Optimizing the Agilent 1100 Series well-plate sampler

Application

Introduction

Autosamplers are more and more used in HPLC to improve laboratory productivity and consistency as well as accuracy of analytical results. The demands on autosamplers has increased during the last few years because of the need for higher precision, lower carry-over and higher sample throughput.

The recent demands for a state-of-the-art HPLC autosampler are:

- lowest carry-over for reliable quantitative data in trace analysis,
- fast injection cycles for high throughput of samples,
- low delay volume for fast gradient operations,
- precise injection over a wide injection volume range for reliable quantitative data,
- linearity over a wide injection volume range for highest flexibility regarding sample volume,
- usable for vials as well as for microtiter and deep-well-plates for highest flexibility in sample preparation,
- vial/well-bottom sensing for access to minimal sample amounts,
- coolable for temperature labile samples,
- full integration of all injector parameters in methods and reports for fast sample tracking and compliance with GLP and cGMP,
- on-line diagnostic tools for quick determination of failures, and
- integrated OQ/PV tools for fast performance checkouts.

In this Application Note we demonstrate how to set up a modern well-plate sampler to fulfill these demands. It covers the following topics:

- how to optimize the autosampler for lowest carry-over and lowest cycle times,
- performance data such as linearity and area precision at different injection volumes,
- how well-plate parameters such as well-plate position can be implemented in customized report forms,.
- what maintenance, diagnostic features and automated OQ/PV procedures should be implemented to provide high instrument uptimes.

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

Agilent Technologies
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Instrumentation

The following Agilent Series 1100 instrumentation was used for all experiments:

- Agilent 1100 Series high pressure gradient pump with lowest delay volume for fast gradient analysis
- Agilent 1100 Series micro vacuum degasser for optimum baseline stability and fast sol vent change
- Agilent 1100 Series well-plate sampler with fast injections, overlapped injection mode for shortest cycle times and bypass position of autosampler for lowest system delay volume. Optionally, the Agilent 1100 Series autosampler can be equipped with a sample thermostat for cooling and heating. 96 well-plates and 96 deep-well-plates can be used as well as 384 well-plates and 2-ml vials. It is also possible to use 96 well-plates with glass inserts and caps. Closing mats are available.
- Agilent 1100 Series thermostatted column compartment for highest stability from 10° C below ambient up to 80° C,
- Agilent 1100 Series DAD with fast sampling rate and for additional spectral information, and
- Agilent ChemStation Plus for instrument control, data handling, sample tracking and fast automated reporting and batch reviewing
- data base module of Agilent ChemStation Plus (ChemStore) for data archiving and reviewing data independently from ChemStation.

Results and discussion

Optimizing lowest carry-over

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 when using highly-sensitive MS methods. If samples of very high (mg/ml) and very low (ng/ml) concentration are analyzed consecutively, carry-over becomes a critical parameter when using these highly sensitive detectors.

Several parts of an injection system can contribute to carry-over:

- needle inside and outside
- needle seat
- sample loop
- seat capillary
- injection valve

The injection routine of the Agilent 1100 Series well-plate sampler is such that sample loop, needle inside, seat capillary and the main channel of the injection valve are always in the flow path. So these parts are continuously flushed during the complete analysis (figure 1).

The outside of the needle can be flushed prior to an injection with fresh solvent. This outside wash of the needle also prevents contamination of the needle seat. The flush port of the Agilent 1100 Series is refilled with fresh solvent by a peristaltic pump, which is installed in the autosampler housing. The volume of the flush port is about 680 µl and the pump delivers 6 ml/min. Setting the wash time to 10 seconds means the flush port volume is refilled more than once with fresh solvent, which is sufficient in most cases to clean the outside of the needle.

