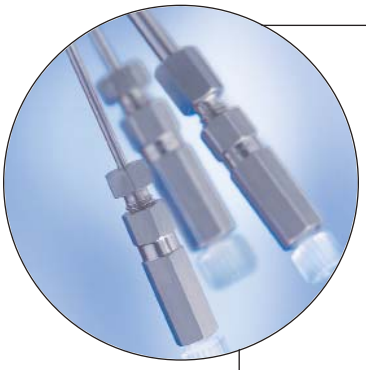
Performance characteristics of the Agilent 1100 Series capillary LC system using diode-array UV and MS for detection

Technical Note



Abstract

Capillary LC is the method of choice, when sample volumes are limited or/and higher sensitivity is essential. A LC capillary/MS combination fulfills these requirements and is a typical solution in the biopharmaceutical research environment. This Technical Note describes the excellent performance of the Agilent 1100 Series capillary LC system using a diode-array detection system and an Agilent 1100 Series MSD Ion Trap as detectors. The system offers robustness, easy set-up and optimized control and data handling software.

This application has been verified using an Agilent 1200 Series LC system, and showed comparable or even better performance.

Agilent Technologies

Introduction

The combination of capillary LC using mass spectrometers and UV diode-array detectors is a technique, which is being more and more applied in bio-analytical work to analyze peptides and proteins. Furthermore, capillary LC is also frequently used for the analysis of drugs and its metabolites. The reason is that in many cases it is necessary to gain sensitivity when sample volume is limited or if the sample concentration is very low. Columns with smaller internal diameter offer the benefit of increased sensitivity, since sensitivity or limit of detection varies inversely with column diameter. The effect can be understood by scaling down of volumes, in this case peak volumes, while keeping the mass constant. Thus, compounds are eluted in smaller volume, resulting in an increase of peak concentration, an increase in peaks height and consequently better signal-to-noise ratio. Table 1 indicates the theoretical gain in limit of detection of lower internal diameter columns compared to 4.6-mm id columns.

Figure 1 is an example, showing the influence of decreased column diameter on the limit of detection. The same amount of sample was injected on two different columns. A comparison was made between a microbore column with an internal diameter of 1.0 mm and a capillary column with an id of 0.3 mm. The limit of detection is better by a factor 10 to 28 on the 0.3-mm id capillary for the three peptides analyzed.

Column id (mm)	Factor for increase in sensitivity
4.6 to 1.0	21.2
4.6 to 0.3	235.0
4.6 to 0.05	8816.0

Table 1
Internal diameter vs. gain in limit of detection

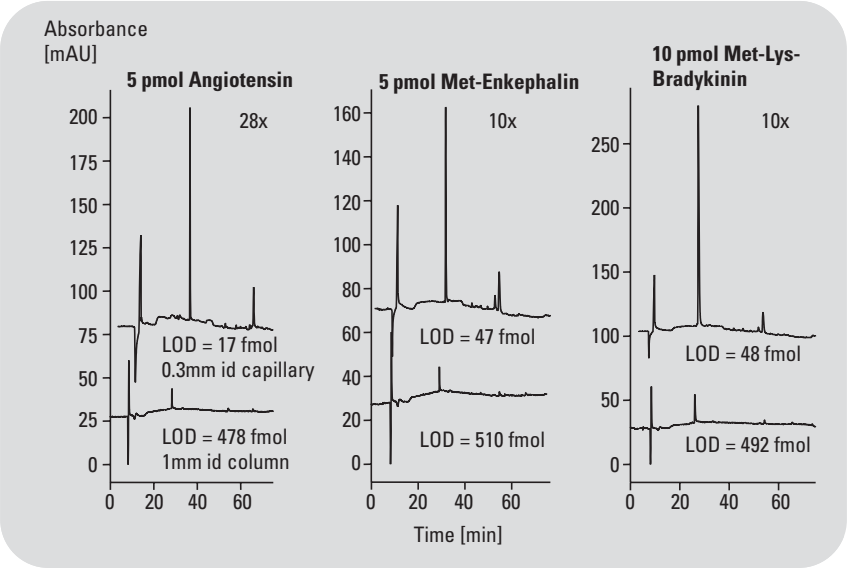


Figure 1
Analysis of three peptides on a 1-mm id column and a capillary column of 0.3-mm id. The limit of detection is better by a factor 10 to 28 on the capillary compared to the 1-mm id column.

Chromatographic conditions

Solvent:	A = H_2O + 0.01 % formic acid
	B = ACN + 0.009 % formic acid
Capillary Pump gradient:	5 min 5 % B, 60 min 60 % B, 75 min 60 % B
Stop time:	75 min;
Post time:	15 min
Flow:	Capillary column 4 µl/min, 1 mm column 30 µl/min
Column:	ZORBAX 300SB-C18, 0.3 x 150 mm, 3.5 µm
	ZORBAX 300SB-C18, 1.0 x 150 mm, 3.5 µm
Pre-Column:	ZORBAX 300SB-C18, 0.5 x 3.5 mm, 3.5 µm
Column temp.:	25 °C
Pre-Column switch:	5 min
Injection volume:	1 µl
DAD:	210/8 nm (ref.: 360/100 nm)

Conditions for the 1-mm id column

Flow:	30 µl/min, 100 µl sensor for the capillary system

Although this improvement in limit of detection has been known for years, capillary LC was not frequently used due to problems regarding robustness and handling of the instrumentation. Recently capillary instruments, that have overcome these problems have become available commerically. One of the most robust and most easy to handle instruments is the Agilent 1100 Series capillary LC system. Since its introduction two years ago, more than 300 systems have been sold worldwide and feedback regarding robustness, performance and handling is very positive. Besides capillary LC for flow rates from 1 to 100 µl/min, nano LC for flow rates <1 µl/min is also gaining more and more interest for analysis of peptides and proteins using MS and MS^n as detection system. The Agilent Nanoflow Proteomics Solution covers a flow rate range from 0.01 – 1 µl/min (Agilent publication number 5988-3621EN). The nanoflow solvent delivery system combined with the nanospray nebulizer offers additional sensitivity advantages for Agilent mass spectrometers.

This Technical Note describes the excellent performance of the Agilent 1100 Series capillary LC system using 300- and 500-µm id columns. This includes:

- precision of retention times,
- gradient mixing performance,
- system delay volume,
- precision of areas with injection volumes in the nl and µl range,
- injection volume linearity,
- minimum accessible sample volume,
- carry-over with MS Ion Trap, and
- limit of detection using the diode-array detector, MS Ion Trap.

We also describe the expected performance, if the Agilent 1100 Series capillary LC system is modified to tolerate flow rates above 0.1 ml/min and up to a maximum of 2.5 ml/min.

Instrumentation

The Agilent 1100 Series capillary LC system can be used in normal mode and capillary mode. In normal mode flow rates above 100 µl/min up to 200 µl/min can be used. In this mode the electronic flow control is bypassed with a capillary. In addition, the 1100 Series capillary LC system can be modified to even tolerate flow rates up to 2.5 ml/min. This upgrade involves change of flow capillaries, change of UV detector cell and installation of the standard purge valve. In the capillary mode column flow rates from 1 up to 100 µl can be set using two different flow sensors:

- The 20-µl sensor is used for a flow rate range between 1 – 20 µl/min, for columns with internal diameters < 800 µm
- The 100-µl sensor is used for a flow rate range between 20 up to 100 µl/min, for columns with internal diameter ≤ 1 mm - 2 mm columns can be also used, if flow rates ≤ 100 µl/min are applicable.
- The primary flow rate, which determines the flow rate of the pump before the flow splitting point can be varied between three flow rate ranges:
  - low solvent consumption — 200 to 500 µl/min
  - medium solvent consumption — 500 to 800 µl/min
  - high solvent consumption — 800 to 1300 µl/min for rapid gradient changes

This flow measurement principle ensures highly precise retention times and robustness.

