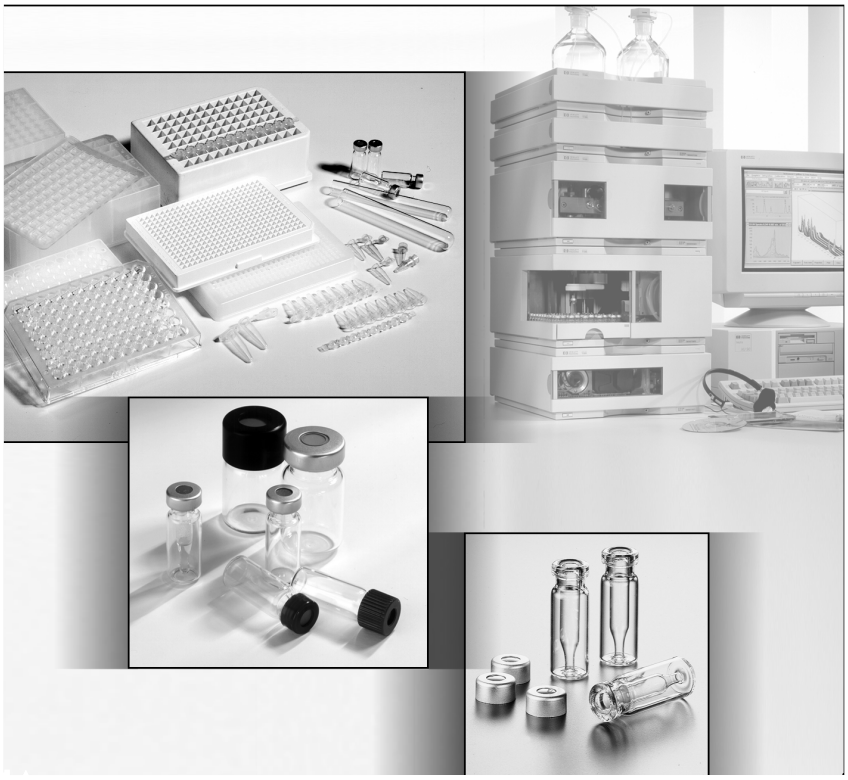
Optimizing the Agilent 1100 Series System for High Sample Throughput

Technical Note



Introduction

High sample throughput is particularly important in the pharmaceutical industry, for example, in combinatorial chemistry and metabolite studies. In combinatorial chemistry a large number of organic compounds per unit time are purposely produced in order to be screened against a variety of biological targets for drug detection.

An ideal instrument configuration for high sample throughput comprises:

- an autosampler such as a standard HPLC autosampler for 2-ml vials or a sampler with microtiter and deep-well plates,
- a high-pressure gradient pump for lowest delay volume,
- a UV detector such as a diode array detector or variable wavelength detector, and
- a mass selective detector for additional mass and structural information (optional).

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The limiting factor for high throughput in such systems is the speed of the HPLC analysis. Standard HPLC cycle times from injection to injection for gradient analysis lie between 15 and 20 min using columns of 100 to 200 mm in length.

To significantly increase the sample throughput, cycle times must be shortened. This can be achieved using fast gradients with short columns and high flow rates. In the following example we demonstrate how to optimize the Agilent 1100 Series high-pressure gradient system to obtain rapid gradients and high sample throughput. Hints are given on the influence of chromatographic parameters on cycle times, and on how run times of less than two minutes can be expected to affect performance.

Equipment

All HPLC experiments were carried out on the Agilent 1100 Series high-pressure gradient system comprising:

- Agilent 1100 Series high-pressure pump for lowest delay volume. In this design each solvent is pumped by its own pump assembly, and mixing takes place on the high-pressure side. This means gradient changes reach the column much faster than in low-pressure gradient systems where mixing takes place on the low-pressure side.
- Agilent 1100 Series vacuum degasser for optimum baseline stability.
- Agilent 1100 Series autosampler for sampling from 2-ml standard vials.
- Optional Agilent 220 micro plate sampler for flexible sampling from deepwell and/or microtiter plates.
- Agilent 1100 Series thermostatted column compartment for highest stability from 10 °C below ambient up to 80 °C.
- Agilent 1100 Series diode array detector with standard flow cell (10-mm pathlength, 13-microliter volume).
- Optional Agilent 1100 Series variable wavelength detector.
- Optional Agilent 1100 Series LC/MSD module for mass and structural information.
- Agilent ChemStation with 3D HPLC single instrument software for instrument control, data handling and sample tracking.