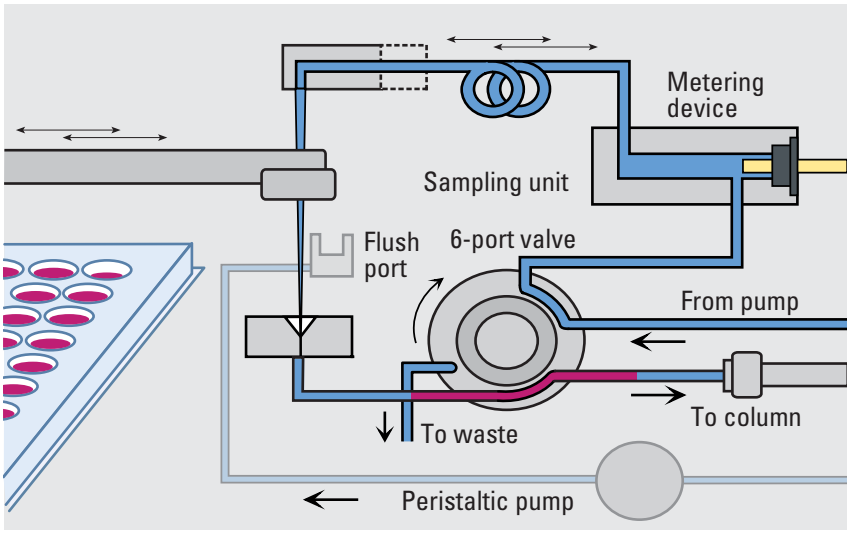

Figure 1
Schematic of injector design

Using this wash procedure the carry-over can be kept below 0.01 % for most compounds. Figure 2 shows the analysis of propylparabene. Propylparabene is soluble in methanol and methanol was therefore selected as wash solvent for cleaning the outside of the needle. 1 µl of a 1-mg/ml concentration was injected, followed by a 5-µl injection of methanol. The carry-over was found to be as low as 0.004 %. This corresponds to a carry-over of 40 pg absolute related to a 1000 ng injection. A blank run previous to the carry-over experiments showed that the system was clean.

Figure 3 shows the analysis of caffeine. Caffeine is highly soluble in water and consequently water was selected as wash solvent for the outside of the needle. As before 1 µl of a 1-mg/ml concentration of caffeine was injected followed by a 5-µl injection of water. The carry-over was found to be 0.007 %. This corresponds to 70 pg absolute related to the 1000 ng injection and using the Agilent 1100 Series Series DAD.

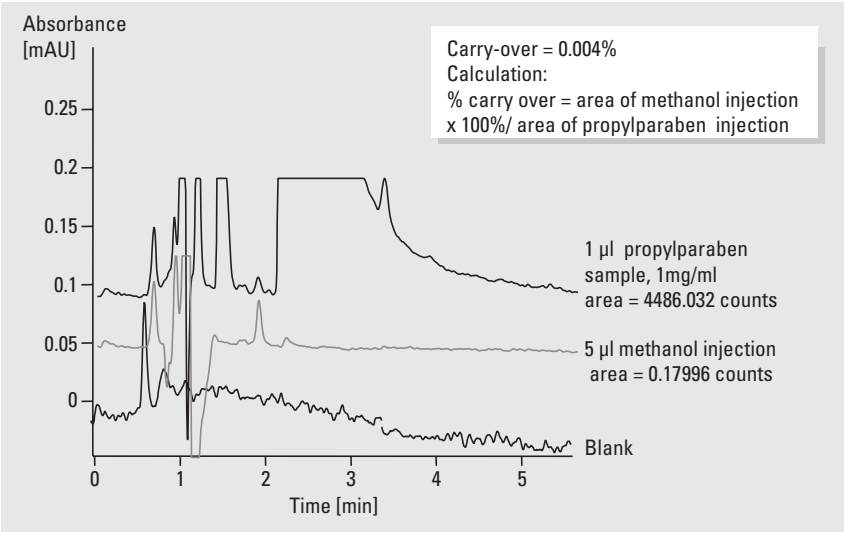

Figure 2
Carry-over measurements using standard wash precedure for propylparabene. Wash solvent is methanol

Column:	125 × 4 mm Hypersil ODS, C-18, 5 µm
Mobile phase:	water/acetonitrile 50/50 for 5 min, gradient to 99 % acetonitrile at 8 min
Flow rate:	1 ml/min
Stop time:	8 min
Post time:	4 min
Injection:	5 µl propylparabene (1 mg/ml), 5 µl methanol to test carry over
Outside wash of needle before injection:	25 s with methanol using flush port
Column temp.:	40 °C
UV detector:	DAD 257/20 nm reference 450/80 nm