The Agilent 1100 Series capillary LC system used included:

- Micro vacuum degasser with an internal volume of 1 ml for fast solvent changeover.
- High-pressure capillary pump offering stable flow rates in the low µl range.
- Micro well-plate sampler for precise injections in the nl and µl range. A sample cooling device was used to avoid sample decomposition.
- Thermostatted column compartment for stable retention times at any temperature between 10 degrees below ambient and 80 °C.
- Diode-array detector with a 500 nl cell for sensitive analysis of small peak elution volumes.
- MSD/SL Ion Trap with optimized capillary sprayer for enhanced sensitivity and MS^n capabilities.
- Modification kit to upgrade for 2.5 ml/min flow rate.

Results and Discussion

Precision of retention times

Figure 2 shows the analysis of peptides. 1.4 µl of a HPLC peptide standard containing 5 ng/µl each were injected. The standard includes small molecules and molecules above MW= 500. The precision of retention times is typically < 0.5 % RSD over the complete range. The delay volume of the autosampler was bypassed after the sample had reached the top of the column. This saves run and cycle time.

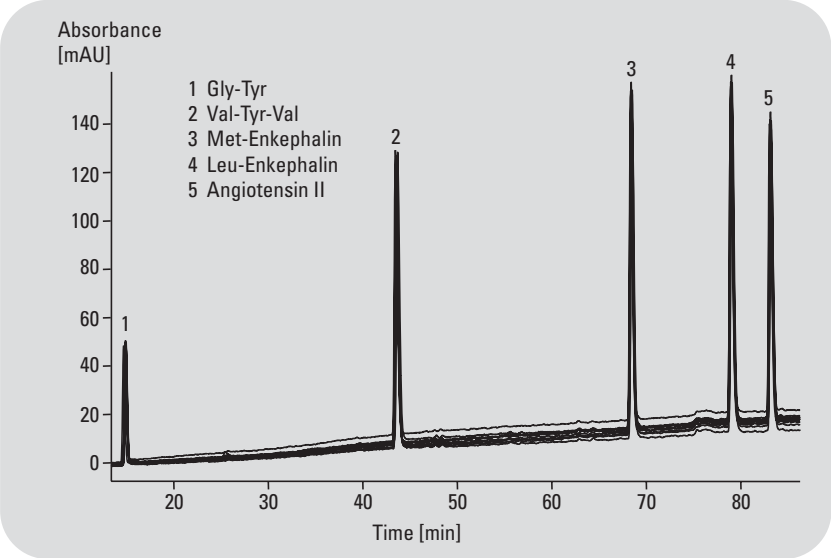


Figure 2
Precision of retention times

Impact of backpressure fluctuations on retention times

The Agilent 1100 Series capillary pump uses proven microbore high pressure mixing technology – the best way to generate both precise shallow as well as rapid gradients for screening applications.
Flow rates down to 1 µl/min are delivered using an active splitting mode, the so-called electronic flow control (EFC), which involves a flow sensor together with an electromagnetic proportioning valve (EMPV). The column flow is actively measured and kept stable even at changing pressures. This becomes important when the column back-pressure increases, due to sample particles which may partly block column frits (figure 3).

In the flow sensor the column flow is measured precisely and the flow sensor signal triggers the electromagnetic proportioning valve (EMPV), which acts as an active splitter system. Because the regulation process is based on heat capacities each solvent mix needs its individual calibration curve, which is available from

Chromatographic conditions

Sample:	Sigma HPLC Peptide Standard H-2016
Column:	0.3 x 250 mm, ZORBAX 300SB-C18, 5 µm, 300 Å
Solvent A:	0.05 % TFA in water
Solvent B:	0.045 % TFA in acetonitrile
Mixer:	420 ml standard mixer
Flow rate:	200 µL/min main, 5.5 µL/min column flow
Temp.:	30°C
Detection:	206/10 nm, 450/80 nm, 500 µL / 10 mm DAD flow cell
Pump piston stroke:	20 µL

Pump gradient

0 min,	1 %B
120 min,	31 %B
130 min,	85 %B
132 min,	85 %B
140 min,	1 %B
175 min,	1 %B

Injector program for bypassing the autosampler delay volume after sample load onto the column

Draw
Inject
Wait 2 min
Valve bypass
Wait 168 min
Valve mainpass

the ChemStation or the control module for most common solvents and solvents mixtures.

Gradient performance – composition accuracy, precision and mixing noise

To perform highly reproducible separations based on gradient runs it is important that the pump mixes the solvents accurately and precisely. To determine the composition accuracy, precision and mixing noise of the capillary pump, step gradients with water and a water/acetone tracer were used. As detector, a diode-array detector was used and sample wavelengths at 265 nm were selected. A restriction capillary replaced the column. The experiments were performed for a flow rate of 4 µl/min.

Different instrument configurations were tested changing mixer, injector parameters and the primary flow rate range. For the experiments all instrument configurations were tested for step height accuracy, step height precision, step mixing noise and delay volume.

Step height accuracy

The way step height accuracy is calculated here indicates whether the gradient provides the correct % of the binary gradient.

Step height accuracy calculation

The height of the 100 % B (tracer) is measured and the chromatogram is rescaled from 0 % to 100 % B. For each step, the differences between the theoretical and the measured step height is determined as percentage

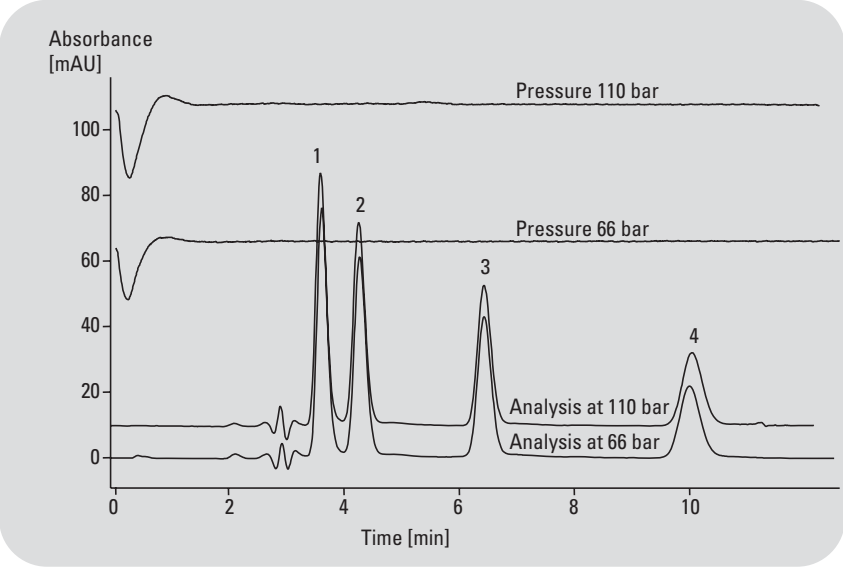


Figure 3
Analysis of phthalates, biphenyl and o-terphenyl at different pressure values: 66 respectively 110 bar. An important criterion is the flow stability for different pressure levels indicated by stable retention time data.

