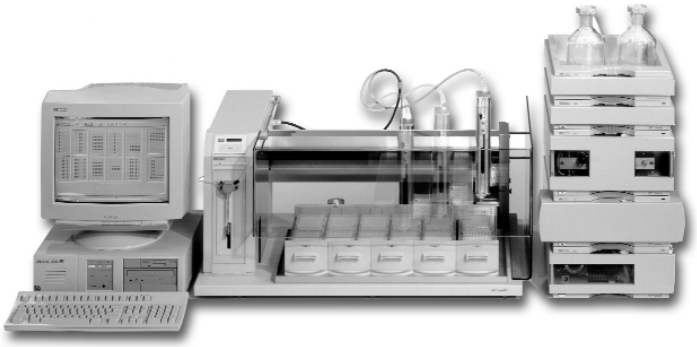
Optimizing the Agilent high-throughput analysis system for high performance and precision

Technical Note



Abstract

The Agilent 220 micro plate sampler—integrated into the Agilent 1100 Series LC system—is the ideal tool to analyze large numbers of structurally distinct compounds. It offers the full potential of well plate technology:

- High sample capacity to increase sample throughput and shorten response times. This is especially useful for drug discovery, combinatorial chemistry, medicinal chemistry and natural product analysis.
- The flexibility of having a sampler mode for high-speed sample analysis and a fraction collector mode for sample isolation and purification.
- An automated injector program for sample preparation such as derivatization, dilution and mixing.
- The ability to store and inject over 4,600 samples (using 384-well plates) for unattended high-throughput.

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Introduction

The Agilent 220 micro plate sampler (MPS)[1] is an essential part of Agilent's system for combinatorial chemistry and high throughput HPLC analysis. It combines high sample capacity, high speed and sampling/fractionation capabilities in one system. In combination with the Agilent 1100 Series HPLC system using mass selective detection it is a complete system that fulfills the requirements of combinatorial chemistry and high-throughput analysis, offering robustness, ruggedness, sensitivity, selectivity and speed. The integrated system plus the single software platform simplifies system setup, operation and management of large amounts of data.
Details of the Agilent 220 MPS and how it works in an Agilent system for combinatorial chemistry or high throughput screening is described in another Agilent technical note[2]. In this note we explain how to further optimize the hardware and software setup of the Agilent 220 MPS to achieve higher performance and precision.

Equipment

All experiments were carried out on the Agilent 1100 Series HPLC system consisting of

- Agilent 1100 Series vacuum degasser,
- Agilent 1100 Series binary pump,
- Agilent 1100 Series thermostatted column compart ment,
- Agilent 1100 Series diode array detector, and
- Agilent 220 micro plate sampler.

The system was controlled using the Agilent ChemStation (version A.07.01) and the micro plate sampling software (version A.03.01).

Injection principle

In contrast to the Agilent 1100 Series autosamplers the Agilent 220 MPS works with a fixed-size sample loop injection (figure 1). While the valve is in loading position the amount of drawn sample is injected into the fixed size sample loop capillary and the surplus of sample is flushed through the loop into waste. To achieve optimal performance overfilling of the sample loop is recommended. The overfill volume depending on loop size is shown in table 1.

Loop Volume [µl]	Sample Volume [µl]
5	35
10	40
20	55
50	95

Table 1
Sample loop sizes and recommended sample volumes

The reason why overfilling is necessary is the hydrodynamic behavior of fluids as they pass through tubing. A process called laminar flow takes place under conditions in which molecules close to the tubing walls are slowed by frictonal forces. The result is a bullet-shaped profile in which the molecules in the center of the stream travel roughly twice the velocity of those at the tubing wall[3].
The surplus of sample volume also ensures that the dead volume of the injector port capillary of approximately 4.2 µl is completely flushed with sample. After switching the valve into the injection and run position the amount of sample in the loop is applied to the system.

To apply sample amounts smaller than the loop volume the *Centered Loop Fill* or the *Partial Loop Fill & Inject* commands can be used. For the *Centered Loop Fill* technique a sandwich of 1-µl air gaps and sample is injected into the sample loop. The volume drawn before the air gaps and the sample volume is calculated by the software to position the sample volume in the middle of the sample loop. The precision for this technique is lower than for complete loop fill because only the

