

# Easy and Fast Automation of Sample Preparation

Sample preparation using injector workflows with the Agilent 1260 Infinity III Multisampler and Vialsampler



## Author

Adem Cakar  
Agilent Technologies, Inc.

## Abstract

The demand for automated workflows is increasing as the number of samples in the lab continues to grow, but implementing automation in the lab environment is difficult, time-consuming, and costly. This technical overview describes an alternative method for implementing an automation method quickly and easily for sample preparation. This includes derivatization of amino acid samples, sample dilution, and preparation of calibration standards using an Agilent 1260 Infinity III Multisampler or Agilent 1260 Infinity III Vialsampler in combination with injector workflows. The results obtained were excellent in terms of precision, repeatability, and sensitivity, and are independent of the operator's skills.

## Introduction

Manual sample preparation workflows, such as derivatization and the preparation of calibration standards are labor- and time-intensive, and the results are highly dependent on operator skills. An automated system avoids manual liquid handling steps, saving time and cost. In addition, results from an automated system are independent of operator skills. Unfortunately, setting up an automation system to handle liquids in a laboratory environment is a difficult and expensive process.

Agilent InfinityLab autosamplers, such as the 1260 Infinity III Vialsampler and 1260 Infinity III Multisampler, offer the possibility to use an injector program that allows a series of operations to be defined and performed sequentially, such as the preparation of calibration standards, the dilution of samples, or sample derivatization. This means that no additional equipment is required to set up an automated workflow for sample preparation. Until now, creating an injector program required some expertise and experience, but with injector workflows this is becoming much easier and faster for all levels of LC users.

This technical overview demonstrates the advantages of injector workflows compared to the conventional injector program. Liquid handling workflows, such as precolumn amino acid derivatization and preparation of calibration standards, were performed with injector workflows in combination with the Agilent 1260 Infinity III LC System with the 1260 Infinity III Vialsampler or 1260 Infinity III Multisampler.

## Experimental

### Equipment

The 1260 Infinity III LC comprised the following modules:

- Agilent 1260 Infinity III Binary Pump (G7112B)
- **Either:** Agilent 1260 Infinity III Vialsampler (G7129A) with Integrated Column Compartment (G7130A), 100  $\mu$ L analytical head and 100  $\mu$ L sample loop (default setup)
- **Or:** Agilent 1260 Infinity III Multisampler (G7167A) with 100  $\mu$ L analytical head and 100  $\mu$ L sample loop (default setup) and additional Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity III Variable Wavelength Detector (VWD) (G7114A), equipped with a 10 mm standard flow cell (14  $\mu$ L, option #018)
- Agilent 1260 Infinity III Fluorescence Detector Spectra (FLD) (G7121B), equipped with an 8  $\mu$ L FLD cell (G1321-60005)

### Software

Agilent OpenLab CDS version 2.8 or later

### Columns

- Agilent InfinityLab Poroshell 120 EC-C18, 2.1  $\times$  50 mm, 2.7  $\mu$ m (part number 699975-902)
- Agilent AdvanceBio AAA LC column, 3.0  $\times$  100 mm, 2.7  $\mu$ m (part number 695975-322) with Agilent AdvanceBio AAA guard column, 3.0  $\times$  5 mm, 2.7  $\mu$ m (part number 823750-946)

### Chemicals

Agilent InfinityLab gradient grade acetonitrile for LC (part number 5191-5100\*) and Agilent InfinityLab gradient grade methanol for LC (part number 5191-5110\*) were

used for all experiments. Fresh ultrapure water was obtained from a Milli-Q integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) and disodium tetraborate decahydrate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (HCl) (37%) and ortho-phosphoric acid (85%) were obtained from Merck (Darmstadt, Germany).

