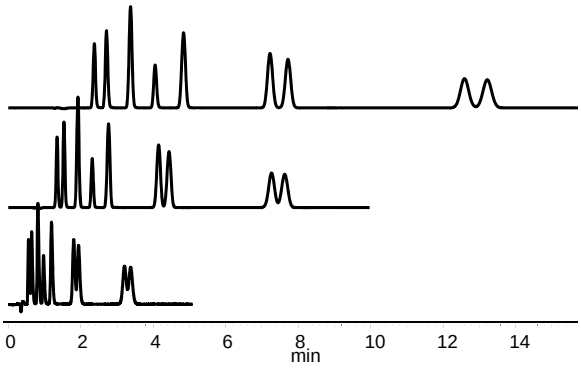
How to speed up separations?

Scaling down the column length

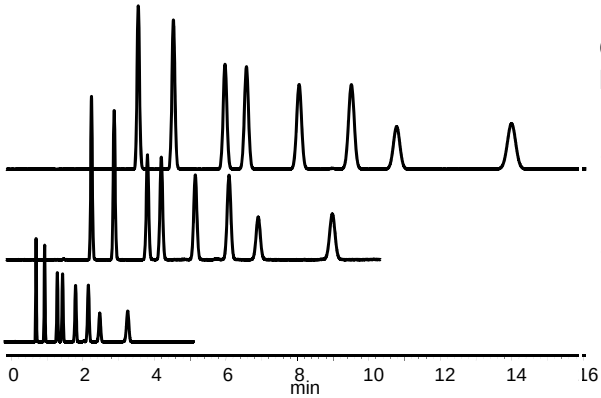
In HPLC, speed of analysis is mainly related to the column length. Under same conditions a long column needs more time for a separation than a short column. The easiest way to reduce the separation time is therefore to reduce the column length.
However a shorter column using the same particle size material of same efficiency (plates per meter = N/m) will have less plates per column (N/column). Therefore, a very short column shows less resolution than a long column of the same particle size material.



Column length	Particle size	Efficiency
150 mm	5 µm	15 000 N/column
100 mm	5 µm	10 000 N/column
50 mm	5 µm	5 000 N/column

This lack of efficiency and resolution for shorter columns is compensated by the reduction of particle sizes. Efficiency of the column could be doubled for the same column length if 2 µm particles instead of 5 µm are used.

Short columns = increase analysis speed
High efficiency (small particles) = high resolution



Column length	Particle size	Efficiency
150 mm	5 µm	15 000 N/column
100 mm	3 µm	14 500 N/column
50 mm	2 µm	10 000 N/column

Particle size: 5 µm = 100,000 N/m; 3 µm = 145,000 N/m; 2 µm = 200,000 N/m

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Scaling from HPLC to UHPLC

A transfer of HPLC methods to UHPLC requires scaling down from bigger inner diameter columns (e.g. 4.6 mm i.d.) to smaller inner diameter columns (e.g. ~2.1 mm i.d.) and from long columns (e.g. 150 mm length) to short columns (e.g. 50 mm length) in addition to the reduction of particle sizes (from e.g. 5 µm to 2 µm).

To ensure equivalent chromatographic separation, it is also necessary to scale the flow rate, injection volume and the gradient parameters.

Adjusting the column length

The first step is to determine the appropriate column length in order to maintain the same separation. Keeping the same column length while decreasing the particle size will increase the number of theoretical plates as well as backpressures. Therefore, when decreasing particle size, column length can be shortened without losing resolution.

$$\text{Column length } L_2 = L_1 \times dp_2 / dp_1$$

L_1 - HPLC column length
L_2 - UHPLC column length
dp_1 - HPLC particle size
dp_2 - UHPLC particle size

Scaling the flow rate

Decreasing the internal diameter of the column (e.g. from 4.6 mm to 2.1 mm) requires recalculating column flow rate in order to maintain linear velocity. Linear velocity is defined as the distance which mobile phase travels over time (cm/min), whereas flow rate is the volume of mobile phase that travels over time (mL/min). To maintain the same linear velocity through a column with a smaller internal diameter, the flow rate must be decreased proportionally to the column internal diameter according to the equation below.

$$\text{Flow rate } f_2 = f_1 \times (d_2)^2 / (d_1)^2$$

f_1 - HPLC flow rate
f_2 - UHPLC flow rate (mL/min)
d_1 - HPLC column ID
d_2 - UHPLC column ID (mm)

Scaling the injection volume

Decreasing the column internal diameter and length, decreases the overall column volume and sample capacity. Therefore, we must alter the injection volume. Please note that since overall column volume has decreased, it is more important to match the sample solvent to the starting mobile phase composition. Mismatched sample solvents can cause irreproducible retention times, efficiencies, and even changes in selectivity. If using a larger injection volume than calculated, check for peak abnormalities and irreproducibility that could result from phase overload.

$$\text{Inject. volume } V_2 = V_1 \times (d_2^2/d_1^2) \times (L_2 / L_1)$$

V_1 - HPLC Injection volume
V_2 - UHPLC Injection volume
d_1 - HPLC column ID
d_2 - UHPLC column ID (mm)
L_1 - HPLC column length
L_2 - UHPLC column length

Adjusting gradient time

When an analytical method is scaled down, the time program of the gradient also needs to be scaled down to keep the gradient volume the same.

$$\text{Time: } t_2 = t_1 \times (f_1/f_2) \times (d_2^2/d_1^2) \times (L_2 / L_1)$$

t_1 - HPLC time
t_2 - UHPLC time
f_1 - HPLC flow rate
f_2 - UHPLC flow rate (mL/min)
L_1 - HPLC column length
L_2 - UHPLC column length

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Scaling a HPLC method

from Purospher® STAR 5 µm column dimension 150-4.6 mm
to Purospher® STAR 2 µm column dimension 50-2.1 mm

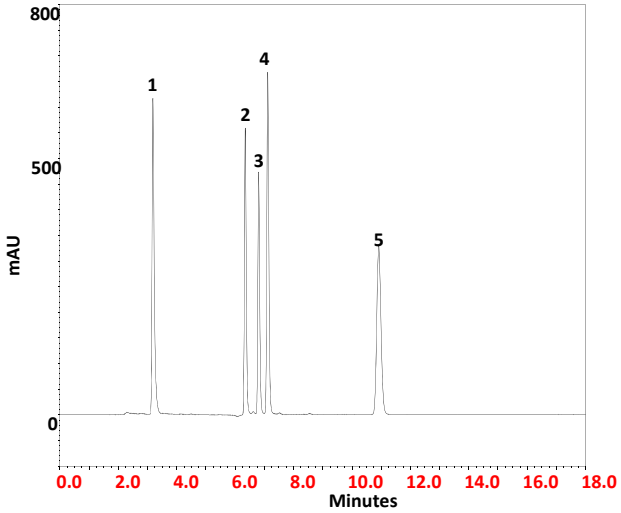
Separation of a mixture of 5 hormones was scaled from HPLC to UHPLC conditions. All calculations were following the equations on the previous page.

Flow rate $f_2 = 0.5 \times 2.1^2 / 4.6^2 = 0.105$ mL/min

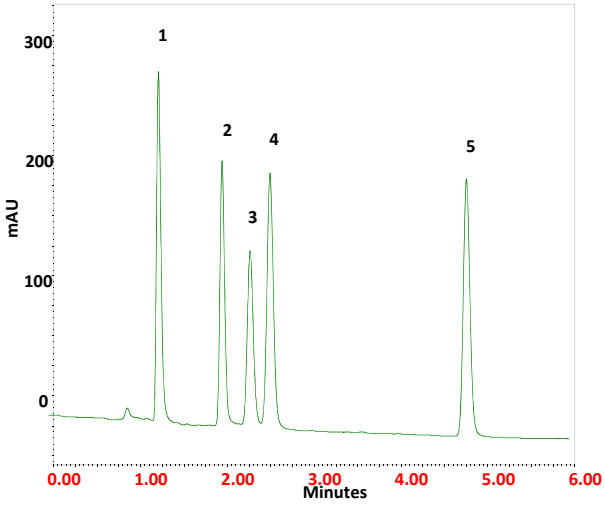
Time: $t_2 = 0.9 \times (0.5/0.105) \times (2.1^2/4.6^2) \times (50 / 150) = 0.3$ min

Inject. volume $V_2 = 7 \times (2.1^2/4.6^2) \times (50 / 150) = 0.5$ µL

Purospher® STAR RP-18e (5 µm)
LiChroCART® 150-4.6 mm



Purospher® STAR RP-18e (2 µm)
Hibar® HR 50-2.1 mm



Mobile phase:
A: 10 mM Na-Phosphate buffer pH=7 + 10% Acetonitrile
B: Acetonitrile

A: 10 mM Na-Phosphate buffer pH=7 + 10% Acetonitrile
B: Acetonitrile

Gradient:

Time/min	%A	%B	Flow_mL/min
0.0	55	45	**0.5**
0.9	55	45	0.5
1.2	65	35	0.5
4.5	65	35	1.5
4.8	45	55	1.5
18.0	45	55	1.5

Time/min	%A	%B	Flow_mL/min
0.0	55	45	**0.105**
0.3	55	45	0.105
0.4	65	35	0.105
1.5	65	35	0.310
1.6	45	55	0.310
6.0	45	55	0.310

Equilibration: **7.14 min with 1.5 mL/min A/B 55/45 v/v**
Temperature: 30 °C
Detection: UV 220 nm
Injection vol.: 7 µL

2.38 min with 0.31 mL/min A/B 55/45 v/v
50 °C
UV 220 nm
0.5 µL

		N/m 150-4.6	T_{USP} 150-4.6	N/m 50-2.1	T_{USP} 50-2.1
1	Prednisolon	93227	1.58	58660	1.34
2	β-estradiol	357827	1.18	128520	1.20
3	17-α-Ethinyl-estradiol	452253	1.23	100800	1.17
4	Estron	447100	1.19	110500	1.11
5	Progesteron	204053	1.10	369760	1.14

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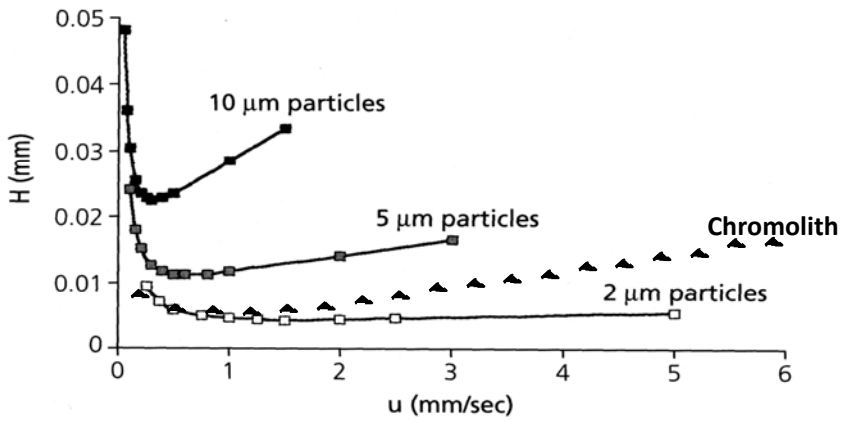
How to achieve maximum speed?