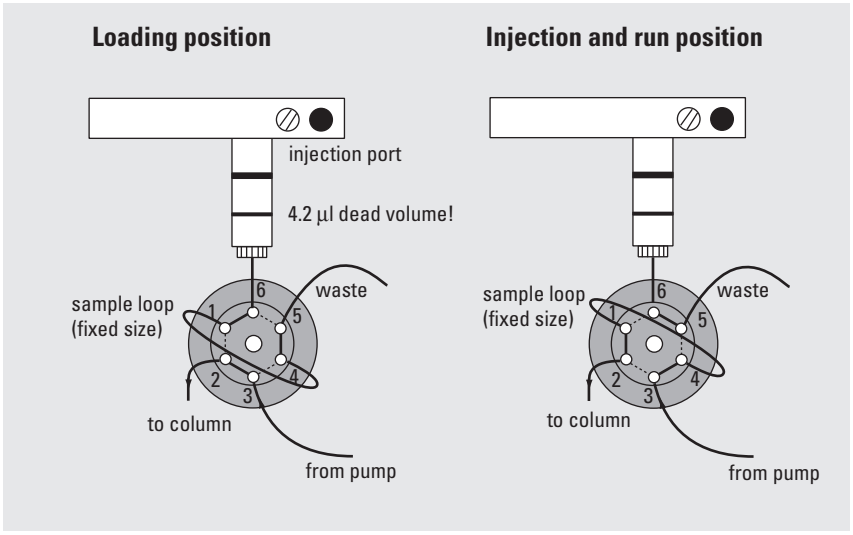

Figure 1
Injection principle of the Agilent 220 micro plate sampler

mechanical precision of the dilutor syringe determines the injected sample volume. For *Partial Loop Fill & Inject* the sample volume plus a relatively large rinse volume is drawn from the sample. The rinse volume is used to rinse the injection port and the sample loop before the actual sample volume is pushed into the sample loop. The precision is higher than for *Centered Loop Fill* but also more sample volume is required.

Parameters to optimize

Parameters influencing the performance of the sampler in sampling mode are:

1. Drawn sample volume (complete loop fill)
2. Draw speed (complete loop fill)
3. Size of air gap (complete loop fill)
4. Dilutor syringe size and sample loop size (centered loop fill)

1. Drawn sample volume (complete loop fill)

Although the size of the sample loop determines the injected sample volume the amount of drawn sample volume also influences the precision of the measurement[3]. Figure 2 shows the precision of peak areas for ten measurements for different drawn sample volumes

For the drawn sample amount of 5 µl the precision of peak area is very high because 5 µl is just enough sample to fill the injection port capillary (dead volume 4.2 µl). Increasing the sample volume also increases the precision of peak area. When the recommended sample volume is used the precision is usually optimized. More sample volume does not increase the precision any further. If not enough sample volume for sufficient overfill of the sample loop is available, a lower precision of peak area results.

2. Draw speed (complete loop fill)

The draw speed for the sample as well as the inject speed influences the precision of the measurement as shown in figure 3.

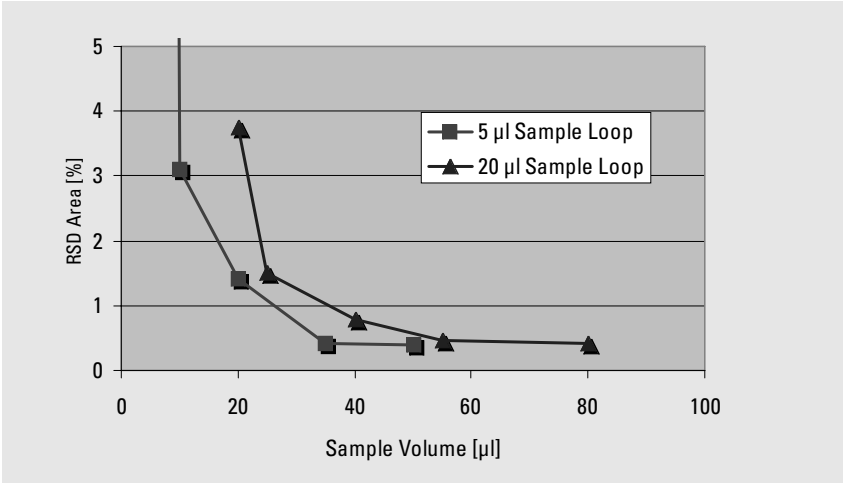

Figure 2
Precision of peak area for different sample volumes (5 and 20-µl sample loop, complete loop fill)