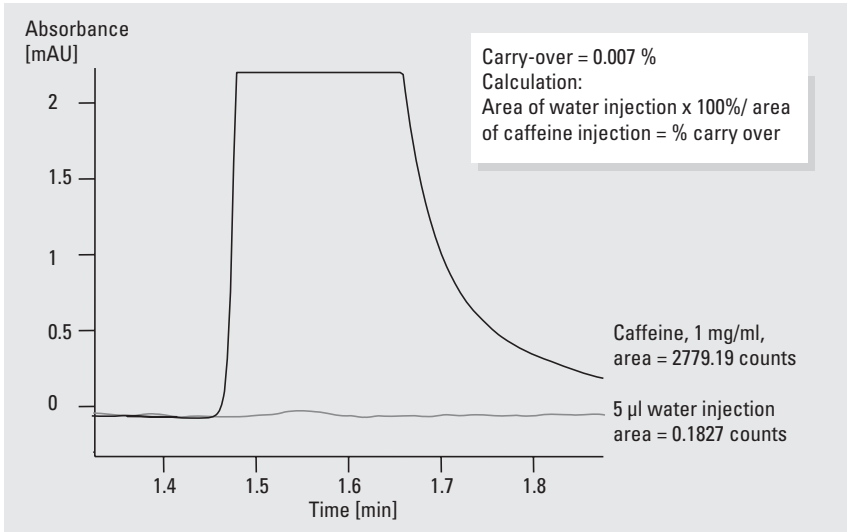

Figure 3
Carry-over measurements using standard wash precedure for caffeine. Wash solvent is water

Column:	125 × 4 mm Hypersil ODS, C18, 5 µm
Mobile phase:	water/acetonitrile 80/20
Flow rate:	1 ml/min
Stop time:	2.5 min
Injection:	1 µl caffeine (1 mg/ml), 5 µl water to test carry-over
Outside wash of needle before injection:	20 s with water using flush port
Column temp.:	40 °C
UV detector:	DAD 273/10 nm reference 450/80 nm

For some applications it can happen that carry-over is caused by residues in the injection valve. In this case it is recommended to use an injector program to reduce carry-over. The injector program switches the injection valve in the so-called bypass position. In this position the bypass channel of the injection valve is switched into the flow path for cleaning. This switching event should take place at the end of the equilibration time to ensure that the bypass channel is filled with the start concentration of the mobile phase. Otherwise the separation could be influenced, especially if microbore columns are used.

In figure 4 a comparison is made between carry-over for an injection with standard wash and an injection using an injector program with additional injection valve switching. The compound used was beclomethasone and 1000 ng were injected followed by the injection of 5 µl methanol. Beclomethasone was selected because carry-over is critical for this compound. With standard injection the carry-over was found to be 0.03 %, Using the injector program the carry-over was down to 0.003 %.

In summary it can be stated that for the Agilent 1100 Series wellplate sampler carry-over will be typically below 0.01 %, if the outside of the needle is cleaned with an appropriate solvent. Further reduction of carry-over can be obtained using an injector program with additional injection valve switching for cleaning the bypass channel. Injector programs also offer the possibility to use additional wash vials for cleaning the outside of the needle with more than one solvent.

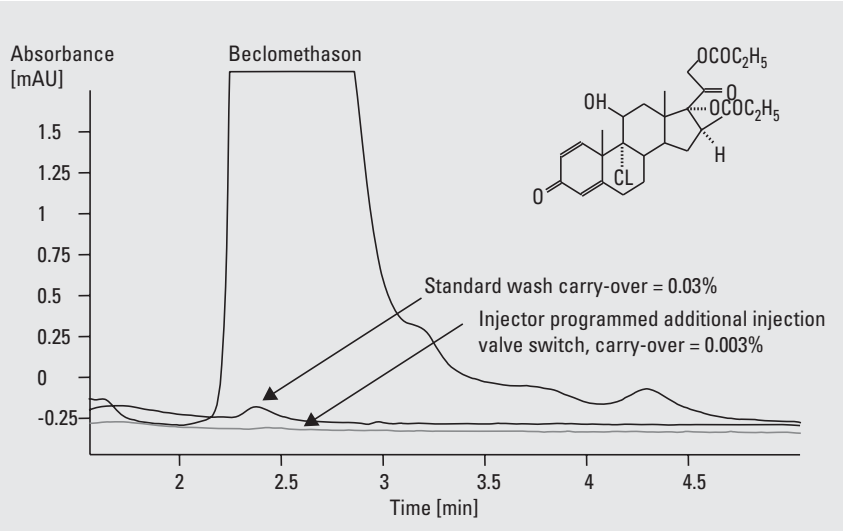

Figure 4
Optimization for lowest carry-over using an injector program

Column:	2.1 × 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 program:
Draw 0.5 (5) µl from sample
Needle wash as method
Inject
Wait 7 minutes
Valve bypass
Wait 0.2 minutes
Valve mainpass
Valve bypass
Valve mainpass

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 steepening gradients. Having optimized these parameters the speed of the autosampler can become a limiting factor where drawing and ejecting a sample takes about two to three minutes and/or overlapped injection is not possible.

