Performance characteristics of the Agilent 1200 Series LC system

Technical Note

Introduction

The Agilent 1200 Series LC system is based on the industry leading Agilent 1100 Series LC system and both systems share many common performance characteristics. However, a new design, manufacturing and software enhancements in the Agilent 1200 Series provide significant advances in overall system robustness and reliability. Reengineering and improvements to critical internal components of the pump, autosampler and detectors of the Agilent 1200 Series provide substantial gains in performance, ease of use and uptime. This Technical Note describes the steps for a performance evaluation of the Agilent 1200 Series LC system with various configurations. Module specific and complete system performance was evaluated using different separation methods according to pertinent criteria. Samples were selected that matched particular aspects of system evaluation, for example, peptide maps—where separation is particularly dependent on the solvent composition, and analysis of PNAs that are especially sensitive to changes in column temperature.

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Equipment and materials

The performance evaluation was carried out on an Agilent 1200 Series LC system using various configurations. The following modules were used interchangeably:

- Vacuum degasser for optimum removal of air in the mobile phases
- Isocratic pump for routine analysis
- High-pressure binary pump for gradient operation from 0 to 100 % of mobile phase B
- Low-pressure quaternary pump for flow rates from 0 to 10 mL/min
- High performance autosampler for fast and precise injections from 0.5 up to 100 µL
- Thermostatted column compartment for stable temperature control from 10 °C below ambient up to 80 °C
- Variable wavelength UV-Vis detector for routine analysis of known compounds
- Diode array detector (DAD) in its B version, a UV-Vis detector for multi signal acquisition and compound identification using spectral information

Agilent ChemStation software was used for instrument control, data acquisition and evaluation.

Key measurements for performance characterization

Several key measurements are necessary to evaluate the performance of an HPLC system. Some characteristics are influenced by only one part of the system. For example, linearity, spectral resolution and detection limits are influenced mainly by the detector, whereas delay volume and composition accuracy are influenced by the pump, and carry-over by the autosampler. In contrast, other characteristics such as baseline noise and precision of retention times and peak areas are influenced by the complete system. Technical Note describes the following key measurements:

- Detector – baseline noise, drift, wander, linearity, spectral resolution, sensitivity
- Pump – composition accuracy, precision, ripple, precision of retention times, delay volume
- Column compartment – temperature stability
- Autosampler – precision of peak areas, linearity, carry-over

In this list certain measurements are assigned to a particular module although the results could be influenced by several modules.

Delay volume

The delay volume is defined as the volume between the point of solvent mixing and the column. Parameters affecting delay volume include the pump, mixers, injectors and tubing and fittings that connect the modules of the system. Large delay volumes affect the sharpness of the gradient and therefore the selectivity of an analysis – they also increase the run-time cycle, especially at low flow rates. The delay volume depends on the back pressure and was measured by running a tracer gradient. The delay volume for an Agilent 1200 Series LC system equipped with a quaternary pump is approximately 800 to 1100 µL The delay volume for an Agilent 1200 Series LC system equipped with a binary pump is about 180 to 480 µL if no mixer is installed and 600 to 900 µL if the mixer is installed

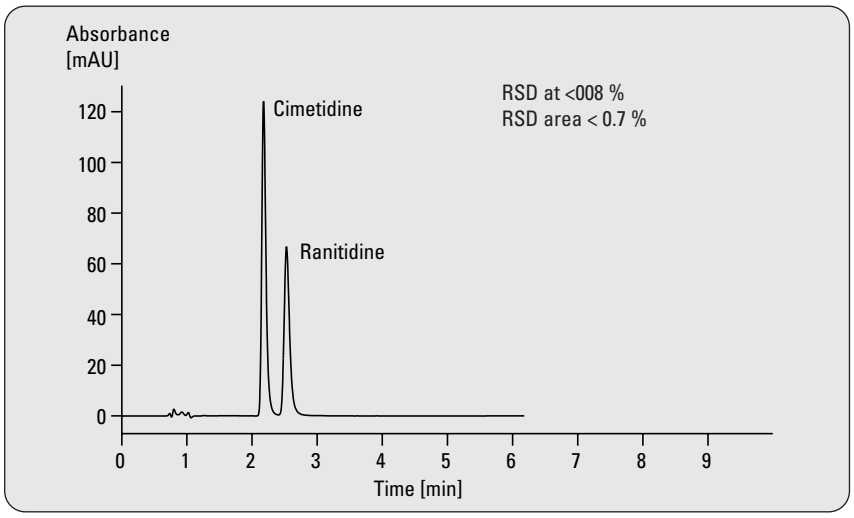

Figure 1
Analysis of H_2-antagonists.

Column:	4.6 x 75 mm ZORBAX SB-C18, 3.5 µm
Mobile phase:	A = 0.025 M KH_2PO_4 in water, (pH = 3), B = acetonitrile
Flow rate:	1.0 mL/min
Isocratic:	At 0 min 8 % B at 10 min 8 % B
UV detector:	Variable wavelength detector 225 nm, standard cell
Column compartment temperature:	25 °C
Stop time:	10 min
Injection volume:	5 µL

Isocratic pump, precision of retention times

Precision of retention times is an important characteristic that influences qualitative and quantitative results. The precision depends on the performance of the pump and degasser, and on the stability of the column temperature. To measure precision, a standard sample was injected several times and the absolute and relative standard deviations of retention times were calculated. The isocratic pump – with one pump head – showed excellent reproducibility of retention times and peak areas over a wide flow range up to 10 mL/min. A chromatogram of the evaluated sample is shown in figure 1.

Isocratic, quaternary and binary pump, composition accuracy, precision and ripple

To perform highly reproducible separations based on gradient runs it is important that the pump mixes the solvents accurately and precisely. A step gradient from 0 to 10 % tracer with methanol and a methanol/propylparabene tracer was used, while monitoring the detector signal at 254 nm, to determine the composition accuracy, precision and ripple of the binary and quaternary pumps. The column was replaced by a restriction capillary. Figures 2 and 3 show the step gradients of the pumps and table 1 shows the averages of the results of the composition test (n = 3). The accuracy of each step, expressed as units of % B, was calculated as the difference between theoretical and the measured step heights. The ripple (mixing noise) was calculated as the peak-to-peak noise of each step expressed in units of % B.