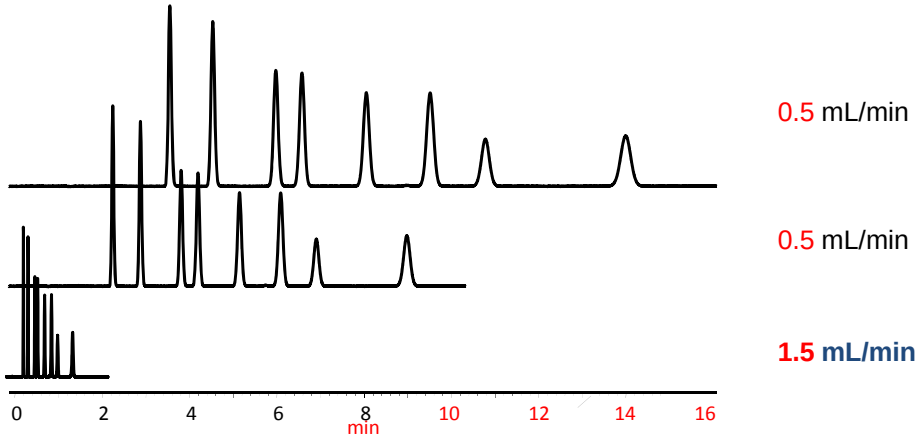
Separation speed is also flow rate dependant. The higher the flow rate is, the faster the separation is. Chosen flow rate is affecting the separation efficiency according to the **Van Deemter equation**.
The **Van Deemter equation** relates separation efficiency of the column to the linear velocity of the mobile phase by considering physical, kinetic, and thermodynamic properties of a separation. These properties include diffusion and mass transfer kinetics between stationary and mobile phases. The Van Deemter equation predicts that there is an optimum velocity interval at which there will be the minimum variance per unit column length and therefore, a maximum efficiency. The more flat the Van Deemter equation is, the more freedom we have choosing flow rates without loss of efficiency.



Purospher® STAR 2 µm particle packed columns shows an extremely flat Van Deemter curve. This makes this material perfectly suitable to use at increased flow rates in order to speed up the separations without loss of efficiency.

Chromolith® columns are very much suitable for higher flow rates as well.

Short columns with high efficiency at high flow rates = maximum speed



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Speed up a scaled method

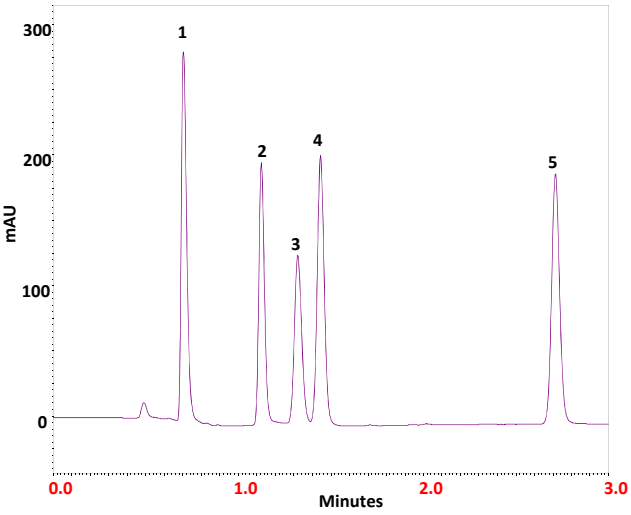
Flow rate was doubled (Chromatogram to the left) or increased by 3 times (Chromatogram to the right) in order to gain in throughput for the sample containing 5 hormones. The gradient was adjusted according to the equation :

$$\text{Time: } t_2 = t_1 \times f1 / f2$$

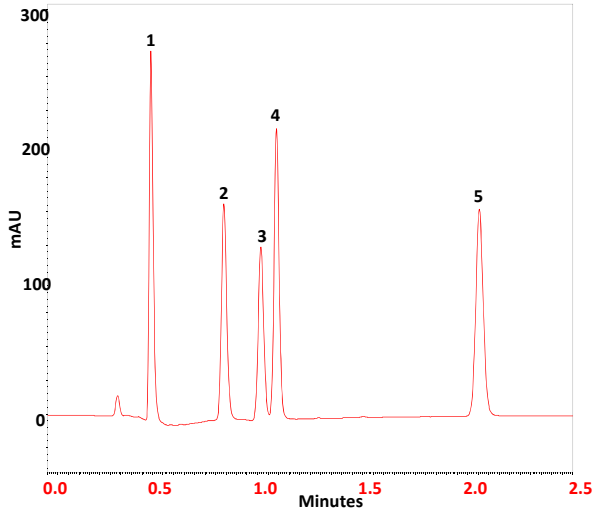
All 5 hormones were separated with good resolution within 2.5 min.
The table below shows very similar separation efficiency for all flow rates: the original scaled method and the flow rate x2 and the flow rate x3.

Purospher® STAR 2 µm = No loss of efficiency at high flow rates

Purospher® STAR RP-18e (2 µm)
Hibar® HR 50-2.1 mm



Purospher® STAR RP-18e (2 µm)
Hibar® HR 50-2.1 mm



Mobile phase:

A: 10 mM Na-Phosphate buffer pH=7 + 10% Acetonitrile
B: Acetonitrile

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B: Acetonitrile

Gradient:

Time/min	%A	%B	Flow mL/min
0.00	55	45	0.210
0.15	55	45	0.210
0.20	65	35	0.210
0.75	65	35	0.620
0.80	45	55	0.620
3.00	45	55	0.620

Time/min	%A	%B	Flow mL/min
0	55	45	0.315
0.1	55	45	0.315
0.2	65	35	0.315
0.5	65	35	0.930
0.6	45	55	0.930
2.5	45	55	0.930

Equilibration: **1.19 min with 0.62 mL/min A/B 55/45 v/v**
Temperature: 50 °C
Detection: UV 220 nm
Injection vol.: 0.5 µL

0.79 min with 0.93 mL/min A/B 55/45 v/v
50 °C
UV 220 nm
0.5 µL

		N/m 50-2.1	N/m 50-2.1 Speed up x2	N/m 50-2.1 Speed up x3
1	Prednisolon	58660	56480	66040
2	β-estradiol	128520	133040	103120
3	17-α-Ethinyl-estradiol	100800	101780	134560
4	Estron	110500	138260	193140
5	Progesteron	369760	369200	300780

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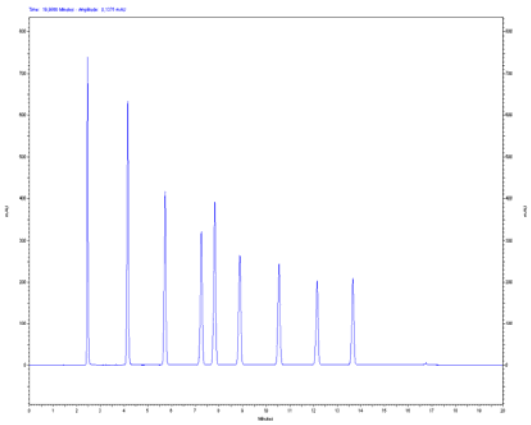
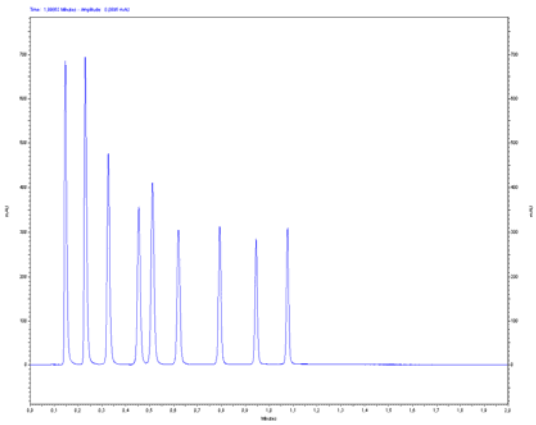
Speed up your Separation by 10 fold

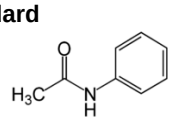
Scaling from HPLC to UHPLC can speed up the separation up to 10 times and save solvent by up to 90% at the same time.
The separation of 9 alkylphenones shown below was achieved in 20 minutes using a 150-4.6 mm Puropsher® STAR 5 µm column at 1.0 mL/min flow rate (20 mL per run). The method was scaled to a column dimension of 50-2.1 mm using a 2 µm material of the same sorbent. The new UHPLC method total run time is 2 minutes including re-equilibration of the gradient at a flow rate of 1.1 mL/min (2.2 mL per run).

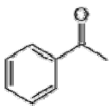
Purospher® STAR 2 µm = 10 times faster with 2.2 mL solvent per run

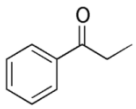
	Purospher® STAR RP-18e (**5 µm**) LiChroCART® **150-4.6** mm	Purospher® STAR RP-18e (**2 µm**) Hibar® HR **50-2.1** mm
		
Run Time:	**20 min** incl. re-equilibration	**2 min** incl. re-equilibration
Column temperature:	40 °C	40 °C
Eluent A:	Water	Water
Eluent B:	Acetonitrile	Acetonitrile
Gradient:	0 min 45% B, from 45 to 95% B in 15 min, from 15.1 to 20 min re-equilibration with 45 % B	0 min 55% B, from 55 to 100% B in 0.8 min, from 0.9 to 2 min re-equilibration with 55 % B
Flow rate:	**1.0 mL/min**	**1.1 mL/min**
Pressure:	105 bar	505 bar
UV:	247 nm	247 nm
Injection volume:	10 µL	1 µL

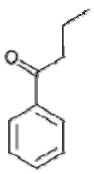
Sample: Alkylphenone standard

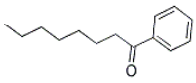
1. Acetanilide
2. Acetophenone
3. Propiophenone
4. Butyrophenone
5. Benzophenone
6. Valerophenone
7. Hexanophenone
8. Heptanophenone
9. Octanophenone

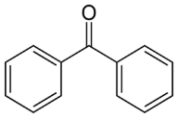

Acetanilide

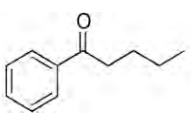

Acetophenone

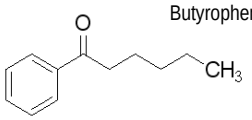

Propiophenone

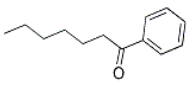

Butyrophenone


Octanophenone


Benzophenone


Valerophenone


Hexanophenone


Heptanophenone

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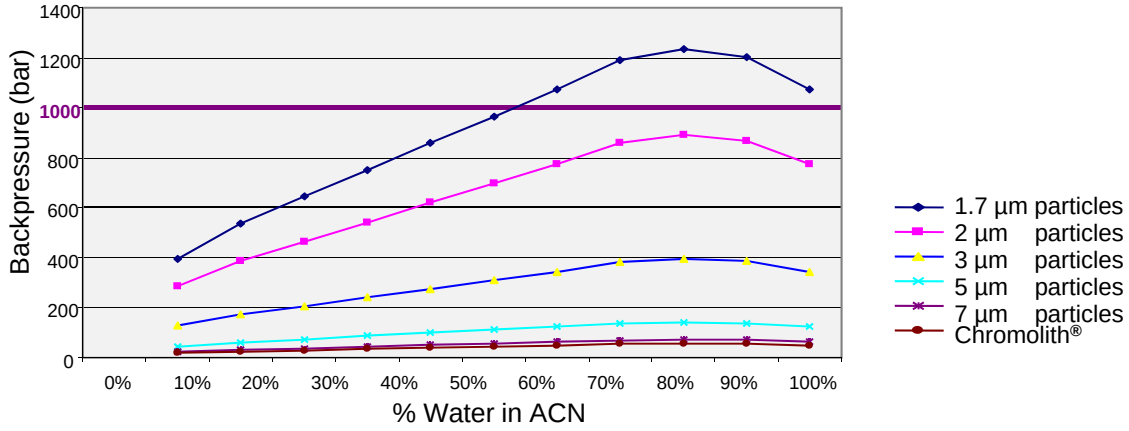
Why 2 µm particles, not sub 2 µm?