Compounds and chromatographic conditions

For our experiments we selected the following compounds which differ considerably in polarity:
- caffeine
- primidone
- phenacetin
- mandelic acid benzylester
- biphenyl

The chromatographic conditions are listed next to the figures.

Optimization of chromatographic parameters

The following parameters have to be adapted to obtain short cycle times, sufficient resolution and best performance over a wide range of polarity:
- column length
- gradient
- flow rate
- delay volume
- data rate of detector
- column temperature

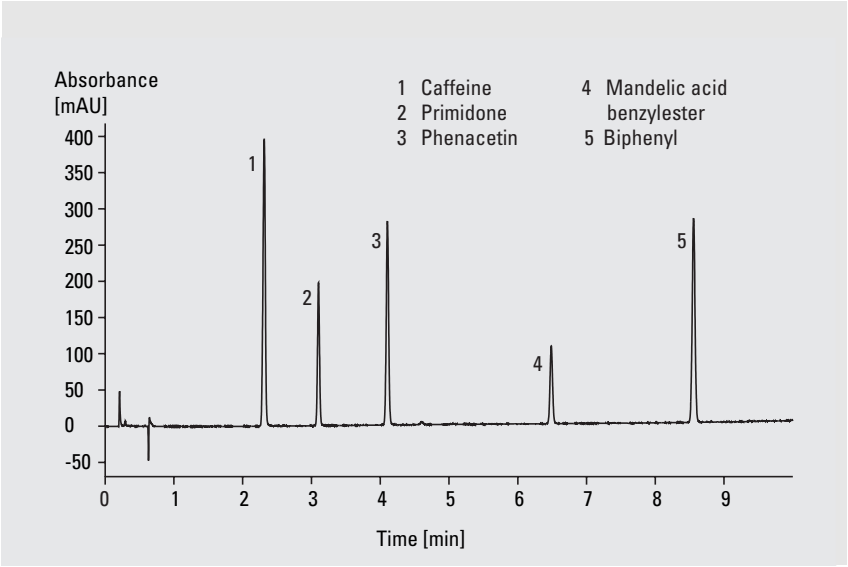
The aim was to achieve cycle times of about 2 min and baseline separation for all compounds.

Influence of column length on run time

For a standard column with a length of 100 mm and an id of 4.6 mm, run time cycles of about 15 min are good practice. In figure 1, the compounds mentioned in the previous paragraph were analyzed.

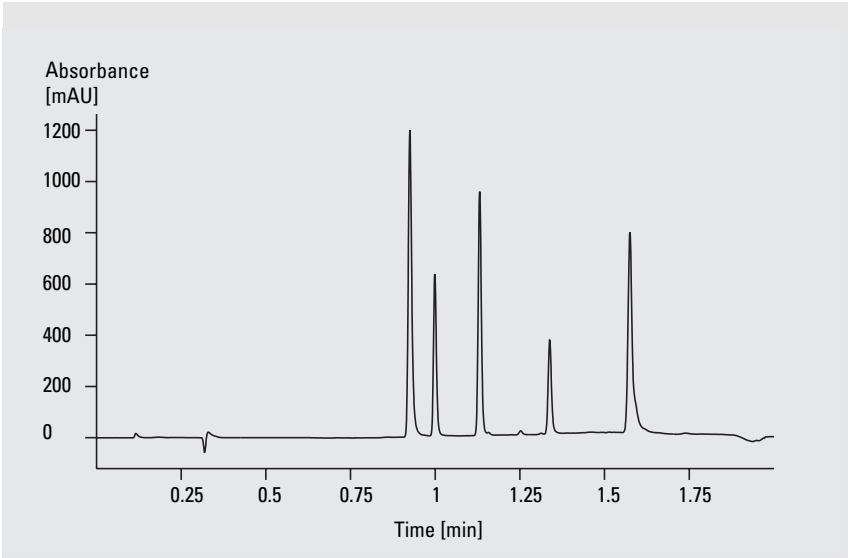
Cycle times of 14 min were obtained with excellent resolution for all compounds.

Shorter cycle times are obtained using a short column. In figure 2, the analysis of the same compounds is shown using a 50-mm colum. Cycle times are down to 2.8 min and baseline separation for all compounds is given, despite decrease in resolution. Shortening the column length was the principal step in achieving reduced cycle times. The example on the next page demonstrates how to shorten the cycle even further.