In table 1 the performance data regarding ripple, composition accuracy and precision are shown.

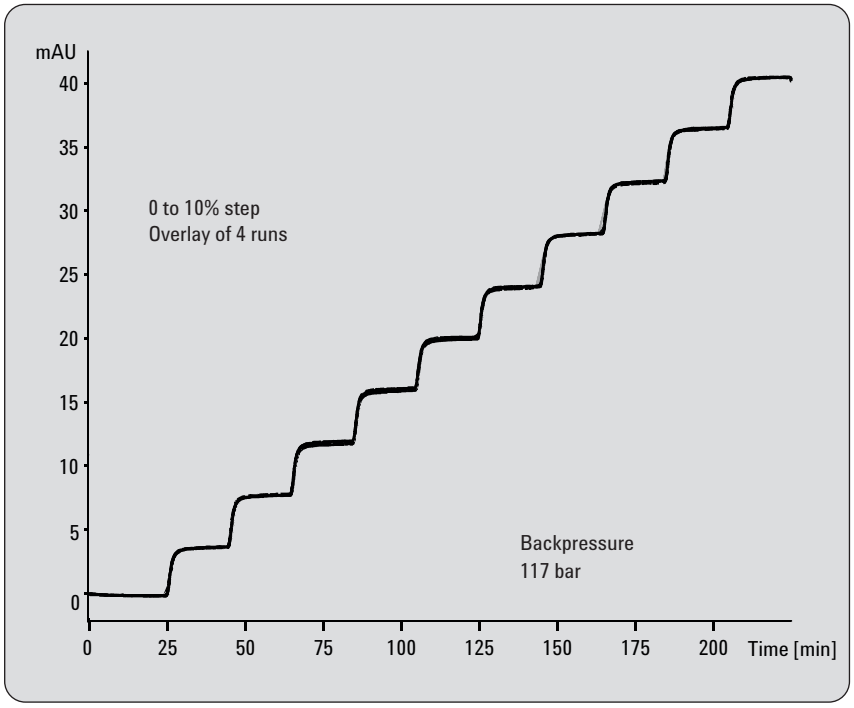

Figure 2
Step gradients results, quaternary pump at 0.2 ml/min flow rate, step gradient from 0 to 10 %.

Conditions:	4.8 m x 75 µm id Peek restriction capillary with dead volume of 20 µL
Solvent A:	Methanol
Solvent B:	Methanol + propylparaben
Flow rate:	0.2 mL/min
Back pressure:	117 bar
Step gradient:	Start with 0 % B, up to 10 % B in 1 % steps, each step 20 min
Compressibility:	120 x 10^{-6} 1/bar
Minimum stroke setting:	auto
Primary channel:	auto
DAD:	254/30 nm, ref 400/100 nm

Tested parameter	Quaternary pump Backpressure 117 bar Flow rate 0.2 ml/min	Binary pump Backpressure 117 bar Flow rate 0.2 ml/min
Composition ripple, related to a 100% step	0.01 to 0.03 %	0.01 to 0.03 %
Accuracy	0.23 %	0.23 %
Precision	0.11 %	0.12 %

Table 1
Averages of the results of the composition test for accuracy precision and ripple.

Precision of retention times – binary pump

Precision of retention times is one of the main chromatographic parameters which has to be tested to evaluate the performance of an LC system. The Agilent 1200 Series binary system provides excellent retention time precision for baseline separated peaks; see figure 4. The two dual pistons of the pump are servo-controlled to meet the highest chromatographic demands for gradient formation at low flow rates. A mixer reduces mixing noise, which is important if lowest detection limits are required for solvents containing TFA, for example, and detection is set at 210–220 nm. The binary pump achieved excellent retention-time repeatability with micro-bore columns. Peptide mapping on 1 mm columns puts stringent demands on the pump because small changes in solvent composition can cause considerable changes in the retention times. Under gradient conditions at flow rates of 50 µL/min the solvent delivery system must be capable of precisely delivering 1 µL/min per channel. Good mixing and a small delay volume is a must for smooth

Condtions:

Sample:	Antipyrine, Phenacetine, Diazepam
Column:	4.6 x 150 mm ZORBAX Eclipse XDB-C-8, 5 µm
Mobile phases: water/acetonitrile = 70/30	
Flow rate:	1.2 mL/min
Gradient:	at 0 min 30 % ACN at 5 min 80 % ACN at 5.5 min 30 % ACN at 7 min 30 % ACN
Injection volume:	25 µL
Outside wash of needle before injection: 14 s with methanol using flush port	
Column temp: 40 °C	
Detector:	DAD, 254/20 nm and 350/80 nm reference, peak width > 0.1 min, slit width = 4 nm

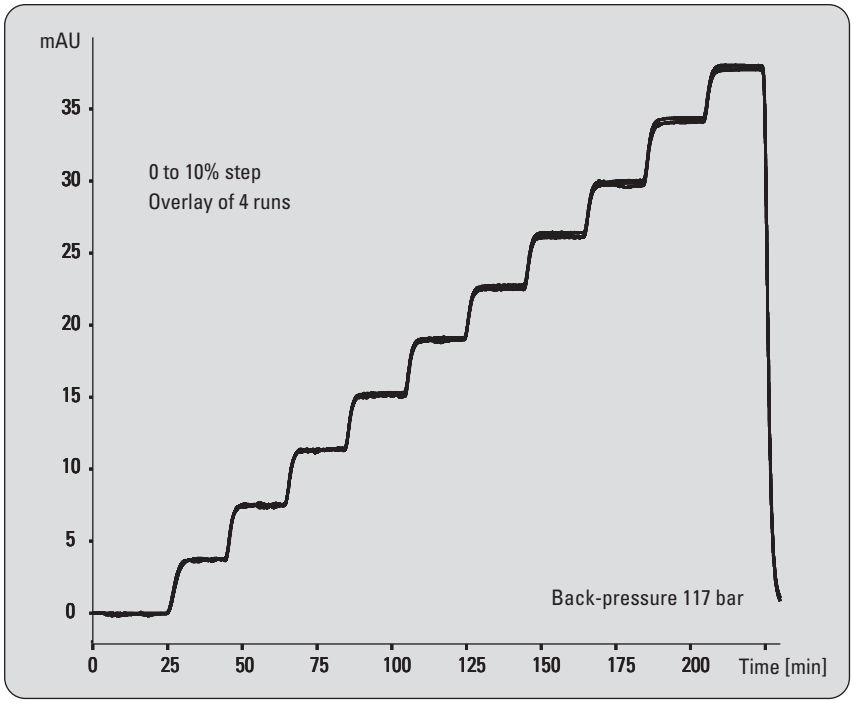

Figure 3
Step gradients results high pressure gradient pump at 0.2ml/min flow rate.

