



ALEXYS Analyzer
for Highest Sensitivity in
Neurotransmitter Analysis

**Monoamines and
Metabolites**

Noradrenaline
Dopamine
Serotonin
5-hydroxyindole acetic
acid (5-HIAA)
3,4-dihydroxyphenylacetic
acid (DOPAC)
homovanillic acid (HVA)

**OPA derivatized amines
and amino acids**

GABA and Glutamate
Histamine (LNAA)
4-aminobutyrate (GABA)
Glutamate (Glu)
LNAA

**Choline and
Acetylcholine**

Choline (Ch)
Acetylcholine (ACh)

**Markers for
oxidative stress**

3-nitro-L-Tyrosine
8-OH-DPAT

**Glutathione and
other thiols**

Micro volume injections

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- **Micro volume sample injection method**
 - **No sample loss to loop 'overflow'**
 - **Reproducible and robust**
 - **Excellent peak efficiency**
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Summary

A method is described for sample injections without the commonly applied overflow. The sample is aspirated between air plugs and transported to the sample loop with minimum sample dilution. The method is reproducible and fully adjustable. It efficiently handles small sample volumes without 'wasting' any precious sample.



Introduction

In standard injection methods - full or partial loop fill - the needle and connecting tubing are filled with sample, prior to the aspiration step to fill the loop. This method of overfill takes about 3 times the loop volume, which means that for a 20 µL full loop injection about 60 µL sample is used. This method ensures a high accuracy and repeatability of injections. However, with really small samples it is not possible and therefore a method has been developed to minimize the sample use.

The ALEXYS LC/EC system comprises a fully programmable AS110 autosampler. In addition to the standard available injection methods, the AS110 has the option to create a User Defined Program. In this note we present a dedicated injection method with only a few microliters overfill, to efficiently inject the available sample while maintaining reproducibility and peak efficiency.



Figure 1: ALEXYS Neurotransmitter Analyzer.

Method

The User Defined Program is designed for use with the newest ALEXYS systems containing an AS110 autosampler (pn. 191.0035x or 191.0036x, with CPU v. 1.30 or higher). These autosamplers are equipped with a 15 µL sampling needle and a sample loop in the range of 1-100 µL. In the Clarity software, the driver 'Alias; including User Program' unlocks the necessary control options. In 3 steps the AS 110 is prepared for User Defined Programming.

Step 1 is to evaluate and install the right sample loop. The method is based on a full loop injection. Prior to installing a loop, column loadability must be evaluated and available sample volume has to be taken into account. Most micro-LC applications only work well with injection sizes of a few µL. The exact column loadability can be measured on forehand using a 20 µL sample loop, partial loop fill method and a series of incrementing injection volumes. The selection of the sample loop should also be at least 3 µL smaller than the total available sample size. Table 1 shows the sample loop sizes that are available from Antec.

Table 1

| Part numbers of different sizes of sample loops for 1/16" and 1/32" injector ports of the AS110 autosampler. | | |
|--|-----------------|-----------------|
| Vol | For 1/16" ports | For 1/32" ports |
| 1 µL | - | 250.1218 |
| 1.5 µL | - | 250.1220 |
| 2 µL | 250.1201 | 250.1222 |
| 5 µL | 250.1200 | 250.1224 |
| 10 µL | 250.1202 | 250.1226 |

Step 2 is to optimize the needle height close to the bottom of the vials to ensure that all available sample can be aspirated from the vial. For this, place an empty vial on the front row of the sample tray, perform an injection with a needle height of 5 mm and freeze the needle in position after it lowered into the vial by switching the autosampler power off. Using a flashlight it should be possible to see the needle tip inside the vial.

- Estimate the size of the space between the tip and the bottom.
- Repeat the injection, but now with a slightly lower needle height and evaluate again.
- Repeat the procedure until the right needle height is obtained (= needle tip close to the bottom, but not through).



Step 3 involves programming the injection program in the Clarity software and finding the best combination of settings in the injection program. The principle of the steps in the injection program is visualized in Fig. 2. Take into consideration that this injection program will be optimized for the actual combination of total sample volume, sample loop, individual autosampler and tolerances of all the involved volumes.

Programming the Clarity software

In the Clarity data system, the injection method is programmed in the window 'Method / AS Control...':

- Under the tab 'Injection', the box 'Use User program' has to be checked.
- Under the tab 'System', the correct syringe volume has to be set, as well as the sample tray temperature (if necessary).
- In the tab 'Spec. Vials' the vial tray type has to be set correctly to prevent a needle crash.
- The tab 'User program' is then programmed with the lines from Table 2:

Table 2

User defined program for efficiently handling small samples.

| Step | Line | Description |
|------|--|---|
| 1 | Injector valve, Load | switch valve to Load position |
| 2 | Aspirate Air, syringe speed 2, vol 5µL | Insert air plug in needle |
| 3 | Aspirate Sample, syringe speed 1, needle height 4.5 mm, vol X µL | Total sample use (X) |
| 4 | Wait, time 0.1 min | |
| 5 | Aspirate Sample, syringe speed 1, needle height 4.5 mm, vol 0 µL | This line keeps the needle in position while the sample enters the needle |
| 6 | Aspirate Air, syringe speed 2, vol Y µL | Move the sample into the loop |
| 7 | Wait, time 0.1 min | |
| 8 | Injector valve, Inject | Inject the sample |
| 9 | Marker, Injection | Start data acquisition |
| 10 | Wash from Port 1, 250 µL | Needle wash |

User defined program for efficiently handling small samples. Volume 'X' has to be programmed as the size of the sample loop + 2µL (or more if there is apparent dead volume in the ports). Volume 'Y' is in principle the size of the sample needle, but a 1-4 µL deviation from this theoretical value may be closer to the real optimum.