Column	100 x 4.6 mm ODS Hypersil, 5 µm
Flow rate	2 ml/min
Mobile phase	A = water, B = acetonitrile (ACN)
Gradient	5 % B to 95 % B in 12 min to 5 % B in 13 min
Run time	13 min
Post run	1 min
Diode-array settings	210/8 nm, ref. wavelength 360/100 nm
Injector volume	5 µl
Column temperature	50 °C

Figure 1
Analysis of selected compounds using a 100-mm column



Column	50 x 4.6 mm Zorbax SB-C18, 3.5 µm
Flow rate	2 ml/min
Mobile phase	A = water, B = acetonitrile (ACN)
Gradient	5 % B to 95 % B in 1 min 95 % up to 1.5 min to 5 % B in min
Run time	2 min
Post run	0.8 min
Diode-array settings	210/8 nm, ref. wavelength 360/100 nm, response time 0.1 s
Injector volume	5 µl, autosampler in bypass mode
Column temperature	50 °C

Figure 2
Analysis of selected compounds using a 50-mm column

Influence of gradient on run time

In order to achieve a good separation for the polar and the non-polar compounds, a gradient from 5 to 95 % of the organic phase was chosen. The evaluation of the steepest possible gradient with baseline separation for all compounds is shown in figure 3. The flow rate was 2 ml/min.

As a result, the gradient from 5 to 95 % ACN in 1 min met the demands of shortest run time and baseline separation for all peaks.

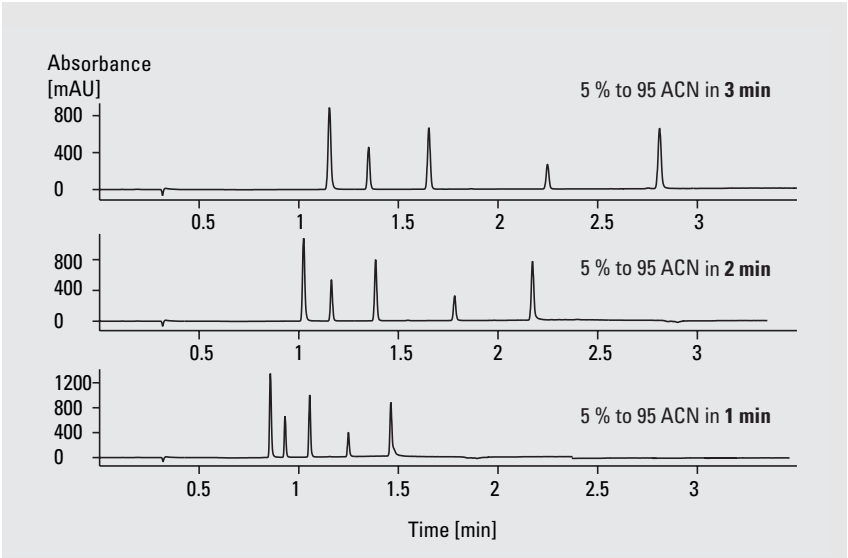


Figure 3
Evaluation of gradient steepness

Influence of flow rate on run time

As well as the column length, the flow rate can also be used to shorten run times or achieve better cycle times. In figure 4, the analysis of the selected compounds at 4 different flow rates is shown using the gradient from 5 to 95 % ACN in 1 min.

Cycle time can be reduced from 3 min at a 1 ml/min flow rate, down to 1.3 min at a flow rate of 4 ml/min. All peaks are baseline separated, also at a flow rate of 4 ml/min. In the evaluation of peak area counts, it is apparent that there are about four times less area counts in the 4-ml/min run than in the 1-ml/min run.