Conditions:	4.8 m x 75 µm id Peek restriction capillary with dead volume of 20 µL
Solvent A:	Methanol
Solvent B:	Methanol + propylparaben
Flow rate:	0.2 mL/min
Back pressure:	117 bar
Step gradient:	Start with 0 % B, up to 10 % B in 1% steps, each step 20 min
Compressibility:	A and B = 120 x 10^{-6} 1/bar
Minimum stroke:	A and B = 20 µL
DAD:	254/30 nm, ref 400/100 nm

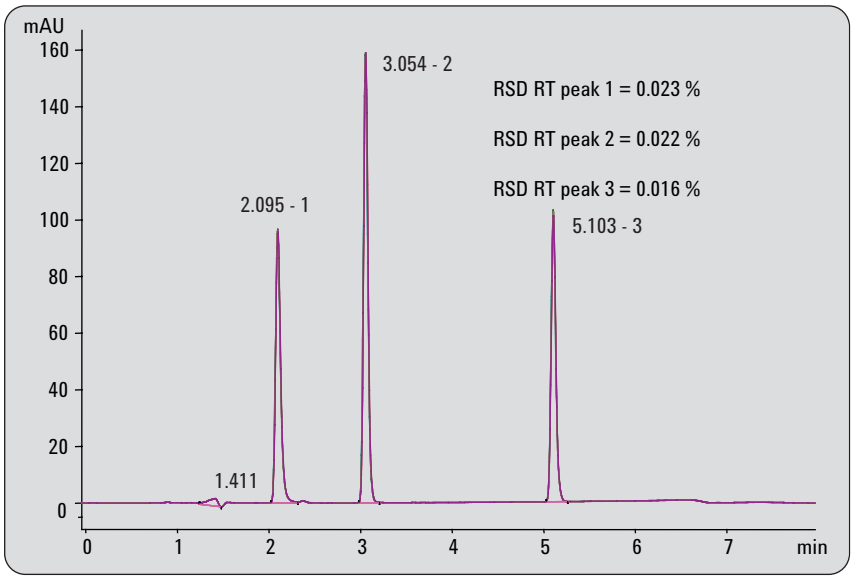

Figure 4
Step gradients results high pressure gradient pump at 0.2ml/min flow rate, overlay of 6 runs.

Column:	Vydac TP218, 5 µm, 1 x 250 mm
Solvent A:	0.05 % TFA in water
Solvent B:	0.043 % TFA in acetonitrile
Flow rate:	50 µL/min
Temperature:	40 °C
Gradient:	0 min, 1 % B
	84 min, 33 % B
	152 min, 51 % B
Injection:	1.1 µL, 190 pmol
Detection:	214/8 nm, reference 450/80 nm

baseline and non-distorted gradient profiles. Figure 5 shows six repetitive overlaid runs of a tryptic digest of myoglobin with retention-time precision in the range of 0.07 to 0.5 % RSD.

Precision of retention times – quaternary pump

The quaternary pump showed excellent low-pressure mixing capabilities made possible by the electronically activated inlet valve, pulse damper and dual-piston, in series design with variable stroke volume and high-speed proportioning valve. A wide flow range up to 10 mL/min supports standard and semi-preparative applications. In figure 6 the analysis of PNAs is shown using the quaternary pump and a 3 mm i.d. column. The separation of all PNAs is excellent and the retention time precision is very good. The area precision in the low mAU range is also very good. The precision for retention time is typically < 0.1 % and for areas typically < 2 % for this type of application.

Thermostatted column compartment

The column-temperature stability is another dominating factor because retention times are short-

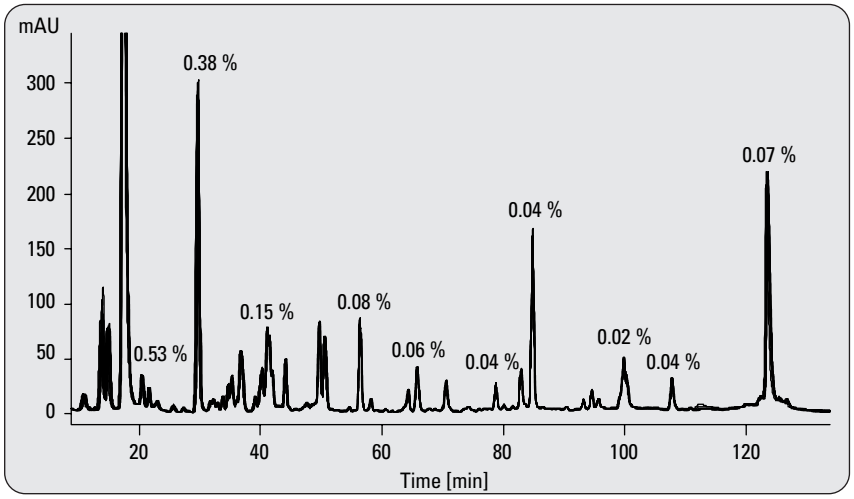

Figure 5
Retention-time precision (%RSD) of 6 injections of a tryptic digest of myoglobin.