Standards and derivatization reagents were obtained from Agilent, including:

- Amino acids supplement kit (part number 5062-2478) containing: L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline, and sarcosine (1 g each)
- Amino acid standard, 100 pmol/ $\mu$ L (part number 5061-3332)
- Borate buffer, 0.4 N in water, pH 10.2, 100 mL (part number 5061-3339)
- FMOc reagent, 2.5 mg/mL 9-fluorenylmethylchloroformate in acetonitrile, 10  $\times$  1 mL (part number 5061-3337)
- OPA reagent, 10 mg/mL each of o-phthalaldehyde and 3-mercaptopropionic acid in 0.4 M borate buffer, 6  $\times$  1 mL (part number 5061-3335)
- Caffeine standards kit, 125  $\mu$ g/mL (part number 8500-6762)

### Solvents for amino acid analysis

**Mobile phase A:** Dissolve 2.8 g of  $\text{Na}_2\text{HPO}_4$  and 7.6 g of  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  in 1.9 L of water and add 1.5 mL of HCl (37%). Mix until homogeneous, adjust the pH to 8.2 with HCl, and fill up to 2 L with water. It is recommended to use an amber 2 L solvent bottle (part number 9301-6341) to avoid algae growth.

\* Only available in select countries

### Mobile phase B:

Acetonitrile:methanol:water 45:45:10 (v:v:v)

**Injection diluent:** 10 mL of mobile phase A + 200 µL of orthophosphoric acid (85%)

After opening an OPA or FMOC ampoule, the reagents were distributed to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024) and stored for no longer than a week. Borate buffer and injection diluent were transferred to vials without inserts. All reagents should be stored at 4 to 8 °C, and reagents in the autosampler should be exchanged daily.

### Amino acid standard solutions

To prepare the extended amino acid (EAA) stock solution containing 1.8 nmol/µL of asparagine, glutamine, and tryptophan, weigh 59.45 mg of asparagine, 65.77 mg of glutamine, and 91.95 mg of tryptophan into a 25 mL volumetric flask and fill it with 0.1 M HCl. Dilute the solution 1:10 with 0.1 M HCl.

For the internal standard (ISTD) stock solution containing 1 nmol/µL of norvaline and sarcosine, weigh 58.58 mg of norvaline and 44.54 mg of sarcosine into a 50 mL volumetric flask and fill it with 0.1 M HCl. Dilute the solution 1:10 with 0.1 M HCl.

It is recommended to distribute the stock solutions before freezing them at -20 °C to avoid freeze-thaw cycles.

For the preparation of the calibration standards, the EAA stock solution was diluted to 900, 450, 225, 90, 45, 22.5 and 9 pmol/µL and combined with 1:1 ISTD stock solution. This was later mixed at a ratio of 1:10 with respective concentrations of amino acid standard to obtain final concentrations of 90, 45, 22.5, 9, 4.5, 2.25, 0.9 and 0.45 pmol/µL of each amino acid and 50 pmol/µL ISTD.

### Sample preparation method for derivatization of amino acids with injector workflows

To set up an amino acid derivatization in the injector workflows, the workflow template "OPA/FMOC-protocol for AAA" (Figure 1) opens a user interface (Figure 2). In this user interface, choose the sampler positions and volume of sample, buffer, and reagent. There are also options to optimize the mixing parameters. If further optimizations are required, it is possible to change all necessary parameters needed in the advanced view (Figure 3). The advanced view in the injector workflows has the same interface as the conventional injector program.

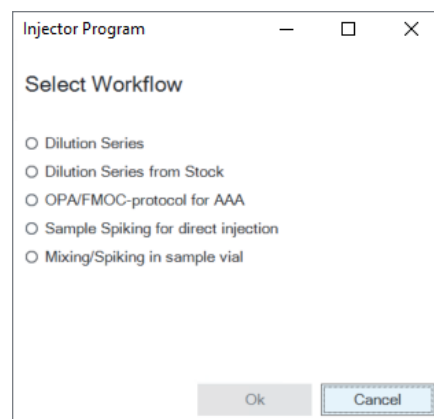


Figure 1. Selection interface with different workflow templates.

| Workflow                  |          |                 |          |
|---------------------------|----------|-----------------|----------|
| OPA/FMOC-protocol for AAA |          | Select Workflow |          |
| Parameters                |          |                 |          |
| Sample Volume:            | 1.00 µL  | Mix 1 Volume:   | 7.00 µL  |
| Borate Buffer Volume:     | 5.00 µL  | Repetitions:    | 10       |
| From Location:            | D1F-A1   |                 |          |
| OPA Reagent Volume:       | 1.00 µL  | Mix 2 Volume:   | 7.40 µL  |
| From Location:            | D1F-A2   | Repetitions:    | 10       |
| FMOC Reagent Volume:      | 0.40 µL  | Mix 3 Volume:   | 20.00 µL |
| From Location:            | D1F-A3   | Repetitions:    | 5        |
| Injection Diluent Volume: | 32.00 µL |                 |          |
| From Location:            | D1F-A4   |                 |          |

Figure 2. Workflow template for precolumn OPA/FMOC derivatization of amino acids.