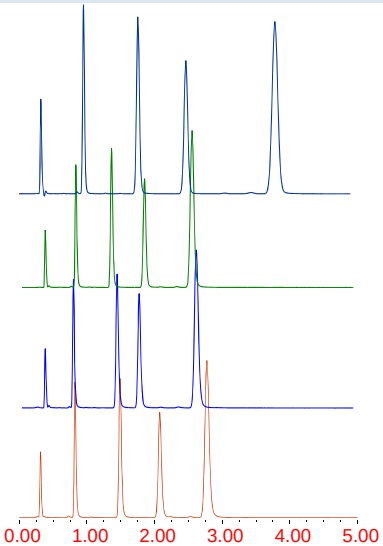
UHPLC users mostly expect sub-2 µm particles in column. We at Merck Millipore offer 2 µm particles instead for very good reasons:
Column backpressure is dependent on the particle size of a column material, and column backpressure increases dramatically with the decrease in particle size.

Effect of particle size on backpressure



Column efficiency depends on particle size: the smaller the particles, the higher the column efficiency. On other hand efficiency is influenced by many other parameters such as the dwell volume of the instrument, the detector cell volume, injection volume, and etc.
Comparing sub-2 µm UHPLC columns on the same instrument and under the same conditions, no real difference in efficiency of 1.7 µm, 1.8 µm, 1.9 µm or 2 µm particles could be seen. However the pressure drop of the different particle size materials shows very big differences. A 1.7 µm particulate material has more than 100 bar higher column backpressure than the 2 µm material.

Purospher® STAR 2 µm = similar efficiency as sub-2 µm, but lower backpressure



Purospher® STAR RP-18 endcapped, 2 µm
174080 N/m
130 bar

Waters, 1.7 µm
187620 N/m
225 bar

Restek, 1.9 µm
172840 N/m
209 bar

Agilent, 1.8 µm
173260 N/m
175 bar

Column dimension: 50-2.1 mm
Mobile phase: Acetonitrile/Water **60/40**
Detection: UV 254 nm Response: fast
Temperature: RT
Flow: **0.350 mL/min**
Injection: 0.2 µL
Sample:

Thioharnstoff	2.1 mg
Biphenyl-2-ol	5.4 mg
Progesteron	11.6 mg
Hexanophenon	12.3 mg
Anthracen	1.8 mg

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Determination of Cefaclor

A UHPLC method was used for the separation of Cefaclor on two different particle size columns with same column dimensions.
Both columns show a similar retention for Cefaclor. However Purospher® STAR RP-18e (2 µm) has better efficiency compared with ZORBAX® 1.8 µm column as well as significantly lower column backpressure.

Purospher® STAR 2 µm = very high efficiency at lower backpressure

Purospher® STAR RP-18e 2 µm Hibar® HR 50-2.1 mm

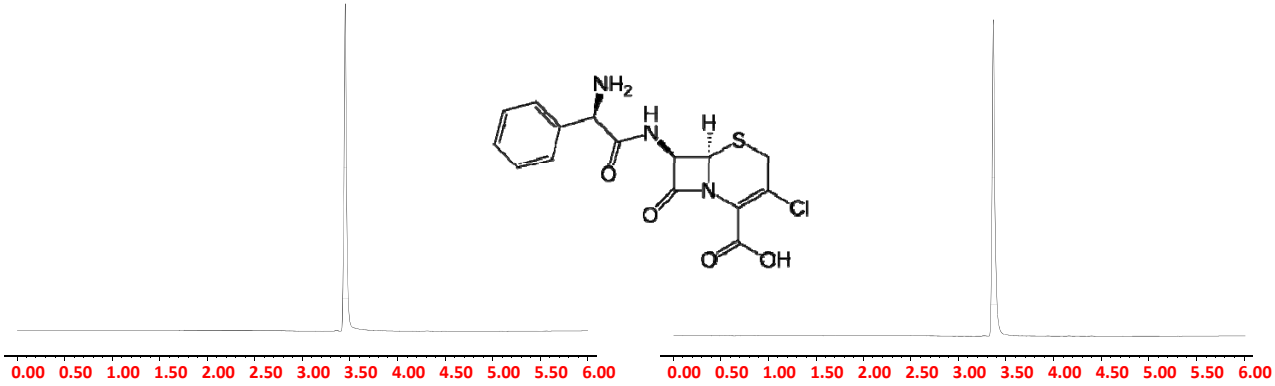
Efficiency: **1736680** N/m

Column backpressure: **161** bar

ZORBAX® SB C18 1.8 µm 50-2.1 mm

Efficiency: **1411120** N/m

Column backpressure: **238** bar



Mobile phase:	**A: 10 mM Na-Phosphate buffer pH=3.5**		
	B: Acetonitrile		
Gradient:	Time/min	%A	%B
	0	5	95
	1	5	95
	6	95	5
Flow rate:	250 µL/min (50-2mm) , 276 µL/min (50-2.1 mm)		
Temperature:	30 °C		
Detection:	UV 254 nm		
Injection vol.:	0.11 µL		

Sample: **Cefaclor capsule 500 mg**

Cefaclor is also known as **cefachlor** or **cefaclorum**. Brand names are: **Ceclor**, **Distaclor**, **Keflor**, **Raniclor**.

Cefaclor belongs to the family of antibiotics known as the cephalosporins used for the treatment of septicaemia, pneumonia, meningitis, biliary-tract infections, peritonitis and urinary-tract infections.

Sample preparation:
One capsule was dissolved in 100 mL water, ultra sonic after 20 min, and filtered through a 0.2 µm filter directly into a vial.

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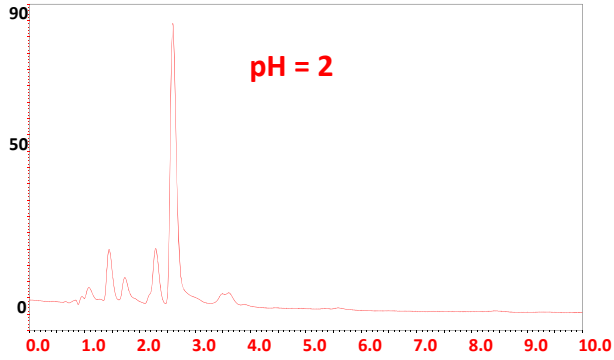
Separation of Omeprazole

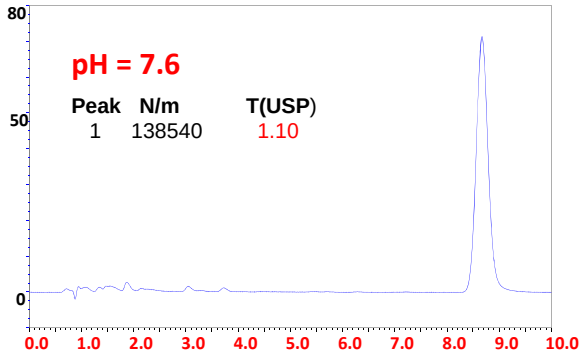
The separation of Omeprazole requires basic conditions due to the fact that this compound is unstable under acidic conditions. Examples of omeprazol sample separation under acidic and basic conditions are given bellow. The separation at pH 2 clearly shows the decomposition products of omeprazole. Separation of omeprazole at high pH shows good stability.

Purospher® STAR RP-18e 2 µm = perfectly suitable at high pHs

Omeprazole (OMZ) is a proton pump inhibitor used in the treatment of dyspepsia, peptic ulcer disease, gastroesophageal reflux disease and Zollinger-Ellison syndrome.
Its mechanism of action is by selectively inhibiting the hydrogen-potassium adenosine triphosphatase enzyme (H^+/K^+ ATPase) of the parietal cells, leading to a reduction of the gastric acid secretion.
Omeprazole is one of the most widely prescribed drugs internationally.

OMZ is available as tablets and capsules (containing omeprazole or omeprazole magnesium) in strengths of 10 mg, 20 mg, 40 mg, and in some markets 80 mg; and as a powder (omeprazole sodium) for intravenous injection.



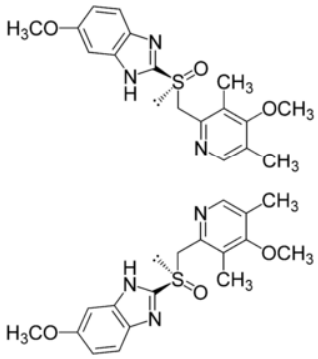


Column: **Purospher® STAR RP-18e 2 µm Hibar® HR 50-2.1 mm**

Mobile phase: **1.)** Acetonitrile/ 20 mM Na-Phosphate buffer **pH = 2** 25/75 v/v
2.) Acetonitrile/ 20 mM Na-Phosphate buffer **pH = 7.6** 25/75 v/v

Flow rate: 167 µL/min
Temperature: 30 °C
Detection: UV 280 nm
Injection vol.: 1.1 µL

Sample: **Omeprazole 20 mg**



Sample preparation:
20 mg Omeprazole were dissolved in 75 mL water, ultra sonic after 20 min, add 25 mL Acetonitrile and filter through a 0.2 µm filter directly into a vial.

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Determination of “c-Met blue” (MSC2156119J)

For pharmaceutical studies the main compound peak is often of less interest. Very low level of byproducts or decomposition products need to be detected with high precision and reproducibility. With Purospher® STAR UHPLC columns, it is possible to achieve lower detection limits and hence build methods with higher sensitivity.

Purospher® STAR 2.1 mm i.d. UHPLC columns = highest sensitivity

Purospher® STAR RP-18e 2 µm Hibar® HR 50-2.1 mm

Mobile phase: **A:** Water/Acetonitrile 95/5 + 0.1% TFA
B: Water/Methanol/Acetonitrile 10/85/5 + 0.1% TFA

Gradient:

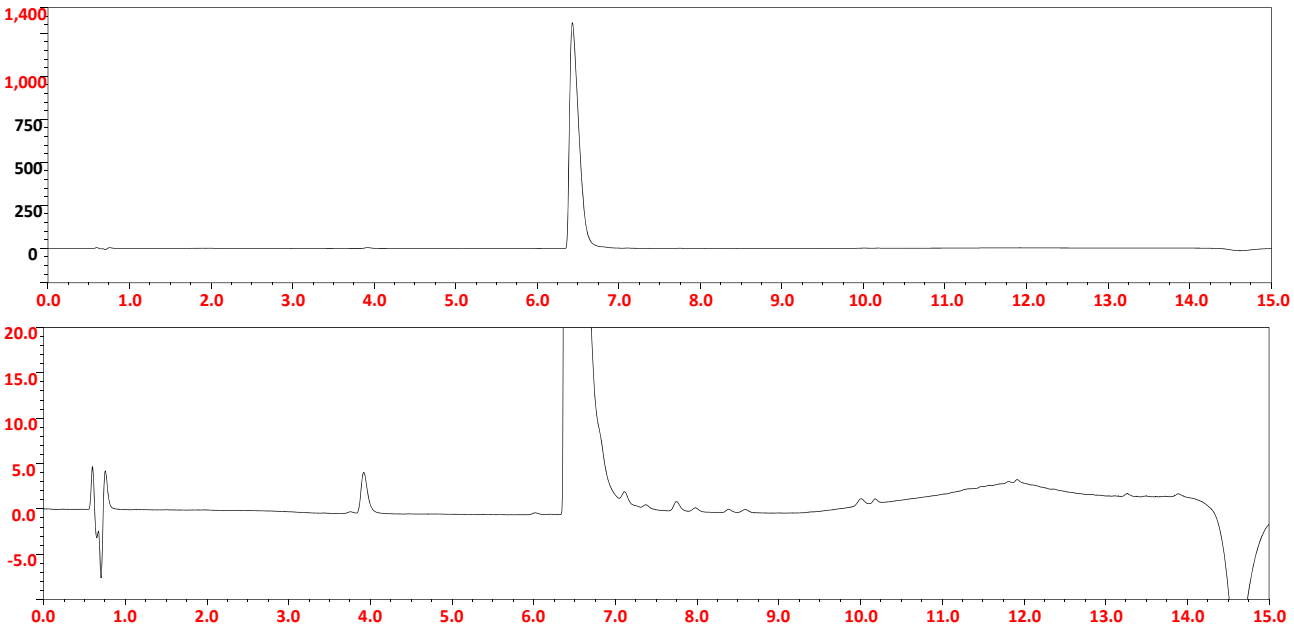
Time/min	%A	%B
0.0	62	38
1.0	62	38
2.5	42	58
7.5	37	63
10.0	5	95
12.5	5	95
12.6	62	38
15.0	62	38

Flow rate: 210 µL/min (50-2.1 mm)
Temperature: 45 °C
Detection: UV 260 nm
Injection vol.: 1.1 µL (50-2.1 mm)

c-Met kinase inhibitor in solid tumors – an investigational small molecule inhibitor of the c-Met receptor tyrosine kinase. Alterations of the c-Met signaling pathway are found in various cancer types and correlate with aggressive tumor behavior and poor clinical prognosis. Currently in a Phase I trial to investigate dose-dependent safety profile and first signs of clinical activity in solid tumors, in collaboration with the M.D. Anderson Cancer Center.

Sample: **1 Tablet 100 mg**

Sample preparation:
One tablet was put in a 100 mL volumetric flask, add 20 mL water, ultra sonic bath after 15 min, add 50 mL acetonitrile, stir with a magnetic stirrer at 250 r/min for 20 min and fill up to mark with water. Filter through a 0.45 µm filter.
Move 4 mL of the filtrate into a 10 mL volumetric flask and then fill up to mark with water.



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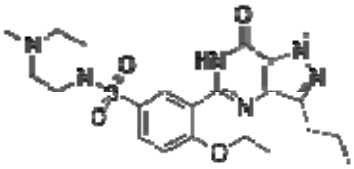
Determination of Sildenafil “Viagra” + Byproducts

Pharmaceutical formulations like Viagra contain a lot of substances in addition to the main compound. Although single compound could be easily separated, a higher resolution is needed for separation of all byproducts. A longer column of 100 mm length was used for separation of Sildenafil and all byproducts as an example.

Purospher® STAR 2.1 mm i.d. UHPLC columns = precise results for separation of formulations with many byproducts

Sildenafil citrate, sold as **Viagra**, **Revatio** and under various other trade names, is a drug used to treat erectile dysfunction and pulmonary arterial hypertension.



Sildenafil

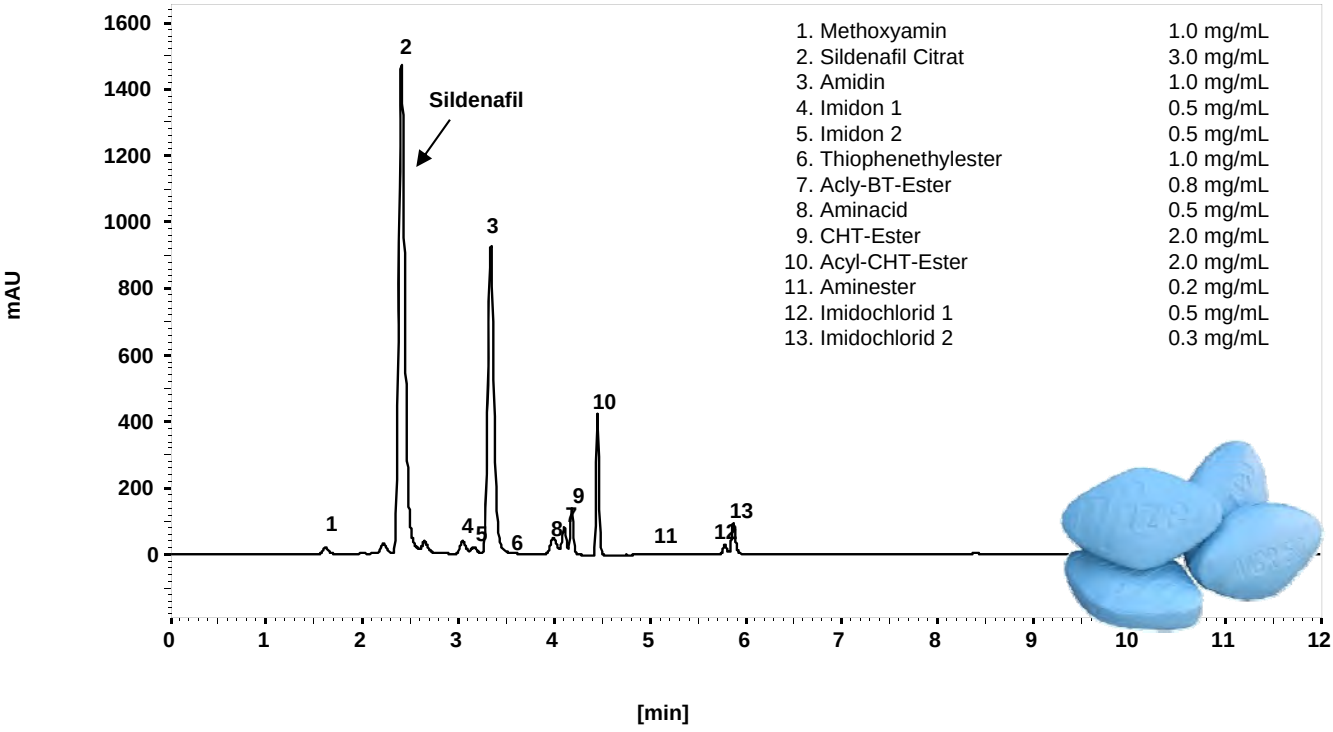
Column: **Purospher® STAR RP-18e 2 µm Hibar® HR 100-2.1 mm**

Eluent:
A: Acetonitrile
B: 20 mM Na-Phosphate buffer pH = 3.0

Gradient:

Time/min	%A	%B	Flow µL/min
0.0	65	55	104
4.0	65	35	208
4.1	75	25	625
10.0	75	25	625

Pressure: 440 bar
Det.: 254 nm
Cell: 3.2 µL
Temp.: 60 °C
Inj.: 0.2 µL



1.	Methoxyamin	1.0 mg/mL
2.	Sildenafil Citrat	3.0 mg/mL
3.	Amidin	1.0 mg/mL
4.	Imidon 1	0.5 mg/mL
5.	Imidon 2	0.5 mg/mL
6.	Thiophenethylester	1.0 mg/mL
7.	Acly-BT-Ester	0.8 mg/mL
8.	Aminacid	0.5 mg/mL
9.	CHT-Ester	2.0 mg/mL
10.	Acyl-CHT-Ester	2.0 mg/mL
11.	Aminester	0.2 mg/mL
12.	Imidochlorid 1	0.5 mg/mL
13.	Imidochlorid 2	0.3 mg/mL

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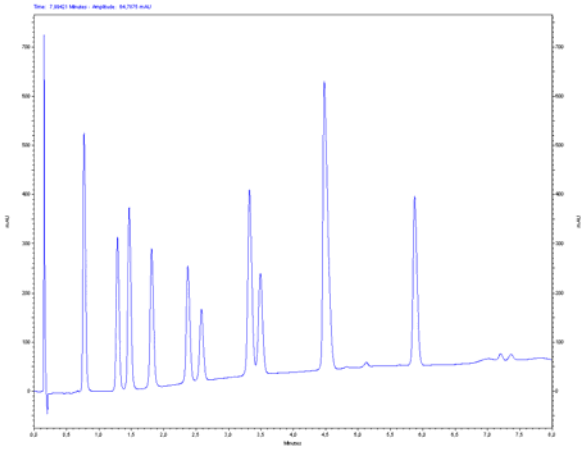
Separation of Lamotrigine & related substances

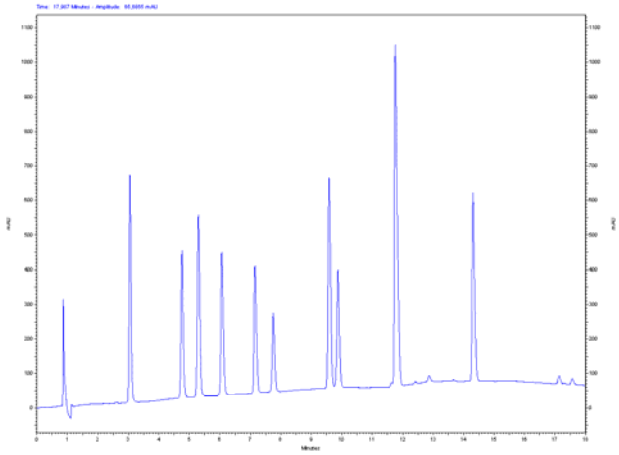
The baseline separation of all 10 Lamotrigines was not possible on a short column (50-2.1 mm). A longer column can improve the separation due to higher column efficiency. Column of 150-2.1 mm packed with the same material shows sufficient resolution for the same compounds.