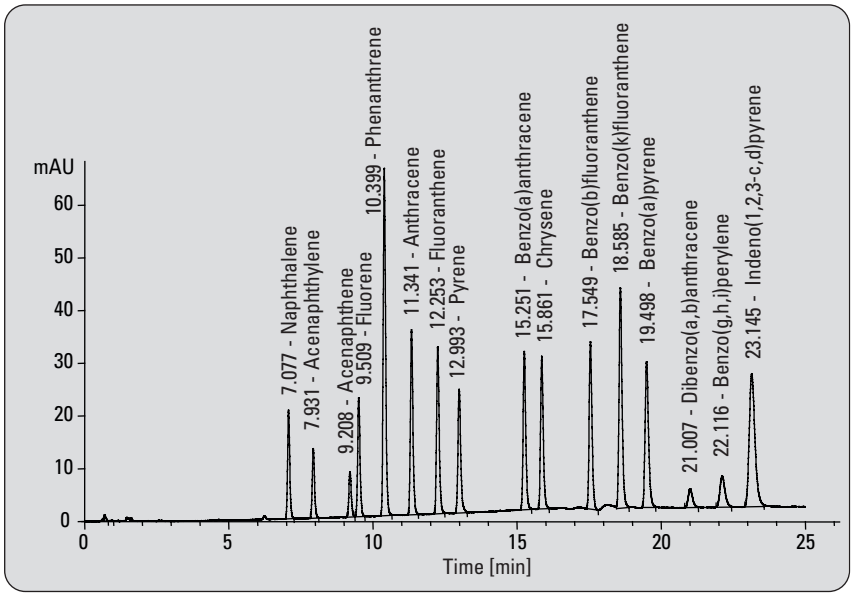

Figure 6
Analyses of PAHs using a 3 mm x 250 mm LiChrospher PAH column for optimum resolution.
Sample:
1 naphthalene, 2 acenaphthylene, 3 acenaphthene, 4 fluorene, 5 phenanthrene, 6 anthracene
7 fluoranthene, 8 pyrene, 9 benzo(a)anthracene, 10 chrysene, 11 benzo(b)fluoranthene,
12 benzo(k)fluoranthene, 13 benzo(a)pyrene, 14 dibenzo(ah)anthracene, 15 benzo(ghi)perylene
16 indeno(123-cd)pyrene.

Chromatographic parameters:

Eluent:	A: water, B: acetonitrile
Column:	Lichrospher PAH 250x3mm
Gradient:	0-3 min 50-60 %B, 3-15.4 min 60-100 %B, 15.4-23.5 min 100 % B,
	23.5-25 min 100-50 %B
Flow:	0.8 mL/min
Detector:	DAD 250 nm

ened at elevated temperatures. A thermostatted column compartment using Peltier control with good ambient temperature rejection ensures stable chromatographic conditions that are not influenced by periodic fluctuations in room temperature during 24-hour use. Figure 7 demonstrates the advantage of Peltier control as compared to conventional air cooling.

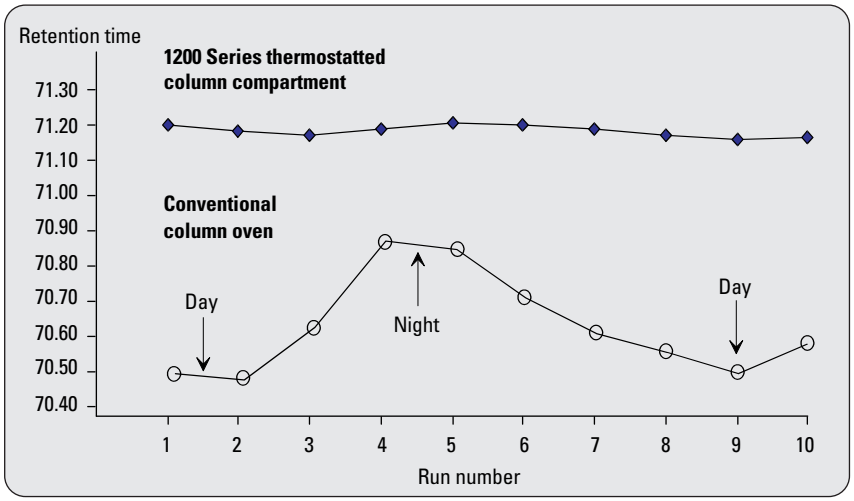

Figure 7
Comparison of Peltier and conventional cooling demonstrated by retention-time fluctuations of a peptide peak over a sequence of 10 consecutive tryptic peptide maps (conditions as in figure 4).

Agilent 1200 Series high performance autosampler – Precision of peak areas and linearity

Precision of peak areas depends on the injection volume and is an important characteristic that influences quantification. The precision deteriorates at low volumes and therefore should be measured at different volumes.

Column:	ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm
Mobile phase:	Water/acetonitrile 70/30
Flow rate	1.2 mL/min
Gradient	at 0 min 30 % ACN at 5 min 80 % ACN at 5.5 min 30 % ACN at 7 min 30 % ACN
Injection:	0.1 µL to 100 µl
Outside wash of needle before injection:	14 s with methanol using flush port
Column temp.:	40 °C
UV detector:	DAD 254/20 nm reference 350/80 nm

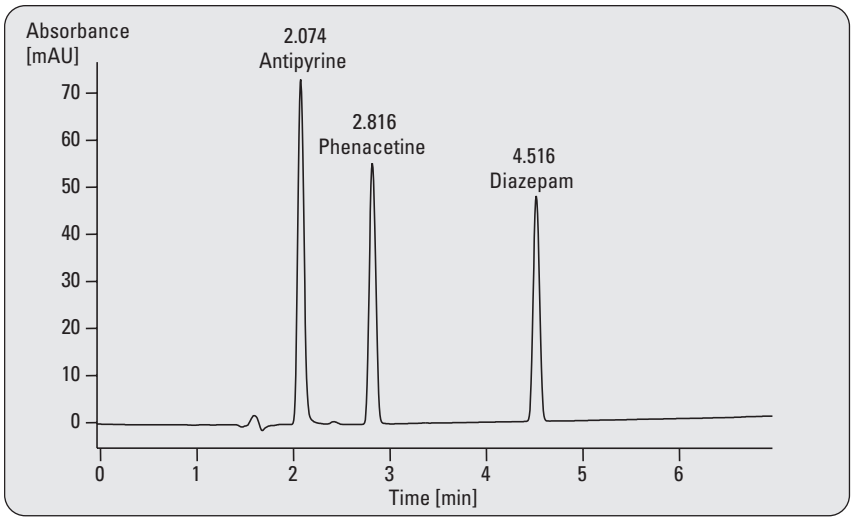

Figure 8
Chromatogram of compounds used for precision.