### Caffeine stock solution and control sample

Caffeine solution (125 µg/mL) was used as a stock solution to prepare the calibration standards from 25 to 0.04 µg/mL with injector workflows. Additionally, a 12.5 µg/mL manually prepared caffeine control sample was used to evaluate the calibration.

### Preparation of caffeine calibration standards with injector workflows

The "dilution series" option (Figure 1) in the injector program was used to prepare the caffeine calibration standards by serial dilution. It is important to note that the use of flat glass vial inserts (part number 5181-3377) is necessary to ensure proper mixing of the dilution. In this workflow template (Figure 4), the positions of sample and diluent must be assigned. The dilution factor and the number of dilutions can be selected. Also here, if further optimizations are required, it is possible to change and optimize all necessary parameters needed in the advanced view (Figure 5).

| Function  | Parameter  |
|-----------|--|
| ▶ Comm... | Comment: Workflow derived from template: OPA/FMOC-protocol for AAA           |
| Draw      | Draw 5.00 µL from location "D1F-A1" with default speed using default offset  |
| Wash      | Wash needle as defined in method   |
| Draw      | Draw 1.00 µL from sample with default speed using default offset             |
| Wash      | Wash needle as defined in method   |
| Draw      | Draw 1.00 µL from location "D1F-A2" with default speed using default offset  |
| Wash      | Wash needle as defined in method   |
| Mix       | Mix 7.00 µL from air with default speed for 10 times                         |
| Draw      | Draw 0.40 µL from location "D1F-A3" with default speed using default offset  |
| Wash      | Wash needle as defined in method   |
| Mix       | Mix 7.40 µL from air with default speed for 10 times                         |
| Draw      | Draw 32.00 µL from location "D1F-A4" with maximum speed using default offset |
| Wash      | Wash needle as defined in method   |
| Mix       | Mix 20.00 µL from air with maximum speed for 5 times                         |
| Inject    | Inject   |

Figure 3. Advanced view of injector workflows and the individual commands for precolumn OPA/FMOC derivatization of amino acids.

Template  
Dilution Series V2 Select Template

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Parameters

| Draw Sample           | From Location: <span style="border: 1px solid gray; padding: 2px;">Location</span> <span style="border: 1px solid gray; padding: 2px;">D1F-A2</span>   | Sample Volume: <span style="border: 1px solid gray; padding: 2px;">18.00</span> µL   |   |          |       |        |      |        |      |        |      |        |      |   |  |
|-----------------------|--|--|---|----------|-------|--------|------|--------|------|--------|------|--------|------|---|--|
| Draw Dilution Solvent | From Location: <span style="border: 1px solid gray; padding: 2px;">D1F-A1</span>   | Dilution Factor 1: <span style="border: 1px solid gray; padding: 2px;">5</span>  | Diluent Volume: <span style="border: 1px solid gray; padding: 2px;">72.00</span> µL |          |       |        |      |        |      |        |      |        |      |   |  |
| Dilute                | <table border="1" style="width: 100%; border-collapse: collapse; text-align: center;"> <thead> <tr> <th>Target Location</th> <th>Sample Volume in Target [µL]</th> </tr> </thead> <tbody> <tr><td>▶ D1F-B1</td><td>18.00</td></tr> <tr><td>D1F-B2</td><td>3.60</td></tr> <tr><td>D1F-B3</td><td>0.72</td></tr> <tr><td>D1F-B4</td><td>0.14</td></tr> <tr><td>D1F-B5</td><td>0.03</td></tr> </tbody> </table> | Target Location  | Sample Volume in Target [µL]  | ▶ D1F-B1 | 18.00 | D1F-B2 | 3.60 | D1F-B3 | 0.72 | D1F-B4 | 0.14 | D1F-B5 | 0.03 | Mix Mode: <span style="border: 1px solid gray; padding: 2px;">Air bubble</span> | Repetitions: <span style="border: 1px solid gray; padding: 2px;">10</span> |
| Target Location       | Sample Volume in Target [µL]   |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
| ▶ D1F-B1              | 18.00  |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
| D1F-B2                | 3.60   |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
| D1F-B3                | 0.72   |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
| D1F-B4                | 0.14   |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
| D1F-B5                | 0.03   |  |   |          |       |        |      |        |      |        |      |        |      |   |  |
|                       | <span style="border: 1px solid gray; padding: 2px;">Add</span> <span style="border: 1px solid gray; padding: 2px;">Remove</span>   | Speed: <input type="radio"/> Default <input checked="" type="radio"/> Maximum <input type="radio"/> <span style="border: 1px solid gray; padding: 2px;">20.0</span> µL/min |   |          |       |        |      |        |      |        |      |        |      |   |  |