Longer UHPLC columns (150 mm length) = higher resolution

Purospher® STAR RP-18e, (2 µm)
Hibar® HR 50-2.1 mm

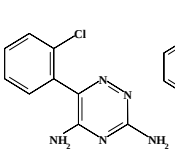


Purospher® STAR RP-18e, (2 µm)
Hibar® HR 150-2.1 mm

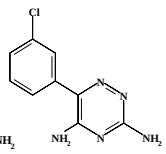


Column temp.:	40 °C	
Eluent A:	Buffer (14 mL triethylamine add 1 L water, adjusted to pH 1.9 with perchloric acid)	
Eluent B:	Acetonitrile	
Flow rate:	**0.8 mL/min**	**0.38 mL/min**
Pressure:	**495 bar**	**530 bar**
Gradient:	0 min 12% acetonitrile, from 12-27% B in 7.1 min, re-equilibration with 12% B from 7.2 up to 12 min.	0 min 17% acetonitrile, from 17-34% B in 16 min, re-equilibration with 17% B from 16.1 up to 25 min.
Injection volume:	2 µL	

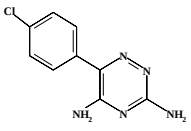
Sample:
Lamotrigine & related substances standard
1. 2-Chloro-Lamotrigine, 2. 3-Chloro-Lamotrigine, 3. 4-Chloro-Lamotrigine, 4. 2.5-Dichloro-Lamotrigine, 5. Lamotrigine, 6. 2.4-Dichloro-Lamotrigine, 7. 3.5-Dichloro-Lamotrigine, 8. 3.4-Dichloro-Lamotrigine, 9. 2.3.5-Trichloro-Lamotrigine, 10. Lamotrigine – open form



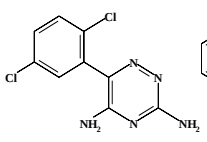
2-Chloro-Lamotrigine



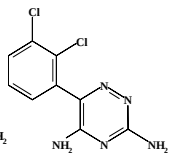
3-Chloro-Lamotrigine



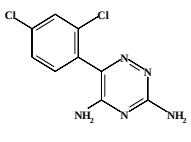
4-Chloro-Lamotrigine



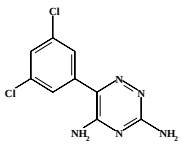
2.5-Dichloro-Lamotrigine



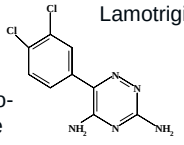
Lamotrigine



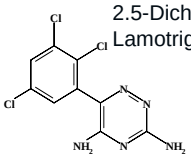
2.4-Dichloro-Lamotrigine



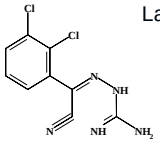
3.5-Dichloro-Lamotrigine



3.4-Dichloro-Lamotrigine



2.3.5-Trichloro-Lamotrigine



Lamotrigine – open form

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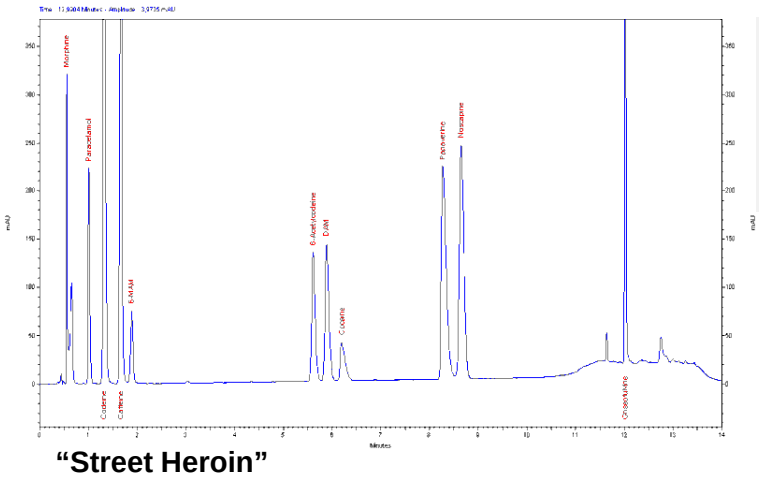
Determination of illegal drugs

The chromatographic determination of illegal drugs is crucial. Diacetylmorphine (DAM, Heroin) and cocaine coelute on many stationary phases.
Purospher® STAR RP-18 endcapped (3 µm) 150-2.1 mm column was able to baseline separate all relevant compounds. Moreover, 3 µm particle size ensured that the UHPLC separation was achieved at moderate column backpressure.
The “Street Heroin” sample shows the same good separation as the standard mixture.

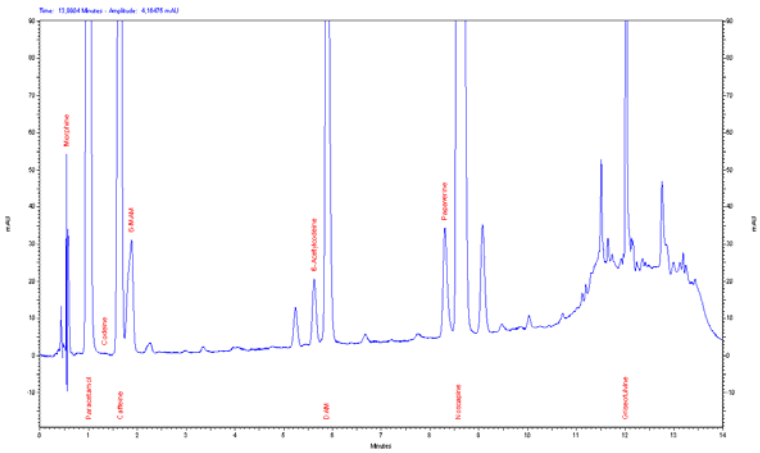
3 µm particles in may cases provide enough column efficiency for a good baseline separation.

Purospher® STAR RP-18e (3 µm), Hibar® HR 150-2.1 mm

Standard Mixture of 11 illegal drugs



“Street Heroin”

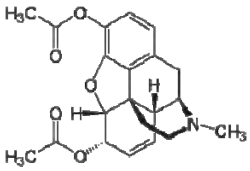


Heroin (**diacetylmorphine)** is typically used to treat severe pain, such as that resulting from a heart attack or a severe injury. Illegally supplied heroin is more often in freebase form, dulling the sheen and consistency to a matte-white powder.

Column temperature: 40 °C
Eluent A: Phosphate buffer, pH 2.5 (2.4 g NaH_2PO_4 + 20 mL ACN add 1 L water and adjust with 85% H_3PO_4 to pH 2.5)
Eluent B: Acetonitrile

Time	A	B	Flow
0.0	89	11	0.8
5.8	82	18	0.8
7.0	81.5	18.5	0.8
9.5	75	25	0.8
12.0	30	70	0.8
13.0	30	70	0.8
13.1	89	11	0.8
16.0	89	11	0.8

Flow rate: 0.8 mL/min
UV: 210 nm
Pressure: 520 bar



Sample:
1.Morphine, 2.Paracetamol, 3.Codein, 4.Coffeine, 5.6-Mono-acetylmorphine (MAM), 6.6-Acetyl-codeine, 7.Diacetylmorphine (DAM, Heroin), 8.Cocaine, 9.Papaverine, 10.Noscapine, 11.Griseofulvine

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Quantification of Buprenorphine and Norbuprenorphine with UHPLC-MS/MS

Purospher® STAR RP-18e, 2 µm columns fulfill all requirements for fast and modern UHPLC-MS analysis. Identification and quantification of Buprenorphine and its metabolites can be done in a few minutes. The analysis time for Buprenorphine is 1.4 min.

Purospher® STAR UHPLC columns = highly suitable for LC-MS

Buprenorphine is a synthetic derivative of the alkaloid thebaine and has partial agonistic properties at the opiate receptor. It is used for pain treatment and aversion therapy for heroin dependence.

Buprenorphin

MS intrument: AB Sciex API 4000™

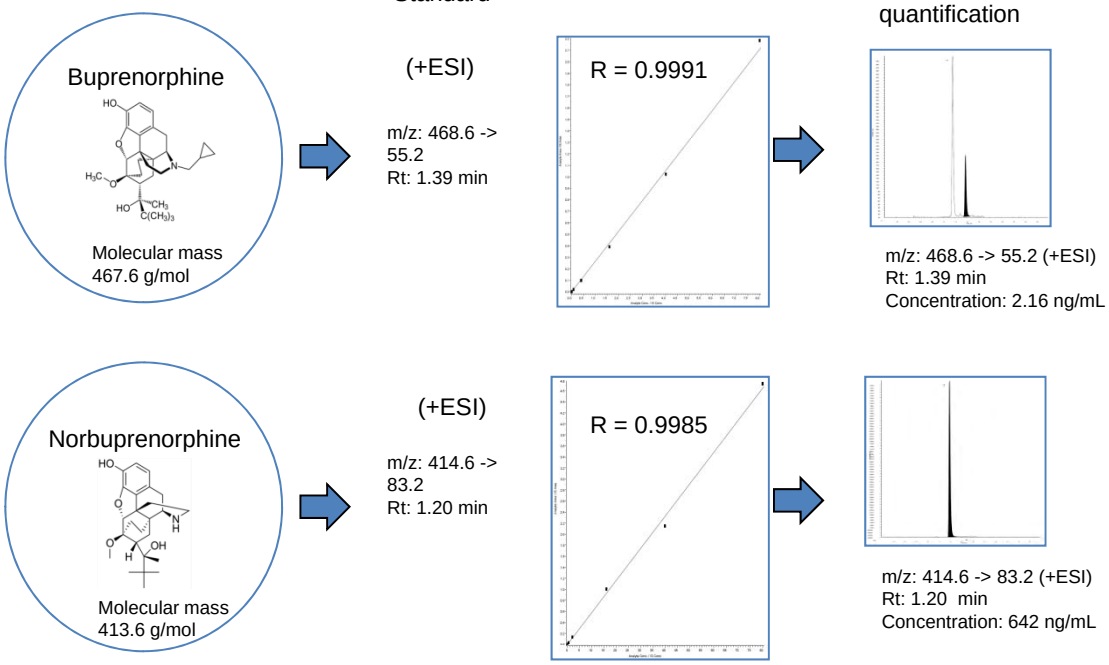
UHPLC Column: **Purospher® STAR RP-18 endcapped, (2 µm) Hibar® HR 50-2.1 mm**

Mobile phase A: 0.1% formic acid in Milli-Q water
Mobile phase B: 0.1% formic acid in acetonitrile

Flow rate: 0.7 mL/min
Mobile phase start: 90/10 A/B
Column backpressure at start: 230 bar

Gradient

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (mL/min)
0.00	90	10	0.7
0.25	90	10	0.7
2.00	10	90	0.7
2.10	90	10	0.7
3.00	90	10	0.7



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Quantification of Tramadol and N-Desmethyltramadol with UHPLC-MS/MS

Tramadol (Ultram, Tramal) is a centrally acting opioid analgesic, used in treating moderate to severe pain. The drug has a wide range of applications, including treatment for restless legs syndrome and fibromyalgia.

Tramadol

MS instrument: AB Sciex API 4000™

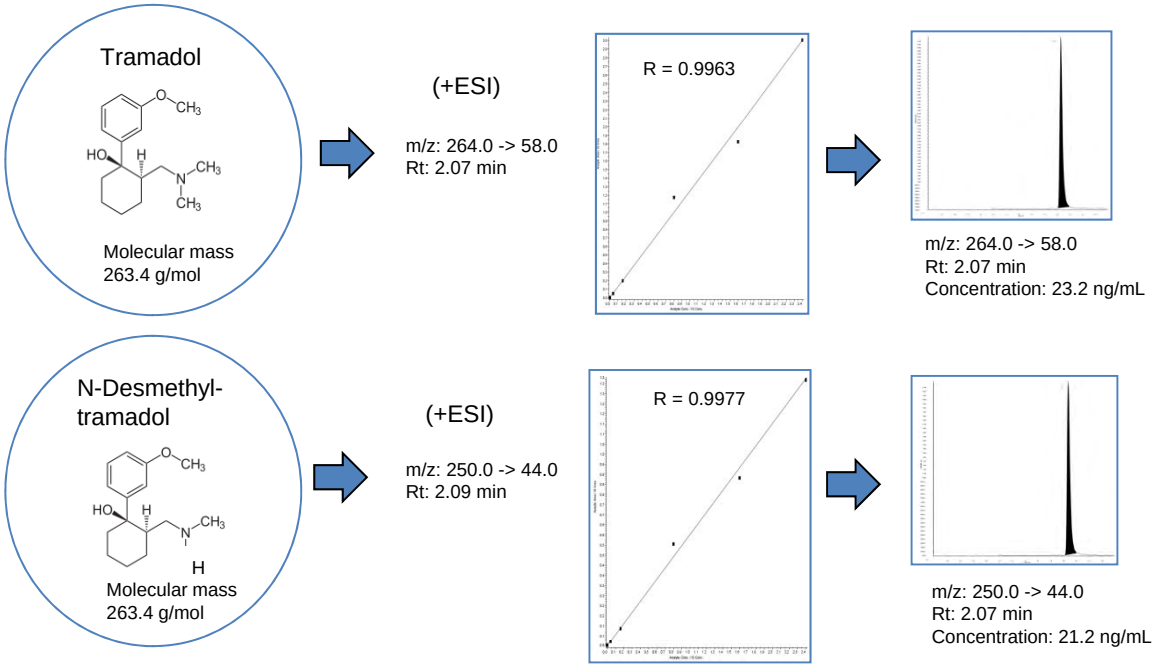
UHPLC Column: **Purospher® STAR RP-18 endcapped, (2 µm) Hibar® HR 50-2.1 mm**