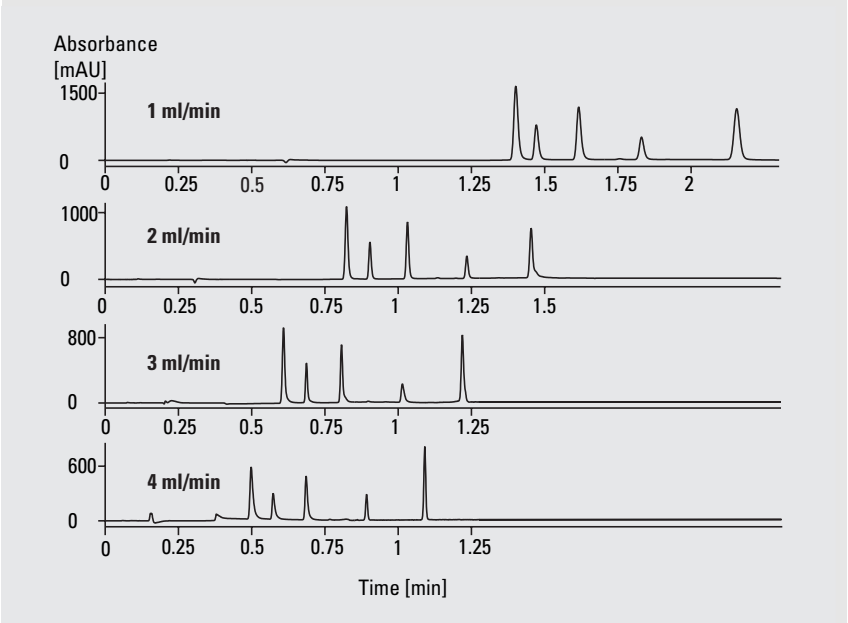


Figure 4
Analysis of selected compounds at four different flow rates

Influence of high flow rates on minimum detectable concentrations

If a high flow rate is run, peak heights and area counts for UV detection are reduced. Consequently, for UV detectors, which are able to detect compounds in the 0.1 ppm range, the compound concentration in the analyzed sample should be in the low ppm or high ppt range to ensure that the UV detector is able to "see" the compounds. It must also be taken into account that a mass selective detector (MSD) cannot handle flow rates of 4 ml/min. The maximum flow rate range is about 1 to 1.5 ml/min. Optimum flow rates for most MSD instruments are about 0.5 ml/min. The column eluent must therefore be split 1 to 10 for optimum conditions. The MSD is able to detect masses in the 100 ppt range. If the compound concentration of the evaluated sample is in the low ppb range, the MSD should still be able to detect the masses, even though the column eluent has to be split before entering the ion source.

Influence of delay volume on run time

Modern high-pressure gradient systems, such as the Agilent 1100 Series, offer system delay volume below 1 ml without modifying the standard system. The Agilent 1100 Series also offers the possibility to switch the Agilent 1100 Series autosampler into bypass mode after the sample has reached the column. This is done using an injector program saving another 300 µl of delay volume. In figure 5, the effect when switching the autosampler into bypass mode is shown. The experiment was done at a flow rate of 2 ml/min, with a gradient of 5 to 95 % ACN in 1 min.

The following injector program used was used:

1. DRAW 5 µl from sample
2. INJECT
3. WAIT 0.03 min
4. VALVE bypass
5. WAIT 1.75 min
6. VALVE mainpass

The wait time before the valve is switched to the bypass position depends on the injection volume, according to the equation:

Wait time = 6 (injection volume + 5 µl)/flow rate.

The run time difference for the last peak is about 0.16 min which corresponds to a volume of 320 µl at 2 ml/min. The influence of the delay volume is not significant in this case and the higher the flow rate the lower the gain in time.

Influence of data rate on precision

Assuming that 20 to 30 data points per peak are needed for a precise measurement, the set data rate of a response time of 0.1 s is sufficient for a peak width of 2 s. The peak width is measured at peak bottom (4 s). This peak width produces 20 data points. All peaks, even those measured at a flow rate of 4 ml/min, are broad enough to produce at least 20 data points per peak.

Influence of column temperature on run time

The influence of column temperature was tested for 50 °C and 25 °C at a 2 ml/ min flow rate, using the gradient from 5 to 95 % ACN in 1 min. The influence is low in this case. The retention time difference for the last peak is only 0.052 min.

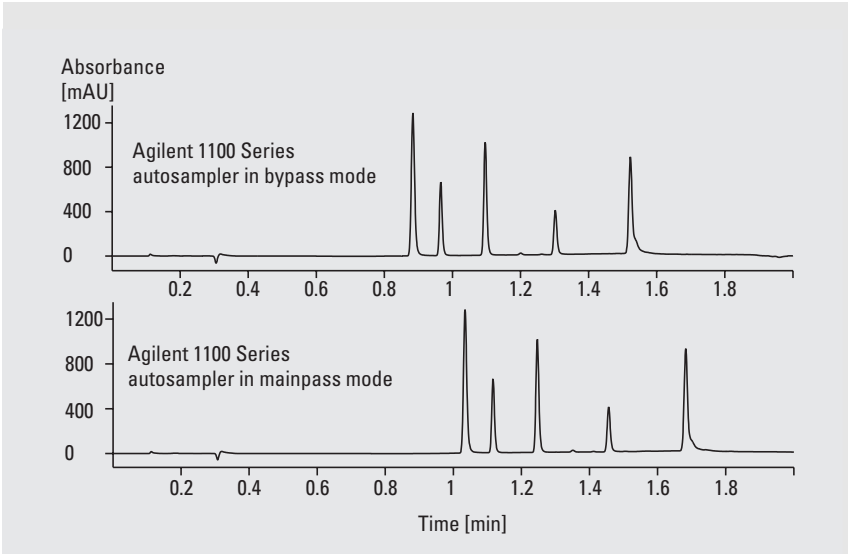


Figure 5
Analysis of selected compounds at different delay volumes

Results of optimization process

The aim was to obtain cycle times of about 2 min and baseline separation of all analyzed compounds. This was achieved as shown in figure 6.