Injection volumes depend on the concentration of samples and a wider injection volume range of an autosampler is better for different sample concentrations. For a high performance autosampler it is important to show good precision over the complete injection volume range. For the Agilent 1200 Series high performance autosampler an injection volume range from 0.1 up to 100 µL is possible without the

Injection volume (µL)	%RSD of areas for antipyrine	%RSD of areas for phenacetin	%RSD of areas for diazepam	Areas counts mean value
0.5	1.54	1.44	1.90	220
1	0.50	0.49	0.48	200
3	0.17	0.19	0.22	500
5	0.13	0.13	0.13	1000
10	0.23	0.20	0.21	450
25	0.33	0.31	0.33	1200
50	0.02	0.04	0.16	700
100	0.02	0.03	0.07	5000

Table 2
Precision for different injection volumes.

need to change hardware. Figure 8 shows the chromatogram of the tested compounds together with the chromatographic conditions applied. The precision for different injection volumes was measured for 0.5, 1, 5, 10, 25 50 and 100 µL injection volumes. For each injection volume, 10 injections were evaluated. Results are shown in table 2. To be independent from integration or/and detection problems, different concentrations were used for the different injection volumes.

Linearity

The linearity of the injector was measured from 0.5 to 100 µL and the results are graphically shown in figures 9 and 10. The data demonstrates the excellent injection volume linearity of the system up to 100 µL. Good linearity in the low and in the high micro-liter injection volume range is another demand that should be fulfilled by a high performance autosampler. Figure 9 shows the volume linearity over an injection volume range from 0.5 µL up to 10 µL. The system is linear from 0.5 µL. The injections were done using a 300-µL well-plate and as sample compounds the compounds shown in figure 8 were used. The result for diazepam was selected as an example in figure 9. The coefficient of correlation for all three compounds is greater than 0.9999 from 0.5 up to 10 µL injection volume. Figure 10 shows the linearity of injection volume from 10 to 100 µL. In this example linearity is given over the complete range. Injections were done from 1.5 mL vials and the result for diazepam was used.

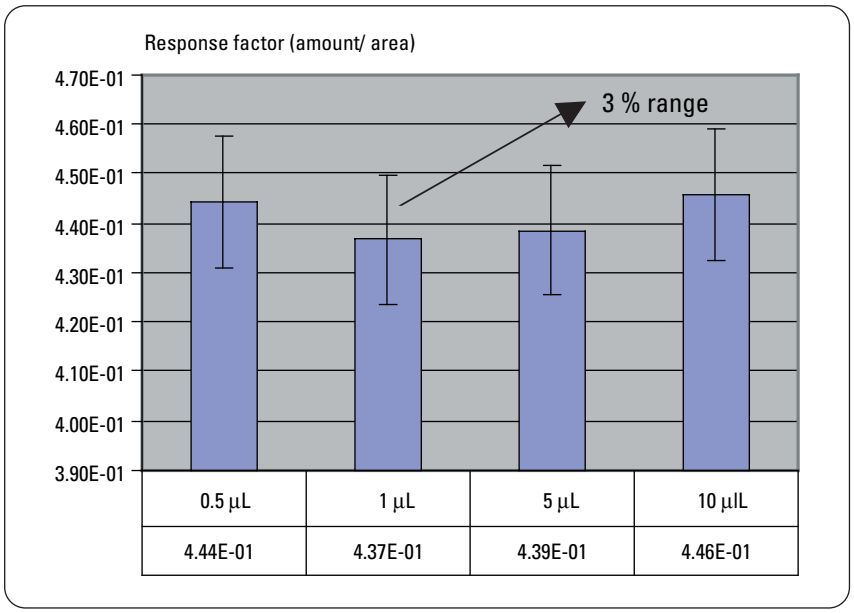

Figure 9
Linearity of injection volume in the low micro-liter range, injected from a 300-µL well plate.

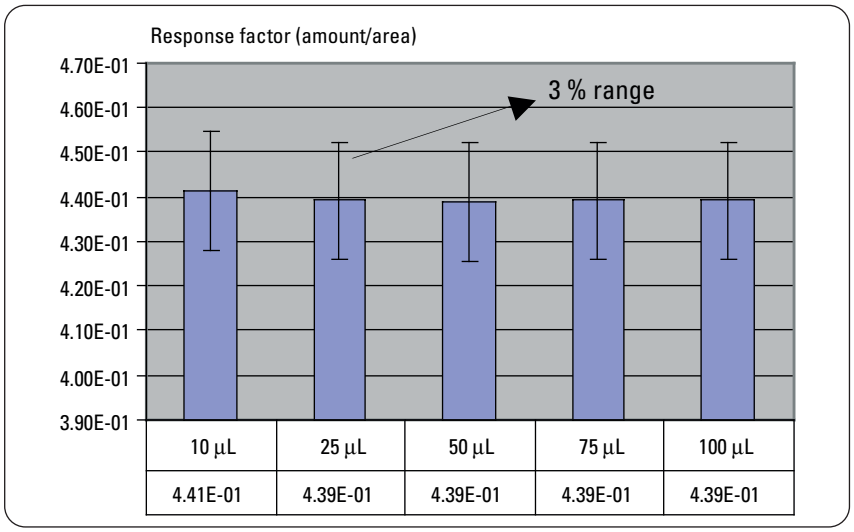

Figure 10
Linearity of injection volume in the middle and high micro-liter range, injected from 1.5 ml vial.