Chromatographic conditions

Sample:	(1) methylphthalate-, (2) ethylphthalate and (3) biphenyl o-terphenyl (4)
Column:	ZORBAX Eclipse SB-C18, 150 x 0.5 mm, 5 µm
Flow rate:	0.01 ml/min
Mobile phases:	Water/ Acetonitrile = 30/70
Injection volume:	200 nl
Column temperature:	30 °C
Diode array detector wavelength:	225/20 nm, ref 550/100 nm

Step height precision

This is an indication whether the gradient formation is the same for several experiments performed under the same conditions.

Step height precision calculation

This calculation is based on several step gradient experiments, using the same chromatographic conditions. For each step the step height accuracy is determined for at least three runs. From these values the average value and the standard deviation for each step are calculated.

Step mixing noise

An indication on how complete mobile phases are mixed for an isocratic step. Theoretically, if solvents are mixed 100 %, no noise should be observed.

Mixing noise calculation

The peak-to-peak noise in absorbance units for several steps is measured and percentage related to a 100 % absorbance step is calculated for each step.

In figure 4 the overlay of three consecutive step gradients are shown for 4-µl/min flow rate. The step gradient started at 0 % tracer – in 1 % steps the tracer percentage was increased up to 10 %. This is the most critical performance range is for every pump. The injection valve was switched into the bypass mode after 1 min to exclude the well plate sampler volume from the system delay volume. It is good practice to shorten cycle times in capillary LC. The primary flow rate was set to low solvent consumption and the column flow rate to 4 µl/min.

- Accuracy of step height for steps from 1 to 10 % in 1 % steps has an average deviation of 0.036 to 0.066 %, close to theoretical values.
- The step height precision for 1 % steps between 0 to 10 % tracer typically shows a standard deviation of 0.024-0.041 %.
- Mixing noise was typically < 0.027 % with autosampler in mainpass, 420 ml mixer installed and low solvent consumption.
- Mixing noise was typically < 0.064 % with autosampler in bypass, no mixer and high solvent consumption

Table 2 provides a short overview of the parameters measured , calculation procedures and results.

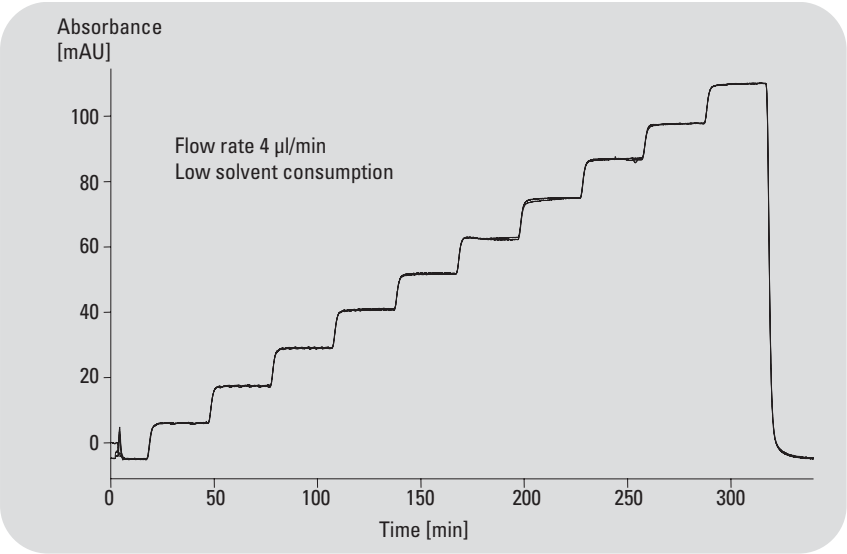


Figure 4
Composition accuracy and precision, mixing noise, overlay of 3 runs. Gradient from 0 to 10 % in steps of 1 %.

Chromatographic conditions

Column flow:	4 µl/min
Mobile phase:	A = water, B=water + 0.5% acetone
Gradient:	from 0 to 10 % tracer in 1% steps – each step was held for 30 minutes, backpressure: approx. 100 bar for 4 µl/min
Compressibility:	46 x 10^6 bar for water as mobile phase
Pump piston stroke:	20 µl
Run time:	340 min
DAD signal:	267/10nm, ref. 360/100nm
Switch of injection valve in bypass:	after approx. 1 min

Parameter to be measured	Principle of measurement	Calculation procedure	Result
Accuracy of binary gradient	1 % step height accuray from tracer experiment for 0 to 10 % in 1 % steps	1. Calculation of deviation from theoretical value in mAU units 2. Calculation of deviation in percentage relative to a 100 % step	Typically the average deviation for 1 % steps is in the 0.036 to 0.066 % range
Gradient precision	Standard deviation of step heights in absorbance units for at least 3 tracer experiments	1. Measurement of step heights accuracy for at least 3 tracer experiments for steps from 0 to 10 % tracer in 1 % steps 2. Standard deviation of the average deviation for at least 3 experiments	The standard deviation for 1 % steps is typically in the range of 0.024 to 0.041%
Performance of isocratic solvent mixing	Mixing noise on different steps of a tracer experiment from 0 to 10 % tracer is measured	Peak-to-peak noise is measured for several steps in absorbance units and percentage to a 100 % absorbance step is calculated	Mixing noise with mixer typically < 0.027 % Mixing noise without mixer typically < 0.064 %

Table 2
Performance of high pressure gradient Agilent 1100 Series capillary pump

System delay volume

The lower the flow rates the more important the system delay volume. For a capillary system small delay volumes between point of injection and column head, column end and detector cell are of utmost importance for low peak dispersion. Also, the detector cell should have a low volume. The delay volumes for the 1100 Series capillary LC system are 0.08 µl before and after the column. The internal volume of the detector is 500 nl. The complete system delay volume is about 600 µl.

The delay volume indicates the internal volume of an LC system, which has to be swept until a gradient change can reach the top of the column.

In the following delay time is calculated instead of delay volume. This is due to the fact that the primary flow is split and exact delay volume calculation is not practical.

Delay time calculation

A step gradient is programmed and the time needed to see this change in the detector is measured. The time when the gradient change is seen by the detector was selected to be approximately 10 times the noise. For these experiments no column is used.

Using gradient analysis the delay volume between start of gradient and reaching the top of the column is the key factor for decreasing cycle times and consequently saving labor time. The system delay time for the 1100 Series capillary LC system was tested using tracer experiments with 4- µl/min flow rate and different instrument configurations (table 3). The last configuration is the standard configuration, which is best suited for lowest carry over. Here the autosampler delay volume stays in the flow path during the complete run time and the delay time is 16 min. This configuration should be used when highly concentrated samples (mg/ml) are injected. For all other applications switching the micro well-plate sampler in the bypass mode can significantly reduce the delay volume. The delay volume of the autosampler is purged with the set column flow after the flow splitting point in the electromagnetic proportioning valve. Switching the injection valve into the bypass mode saves 300 µl delay volume of the autosampler and is the main step to reduce the system delay volume. The delay time is reduced by 50 %. To further reduce the delay time the primary flow rate was increased to its maximum. This means the delay volume in front of the splitting point is flushed more rapidly and the delay time is down to 5.7 min. To obtain lowest delay times the standard mixer, which is located before the splitting point of the primary flow was removed to save 420 µl delay volume and to reduce the delay time down to 5.4. As a result it can be stated that one of the most important parameters to reduce delay time is bypassing the autosampler delay volume, which is situated after the split point of the primary flow.