The time the autosampler needs to inject the next sample is sufficient to equilibrate the system for the start conditions.

Method performance was tested over 10 runs and relative standard deviations (rsd) are listed in table 1. The injection volume was 5 µl. The precision of areas is worse than normally expected in HPLC. This is due to the low number of data points that can be acquired at such narrow peak width.

Compared to standard cycle times of about 15 to 20 min, the cycle time could be reduced by a factor of 10.

Column	50 x 4.6 mm Zorbax SB-C18, 3.5 µm
Flow rate	4 ml/min
Mobile phase	A = water, B = acetonitrile (ACN)
Gradient	5 % B to 95 % B in 1 min, 95 % until 1.09 min to 5 % B at 1.1 min
Run time	1.2 min
Post run	time needed to inject the next sample
Diode-array settings	210/8 nm, ref. wavelength 360/100 nm, response time 0.1 s
Injector volume	5 µl, autosampler in bypass mode
Column temperature	50 °C

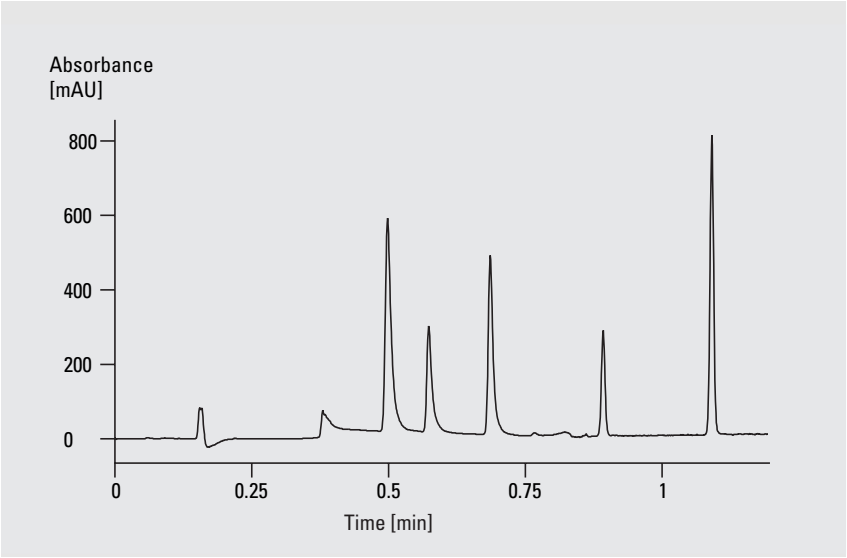


Figure 6
Optimized chromatogram for high throughput and cycle run times below 1.5 min

Compound	Precision of retention time	Precision of areas
Caffeine	0.30	2.60
Primidone	0.29	2.58
Phenacetin	0.19	2.51
Madelic acid benylester	0.16	2.58
Biphenyl	0.06	2.32

Table 1
Precision of rentention times and areas

Conclusions

High-sample throughput in an HPLC system can be achieved by optimizing the cycle times from injection to injection using short columns, high flow rates and steep gradients. Such an HPLC system should include a high-pressure gradient pump. These pumps have low delay volumes and gradient changes almost immediately affect the column. The column length should be 50 mm or less, and the internal diameter should be approximately 4.6 mm. Start and end concentration of the gradient composition should be selected such that it has an impact on all compounds. Otherwise the unaffected compounds tend to broaden, especially at higher injection volumes. The gradient slope should be as steep as possible, however, peak resolution is the limiting factor. A high flow rate of approximately 2 to 4 ml/min is recommended in order to further reduce run times and equilibration times. The data rate must be set as high as possible to guarantee at least 20 to 30 data points per peak. If all parameters are optimized, the sample throughput can be increased at least by a factor of 10 with good precision for retention times and areas.

Literature Reference

Wolfgang K. Goetzinger and James N.Kyranos, “Fast gradient RP-HPLC for high throughput quality control analysis of spatially addressable combinatorial libraries” *American Laboratory*, *pp 27-37*, **April 1998**.

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