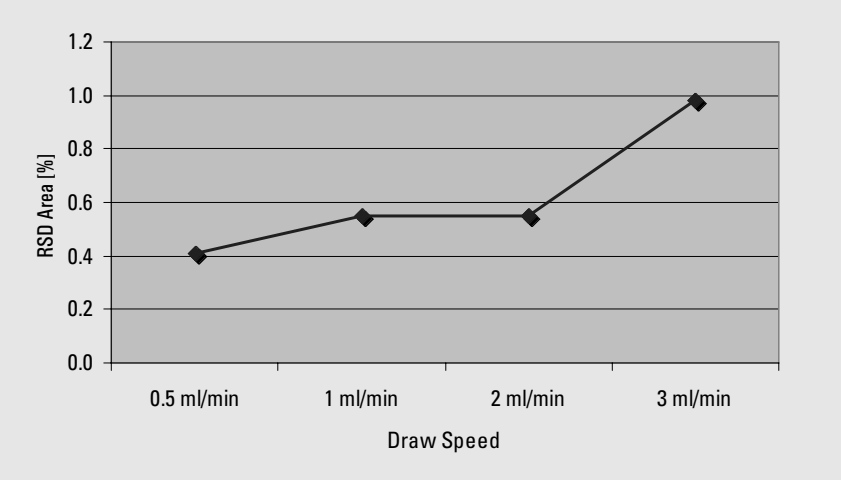

Figure 3
Precision of peak area for different draw and inject speeds (5-µl sample loop, complete loop fill)

The best precision is achieved with the lowest draw speed of 0.5 ml/min, which is still higher than the default draw speed of the Agilent ChemStation (0.2 ml/min). Table 2 shows the necessary combined times for the *Create Air Gap*, *Draw Sample* and *Inject* steps for different sample volumes and different draw speeds.

Draw Speed	0.5 ml/min	1 ml/min	3 ml/min
Sample Volume			
35 µl	8.76 s	4.38 s	1.46 s
55 µl	13.56 s	6.78 s	2.26 s

Table 2
Times for Create Air Gap, Draw Sample and Inject steps for different sample volumes and draw speeds

The times required for the *Create Air Gap*, *Draw Sample* and *Inject* steps are in the range of a few seconds, which shouldn't make much difference for normal chromatographic runs. For high throughput analysis with run times in the range of 60–120 seconds a time of 13.56 seconds (55-µl sample volume, draw speed 0.5 ml/min) is about 10–20 % of the whole run time. Therefore it might be necessary to use higher draw speeds. For a draw speed of 3 ml/min the *Create Air Gap*, *Draw Sample* and *Inject* time of 2.26 seconds is only 2–4 % of the whole run time. The disadvantage of higher draw speed is lower precision of the analysis.

3. Size of air gap (complete loop fill)

It is strongly recommended to insert a *Create Air Gap* command before the *Draw Sample* command in the CC-Mode method. The air gap prevents mixing of the sample with the solvent in the tube connected to the dilutor syringe. The optimal size of the air gap can be seen in figure 4.

An air gap between the sample and the solvent in the tube connected to the dilutor syringe is necessary to avoid mixing of sample and solvent. The size of the air gap should be between 1 and 4 µl, with a recommended size of 2–3 µl. The size of the air gap must not be larger than the dead volume of the injection port capillary of about 4.2 µl. For an air gap size of 5 µl, for example, some of the air gap is moved into the sample loop during injection. The effect is evident for an air gap size of 10 µl. The loop is half filled with air from the air gap. Therefore less sample volume is applied to the system.

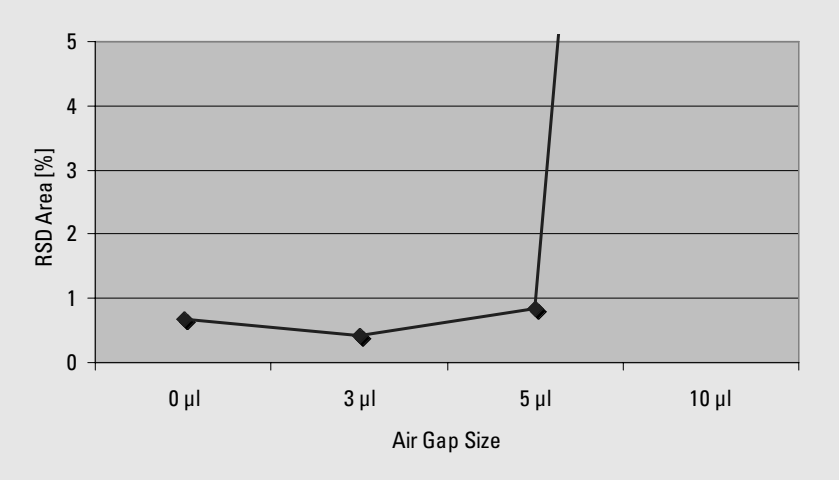

Figure 4
Precision of peak area for different air gap sizes

Dilutor syringe size and sample loop size (centered loop fill)

To inject sample volumes smaller than the sample loop size the *Centered Loop Fill* command is used. Figure 5 shows how a sandwich of air gaps and sample is injected into the sample loop. The sizes of the air gaps are calculated by the software by placing the sample in the middle of the sample loop (figure 5).

