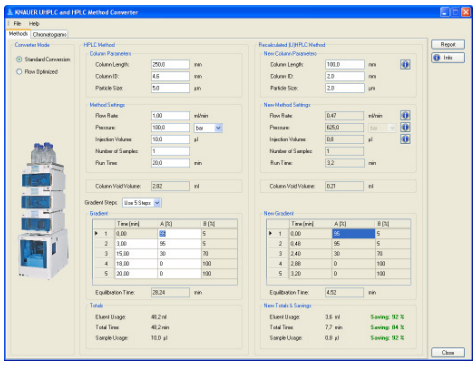
Application Note

► Guidelines for method transfer from HPLC to UHPLC



Category	Ultra High Performance Liquid Chromatography
Matrix	none
Method	HPLC ⟷ UHPLC
Keywords	Method converter, Method Transfer, UHPLC
Analytes	Crude peptides, DNPH aldehydes, phthalates
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Summary

Reducing the particle size, shortening the column length, and increasing the linear velocity of the mobile phase are requirements for successful method transfer from traditional HPLC to UHPLC. Only by applying these utilities the major challenges of most analytical laboratories like fast method development and increased sample throughput can be the way to master the challenges. The availability of HPLC columns with very small particles (< 2 µm) in combination with UHPLC systems opens the new possibilities.
The method transfer can be easily realized by using the KNAUER UHPLC Method Converter software to increase the separation speed and find suitable UHPLC method parameters. With this application note we will share the experiences in HPLC-to-UHPLC method transfer to improve productivity. It is a practical guideline on scaling down separations by taking into account particle size, column ID, and column length.

Introduction

Nowadays high performance liquid chromatography (HPLC) is used in a wide variety of industries and service laboratories as one of the most rugged and reliable techniques for the analysis of organics. Typical fields of application include pharmaceuticals, food, flavors, natural products, environmental contaminants and polymers analyzed in a wide variety of samples.[1] Since the mid 1980-ies, reliable HPLC instrumentation is available that separates complex mixtures on column of typically 250 mm length packed with 5 µm particles, using a pumping system with capacity up to 5000 psi/400 bar. The flow rate is in the range of 0.1 – 10 ml/min. The Van Deemter plot (see fig. 1) indicates the theory of liquid chromatography.[2] In the case of smaller particles not only the separation efficiency increases (lower optimum plate height with smaller particles), but also this optimum is realizable at higher flow rates. Moreover the Van Deemter curve is much more flat at flow rates (or velocities) above the optimum. The same separation efficiency can be achieved on a shorter column when changing to smaller particles. Together with the higher mobile phase velocity this points out the main benefits of sub 2 µm particles used in (U)HPLC. Over the past few years a tremendous interest in approaches to speed up/ and /or increase resolving power of the analytical separation could be recognized. A lot of chromatographers want to transition some of their classical HPLC methods to fast UHPLC methods. With the help of special HPLC calculation software this is no longer depending on the experience of an HPLC user.

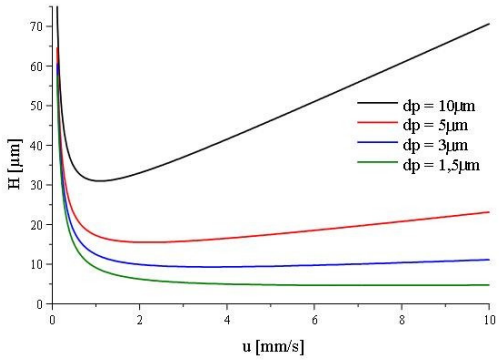


Fig. 1
Van Deemter plot

Improvement in throughput and resolution

As illustrated in table 1 and figure 2 for high throughput separations it is possible to realize equivalent efficiency comparing a 150 mm column packed with 5 µm particles and a 50 mm column packed with sub 2 µm particles. Applying the column replacement a reduction of analysis time by 9-fold is happening. If the column length is not reduced by replacing 5 µm particles with sub 2 µm particles the resolution can be increased. However the particle size reduction generates dramatically higher back pressure, with classical HPLC systems not realizable. But also to benefit from the full potential of sub 2 µm columns, it is highly recommendable to work with a chromatographic system which can realize pressures up to 1000 bar.