Mobile phase A: 0.1% formic acid in Milli-Q water
Mobile phase B: 0.1% formic acid in acetonitrile

Flow rate: 0.4 mL/min
Mobile phase start: 95/5 A/B
Column backpressure at start: 170 bar

Gradient

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (mL/min)
0.00	95	5	0.4
0.20	95	5	0.4
2.00	50	50	0.4
2.50	10	90	0.4
2.80	10	90	0.4
3.00	95	5	0.5
4.50	95	5	0.5



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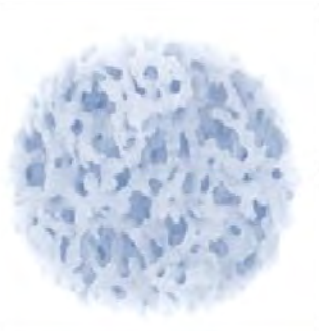
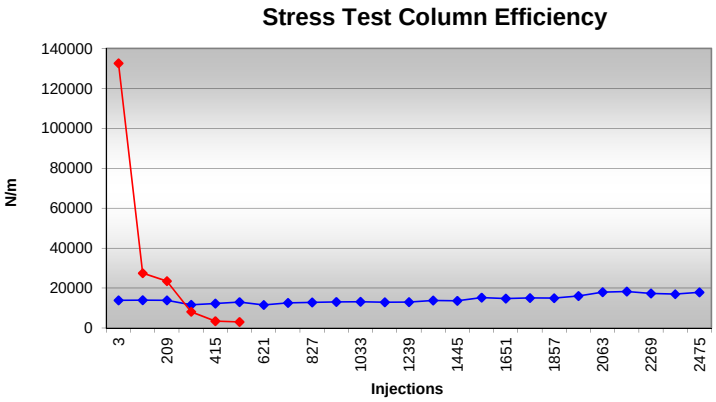
Column lifetime test without sample preparation

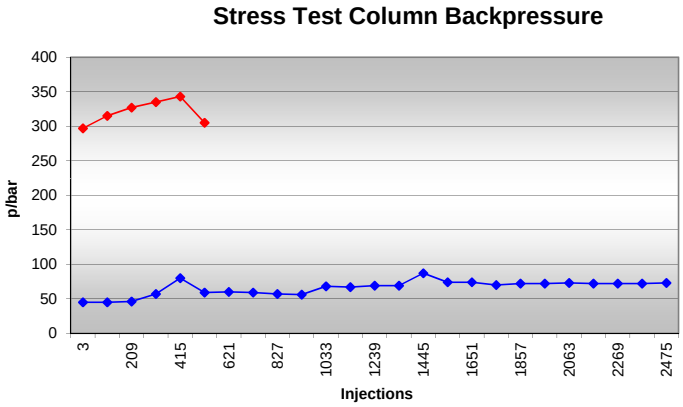


Column lifetime depends on sample preparation and the particle size in the column.
Samples with more complex matrices need intensive sample preparation to avoid column contamination or clogging. For these samples, Chromolith® columns are the best choice to get fast separations with minimum sample preparation, and therefore saves both time and money.

A column lifetime study using Cilengitide infusion solution without sample preparation shows clear lifetime differences in between a sub-2 µm column (ZORBAX® 1.8 µm) and a Chromolith® column – the sub-2 µm column showed a significant drop in column efficiency and an increase in column backpressure after a short amount of time .

Chromolith® HPLC columns are unbeatable in terms of lifetime with "dirty" samples.





Mobile phase:
A: 20 mM Na-Phosphate buffer + 20 mM NaCl pH = 3.6
B: 20 mM Na-Phosphate buffer + 20 mM NaCl pH = 3.6 / Acetonitrile 50/50 v/v

Gradient:	Time/min	%A	%B
	0.0	65	35
	1.3	65	55
	1.5	45	55
	3.0	15	85
	3.1	65	35
	4.0	65	35

Flow rate: 762 µL/min (50-2 mm)
840 µL/min (50-2.1 mm)
Temperature: 50 °C
Detection: UV 215 nm
Injection vol.: 2.5 µL (50-2 mm)
2.8 µL (50-2.1 mm)

Sample: **Cilengitide infusion solution**

Stress test:
3 x Gradient with injection
100 x only injection with A/B 65/35 run time 0.1 min
3 x Gradient without injection (washing)
3 x Gradient with injection
100 x only injection with A/B 65/35 run time 0.1 min
…….etc…….until more than 5000 injections

Chromolith® FastGradient 50-2 mm
ZORBAX® SB C-18 1.8 µm 50-2.1 mm

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www.merckmillipore.com/chromatography
chromatography@merckgroup.com

Determination of matrix-rich samples
Kytta-Balsam® f

UHPLC analysis of samples like creams or balsam requires a significant amount of sample preparation. UHPLC columns packed with small particles are easy to clog due to the very little space between particles, for example, for a sub-2 µm material the between-particle space is only 0.3 µm. Sample preparation is a time consuming step, introduces a lot of errors and is quite costly.
For such samples, a monolithic column like Chromolith® is a much better choice because these columns are more permeable, less prone to clogging and therefore require much less sample preparation.

Kytta-Balsam® f is based on comfrey albums. M2
Comfrey contains allantoin, along with other substances, such as choline, tannins, mucilage, saponins, rosmarinic acid derivatives and pyrrolizidine alkaloids (PAs).
Comfrey albums (?) are used primarily to treat pain and inflammatory conditions as a result of sports injuries (bruises, strains, sprains, hematomas), rheumatic pain, arthritis, muscle and joint pain, tendonitis, back pain, fractures and dislocations, and thrombophlebitis.

Less sample preparation with Chromolith® 2 mm i.d. HPLC columns = significant time and cost savings

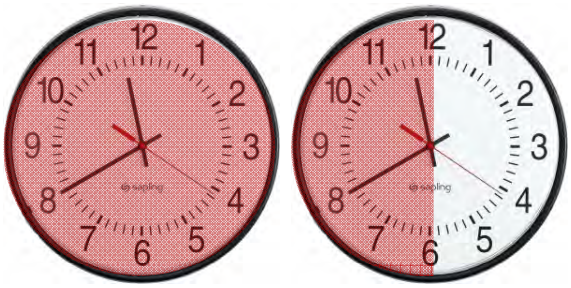
Sample preparation:

2. For ZORBAX® SB C18 1.8 µm
Weight approxymately 0.80 g of Kytta Balsam f in a 50ml volumetric flask, eluate with 5 ml water, make up to the mark with acetonitrile.
The solution is mixed with a megnetic stirrer for one hour and subsequently centrifuged at 4500 U/min (approx. 4000 g) for 10 min. and filtered through 0,2µm into autosampler vial.

1. For Chromolith® FastGradient:
Weight approxymately 0.80 g of Kytta Balsam f in a 50ml volumetric flask, eluate with 5 ml water, make up to the mark with acetonitrile, 15 min. ultra sonic bath, and filled into autosampler vial.

Preparation time:

1h 30 min



25 min

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M2 what is comfrey albums? leaves? ointments?
M203606; 20.02.2013

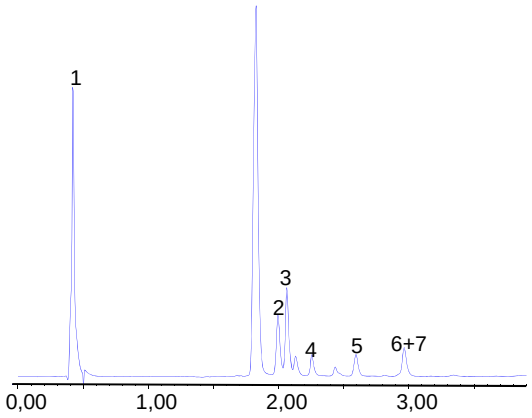
Determination of matrix rich samples
Kytta Balsam f

The UHPLC separation of the prepared sample shows a very similar result with both column Chromolith® or Zorbax 1.8 µm, but the column backpressure is significantly lower using the Chromolith® column.

Chromolith® HPLC columns = significant lower column backpressure for similar chromatographic results

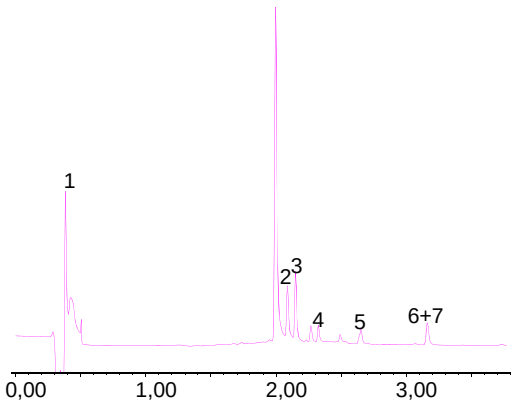
Chromolth® FastGradient RP-18e 50-2mm

Column backpressure: 66 bar



Zorbax SB C18 1.8µm 50-2.1mm

Column backpressure: 339 bar



Mobile phase: **A:** 5 mM Na-Phosphate buffer pH=6
B: Acetonitrile

Gradient:

Time/min	%A	%B
0	100	0
0.1	100	0
1.75	30	70
4	30	70
4.1	100	0
9	100	0

Flow rate: 420µL/min (50-2mm)
463µL/min (50-2.1mm)

Temperature: 30°C

Detection WL-Gradient:

Time/min	WL
0	200
0,4	200
0,5	270
9	270

Injection vol.: 1.9µL (50-2mm) , 2.1µL (50-2.1mm)

Sample: **Kytta Balsam f**

1. Allantoin
2. Phenoxyethanol
3. Methyl-4-hydroxybenzoate
4. Ethyl-4-hydroxybenzoate
5. Propyl-4-hydroxybenzoate
6. n-Butyl-4-hydroxybenzoate
7. iso-Butyl-4-hydroxybenzoate

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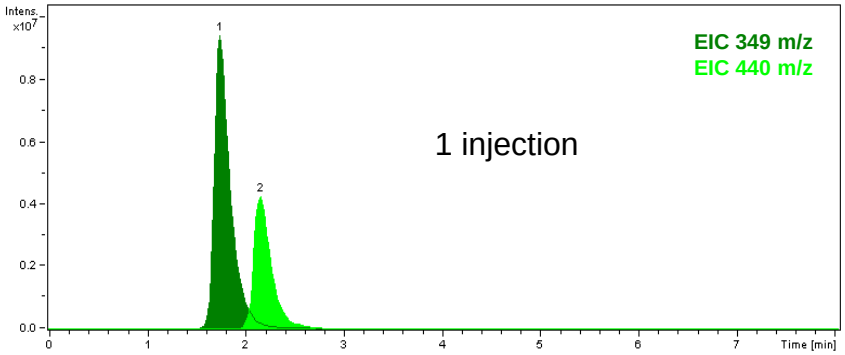
Direct LC-MS analysis of drug and metabolite in serum without sample preparation

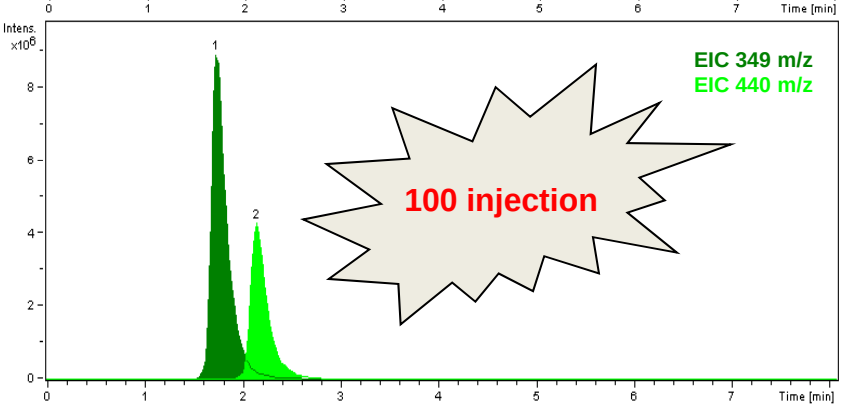
The lifetime of Chromolith monolithic silica columns is typically more than twice that of comparable particle-packed columns, in particular when analyzing matrix-rich samples, such as serum or plasma samples, protein digests or food samples.