The Agilent 1100 Series well-plate sampler described here is able to draw and inject a sample within 36 seconds, including a 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 sample loop, needle and needle seat capillary. Now 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 if low flow rates have to be used as is necessary in narrow-bore and micro-bore HPLC. For the Agilent 1100 Series well-plate sampler reduction of delay volume can be selected from the injector set-up screen, independently from selecting overlapped injection mode, and is then effective for all runs.

Figure 5 shows a fast standard sequence with no overlapped injections. Hydrocortisone, beclomethasone and hydrocortisone-acetate were used as sample. These compounds were separated using a short 4.6-mm id column, 3 ml/min flow rate and a steep gradient. The injector needs 36 seconds and detector balancing takes about 14 seconds before the next sample is injected. The total cycle time including run time and equilibration time is 1 minute 54 seconds. This means 31 runs can be completed in 1 hour.

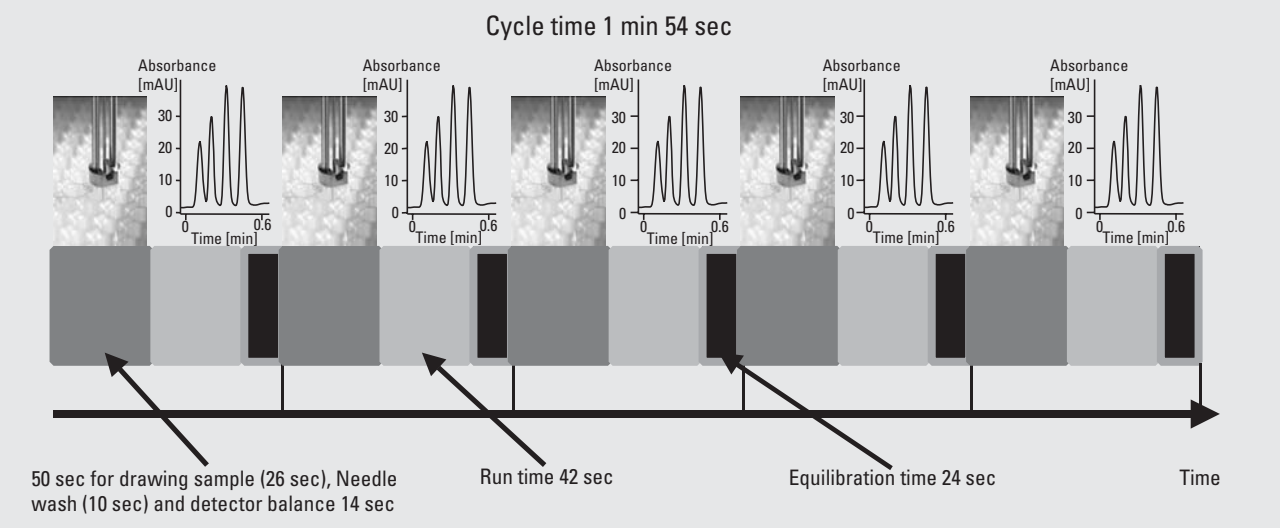

Figure 5
Fast standard sequence

Figure 6 shows the same type of analysis, but here the sequence includes overlapped injections. For this sequence detector balancing performed before each run was switched off. Experiments showed that for well-equilibrated systems no reduction in performance occurs (table 1). The cycle time is now down to 1 minute and 6 seconds. This means 54 runs can be completed in 1 hour.

Precision data for the previous sequences are given in table 1. For all three sequences significant differences cannot be observed. Precision data was evaluated from 10 run each.

Injection cycle times also depend on the injection volume, For the previous experiments 1-µl injection volume was used. If a 100-µl injection is needed the cycle time is prolonged by about 8 seconds. To obtain this, the draw and eject speed of the injection system has to be increased to 1 ml/min. By increasing the draw speed it is