Table 1
Comparison high throughput vs. high resolution

	high throughput			high resolution	
column length	150 mm	50 mm	**column length**	150 mm	150 mm
particle size	5 µm	sub 2 µm	**particle size**	5 µm	sub 2 µm
plates	N = const.	N = const.	**plates**	$N_{5\ \mu m}$	$\mathbf{N_{2\ \mu m} = 3xN_{5\ \mu m}}$
run time	$t_{R\ 5\ \mu m}$	$\mathbf{t_{R\ 5\ \mu m}}$ **/ 9**	**run time**	$t_{R\ 5\ \mu m}$	$t_{R\ 5\ \mu m}=t_{R\ 2\ \mu m}$

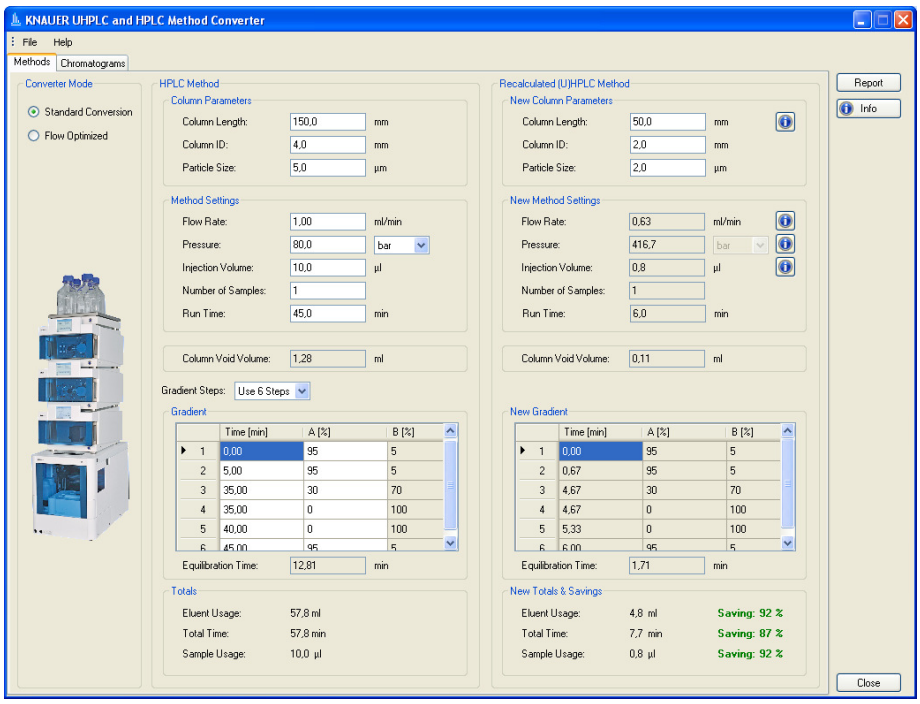


Fig. 2
UHPLC Method Converter

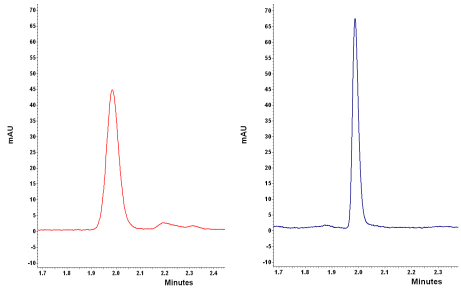
UHPLC instrumentation

To get the full advantage of the sub 2 µm columns, that means to realize the highest plate number, a special adapted HPLC system is needed. The extended pressure capability of the pumping system is only one parameter. A carefull look has to be taken to the system volume by working with reduced column volumes (see table 2). The plate number is directly influenced by peak broadening and can be caused by diffusion of the molecules in the column, by the injection volume and by the dead volume of the (U)HPLC system. It is obvious (see table 2) that small diameter columns reduce the eluent consumption but require small extra column volume, especially focused on detector cell volume, tubing volume and injection volume. The recommended tubing volume should be as small as possible by using short capillaries with small inner diameter (0.12 mm ID) and zero-dead volume fittings are generally preferred. The injection volume should be adapted according to the column dimensions. A recommendation is to maintain the injected volume between 1 – 5% of the column dead volume. A typical column dimension for UHPLC is a 50 x 2 mm column with a volume of 120 µl. The adjusted injection volume should be in the range of 1-5 µl to limit the peak broadening effects. The detector cell volume must be reduced by same sensitivity comparing to classical HPLC and should be ideally in the range of 2-3 µl. But only if the adaptation of data rate up to 100 Hz and time constant is sufficient to detect a suitable amount of data points for very small eluting peaks within the first two minutes. **Due to these statements, the 2 mm ID columns should be considered as optimal for UHPLC operation.**