Precision of areas

Precision of peak areas depends mainly on the precision of injection volume and is an important characteristic that influences quantification. Precision deteriorates at low volumes and should therefore be measured over the complete injection volume range. In our example precision was tested for different injection volumes using diazepam as sample. Different sample concentrations were used to avoid precision difference due to concentration differences. The chromatographic conditions involved a gradient analysis using water and acetonitrile (ACN) as mobile phases. The gradient started at 5 % ACN and was increased to 80 % ACN in 15 min. The column flow rate was 5.5 µl/min and the detection wavelength was 254/10 nm. A 0.3 × 75 mm ZORBAX 300SB C-8, 3.5 µm column was used. For more details refer to chromatographic conditions of figure 5b.

Instrument configuration	Delay time	Recommended usage
Bypass, high solvent consumption, no 420 µl standard mixer	5.4 min	Short cycle time is an issue.
Bypass, high solvent consumption	5.7 min	Short cycle time is an issue, optimum mixing required.
Autosampler in bypass mode, low solvent consumption	7.9 min	Protein, peptide, drug applications.
Autosampler in mainpass, low solvent consumption	16.0 min	Lowest carry over is needed when highly concentrated samples (mg/ml) are injected.

Table 3
Delay times at different instrument configuration. The column flow rate is 4 µl/min.

The following injection volumes were injected 6 times each: 0.03 µl, 0.05 µl, 0.1 µl, 0.5 µl, 1 µl, 5 µl, 8 µl.

The 1100 Series well-plate micro sampler has two different injection loop capillaries for two different injection volume ranges

- up to 8 µl and for a practical injection volume range from 30 nl to 8 µl
- up to 40 µl for a practical injection volume range from 200 nl to 40 µl (for precision data see figure 9)

Table 4 summarizes the results for different injection volumes using the 8-µl loop capillary.

It is quite obvious that between 30- and 50 nl injection volume precision of areas can typically be expected < 10 %. For injection volumes below 500 nl the precision of areas is between 2 and 3 %. From 500 nl upwards the precision of areas is typically ≤ 1 %.

Injection volume of diazepam [µl]	RSD retention times [%] (n=6 each)	RSD areas [%] (n=6 each)	Amount of injected diazepam sample [ng]
0.03	0.07	8.4	0.339
0.05	0.14	2.2	0.565
0.1	0.1	1.6	1.13
0.5	0.07	0.3	5.65
1	0.34	1	1.13
5	0.1	0.2	5.65
8	0.06	0.7	0.904

Table 4
Precision of areas at different injection volumes

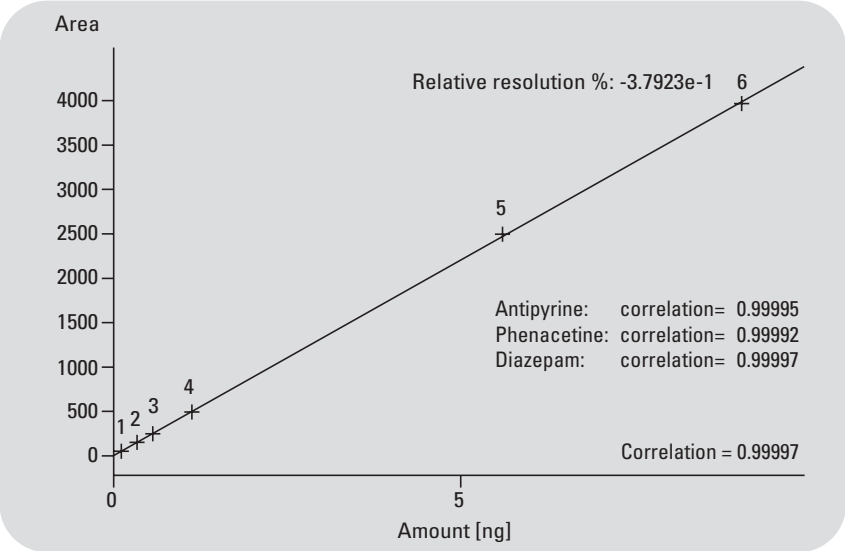


Figure 5a
Injection volume linearity from 0.1 µl up to 8 µl – as an example the linearity curve for diazepam is shown.

Injection volume linearity

Injection volume linearity is another important parameter in chromatography if the injection volume has to be varied to compensate for different sample concentrations. The 1100 Series micro well plate sampler, as mentioned before, has two practical injection volume ranges, from 30 nl to 8000 nl and from 200 nl to 40 µl. Since the main topic of this Technical Note is capillary LC, we have evaluated the injection volume linearity from 0.1 µl up to 8 µl.

Linearity can be expressed in different ways. In figure 5a the correlation between "Area" and "Amount" is evaluated. The correlation is > 0.99991 for the three drugs. The other, more common method is to evaluate the response factor against the injection volume and to determine the range of deviation of the resulting data points (figure 5b). The relative standard deviation of the response factors is in the range of 2 % over the tested injection volume range for diazepam. For the other two compounds the relative standard deviation for the response factors is in the 4 % range.

Minimum accessible sample volume
Especially when analyzing proteins or compounds in biological fluids, sample volume may be limited. In this case it is advantageous, if the micro well-plate sampler is able to draw nearly the complete volume of very low sample volumes. One valuable feature in this case is the ability to find the bottom of a vial or a well. Having found the well or vial bottom the draw position of the needle should be adjustable to have access even to very small amounts of sample. For our experiments we chose 2 different vial types: a 300-µl conical plastic vial and a 200-µl conical plastic insert with polymer feet for 2-ml glass vials. These polymer feet act as shock absorbers. This allows nearly all sample volume to be withdrawn. For these inserts the default settings of the 1100 Series micro well-plate sampler might not be appropriate. To be sure that the draw position of the needle is correct, it is recommended to test it with a standard solution with known concentration. Otherwise, it may happen that the needle is too close to the bottom and the selected injection volume is not drawn completely. For the micro plastic vial the "well/vial bottom sensing" function should be selected and the draw position for the needle should be set to -0.8 mm. Using plastic vials is of advantage if peptide and/or proteins are analyzed, because these compounds tend to adsorb on glass.