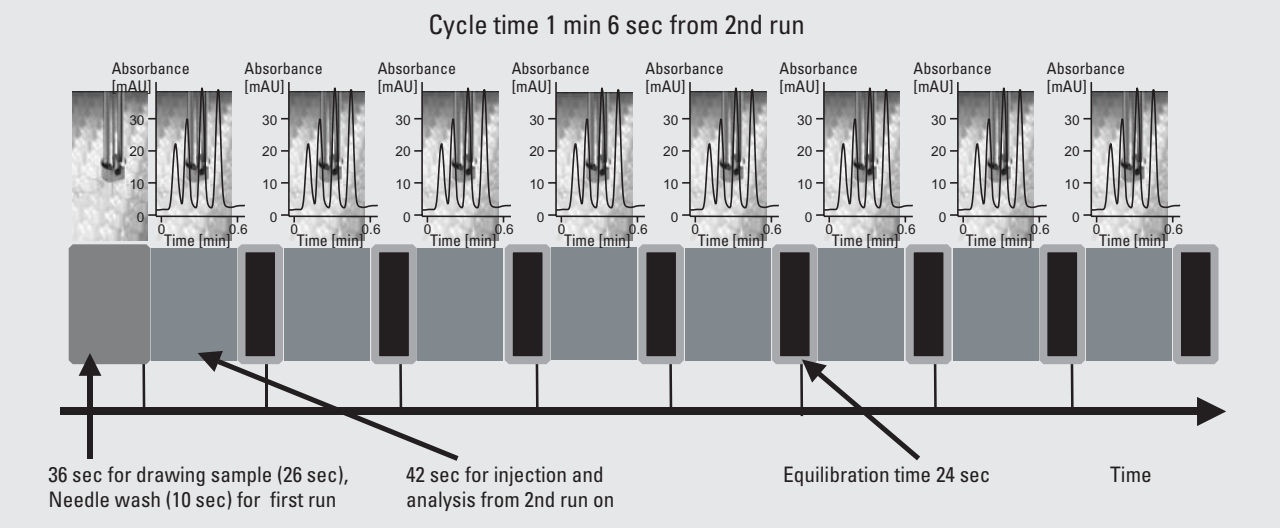

Figure 6
Ultra fast sequence with overlapped injections

Column:	4.6 × 30 mm Zorbax SB-C-18, 3.5 µm
Mobile phase:	water/acetonitrile 60/40
Flow rate:	3 ml/min
Gradient:	at 0 min 40 % ACN at 0.3 min 40 % ACN at 0.6 min 55 % ACN at 0.7 min 40 % ACN
Injection:	1 µl for sample
Outside wash of needle before injection:	10 s with methanol using flush port
Overlapped injection after sample flushed on column (switching time calculated by the instrument based on flow rate and injection volume)	
Column temp.:	40 °C
UV detector:	DAD 254/20 nm reference 350/80 nm

Compound	Standard sequence		Standard sequence no detector balance		Sequence with overlapped injections, no detector balance	
	%RSD RT	%RSD area	%RSD RT	%RSD area	%RSD RT	%RSD area
Hydrocortisone	0.26	1.46	0.28	1.39	0.19	1.28
Beclomethasone	0.19	1.44	0.24	1.34	0.26	1.00
Hydrocortisone-acetate	0.19	1.40	0.29	1.44	0.27	0.97

Table 1
Precision of different sequences

possible that the area precision is lower than for the default draw speed.

In summary it can be stated that gradient analyses times of less than 45 seconds are possible using short columns, high flow-rates and steep gradients. Cycle times of approximately 1 minute are achievable using the high-speed Agilent 1100 Series well-plate sampler which is able to inject a sample in less than 35 seconds including a wash of the needle outside and with the possibility of overlapped injections.

Precision for different injection volumes

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 well-plate sampler it is important to show good precision over the complete injection volume range. For the Agilent 1100 Series well-plate sampler an injection volume range from 0.1 up to 100µl is possible without the need to change hardware. Figure 7 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 volume. 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 have been used for the different injection volumes.