Table 2

Comparing of system volumes and column volumes

Column type	Column volume	HPLC system volume	UHPLC system volume
250 x 4 mm (5 µm)	~ 85 %	~ 15 %	
50 x 2 mm (sub2µm)	~ 20 %	~ 80 %	~ 15-20 %



UHPLC Method transfer strategy

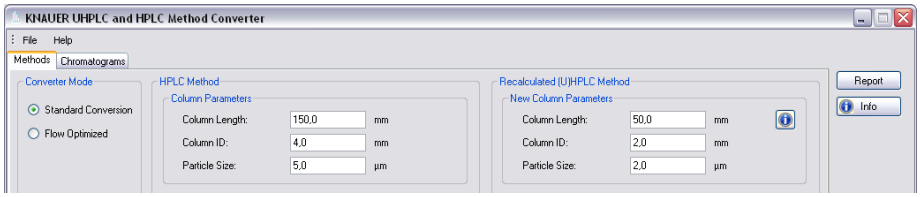
The main focus for UHPLC method adoption is to achieve sufficient resolution in the shortest time. The strategy of the method conversion is to maintain the resolution performance by using shorter columns packed with sub 2 µm particles. The theory for the method transfer is based on chromatographic mechanisms. When performing a scale down procedure, a few calculations can be used to determine equivalent UHPLC conditions. Depending on the supplier, it is possible to find columns dedicated to UHPLC with inner diameter of 1 mm, 2 or 2.1 mm, 3 mm, 4mm and 4.6 mm.

Calculation of column dimensions:

The determination of appropriate column length (ℓ) according to keeping the theoretical plate number constant will maintain the same separation (see formula 1). When decreasing particle size (dp), column length can be shortened without lost of separation power.

$$l_2 = \frac{l_1 \bullet dp_2}{dp_1}$$ Formula 1

As an example from regular 150 x 4 mm, 5 µm column the recalculated new column parameters are 50 x 2 mm with sub 2 µm particle size.



Calculation of injection volume:

UHPLC methods based on sub 2 µm columns are most often transferred to smaller volume columns. The same injection volume will take up a larger portion of the new column and possibly lead to band broadening or potentially overloading effects. It is therefore important to scale down the injection volume to match the chance in the column volume. A simple equation is used to calculate the new injection volume v_2.

$$V_2 = V_1\left(\frac{ID_{C1} \bullet l_2}{ID_{C2} \bullet l_1}\right)$$ Formula 2

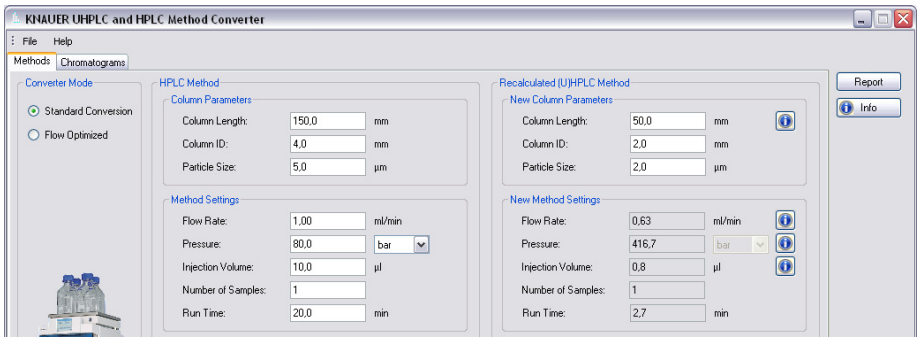
As an example for the 50 x 2 mm column the recommended new injection volume according to formula 2 is 1 µl. The adjusted injection volume can be in the range of 1-5 µl to limit the peak broadening effects.