Method optimization

To find the best combination of settings, a series of injections using slightly different values for X and Y in Table 2 have to be performed and the data compared with results from a regular full loop injection. It is important that the sample completely fills the loop during the injection program, because even the partial injection of air will show up in the chromatogram as a disturbance.

The chromatographic parameters to evaluate during the optimization are:

- Peak height
- Baseline ripples and patterns
- Peak shape

When a satisfactory set of X and Y values are obtained, a set of 6 repeated analyses can be used to check repeatability by calculating the relative standard deviation (%RSD) for height, area and retention time.

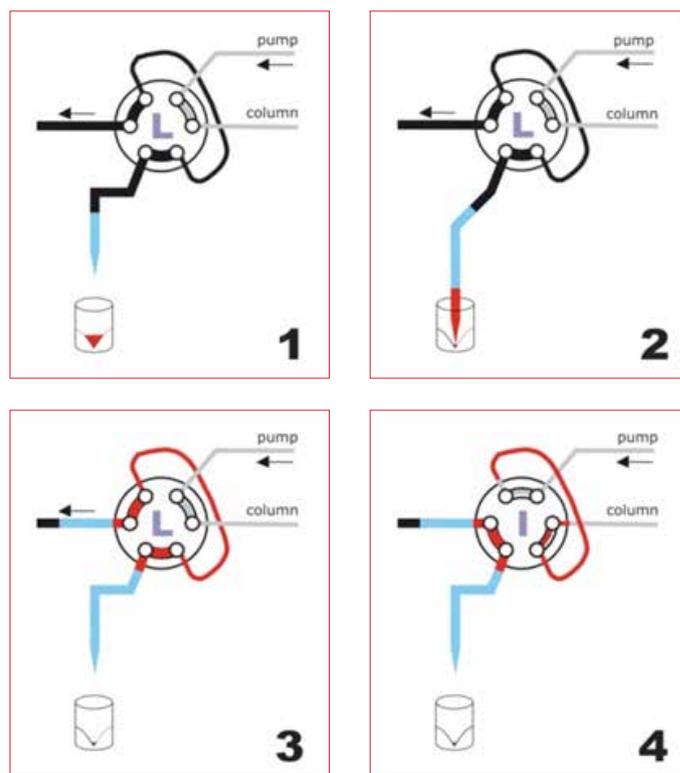


Figure 2: Principle of the described injection method. The sample (red) is aspirated from the vial into the needle tubing (step 1, 2). To minimise dilution at the interface between sample and needle wash solution, an air plug (blue) is inserted. To minimise sample consumption, the sample plug is pulled into the loop while the needle is in mid-air. Front and tail of the sample plug are cut off during injection (step 4). A subsequent automated needle wash removes the air plugs from the injector.



Micro volume injections

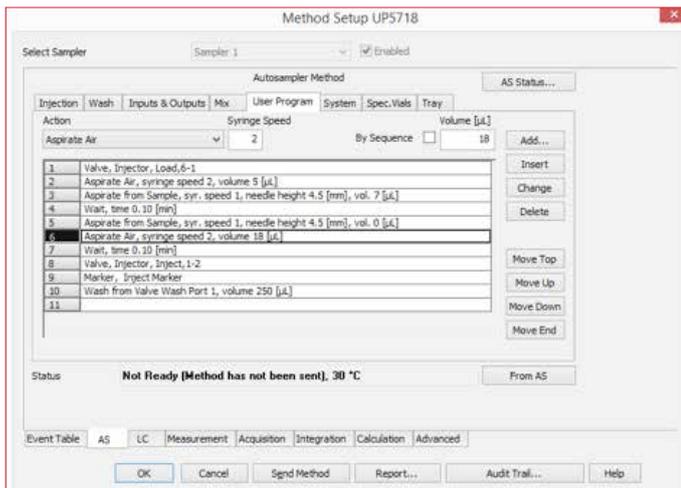


Figure 3: Click 'Add...' to create a user program from the available options in the 'Action' pull down list, or use 'Change' to modify an already programmed line.