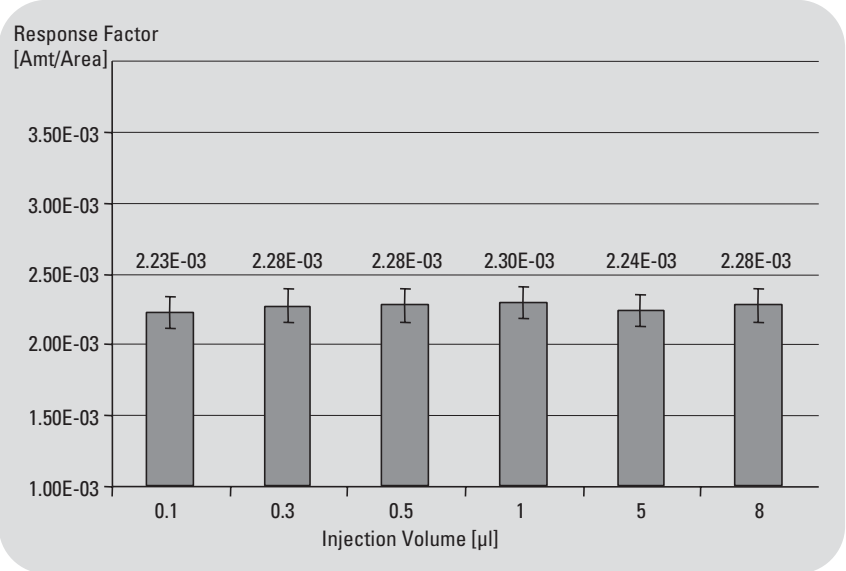


Figure 5b
Injection volume linearity evaluated based on response factors

Chromatographic conditions

Compounds:	antipyrine, phenacetine, diazepam
Column:	75 x 0.3 mm id ZORBAX 300 SB C-8, 3.5 µm
Column flow rate:	5.5 µl/min
Primary flow rate:	200 to 500 µl/min
Mobile phase:	A = water and B = acetonitrile
Gradient:	at 0 min 5 % B, at 3 min 5 % B, at 6 min 40 % B, at 7 min 50 % B, at 15 min 80 % B
Run time:	20 min
Post time:	18 min
DAD signal:	254/10 nm, ref. 350/80 nm

Figure 6a shows the analysis of three drugs. A 200-µl plastic insert for 2-ml vials was filled with 10-µl of sample and five 2-µl injections were performed. The results show that for the next full 2-µl injection 4 ml must be left in the plastic insert.

Nearly the complete amount of sample volume is accessible if the correct injector parameters are used. The optimum draw position in this case was 0.9 mm. In the second experiment (figure 6 b) the 300-µl plastic micro vial was filled with 50-µl sample and 5 µl were injected 10 times.

For the next full 5-µl injection 10 µl must be available. For chromatographic conditions refer to figure 6a (except injector parameters). The injection volume was 5 µl, the draw position -0.8 mm with well/vial bottom sensing.

Carry over using MSD Ion Trap

Carry over is one of the severe problems that can falsify quantitative and qualitative data in HPLC. Today detectors are becoming more and more sensitive and quantitation in the low picogram range is achievable especially using highly sensitive MS detection. When samples of very high (mg/ml) and very low (ng/ml) concentration are analyzed in a row, carry over becomes a critical parameter. We recommend using the 1100 Series micro well-plate sampler for obtaining lowest carry over. Having optimized this sampler, carry over of approximately 0.01 % can be expected even for critical compounds (see following application example). Optimization for lowest carry over involves clean-

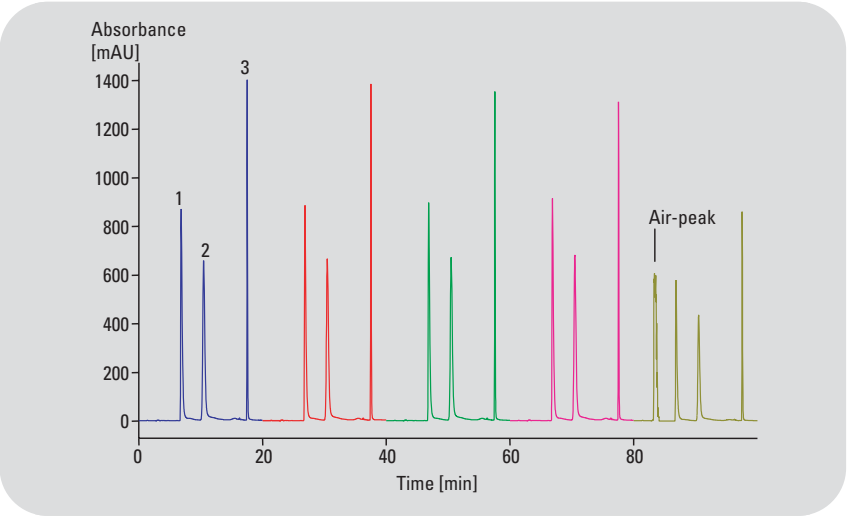


Figure 6a
Minimum accessible sample amount from a 200 µl conical plastic inserts. 5 times 2 µl were injected from a 10 µl sample.

Chromatographic conditions

Compounds:	antipyrine (1), phenacetine (2), diazepam (3)
Column:	75 x 0.3 mm id ZORBAX 300 SB C-8, 3.5 µm
Column flow rate:	5.5 µl/min
Primary flow rate:	200 to 500 µl/min
Mobile phase:	A = water and B = acetonitrile
Gradient:	at 0 min 5 % B, at 3min 5 % B, at 6 min 40 % B, at 7 min 50 % B, at 15 min 80 % B
Run time:	20 min
Post time:	18 min
DAD signal:	254/10 nm, ref. 350/80 nm
Injection volume:	2 µl, draw position 0.9 mm, no well/vial bottom sensing, 200 ml polypropylene inserts with polymer feet for 2-ml glass vials

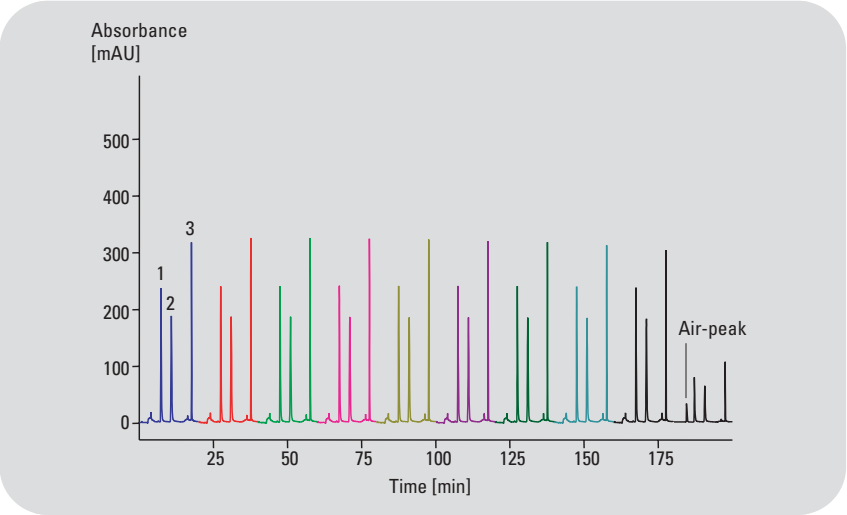


Figure 6b
Minimum accessible sample amount from 300 µl conical plastic vials

ing of the needle exterior and cleaning of the injection valve grooves by switching the valve two or three times after all peaks are eluted. We used azithromycine in our experiments, which is a very large and complex molecule. It is used as wide-band antibiotic. It has a large number of functional groups, which enable the molecule to adsorb at different surfaces. The molecule also shows a cage character, which means it can catch metal ions in its ring following certain transportation processes. All these characteristics are a high risk for carry over of this molecule

To be able to analyze azithromycine with DAD and MS Ion Trap the mobile phase had to be basic and volatile. An isocratic analysis was applied using 85 % methanol and 15 % of an ammonium acetate buffer at pH = 9. The capillary column had an internal diameter of 0.5 mm. The concentration of the injected azithromycine sample was as high as 1 µg/µl and 2 microliter were injected. The wash solvent for the exterior of the injection needle was methanol/water = 1/1, because the molecule dissolves in this solvent mixture. To test carry over the highly concentrated sample was injected, followed by the injection of the same amount of mobile phase solution, and followed by a blank run. Another important factor for obtaining lowest carry over, is not to use overlapped injection mode or the bypass mode for the injection valve when highly concentrated samples are injected. Highly concentrated means samples in the high µg to mg/ml range. For such a demanding sample as azithromycine it is not sufficient to clean the exterior of the needle– the injection valve grooves also have to be cleaned. This cleaning is done using an injector program, shown here. The valve is switched several times after the peak(s) of interest has passed the detector. In addition to external needle wash, this ensures lowest carry over also for critical compounds.