Column:	2.1 x 30 mm ZORBAX Eclipse XDB-C18, 3.5 µm
Mobile phase:	Water/acetonitrile
Flow rate:	0.2 mL/min
Gradient:	at 0 min 30 % ACN at 2.5 min 30 % ACN at 3 min 80 % ACN at 4 min 30 % ACN at 9 min 30 % ACN
Injection:	0.5 µL for sample, 5 µL for methanol injection
Outside wash of needle before injection:	14 s with methanol using flush port
Column temp.:	40 °C
UV detector:	DAD 254/20 nm reference 350/80 nm
Injector:	minimized carry-over mode

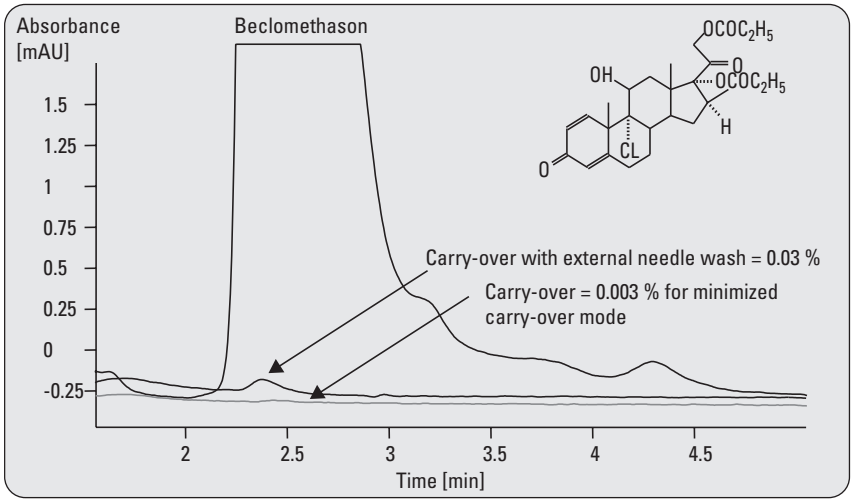

Figure 11
Optimization for lowest carry-over using an injector program.

Carry-over

Peak area reproducibility also can deteriorate when non-reproducible sample carry-over occurs. Therefore a sampling mode which avoids carry-over should be used, for example, a needle wash in pure solvent between two injections. The effect of a needle wash in the flush port and minimized carry over mode is shown in figure 11. For some applications carry-over can occur from residues in the injection valve. In this case it is recommended to use the minimized carry over mode, which can be selected in the autosampler set up screen. This mode is based on an injector program, which switches the injection valve in the bypass/mainpass position. In this mode the injection valve is switched into the flow path for cleaning. In figure 11 a comparison is made between carry-over for an injection with external needle wash in the flush port only and an injection using the minimized carry over mode and external needle wash.

The compound used was beclomethasone and 1000 ng was injected followed by the injection of 5µL methanol. Beclomethasone was selected because carry-over is critical for this compound. With external needle wash only the carry-over was found to be 0.03 %. Using the minimized carry over mode together with external needle wash the carry-over was down to 0.003 %. Finally, an additional enhancement to further reduce carry-over is to use the optional solvent purge kit to clean the injection system with a second solvent using an additional valve[1].

Fast injection cycles, low delay volume

Short injection cycle times for high sample throughput is one of the main issues in an analytical laboratory today. Shortening cycle time starts with shortening column length, increasing flow rates and implementing steep gradients. Having optimized these parameters, the speed of the autosampler can become a limiting factor. Autosampler from third party vendors sometimes need about two to three minutes to draw and eject a sample, which is especially time consuming if overlapped injection is not possible.

The Agilent 1200 Series high performance autosampler described here is able to draw and inject a sample of 1 µL within 25 seconds, including an outside needle wash of 10 seconds. Further reduction of cycle times can be obtained using the overlapped injection mode. This means the injection valve is switched into the bypass position and the mobile phase is directed to the column without passing through the sample loop, needle and needle seat capillary. The next sample is drawn before the previous run has stopped. The sample is kept in the sample loop and is injected directly after the current run is finished. Switching the injection valve into the bypass position reduces the system delay volume by approximately 300 µL. This can help to achieve faster cycle times especially for low flow rate applications using narrow-bore and micro-bore columns.

Detector measurements: Short-term noise and drift (Agilent 1200 Series DAD B for maximum 20 Hz data rate acquisition)

Short-term noise and drift are characteristics which determine a detector's performance. Low

noise is especially important when analyzing compounds at trace levels, whereas drift and wander affect the integration quality. Detector noise, wander and drift were determined according to ASTM (American Society for Testing and Materials) with the overall system noise using water as mobile phase. The noise determination was based on the *"ASTM E-1657-94: Standard practise for testing variable wavelength photometric detectors used in liquid chromatography"*. Table 3 shows the results of the short-term noise and drift measurements for the variable wavelength and diode array detector.

	Noise	Drift [mAU/h]
VWD	0.014	0.052
DAD	0.011	0.182

Table 3
Short-term noise and drift of variable wavelength detector (VWD) and diode array detector (DAD).

The Agilent Series 1200 DAD B has a dual lamp design which insures optimum light intensity from 190 up to 950 nm. In figure 12 the positive influence of this design on noise is shown. The DAD optics are designed to give high signal levels, low baseline noise, high linearity and minimum refractive index effects. Figure 13 shows the analysis of 10 pg anthracene. The calculated limit of detection was 1.2 pg at a signal-to-noise ratio of 2. The comparison of the chromatograms in figure 13

Column:	ODS Hypersil, 2.1 x 100 mm, 5 µm
Mobile phase:	Water/acetonitrile 20/80
Temperature:	36 °C
Flow rate:	0.3 mL/min
Injection:	5 µL
Detection:	Variable wavelength detector: flow cell 14 µL volume and 10-mm path length, wavelength 251 nm, response time 2 s
Diode array detector:	flow cell 13 µL volume and 10-mm path length, wavelength 251 nm, optical slit 4 nm response time 2 s