Example

Optimize the value for Y in the injection procedure

The example in Fig. 3 shows the peak response in an experiment where the sample was moved into the system by varying the volume Y in the user program described in Table 2. The fact that the measured pattern does not match the theoretical pattern is an indicator that the flow path up to the sample loop had a different (larger) volume than expected (sampling needle of 15 μL). When optimizing the injection program to minimize sample use, it is important to check the best value for Y. As can be seen in Fig. 4, the chromatogram baseline is affected when injecting the sample after moving it with more than 18 μL of air, indicating a partial injection of air. Peak shape (asymmetry and theoretical plates) was not affected, and 18 μL was chosen in this case as the optimal value for Y.

Repeatability of optimized injection methods

When a satisfactory set of values for X and Y are obtained, the relative standard deviation (%RSD) for height, area and retention time can be measured using a set of 6 repeated analyses. As an example, we present the results from two optimized injection programs. One method, named '5318', uses a total sample volume of 3 μL (value X in Table 2), and moves it into a 1.5 μL sample loop with 18 μL air (value Y in Table 2). The second method, named '5718', uses a total sample volume of 7 μL (value X in Table 2), and moves it into the 5 μL sample loop with 18 μL air (value Y in Table 2). As can be seen in Table 3, repeatability was shown to be better than 2% for peak area and peak height for both these optimized custom injection methods.

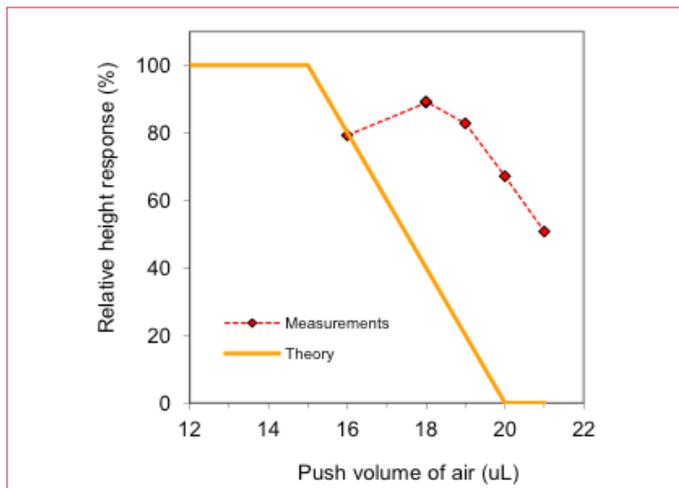


Figure 4: Effect of volume of air (= "Y") on peak response with the use of a custom injection method. The results of using a full loop injection program is set as the 100% response level. Data are from a system equipped with a 15 μL sampling needle and a 5 μL sample loop. For comparison, the theoretic response is plotted as if there would be no contribution of dilution effects during the transport of the sample through the tubing. The tested user program is described in Table 2

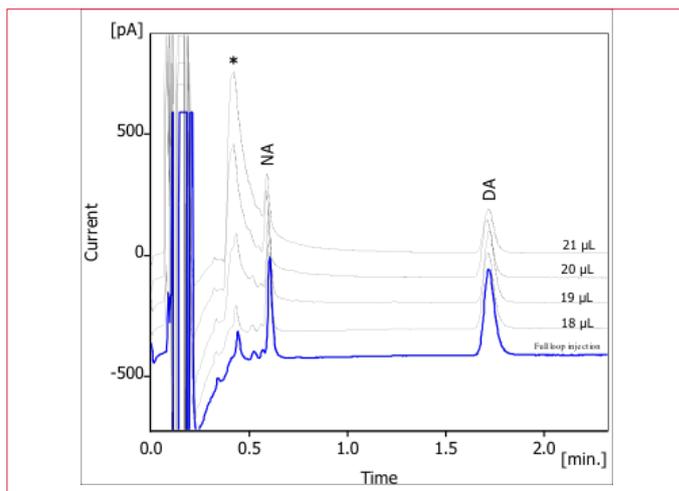


Figure 5: The programming of too large volume of air in custom injection program affects the baseline of chromatograms. The chromatogram from using a full loop injection program is given in blue for comparison. Data are from a system equipped with a 15 μL sampling needle and 5 μL sample loop. Tested standard is 10 nM NA and DA in Ringer's solution with 10 mM acetic acid.



Table 3

| Repeatability values | | |
|-------------------------|-------------------|-----------------|
| Injection method | UP 5318 | UP 5718 |
| Sample loop | 1.5 μL | 5 μL |
| Total sample use (X) | 3 μL | 7 μL |
| Loop filling efficiency | 81% | 87% |
| RSD Peak height | 1.5% | 1.3% |
| RSD Peak area | 1.8% | 1.7% |

Repeatability values (% Relative Standard Deviation) based on 6 injections from 6 different vials each filled with 10 μL of 10 nM 5-HT in Ringer solution and 10 mM acetic acid using the custom injection program '5318' or '5718'. The method transports the sample with 18 μL of air through a sampling needle with an expected volume of 15 μL (injection program details in Table 2). Loop filling efficiency is given relative to response based on a flushed loop injection.

Conclusion

A method is described for efficient small sample injections without over-fill, using only a few additional μL . Plate numbers and peak shapes are similar to those obtained using standard injection procedures. Under optimised conditions, repeatability for peak areas better than 2% RSD are feasible.



Micro volume injections

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

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