Calculation of flow rate:

In most cases the column inner diameter decreases from 4.6 or 4 mm to 2 mm and the flow rate needs to be adjusted to keep the linear velocity constant and maintain an equivalent separation. The linear velocity is related to the flow rate, internal diameter of the column and particle size. A simple equation can be derived to calculate the new flow rate (F_2) (see formula 3).

$$F_2 = \left(\frac{ID_{C2}}{ID_{C1}}\right)^2 \bullet \left(\frac{dp_1}{dp_2}\right) \bullet F_1$$

Formula 3



As an example for the 50 x 2 mm column the recalculated flow rate is in the range of 0.6 ml/min for the standard conversion. The activation of the flow optimized mode enables the user to adjust the flow rate higher than the software has calculated. This can be useful if the analysis time should be more reduced and the limit of the column back pressure is reached.

Calculation of analysis time:

The new expected analysis time of the recalculated method is directly proportional to the change in the column dead time and can be calculated according to formula 4.

$$t_2 = t_1 \bullet \frac{F_1}{F_2} \bullet \frac{ID_{C2}}{ID_{C1}} \bullet \frac{L_2}{L_1}$$

Formula 4

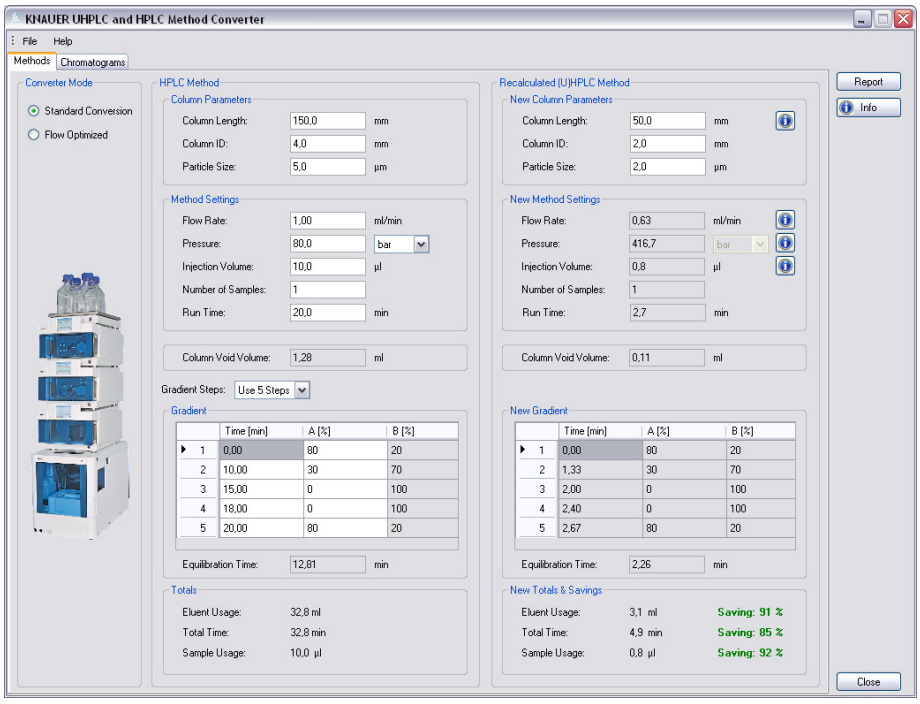
Adjusting gradient profile:

After determination of a proper column dimension a transfer of the gradient according to the new column volume must be realized. So the time program needs to be scaled down to keep the phase interactions constant.

With a simple equation each time segment of the gradient in the new method, including column re-equilibration time can be calculated. The equation takes into consideration the volume of each column, the flow rates and the time segment in the original method (see formula 5).

$$t_{g2} = t_{g1} \bullet \frac{V_{02}}{V_{01}} \bullet \frac{F_1}{F_2}$$ Formula 5

As an example, from a regular 150 x 4.6 mm, 5 µm column to a UHPLC 50 x 2 mm, sub 2 µm column the re-calculated gradient is shown:



Calculation of column void volume V_{0x}:

The void volume of the column is the volume that is not taken by the stationary phase and approximately 68% of the column volume.