Figures 7a and 7b show the analysis of azithromycine. Figure 7a shows an overlay of the 2-µl injection of the 2 mg/l solution followed by injecting 2 µl of mobile phase with optimized cleaning conditions. In this case the carry over is about 0.01 %. For the MS analysis of azithromycine a simple MS ion trap method was used. Here it is most important to exactly specify the target mass, in order to obtain maximum sensitivity. Azithromycine is a doubly positively charged molecule with a mass of 375.

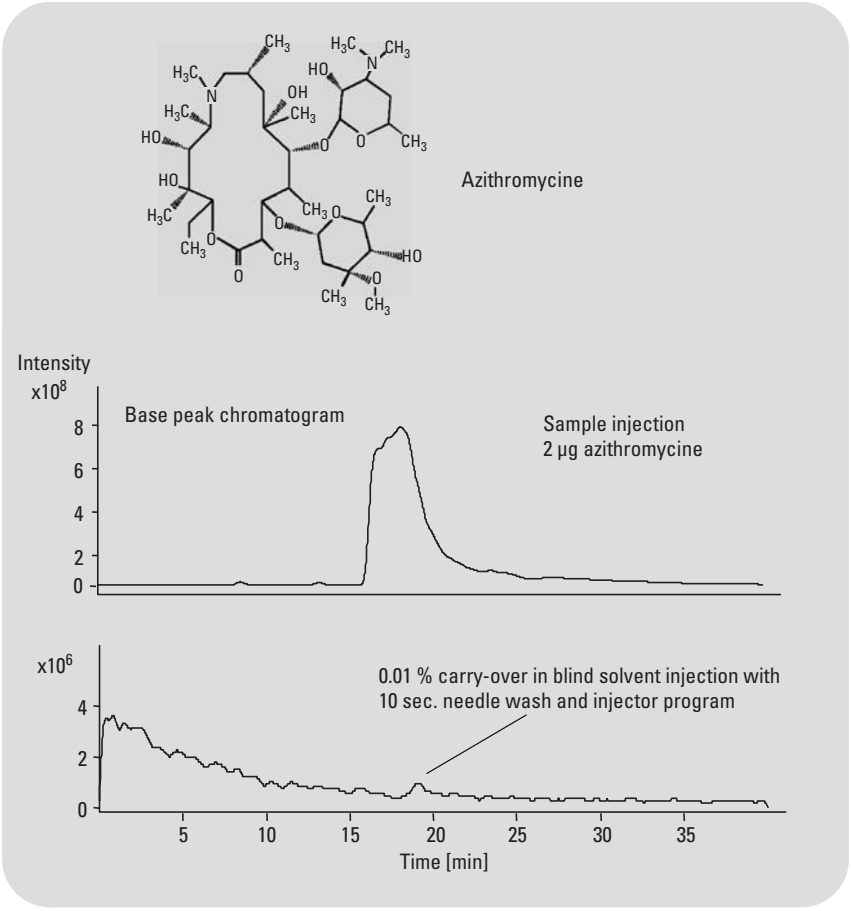


Figure 7a
Analysis of azithromycine and evaluation of carry over without any wash cycles

Figure 7b shows how the different instrument setting influence the percentage of carry over.

Intensive examination showed that using overlapped injection mode or bypass mode, carry over was extremely high with 0.95 % (figure7b). This is due to the fact that in this mode the complete injection system is not flushed during the run time. Without bypassing the autosampler volume carry over is already down to 0.16 %. Washing the exterior of the injection needle reduces the carry over down to 0.04 %. Additional injection valve switches after elution of the peak of interest reduces the carry over further, down to 0.01 %. Even though azithromycine is a very difficult sample regarding carry over, the 1100 Series capillary LC system with the micro well-pate sampler is able to provide a carry over as low as 0.01 %, if some precautions are taken. These include:

- Washing the needle exterior with a solvent that is able to dissolve the sample.
- Using an injector program to wash the injection valve grooves after the peak of interest has passed the detector
- Avoid using the bypass mode for the injection valve to reduce the delay volume of the autosampler over the complete run time.

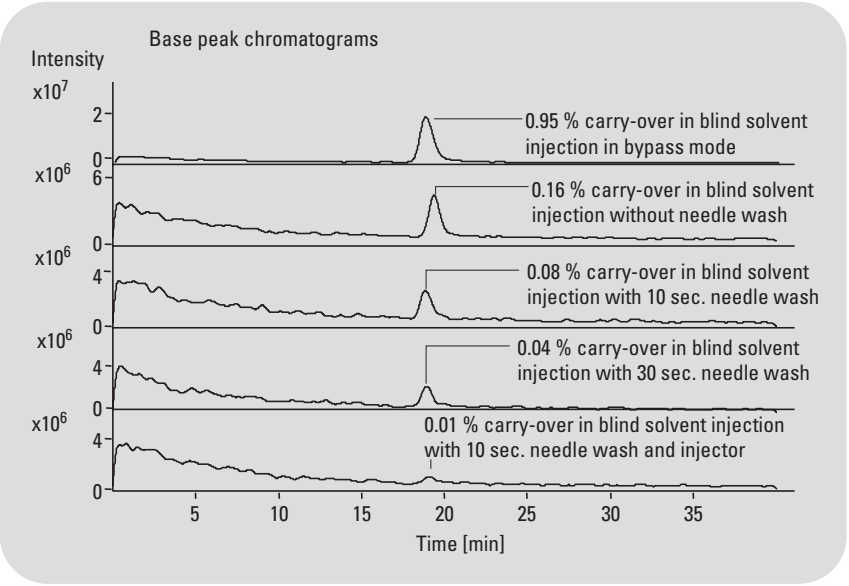


Figure 7b
MS analysis of azithromycine and evaluation of carry over using different instrument set ups and different cleaning methods

Chromatographic conditions

Solvent:	85 % MeOH, 15 % 5 mM NH_4Ac, pH 9 with NH_4OH
Stop time:	40 min
Flow:	5 µl/min
Column:	ZORBAX SB-C18, 0.5 x 150 mm, 5.0 µm
Column temp.:	ambient
Injection volume:	2 µl
Concentration of azithromycin:	1 mg/ml
Wash solvent for needle exterior:	MeOH/water 1/1
DAD:	210/8 nm (ref.: 360/100 nm)
Injection sequence:	1. sample, 2. solvent, 3. blank run

Injector program for lowest carry over:
Draw def. amount from sample
Needle wash as method
Inject
Wait 35 min.
Valve bypass
Wait 0.2 min.
Valve main path
Wait 0.2 min.
Valve bypass
Wait 0.2 min.
Valve main path

MS conditions

Source:	ESI
Drying gas flow:	10 l/min
Nebulizer pressure:	20 psi
Drying gas temperature:	325 °C
ICC:	On
Averages:	5
Max accu time:	300 ms
Target:	20,000
Ion mode:	positive
Target mass:	375 $[M+2H]^{2+}$

Limit of detection using DAD, MSD and MSD Trap

Whenever the compounds of interest are available in very low concentrations or only low sample volumes are accessible, the sensitivity of the detector is one of the most important factors that influences accurate measurement. Nowadays, UV detection, MS quadrupol and MS Ion Trap detectors are able to determine compounds in the low femto-mole range. In the following the limit of detection for three peptides was tested using the Agilent 1100 Series MSD Ion Trap and the Agilent 1100 Series diode-array detector. For these experiments a capillary column with an internal diameter of 0.3 mm was used. The mobile phase was water and acetonitrile with formic acid as modifier instead of TFA (trifluoroacetic acid). TFA is not ideal when using MS detection because it suppresses the signal by forming ion pairs with peptides and proteins. Therefore, formic acid was used which shows better compatibility with mass spectrometers.