For *Centered Loop Fill* the precision of the drawn sample amount depends on the mechanical precision of the dilutor syringe. Therefore, the precision of peak area for *Centered Loop Fill* is lower than for complete loop fill[4,5]. The solvent volumes for different sample volumes are shown in table 3 (100-µl sample loop). While the air gap volumes are fixed (1 µl) the upper solvent volume is calculated by the software by placing the sample volume in the middle of the sample loop. The area precisions for different sample volumes for two different dilutor syringe sizes are shown in figure 6.

The precision of peak area increases by applying more sample volume to the sample loop. It is recommended to fill the sample loop to at least 50 % of the loop size. Figure 6 shows that the precision of peak area is about 1 % at 50 µl sample volume. When using the 500-µl syringe the values are slightly higher than for the 100-µl syringe[4,5].

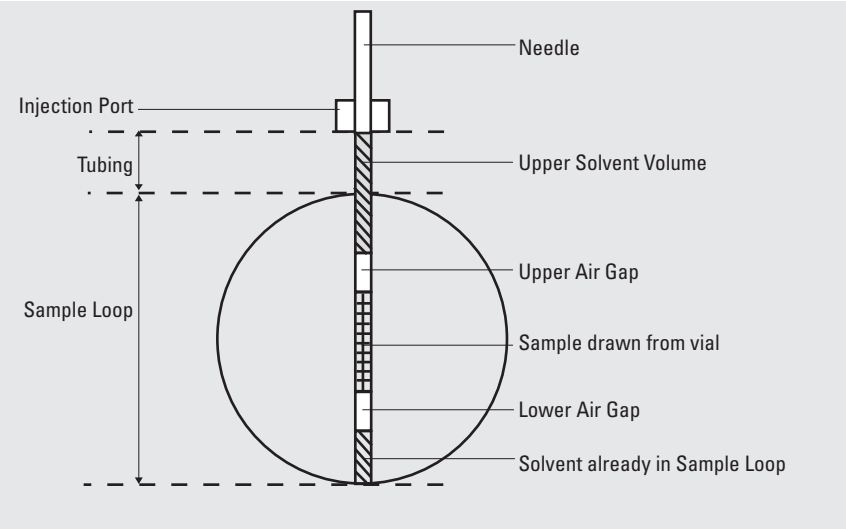

Figure 5
Centered Loop Fill

Sample Volume [µl]	Upper Solvent Volume [µl]	Upper Air Gap Volume [µl]	Sample Volume [µl]	Lower Air Gap Volume [µl]
5	51	1	5	1
10	48	1	10	1
20	43	1	20	1
50	28	1	50	1
80	13	1	80	1

Table 3
Solvent volumes, upper and lower air gap sizes for different sample volumes (100-µl sample loop)

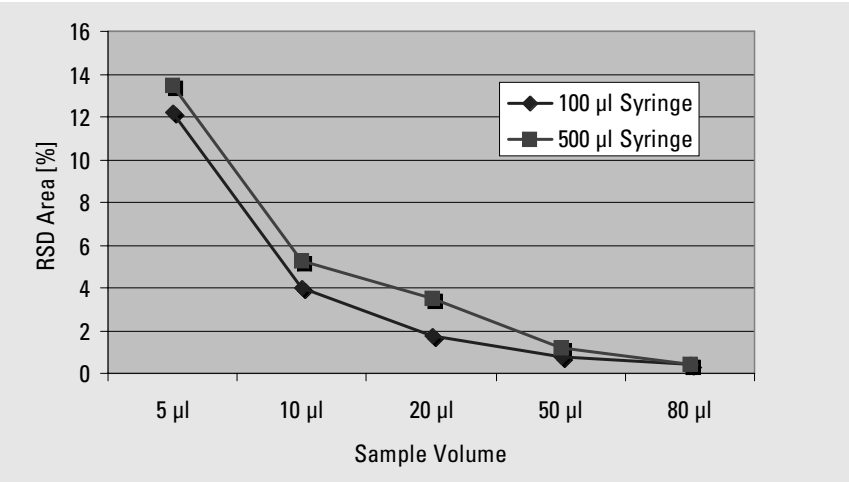

Figure 6
Precision of area for different sample volumes for two different dilutor syringe sizes

Partial Loop Fill

The CC-Mode software (version A.03.01) offers a new *Partial Loop Fill* feature. In addition to the sample volume a rinse volume is drawn from the sample. The rinse volume is used to flush the sample loop before the actual sample volume is placed in it. The advantage over the *Centered Loop Fill* is better precision (figure 7), however, the sample volume required is much higher. Therefore, the decision which partial loop fill mode is used depends on the application. If high reproducibility is required and large sample volume is available the *Partial Loop Fill* should be selected. If only small sample volumes are available the *Centered Loop Fill* with the disadvantage of lower reproducibility has to be used.