Calculation of back pressure:

The new column back pressure is calculated by applying the law of Darcy (see formula 6).

$$\Delta P_2 = \Delta P_1 \bullet \frac{L_2}{L_1} \bullet \frac{dp_1^{\,3}}{dp_2^{\,3}}$$ Formula 6

Calculation of solvent consumption:

Taking into account the change in column inner diameter, particle size and analysis time the solvent savings can be calculated (see formula 7).

$$V_2 = V_1 \bullet \frac{ID_{C2}^{\,2}}{ID_{C1^2}} \bullet \frac{d_{p1}}{d_{p2}} \bullet \frac{t_2}{t_1}$$ Formula 7

Results

On the example of three different applications the method transfer with the KNAUER Method Converter in combination with Smartline and PLATINblue System is demonstrated.

HPLC Method Parameters

Peptides

Column	Eurosil Bioselect C18A, 250 x 4.6 mm, 5 µm		
Eluent A	H_2O (0.1% TFA)		
Eluent B	Eluent A / ACN 40 / 60 (0.1% TFA)		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	100	0
	60.00	0	100
	65.00	0	100
	66.10	100	0
	95.00	100	0
Flow rate	1 ml/min		
Injection volume	20 µl		
Column temperature	40 °C		
System pressure	approx. 100 bar		
Detection	UV at 210 nm (1 Hz)		
Run time	60 min (90 min incl. regeneration)		

UHPLC Method Parameters

Peptides

Column	BlueOrchid C18 A, 100 x 2 mm, 1.8 µm		
Eluent A	H_2O (0.1% TFA)		
Eluent B	Eluent A / ACN 40 / 60 (0.1% TFA)		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	100	0
	10.00	0	100
	11.00	0	100
	11.10	100	0
	15.00	100	0
Flow rate	0.5 ml/min		
Injection volume	5 µl		
Column temperature	40 °C		
System pressure	approx. 550 bar		
Detection	UV at 210 nm (50 Hz)		
Run time	10 min (15 min incl. regeneration)		

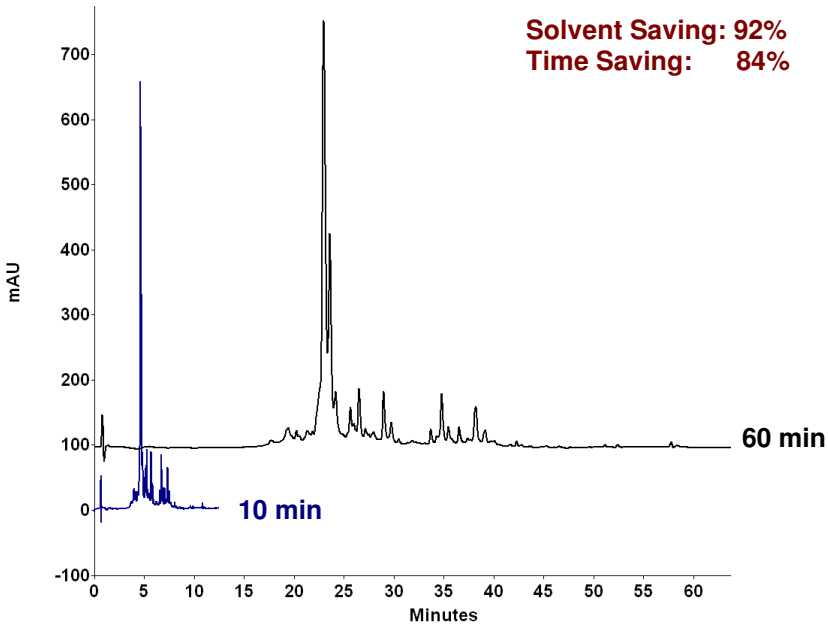


Fig. 3

Separation of crude peptide sample; overlay of HPLC (black) and UHPLC (blue) analysis

HPLC Method Parameters

DNPH aldehydes

Column	Eurospher II C18A, 100 x 3 mm, 3 µm		
Eluent A	H_2O		
Eluent B	ACN		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	60	40
	4.50	45	55
	10.20	0	100
	11.30	0	100
	12.00	60	40
Flow rate	0.8 ml/min		
Injection volume	1 µl		
Column temperature	40 °C		
System pressure	approx. 130 bar		
Detection	UV at 370 nm (5 Hz)		
Run time	11.30 min (15 min incl. regeneration)		