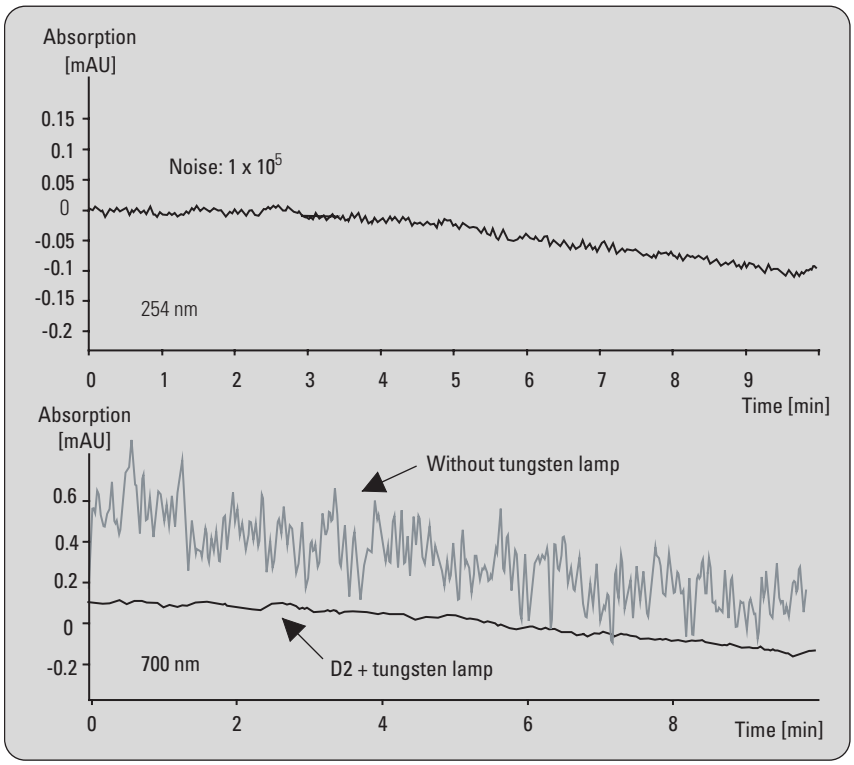

Figure 12
Advantages of twin lamp design.

Chromatographic conditions:	
Mobile phase:	ACN/water 20/80
Flow rate:	0.2 mL/min
Slit:	1 nm
Chromatographic conditions:	
Column:	ODS Hypersil, 4.0 x 125 mm, 5 µm
Mobile phase:	Water
Temperature:	36 °C
Flow rate:	1 mL/min
VWD:	Wavelength 254, response time 2 s
DAD:	Wavelength 254 nm, bandwidth 4 nm, optical slit 4 nm, response time 2 s

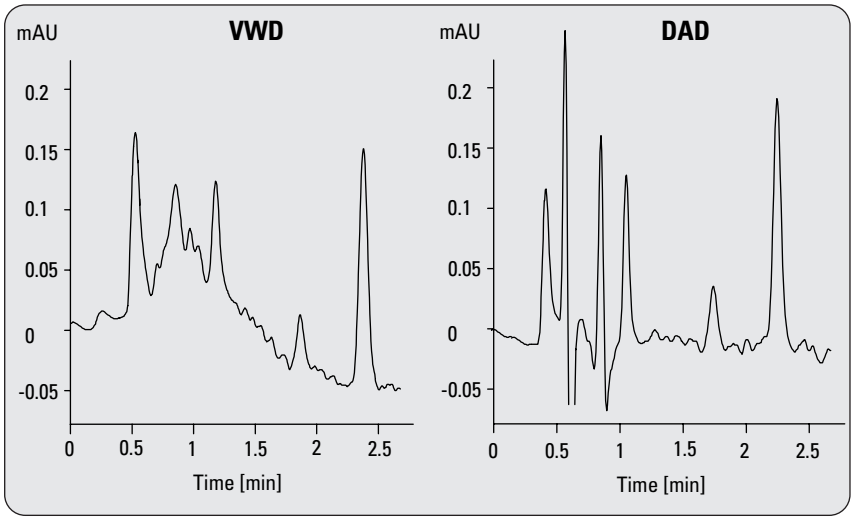

Figure 13
Variable wavelength detector (left) and diode array detector (right) signal of 10 pg anthracene.

Column:	ODS Hypersil, 4.0 x 125 mm, 5 µm
Mobile phase:	Water/acetonitrile 85/15
Temperature:	36 °C
Flow rate:	0.8 mL/min
Injection:	5 µL
Detection:	Wavelength 272 nm response time 2 s

for the variable wavelength and diode array detectors shows that both detectors generated a signal of about 0.25 mAU and a similar limit of detection. This experiment demonstrates that the detection limit of a high-sensitivity variable wavelength detector and a thoroughly optimized diode array detector are similar.

Detector linearity

The linear range of a detector represents the range of concentration of a substance over which the sensitivity is constant within a specified variation, usually ±5 %. Linearity is an important characteristic that influences quantification. The linearity also depends on the type of compound and on the wave length setting. The detector linearity is determined by a series of standards with increasing concentrations that cover the range of interest. The detector linearity was measured by injecting caffeine standards with increasing concentrations over the range 0.1 mAU to 2 AU. Figure 14 shows the linearity plot – this is a plot of detector sensitivity (response [mAU] / concentration [ng/mL]) against the logarithm of concentration – and covers the range 0.24–700 ng/mL, that is, ~1:3000. The linear range of the detector is the concentration range in which the linearity plot lies between the horizontal lines 5 % above and below the line of constant response ratio Rc.