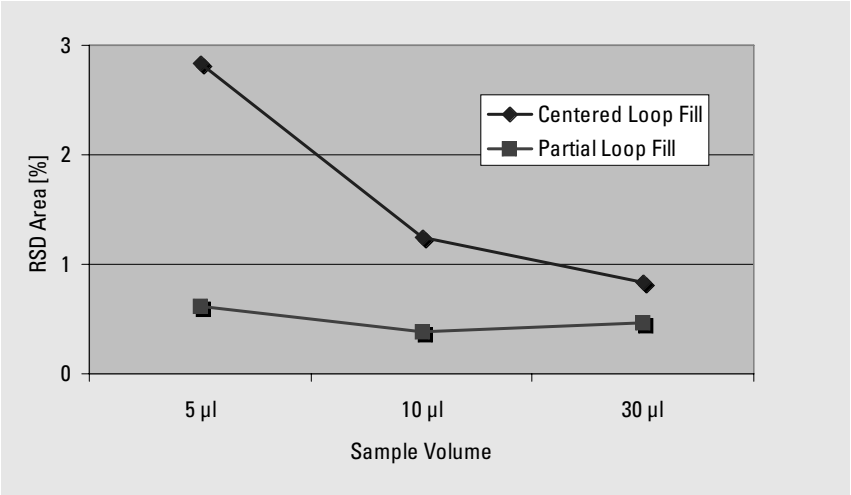

Figure 7
Area precision for Centered and Partial Loop Fill

Carry over

The Agilent 220 MPS and its software offer different options to minimize carry over, such as the wash vial and the rinse options[6]. The effects of these options on carry over are shown in table 4.

Important commands to remove carry over are the rinse functions *Rinse Needle Outside*, *Rinse Needle Inside* and *Rinse Injection Port*. For the sample used in this experiment the effect of the wash vial on the carry over was minimal, however, it becomes very important for samples with higher viscosity.

	Carry over [%]		
Sample concentration [mg/l]	No rinse or wash function	*Wash Vial* for 1 s	*Wash Vial* for 1 s, *Rinse Needle Outside* and *Rinse Injection Port* with 100 µl, 2 ml/min
100	3.2	2.9	0.1
200	1.9	1.9	0.1
300	2.2	1.6	0.1
500	1.9	1.3	0.1
1000	1.7	1.4	0.1

Table 4
Carry over results

Results

The Agilent 220 micro plate sampler is a precise instrument for high throughput analysis of large numbers of samples. To optimize the performance for sample injection the parameters and settings listed in table 5 have to be taken into consideration.

	Parameters/Settings
Drawn sample volume (complete loop fill)	If enough sample volume is available the sample loop should be overfilled with the recommended sample volume. The recommended sample volumes for different sample loop sizes are shown in table 1.
Draw speed (complete loop fill)	The draw speed for *Create Air Gap, Draw Sample* and *Inject* should be set to 0.5 ml/min or less. Higher draw speeds lead to decreasing precision. The time saved is minimal.
Size of air gap (complete loop fill)	Create an air gap before sample is drawn. The size of the air gap should be between 1–4 µl, 2–3 µl are recommended. The size of the air gap must not exceed 4 µl.
Dilutor syringe size and sample loop size (centered loop fill)	When using *Centered Loop Fill* a sample loop filled to at least 50 % should be used. The volume of the dilutor syringe should be the same or higher than the sample loop, but still as small as possible.
Partial Loop Fill	The partial loop filling technique is used when high reproducibili ty is required and large sample volume is available.
Carry over	To avoid carry over use the commands *Rinse Needle Inside*, *Rinse Needle Outside* and *Rinse Injection Port*. Use the *Wash Vial* command, especially for samples with high viscosity.

Table 5
Summary of results

Conclusion

This technical note described the optimization of the Agilent 220 MPS hardware and software for highest precision. The optimal settings for drawn sample volume, draw speed, size of the air gap and dilutor syringe size and sample loop size were evaluated. Furthermore, it was demonstrated how carry over could be minimized. This information should help optimize the Agilent 220 MPS for high performance and precision.

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