The MS conditions were set such that the target mass of the expected peptide mass was selected. The ion mode was selected to be positive. These parameter settings ensure that all different peptide masses were recognized. One analyzed peptide was Met Enkephalin, a relative short peptide with an average mass of 573.7. It forms a positive ion in the MS and is analyzed as single charged molecule with a mass of 574. The next peptide was AngiotensinII with an average mass of 1042.20. It forms a doubly charged

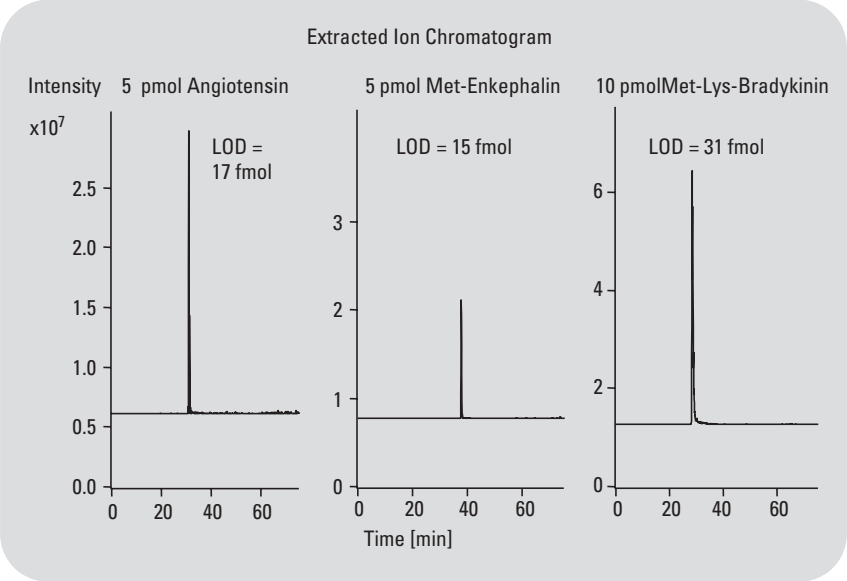


Figure 8a
LODs MSD Ion trap extracted ion chromatogram

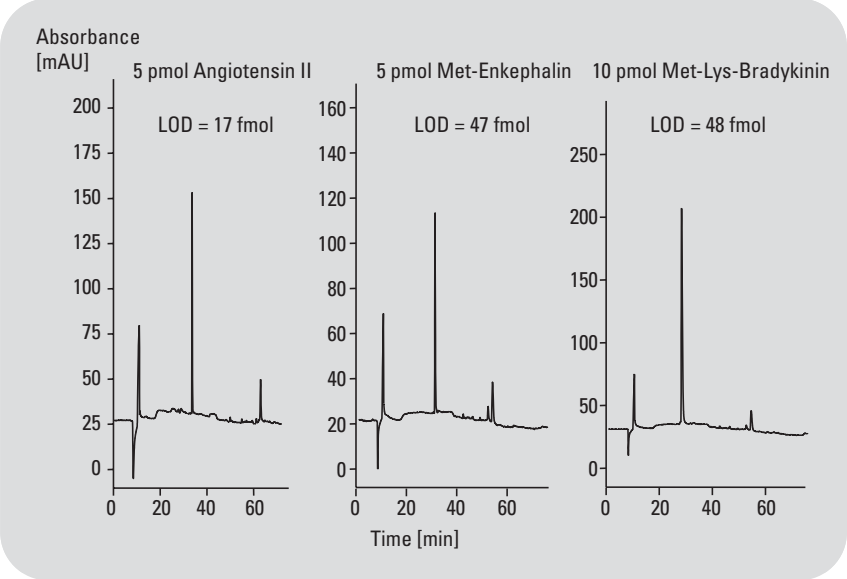


Figure 8b
LODs DAD

positive ion with a mass of 523.8. The last peptide that was analyzed was Met-Lys-Bradykinin with an average mass of 1390.60. This molecule forms a triply charged positive ion with a mass of 440.8.

This comparison confirms that typically the MSD Ion Trap can achieve lower limits of detection compared to a UV detection system. The difference in sensitivity will depend on chromophore properties, ionization properties and others. Another significant advantage of the MSD Ion Trap is the identification of peptides by their masses. Also, with the selected ion mode a peptide can be selectively searched and identified among other co-eluting compounds.

Chromatographic conditions

Solvent:	A = H_2O + 0.01% FA;
	B = ACN + 0.009 % formic acid
Capillary pump gradient:	5 min 5 % B, 60 min 60 % B, 75 min 60 % B
Stop time:	75 min
Post time:	15 min
Flow:	4 µl/min
Column:	ZORBAX 300SB-C18, 0.3 x 150 mm, 3.5 µm
Pre-column:	ZORBAX 300SB-C18, 0.5 x 3.5 mm, 3.5 µm
Column temp.:	25 °C
Column switch:	5 min
Injection volume:	1 µl
DAD:	210/8 nm (ref.: 360/100 nm)

MS conditions

Source:	ESI
Drying gas flow:	10 l/min
Nebulizer:	20 psi
Drying gas temperature:	325 °C
Skim 1:	40 V
Cap exit offset:	135 V
ICC:	On
Averages:	5
Max accu time:	300 ms
Target:	20.000
Ion mode:	positive

Performance of 1100 Series capillary LC system modified for flow rates above 0.1 ml/min

The Agilent 1100 Series capillary LC system can be modified to tolerate higher flow rates. This offers the flexibility of both a capillary LC system and an instrument, which can be used for standard applications with 2.1 and 4.6-mm id columns.

For column ids up to 2 mm and flow rates up to 0.2 ml/min the modification involves bypassing of the electronic flow control using a capillary. If flow rates above 0.2 ml/min are needed the modification involves:

- bypass of the electronic flow control,
- installation of the standard purge valve,
- change of UV detector cell, and
- change of flow path capillaries with small internal diameters to capillaries with bigger internal diameters.

Column (id and length)	Flow rate (ml/min)	Pressure range
100 x 2.1 mm	0.4	38 to 92 bar
	0.8	68 to 174 bar
125 x 4 mm	1	45 to 131 bar
	1.5	67 to 190 bar
100 x 4.6 mm	2	86 to 213 bar
	2.5	112 to 272 bar
6 port micro switching valve with pre-column and analytical column in series	1	76 to 192 bar
6 port micro switching valve with pre-column and analytical column in series	2	347 bar at 50/50 (water/acetonitrile)

Table 5
Pressure drop at different flow rates and different column ids, using water and acetonitrile from 100 % water to 100 % Acetonitrile

Furthermore, in the Agilent ChemStation software, the setup for the loop size has to be changed and the draw speed for sampling has to be increased to avoid long wait times until a sample is drawn up. After this modification the following pressures drops for different column ids and flow rates can be expected (table 5).Water and acetonitrile were used as mobile phase. The gradient applied started with 100 % water and ended with 100 % acetronitrile. The pressure drop was tested at each 10 % step. If more viscous mobile phase are used the flow rate range may be reduced.