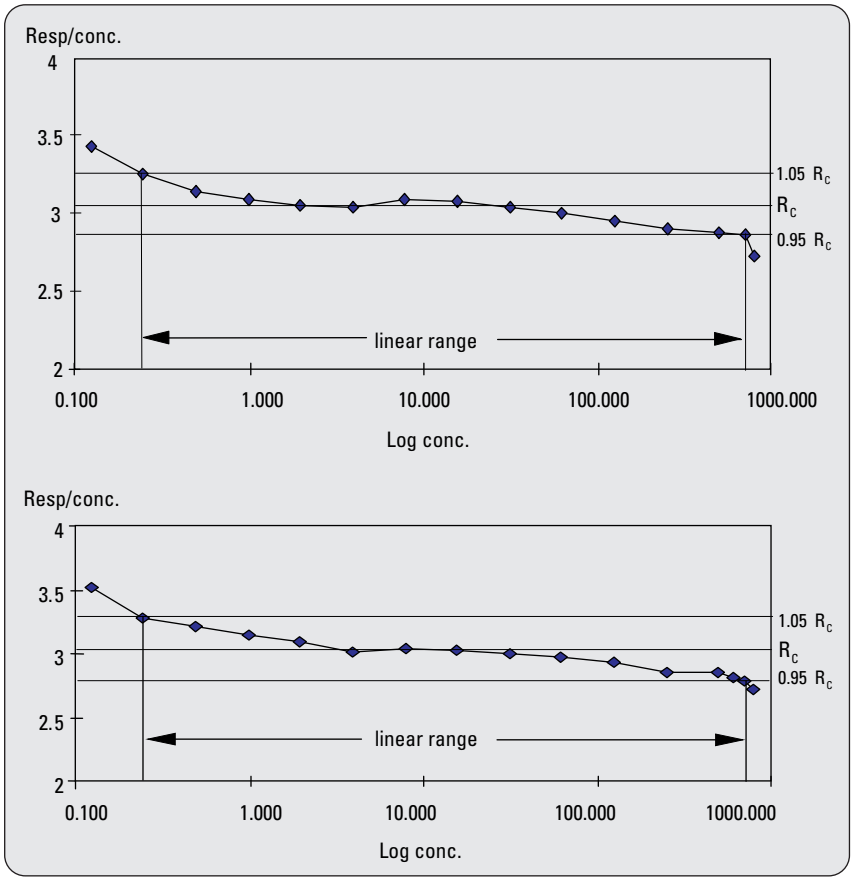

Figure 14
Linearity of variable wavelength detector (above) and diode array detector (below) measured with different caffeine standard concentrations, dissolved in water.

Peak identification at trace level

Retention time and absorption at a single wavelength is not sufficient to prove the identity of compounds in environmental analysis. The complexity of real life samples typically requires additional information to confirm peak iden-

tity. With a diode array detector complete spectral information is available at any time and wavelength during the run. One common practice is to compare the obtained spectra from a peak with a library or to check for peak-purity by comparison of spectra taken throughout a single peak, see figure 15. With the Agilent 1200 Series DAD, both techniques can be applied automatically as part of a method and run routinely for a sequence of samples. Spectra can either be collected throughout the analytical run or peak controlled to save memory space.

Extended wavelength detection

A variety of chemicals and natural products are available for use as colors and dyes to address current interests in fashioned products. In order to prevent health damage arising from this practice, an increasing range of colors are regulated with national directives. The Agilent 1200 Series DAD is well suited for monitoring in the visible wavelength range. The addition of a tungsten lamp leads to superior baseline stability and more complete spectra (figure 16).

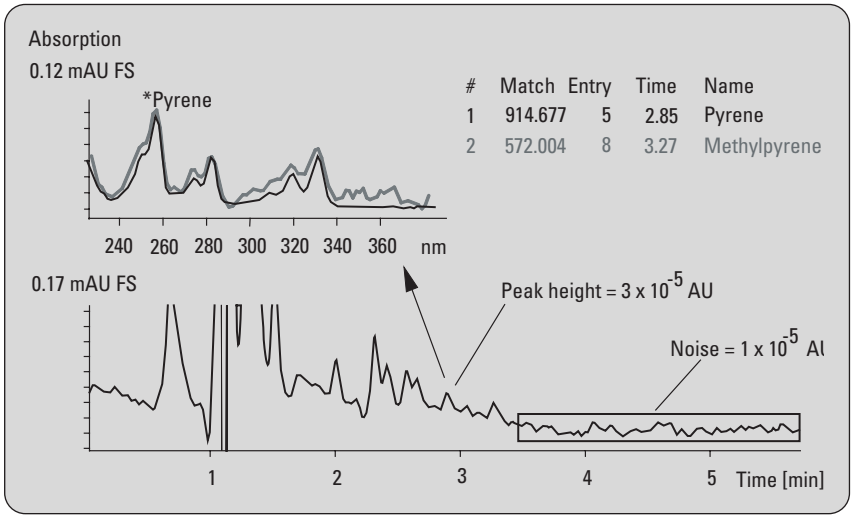

Figure 15
Spectral identification by library search at trace level concentrations.

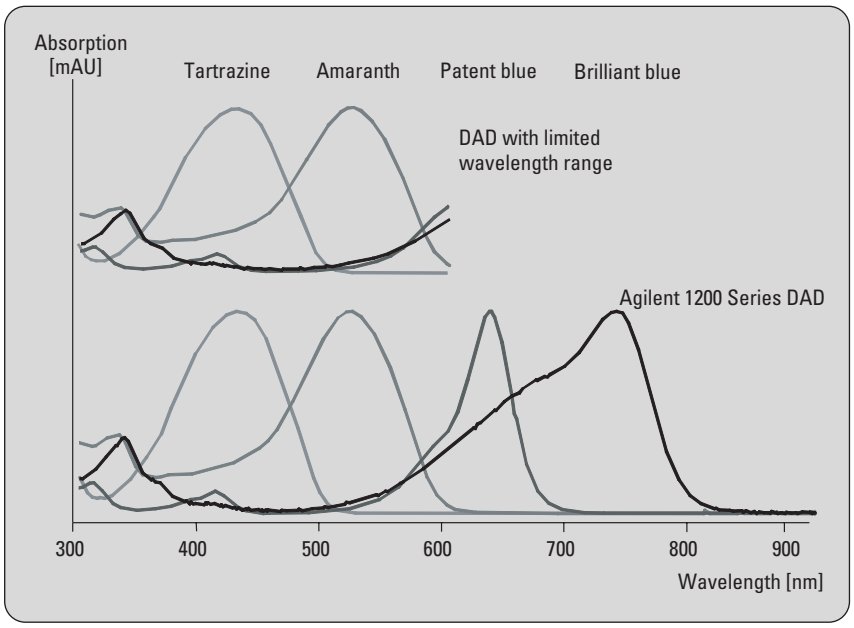

Figure 16
The Agilent 1200 Series DAD gives more complete spectra.

Conclusion

The Agilent 1200 Series HPLC system combines the well known excellent performance of the Agilent 1100 Series with new design elements, which lead to an improvement in retention time and area precision.

References

1.

A.G.Huesgen, “Optimization of the Agilent 1100 Series well-plate autosampler for lowest carry-over using an optional injector purge kit”, *Agilent Application Note*, *Publication number 5989-3357EN*, **2005.**

www.agilent.com/chem/1200

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