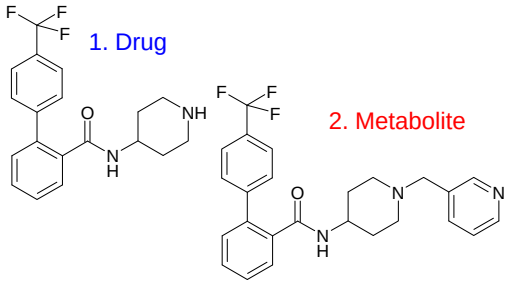
The example in this chart shows the LC-MS analysis of a drug and its metabolite in serum without any sample preparation. The serum sample was simply diluted one to one with water before direct injection into the LC-MS system.

Higher flow rates speed up the overall analysis, giving faster separation, faster washing and shorter re-equilibration times.

Chromolith® HPLC columns = perfectly stable and suitable for LC-MS







Sample:	1. LR 3384 9 µg/ml 2. LR 19370 7 µg/ml in bovine serum, diluted 1:1 with water
Column:	**Chromolith® RP-18e 50-2 mm**
Mobile phase:	A: 0.1% Acetic acid in ACN B: 0.1% Acetic acid in water
Detection.	Bruker esquire 3000 plus
Ionisation	ESI (+)
Nebulizer	50 psi
Dry Gas	10 l/min
Dry Temp	365 °C
Target Mass	400 m/z
Scan	50 – 2000 m/z
ICC	Target: 50.000
Max. Accu Time 50ms	

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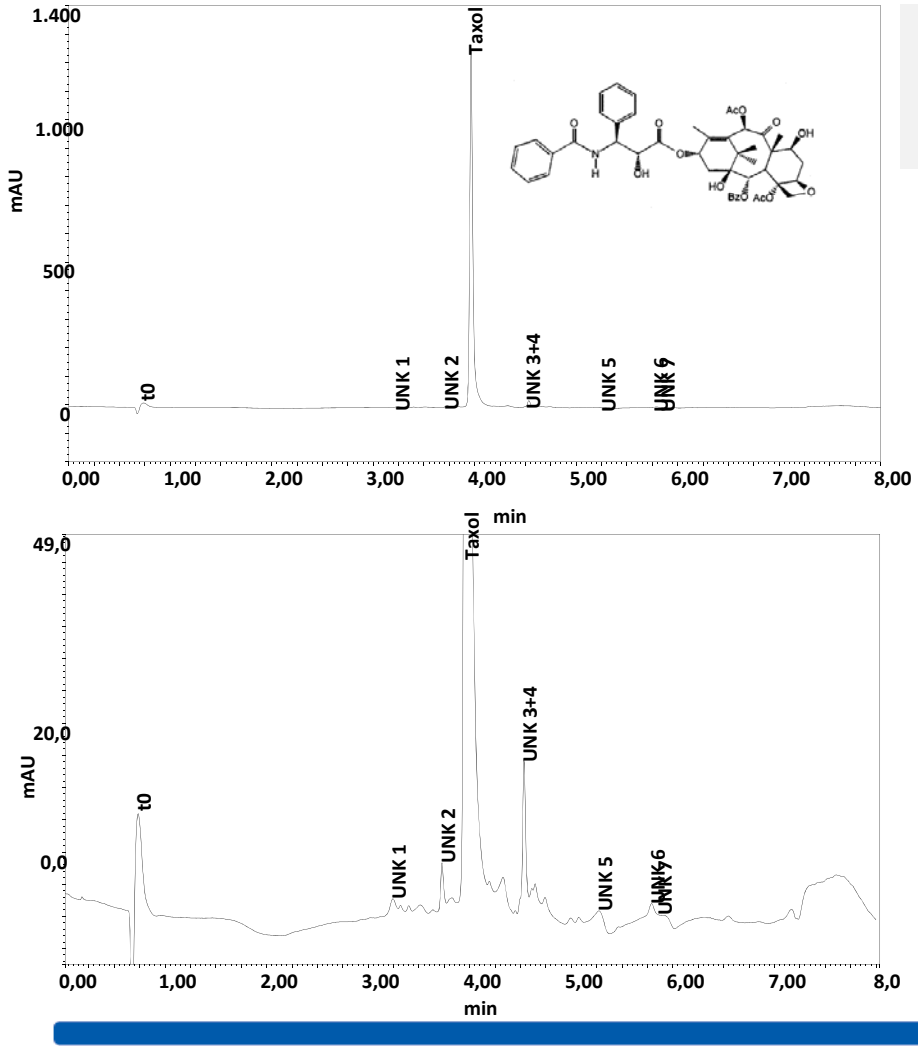
Determination of Taxol with Purospher® STAR Phenyl 2µm

Phenyl HPLC columns show an enhanced selectivity to compared to standard alkyl phases.
They are the first alternative to RP-8 or RP-18 selectivities for separation of aromatic compounds, fatty acids, purines and pyrimidines due to p-p interactions.

Purospher® STAR Phenyl (2µm) UHPLC columns = enhanced selectivity for aromatic compounds



Paclitaxel (**taxol)** is a mitotic inhibitor used in cancer chemotherapy. It was discovered in 1967 by isolation from the bark and needles of the Pacific yew tree, *Taxus brevifolia*.

Purospher® STAR Phenyl 2µm Hibar® HR 50- 2.1 mm

Eluent: A. Acetonitrile
B: Water

Gradient:

Time/min	%A	%B
0	15	85
3.2	85	15
5.6	85	15
5.8	15	85
8	15	85

Flow rate: 271µL/min
Det.: 227nm
Cell: 1.4µL
Temp.: 30° C
Inj.: 1 µL
Backpressure: 154 bar

Sample:

Taxol-Extract from
Taxus breviofolia needles

The right solvent selection

The requirements concerning the performance of a solvent as mobile phase have changed dramatically …

- New detection methods
- Automation of analysis
- New instruments and technical equipment
- New fields of applications
- High degree of authoritative regulations
- New aspects in handling and safety

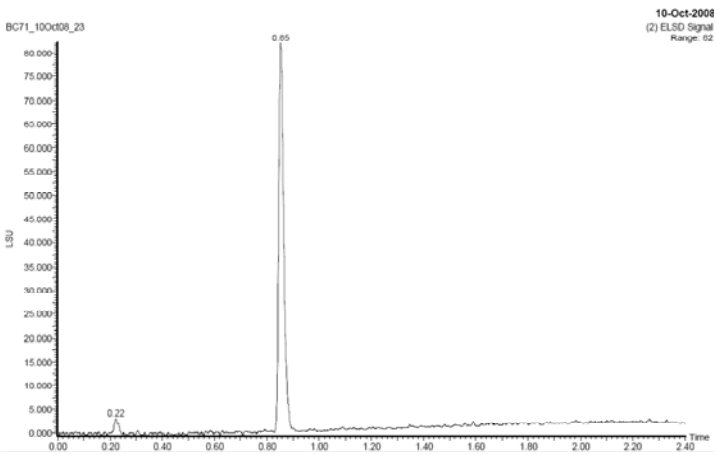
HPLC peak resolution and quantitative results are effected by solvents with lower quality. As important as purity is in a high quality solvent, even consistency is more critical. A solvent that is inconsistent from lot to lot will cause unexpected shifts in results. Even if these anomalies are detected during calibration procedures, they can cause unnecessary confusion and loss of valuable time.

How to meet increasing requirements for UHPLC

UHPLC columns packed with 1.7µm particles tend to block easily, because the space in between the particles is only 0.3µm. Therefore LiChrosolv® gradient grade (hypergrade) solvents are filtered by a 0.2 µm stainless steel filter.

UHPLC Suitability Test (1): ELSD (Evaporative Light Scattering Detector)

Signal: Sarizotan



Condition

Injection: 5 µl

Column: Acquity UPLC BEH C18 1.7µm (2.1x50mm)

Flow rate: 1 ml/min

Eluent A: Acetonitrile LiChrosolv® + 0.1% Formic Acid

Eluent B: Water LiChrosolv® + 0.1% FAC

Gradient: 1.7 min 99% Eluent B to 1% Eluent B

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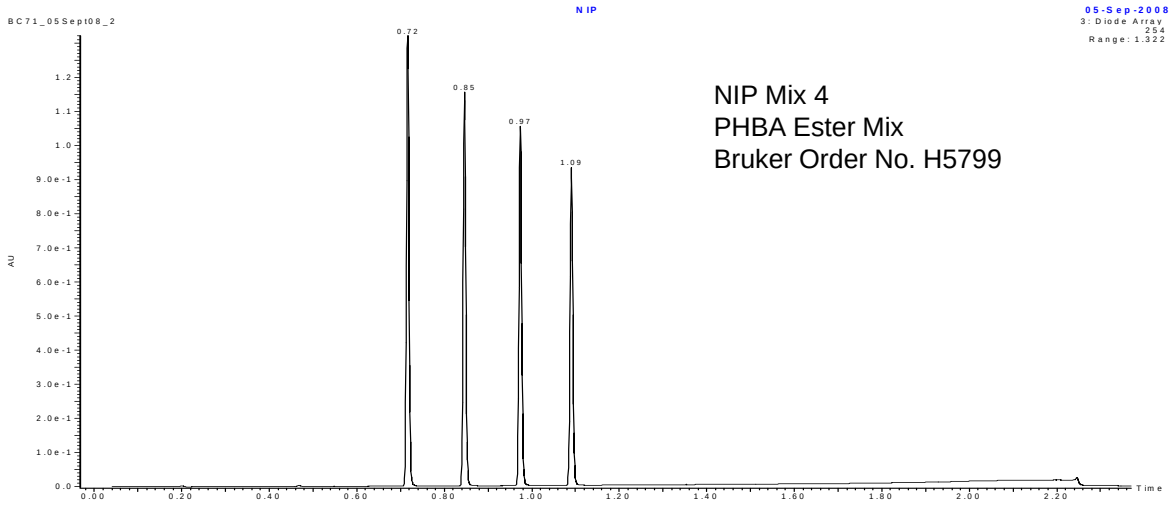
UHPLC Suitability Test (2)