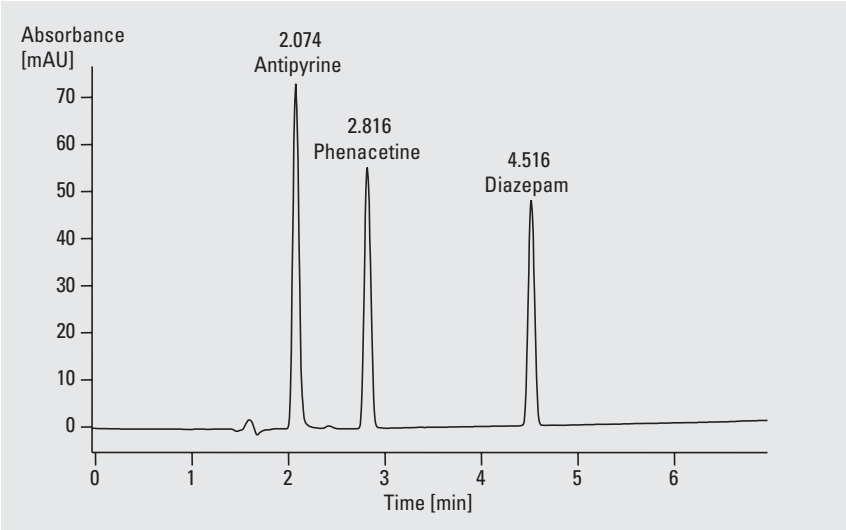

Figure 7
Chromatogram of compounds used for precision and linearity tests

Sample:	antipyrine, phenacetine, diazepam
Column:	Zorbax Eclipse XDB-C8, 4.6 × 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

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

In summary it can be stated that the Agilent 1100 Series well-plate sampler shows excellent area precision over a range from 0.5 µl to 100 µl. One advantage is that no hardware change such as a change of loop or metering device is needed over the settable injection volume range from 0.1 µl up 100 µl. Another advantage is that for optimum precision no waste of sample occurs for rinsing the sample loop. The volume that is drawn will also reach the column. This is in case of limited sample volume a great benefit.

Linearity of injection volume

Good linearity in the low and in the high micro-liter injection volume range is another demand that should be fulfilled by a well-plate sampler. Figure 8 shows the volume linearity over an injection volume range from 0.1 µl up to 10 µl. The system is linear from 0.5 µl. Below 0.5-µl injection volume linearity is not given.
The injections were done using a 300-µl well-plate and as sample compounds the compounds shown in figure 7 were used. The result for diazepam was selected as example in figure 8.

The coefficient of correlation for all three compounds is greater than 0.9999 from 0.5 up to 10 µl injection volume.

Figure 9 shows the linearity of injection volume from 10 to 100 µl. Here linearity is given over the complete range. Injections were done from 1.5-ml vials and the result for diazepam was used as example in figure 9.

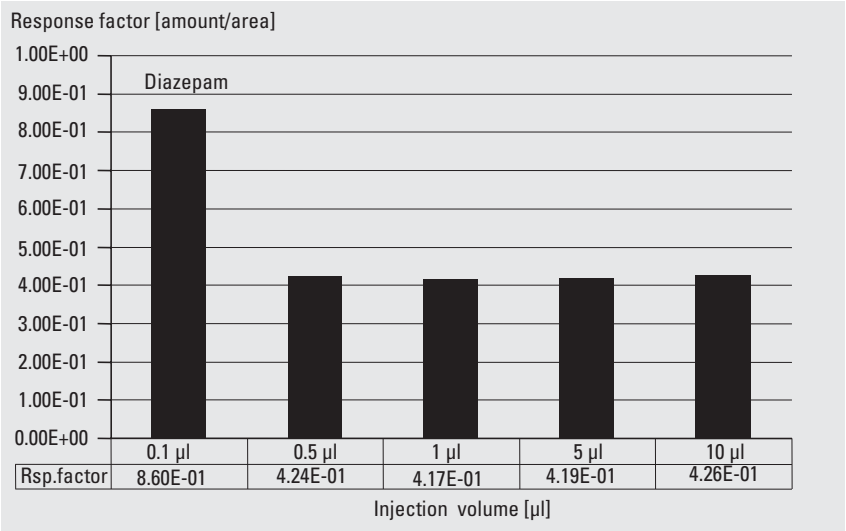

Figure 8
Linearity of injection volume in the low micro-liter range, injected from 300-µl well plate

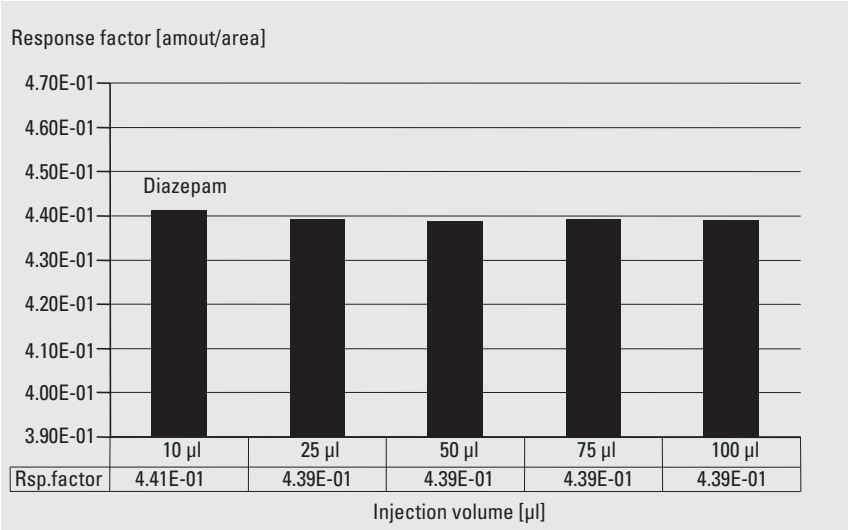

Figure 9
Linearity of injection volume in the middle and high micro-liter range, injected from 1.5 ml vial