UHPLC Method Parameters

DNPH aldehydes

Column	BlueOrchid C18A, 50 x 2 mm, 1.8 µm		
Eluent A	H_2O		
Eluent B	ACN		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	100	0
	1.50	0	100
	3.40	0	100
	3.50	100	0
	5.00	100	0
Flow rate	0.5 ml/min		
Injection volume	1 µl		
Column temperature	40 °C		
System pressure	approx. 250 bar		
Detection	UV at 370 nm (50 Hz)		
Run time	3 min (5 min incl. regeneration)		

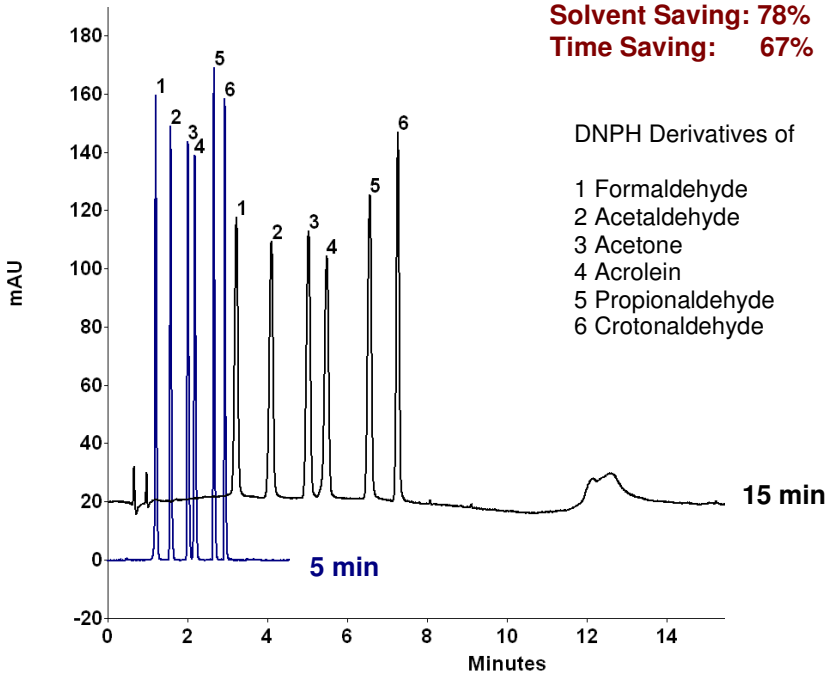


Fig. 4

Separation of DNPH aldehydes; overlay of HPLC (black) and UHPLC (blue) analysis

HPLC Method Parameters

Phthalates

Column	Eurospher II C18H, 250 x 3 mm, 3 µm		
Eluent A	H_2O / ACN 15:85		
Eluent B	ACN		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	100	0
	3.00	100	0
	6.50	0	100
	25.00	0	100
	26.00	100	0
Flow rate	0.6 ml/min		
Injection volume	2 µl		
Column temperature	30 °C		
System pressure	approx. 250 bar		
Detection	UV at 225 nm (5 Hz)		
Run time	25 min (46 min incl. regeneration)		

UHPLC Method Parameters

Phthalates

Column	BlueOrchid C18, 100 x 2 mm, 1.8 µm		
Eluent A	H_2O / ACN 15:85		
Eluent B	ACN		
Gradient	**Time (min)**	**% A**	**% B**
	0.00	100	0
	1.60	100	0
	5.60	0	100
	7.00	0	100
	7.10	100	0
Flow rate	0.4 ml/min		
Injection volume	2 µl		
Column temperature	30 °C		
System pressure	approx. 350 bar		
Detection	UV at 225 nm (50 Hz)		
Run time	7 min (10 min incl. regeneration)		