NIP Mix: UV 254nm



NIP Mix 4
PHBA Ester Mix
Bruker Order No. H5799

Time(min)	Flow	Rate	%A	%B
1.	Initial	1.000	1.0	99.0
2.	1.70	1.000	99.0	1.0
3.	2.00	1.000	99.0	1.0
4.	2.01	1.000	1.0	99.0
5.	2.50	1.000	1.0	99.0

Solvent A% : Acetonitrile LiChrosolv®(gradient grade) + 0.1% Formic Acid
Solvent B%: Water LiChrosolv® + 0.1 % Formic Acid

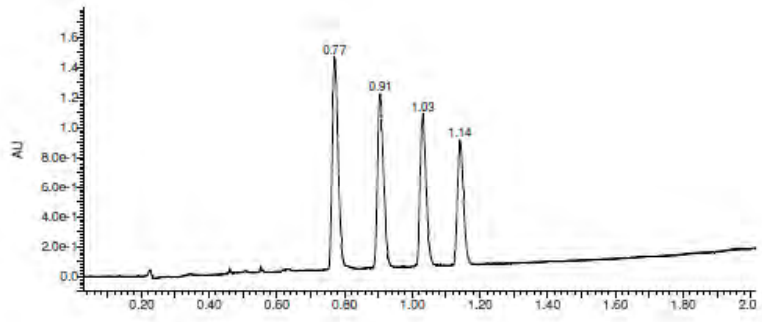
UHPLC suitability of acetonitrile tested under real conditions

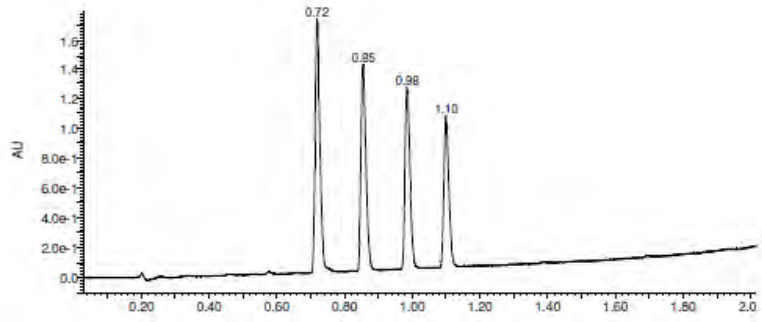
When used for UHPLC in practise there are over 50,000 injections per column possible with the Merck Millipore Acetonitrile hypergrade. This was tested with Acquity UPLC® BEH C18 1.7 µm (2.1x 50 mm) UHPLC columns from Waters with the following high throughput screening method for 50,000 different substances:

time [min]	A [%]	B [%]
0.0	1	99
1.7	99	1
2.0	99	1

flow: 1 ml/min
temperature: 60 °C
A: 99.9% acetonitrile + 0.1% FA
B: 99.9% water + 0.1% FA

The chromatograms show the chromatographic performance regarding the injection of methyl-, ethyl-, n-propyl and n-butyl esters of p-hydroxybenzoic acid into a UHPLC column after 50,000 injections in comparison to a new column.





Conclusions:

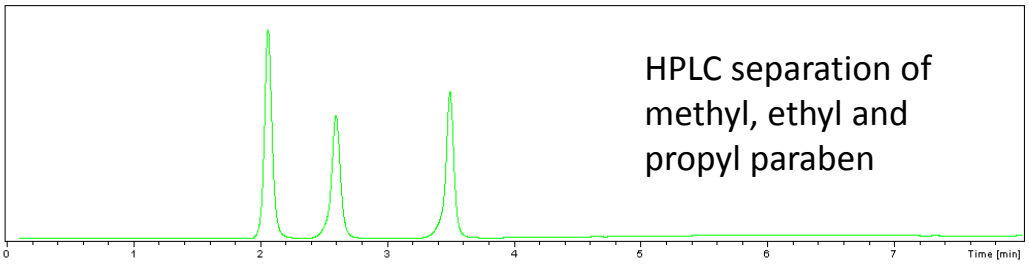
The level of particles in the mobile phase is directly connected to the stability and robustness of the UHPLC system. An increased amount of particles may lead to the blockage of columns, valves and inline filters. A low level of particles in the solvents leads to longer column lifetimes. Merck Millipore has realized a low-particle filling process with a 0.2 µm filtration procedure That meets all the requirements of UHPLC system s on ultrapure solvents.

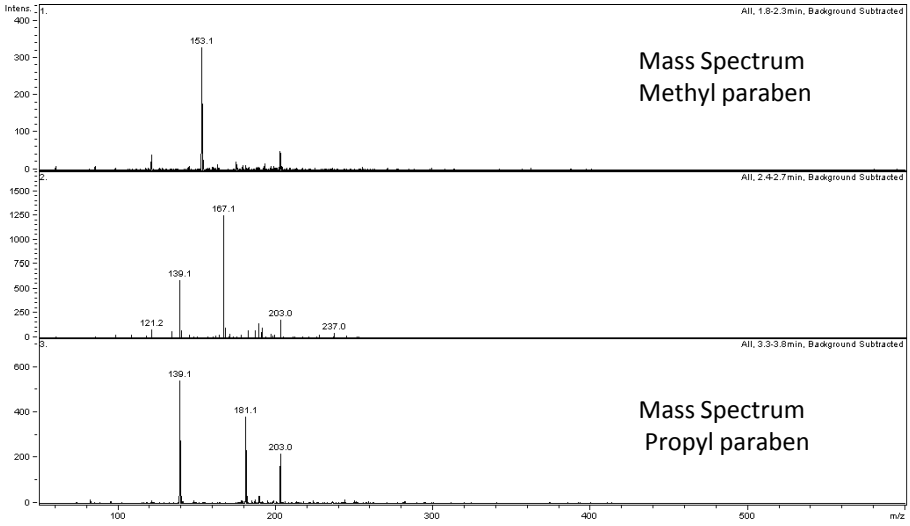
How to meet increasing requirements for LC-MS

With standard solvents and HPLC columns, trace impurities cause unwanted background signals in LC-MS which reduce sensitivity and cause complex spectra and low reproducibility, compared to HPLC with standard UV detectors. Therefore LC-MS requires improved procedures, compared to HPLC with UV detectors.

Criterias for LC-MS:
- High ionisation efficiency
- Simple mass spectrum with low „background“ signals and low adduct formation
- Low „ion suppression“ due to solvents impurities and column „bleeding“
- High reproducibility of the ionisation signal
- Low contamination of the ionisation source

Application: separation of methyl, ethyl and propyl paraben





- Ionisation intensity is low based on high ion suppression due to even low, but mass detectable impurities of unsuitable solvents quality → base peak intensity **< 4 x 10^2**
- Background mass signals appear in the spectra, making interpretation more difficult

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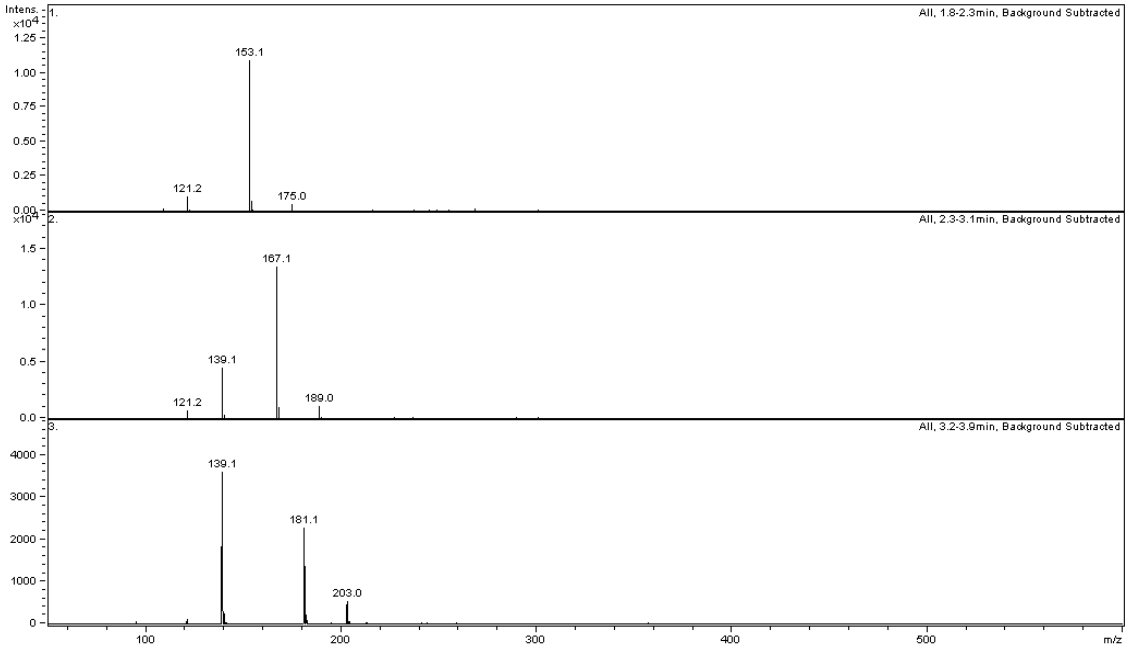
Chromolith® Performance RP-18e 100-3mm with LiChrosolv® Acetonitrile hypergrade



Result:

- Base peak intensity improved 100 times, now > 1 x 10^4
- The combination using an application purified and specified LiChrosolv® hypergrade solvent Acetonitrile or Methanol/Water with a clean, non bleeding Chromolith® RP-18e column gives an optimum LC-MS result

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chromatography@merckgroup.com

Ordering Information for UHPLC Columns and solvents

Purospher® STAR UHPLC columns

Item number	Product name
1.50646.0001	Purospher® STAR RP-18 endcapped 2µm Hibar® HR 50-2.1 UHPLC column
1.50648.0001	Purospher® STAR RP-18 endcapped 2µm Hibar® HR 100-2.1 UHPLC column
1.50649.0001	Purospher® STAR RP-18 endcapped 2µm Hibar® HR 150-2.1 UHPLC column
1.50651.0001	Purospher® STAR RP-18 endcapped 3µm Hibar® HR 50-2.1 UHPLC column
1.50653.0001	Purospher® STAR RP-18 endcapped 3µm Hibar® HR 100-2.1 UHPLC column
1.50654.0001	Purospher® STAR RP-18 endcapped 3µm Hibar® HR 150-2.1 UHPLC column

New selectivities coming soon (June 2013)

Item number	Product name
1.50630.0001	Purospher® STAR RP-8 endcapped (2µm) Hibar® HR 50-2.1 UHPLC column
1.50629.0001	Purospher® STAR RP-8 endcapped (2µm) Hibar® HR 100-2.1 UHPLC column
1.51013.0001	Purospher® STAR Phenyl (2µm) Hibar® HR 50-2.1 UHPLC column
1.51014.0001	Purospher® STAR Phenyl (2µm) Hibar® HR 100-2.1 UHPLC column

Chromolith® columns

Item number	Product name
1.52014.0001	Chromolith® Flash RP-18 endcapped 25-2
1.52007.0001	Chromolith® FastGradient RP-18 endcapped 50-2
1.52006.0001	Chromolith® Performance RP-18 endcapped 100-2

LiChrosolv® Solvents for UHPLC

Item number	Product name
100029	Acetonitrile LC-MS grade LiChrosolv®
100030	Acetonitrile gradient grade LiChrosolv®
106035	Methanol LC-MS grade LiChrosolv®
106007	Methanol gradient grade LiChrosolv®
115333	Water LiChrosolv® for chromatography (enhanced specification also for LC-MS suitability)

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