The coefficient of correlation for all three compounds is greater than 0.99998 from 10 µl up to 100 µl injection volume.

In summary it can be stated that linearity is given in the low as well as in the middle and high injection volume range. It is very convenient if linearity tests can be done over a wide injection volume range without the need to change sample loops or/and metering devices like glass syringes.

Minimum Sample Volume

Especially in the analysis of proteins and of compounds in biological fluids, sample volume might be limited. In this case it is of advantage, if the autosampler is able to find the bottom of a vial or a well. Having found the well/vial bottom the draw position of the needle should be adjustable to have access even to very small amounts of sample. In figure 10 the minimum accessible sample amount was evaluated for 5-µl injections out of 384 well plates. The sample volume was 50 µl and 10 consecutive injections were made.

The required sample amount to provide a full 5-µl injection was 10 µl. Further experiments have shown that from a 5-µl sample volume 2 µl are fully accessible using 384 well plates.

In summary it can be stated that for accessibility of low sample amounts it is of advantage to have the possibility of vial bottom sensing, fine tuning of the draw position of the needle and no need to fill sample loop completely for several times to provide optimum area precision.

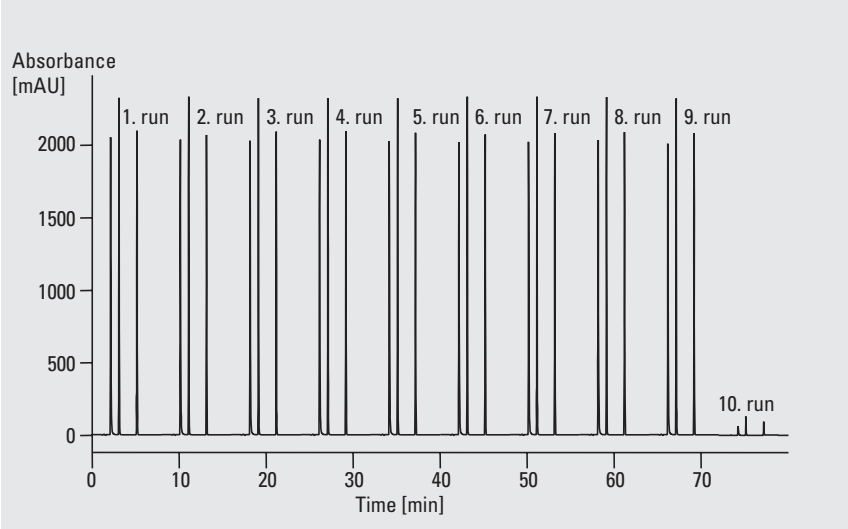

Figure 10
Determination of accessible minimum sample amount. Chromatographic conditions as figure 7

Complete parameter reporting for fast sample tracking in compliance with GLP

We will now show how well-plate sample parameters such as plate position are integrated in a customizable data base report. A report example for the following scenario is given.

A method has been set up for testing an existing library of different drugs. This method has now to be tested for suitability. Figure 11 shows some of the tested samples.

ChemStore Report

For the system used here all data can be transferred to the data base module (ChemStore) of the ChemStation Plus for archiving and reviewing data. The report format can be customized completely, including tables, diagrams, methods, file information and more. Data can also be transferred from the ChemStore data base to spreadsheet programs such as Microsoft Excel® for further calculation.

Well-plate position and data file name can be selected for printing together with the name of the tested compound. In addition well-plate type and the identification name of the well-plate can be included into the report (figure 12).

The ChemStore reporting tool is very flexible regarding data file selection from different sequences, data evaluation and result presentation.