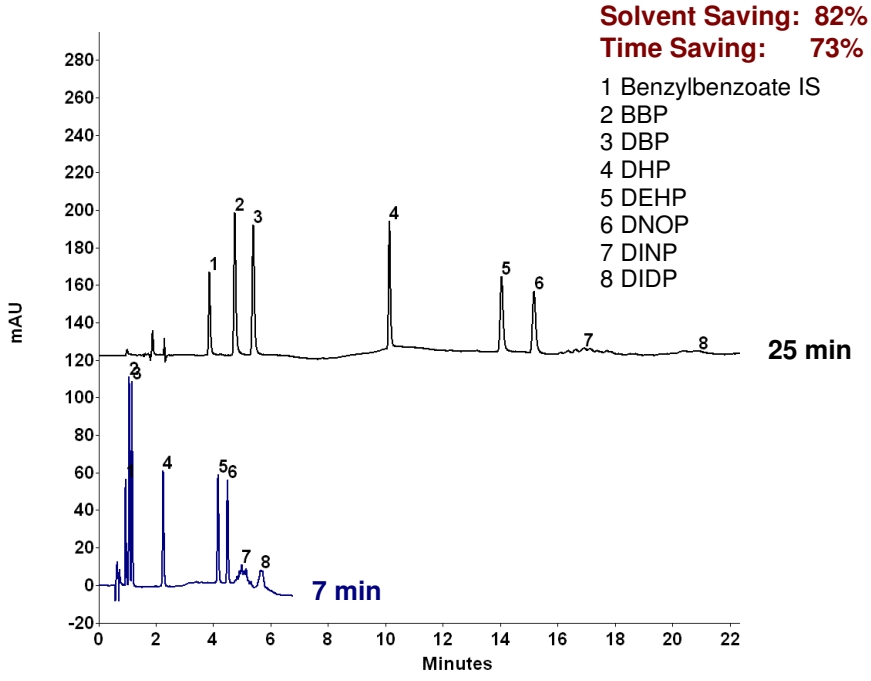


Fig. 5

Separation of phthalate sample; overlay of HPLC (black) and UHPLC (blue) analysis

Conclusion

With the new UHPLC technology the costumer has a powerful tool to improve the chromatographic conditions in terms of throughput and resolution power. The most important factor to obtain a suitable sub 2 µm column performance is the choice of instrumentation. But not only working with HPLC systems that realise higher working pressure than 400 bar is one mandatory. The system must also adapt to realize very low extra-column volumes, limited dwell volumes and a higher data rate than 10 Hz.
The easy method transfer from existing HPLC methods to UHPLC methods is supported by the KNAUER (U)HPLC Method Converter, using basic equations of chromatography. Isocratic runs are more easily to adjust by recalculating only the injection volume and the flow rate. In case of gradient separations additionally gradient time and slope must be adapted. Therefore the dwell volume of the system should be carefully considered.
Some rules for the method development in UHPLC are slightly different comparing to classical HPLC. It has to be taken into account that the back pressure of UHPLC columns packed with sub 2 µm particles is quite high. Temperature gradients inside of the columns can influence the chromatographic separation. To minimize this effect acetonitrile has to be the first choice because of lower viscosity compared with methanol. It can always be beneficial to work at higher temperatures (40 °C).

References

[1] J.W. Dolan, L.R.Snyder, *J. Chromatogr. A* **1998**, 799, 21-34.

[2] A.P.Schellinger, P.W.Carr, *J. Chromatogr. A* **2005**, 1077, 110-119.

[3] D.Guillarme, J.-L. Veuthey, V.Meyer, LC-GC Europe, **2008**, 322-327

Physical Properties of recommended BlueOrchid Columns

BlueOrchid UHPLC columns introduce a new level in ultra high performance LC. The high purity BlueOrchid phases promise faster separations with improved resolution. Outstanding peak symmetry even for basic compounds is realizeable as well as ultra fast separations with superior efficiency, sensitivity and resolution. Narrow particle size distribution minimizes the column back pressure within the range of conventional HPLC systems.

Stationary phase	C18, C18A, C8, PFP, Phenyl, CN, C4, Si
USP code	depending from modification
Particle size	1.8 µm
Form	spherical
pH range	2-8
% C	depending from modification
Endcapping	yes (depending from modification)
Dimensions	150 x 2 mm, 100 x 2 mm, 50 x 2 mm

Recommended Instrumentation

UHPLC applications require the PLATINblue binary gradient UHPLC system equipped with degasser, autosampler, column oven, and diode array detector. Other configurations are also available. Please contact KNAUER to configure a system that's perfect for your needs.

Description	**Order No.**
PLATINblue UHPLC-System	A69420
PLATINblue Pump P-1	
PLATINblue Pump P-1 with Degasser	
PLATINblue Autosampler AS-1	
PLATINblue Column Thermostat T-1 Basic	
PLATINblue Detector PDA-1	
PDA-1 flow cell (10 mm, 2 µl)	
PLATINblue CG Data system	
PLATINblue CG spectra license	
PLATINblue UHPLC method converter	
PLATINblue stainless steel capillary kit	

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