In some cases the 1100 Series capillary LC system may be equipped with a micro-switching valve, which is installed in the column compartment. The capillaries connected to this valve have to be replaced by 100-µm capillaries. The pressure drop after the modification was measured for an analytical and a pre-column switched in series is as follows. At a flow rate of 1 ml/min the pressure drop for 100 % water was found to be 192 bar and for 100 % acetonitrile 76 bar. At 2 ml/min and water/acetonitrile of 50/50 the pressure drop was 347 bar for the previously described column configuration.

The performance of the modified system was tested for:

- precision of retention times and areas,
- delay volume,
- mixing performance, and
- step height differences for 10 % steps.

The precision of retention times and areas was tested using a mixture of 3 compounds and injection volumes between 1 and 40 µl (figure 9). The precision for retention times was found to be < 0.1 % RSD and for areas <0.5 % over the complete injection range.

The delay volume, the mixing performance and the step height differences for 10 % steps was tested using the tracer experiment in figure 10.

The delay volume depends on the mixer. Using the 1100 Series standard mixer the delay volume was found to be 620 µl. Using the low volume Upchurch mixer the delay volume could be reduced to 420 µl. The mixing performance is excellent for all evaluated 10 % steps. The step height varies over ten 10 % steps by 2.14 % RSD.

Results and conditions

System delay volume:	approx. ALS mainpass 620 µl with standard mixer with Upchurch mixer approx. 420 µl at 170 bar
Step height differences:	from 0 to 100 % in steps of 10 % = 2.14 %
Ripple 20 % tracer	= 0.032 %
Ripple 50 % tracer	= 0.004 %
Ripple 90 % tracer	= 0.006 %
Mobile phases:	water/water + 0.5 % acetone
Flow rate:	1 ml/min
Step gradient:	from 0 to 100 % tracer in steps of 10 %, each step was held for 5 min
DAD:	265/10, ref 350/80

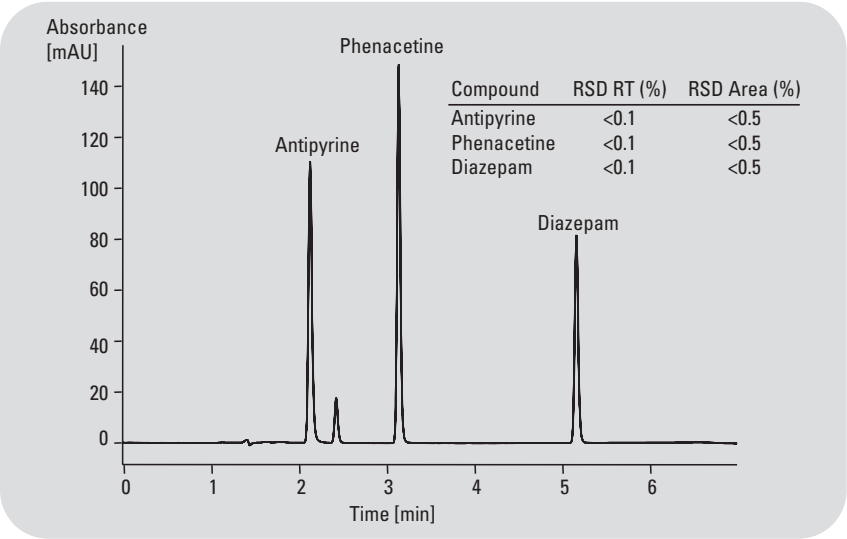


Figure 9
Precision example of retention times and areas for an injection volume of 5 µl

Chromatographic conditions

Sample:	antipyrine, phenacetine and diazepam
Column:	ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm
Flow rate:	1.2 ml/min
Mobile phases:	water/ acetonitrile = 70/30
Gradient:	at 0 min 30 % ACN, at 5 min 80 % ACN, at 5.5 min 30 % ACN, at 7 min 30 % ACN
Injection volume:	5 µl
Column Temperature:	40 °C
Diode array detector wavelength:	254/20 nm, ref 350/80 nm

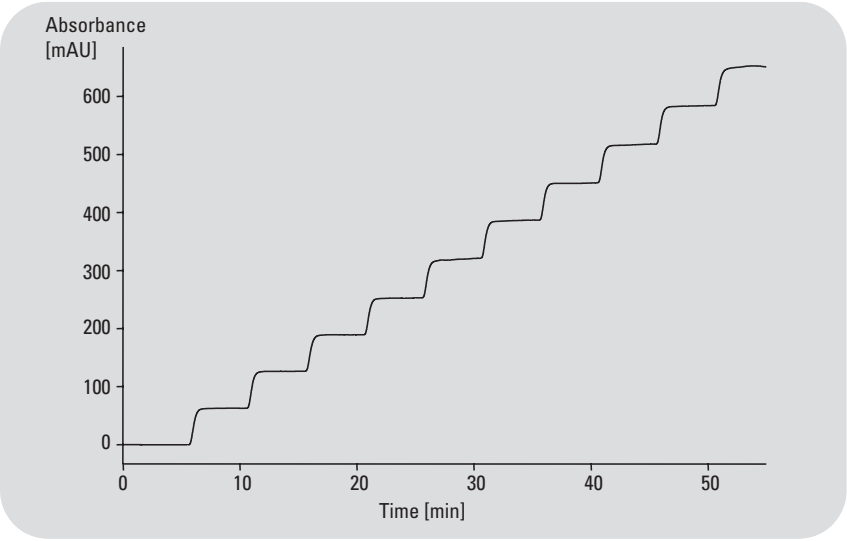


Figure 10
Tracer experiment for testing delay volume, mixing performance and step height precision

Summary

Capillary LC is the method of choice, when sample volumes are limited or/and higher sensitivity is essential. A LC capillary/MS combination fulfills these demands and is a typical solution in the biopharmaceutical research environment. The Agilent 1100 Series capillary LC system offers robustness, easy set-up and optimized control and data handling software. Detectors used are the diode array detection system and a MSD Ion Trap. Typically, the 1100 Series capillary LC system offers the following performance based on previously described experiments:

• RSD of retention times for sigma peptide standard:	< 0.5 % RSD
• System delay volume:	• 5 µl from electronic flow control (EFC) to column head with 20-µl flow sensor, • 14 µl for 100 µl flow sensor, 80 nl from point of injection to column and from column end to detector
• RSD of areas between 30 and 50 nl injections:	< 10 %
• RSD areas for <500 nl injections:	2 to 3 %
• RSD of areas for >500 nl injections:	≤ 1 %
• Carry over:	• < 0.01 % for caffeine with external needle wash on the micro well plate sampler; • 0.01 % for azithromycine if special injector parameter set up is used
• Minimum sample volume:	approx. 2 µl from 4 µl using 100 µl vials and if applicable well/vial bottom sensing and draw position – 0.8 mm
• Limit of detection for peptide standard using DAD:	approx. 50 fmol
• Limit of detection for MS-Ion Trap with capillary sprayer:	< 50 fmol

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