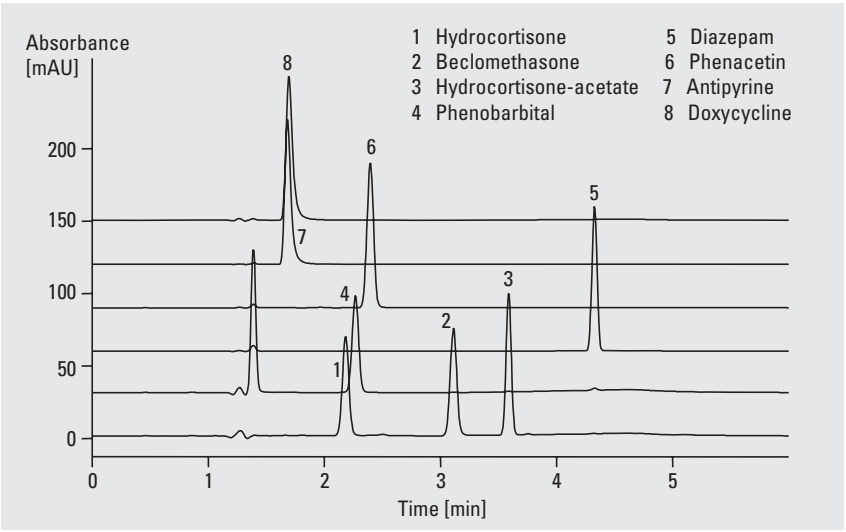

Figure 11
Selectivity test

Column:	Zorbax Eclipse XDB-C8, 4.6 × 150 mm, 5 µm
Mobile phase:	water/acetonitrile 60/40
Flow rate:	1.2 ml/min
Gradient:	at 0 min 40 % ACN at 1 min 40 % ACN at 3 min 80 % ACN at 4 min 40 % ACN at 6 min 40 % ACN
Injection:	0.2 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

Library test report

Run No.	Compound	RT	Amount	Well-Plate ID	Well-Plate	Well-Plate
1	Beclomethasone	3.075	10.00	t405	D-12	*96Agilent*
1	Hydrocortisone	2.145	10.00	t405	D-12	*96Agilent*
1	Hydrocortisone-acetate	3.550	10.00	t405	D-12	*96Agilent*
2	phenobarbital	2.230	10.00	t405	A-08	*96Agilent*
3	Diazepam	4.291	10.00	t405	A-09	*96Agilent*
4	Phenacetin	2.357	100.00	t405	A-11	*96Agilent*
5	Antipyrine	1.642	100.00	t405	B-03	*96Agilent*
6	Doxycycline	1.658	50.00	t405	C-03	*96Agilent*

Figure 12
Example of Agilent ChemStore data base report obtained from Agilent ChemStation Plus

On-line diagnostic tools for quick determination of failure

Nowadays instrument uptime is one of the main concerns, because workload of analytical laboratories does not allow long instrument “down” times. For the Agilent 1100 Series well-plate sampler several tools are implemented to reduce “down” times.
The first step in order to have low failure rates is to follow the maintenance recommendations given in the *Early Feedback Maintenance* screen. Here a time-schedule for changing consumables such as needle seat by number of total injections or the rotor seal by number of injection valve switching events is indicated. If the limit is reached for one part, such as injection rotor seal, a warning appears on the ChemStation and actions can be taken for changing this part before it fails.

Second, if a failure occurs, it is very important that the failure is identified as fast as possible. Here the diagnostic messages and diagnostic tools implemented on the ChemStation for the well-plate sampler can contribute.

Integrated OQ/PV tools for fast performance check outs

For good laboratory practice and good manufacturing practice an instrument has to be validated on a regular basis. This can be very time-consuming if no automated methods are available from the vendor. For all Agilent 1100 Series modules automated OQ/PV procedures can be used. The tests for well-plate sampler include precision of area and height as well as carry-over testing. The user configurable test can be started and all actions needed, such as mobile phase change and required samples are requested from the instrument. If everything is set up, the tests start and at the end a report is given with pass and fail.

Conclusion

The Agilent 1100 Series well-plate sampler is able to provide lowest carry-over and fast injection cycles. It has been proven that in general carry-over is below 0.01 % using the standard wash procedure and below 0.005 % using an injector program with additional steps.

Cycle time for a gradient analysis with high flow rate and a short column can be as low as one to two minutes taking advantage of the fast injection speed of the autosampler and the overlapped injection tool. Linear injection from 0.5 up to 100 µl can be expected as well as high precision for area quantitation.

Using database features all important well plate parameters are combined in customizable report forms for ease of sample tracking. On-line diagnostic and maintenance tools and automated OQ/PV procedures enable high instrument uptime.

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Innovating the HP Way