Figure 4. Workflow template for dilution series.

| Function   | Parameter   |
|------------|---|
| ▶ Comment  | Comment: Workflow derived from template: Dilution Series V2                       |
| Draw       | Draw 72.00 µL from location "D1F-A1" with default speed using default offset      |
| Draw       | Draw 18.00 µL from location "D1F-A2" with default speed using default offset      |
| Wash       | Wash needle as defined in method  |
| Eject      | Eject maximum volume to location "D1F-B1" with default speed using default offset |
| Repeat     | Repeat 10 time(s)   |
| Draw       | Draw maximum volume from air with default speed                                   |
| Eject      | Eject maximum volume to location "D1F-B1" with default speed using default offset |
| End Repeat | End Repeat  |

Figure 5. Advanced view of injector workflows and individual commands for the dilution series.

## Methods

**Table 1.** Chromatographic conditions for analysis of derivatized amino acids.

| Parameter   | Value   |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
|-------------|---|------------|----|----|---|----|---|------|----|---|-------|----|----|----|---|-----|
| Column      | Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 μm with Agilent AdvanceBio AAA guard column, 3.0 × 5 mm, 2.7 μm   |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Solvent     | A) 10 mM Na <sub>2</sub> HPO <sub>4</sub> and 10 mM Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> , pH: 8.2<br>B) Acetonitrile/Methanol/Water (45:45:10; v:v:v)   |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Gradient    | <table border="1"> <thead> <tr> <th>Time (min)</th> <th>%A</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>98</td> <td>2</td> </tr> <tr> <td>0.40</td> <td>98</td> <td>2</td> </tr> <tr> <td>13.60</td> <td>43</td> <td>57</td> </tr> <tr> <td>14</td> <td>0</td> <td>100</td> </tr> </tbody> </table> <p>Stop time: 17 min<br/>Post time: 3 min</p>  | Time (min) | %A | %B | 0 | 98 | 2 | 0.40 | 98 | 2 | 13.60 | 43 | 57 | 14 | 0 | 100 |
| Time (min)  | %A  | %B         |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| 0           | 98  | 2          |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| 0.40        | 98  | 2          |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| 13.60       | 43  | 57         |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| 14          | 0   | 100        |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Flow Rate   | 0.600 mL/min  |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Temperature | 40 °C   |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Detection   | FLD: Excitation: 345 nm; emission: 455 nm<br>After 13.00 min: change excitation: 265 nm; change emission: 315 nm<br>PMT gain: 10<br>Peak width: > 0.025 min (18.52 Hz)  |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |
| Injection   | <p><b>Agilent 1260 Infinity III Vialsampler with 100 μL analytical head and 100 μL loop</b></p> <ul style="list-style-type: none"> <li>– Injection volume: 1 μL</li> <li>– Needle wash: 3 s with acetonitrile: 0.1 M HCl (50:50; v:v)</li> <li>– Sampling speeds: draw speed: 200 μL/min; eject speed: 400 μL/min</li> <li>– Needle height position: offset 0.0 mm</li> <li>– Sample temperature: 8 °C</li> </ul> <p><b>Agilent 1260 Infinity III Multisampler with 100 μL analytical head and 100 μL loop</b></p> <ul style="list-style-type: none"> <li>– Injection volume: 1 μL</li> <li>– Needle wash: 3 s with acetonitrile: 0.1 M HCl (50:50; v:v)</li> <li>– Sampling speeds: draw speed: 200 μL/min; eject speed: 400 μL/min</li> <li>– Needle height position: use vial bottom sensing, offset 0.0 mm</li> <li>– Sample temperature: 8 °C</li> </ul> |            |    |    |   |    |   |      |    |   |       |    |    |    |   |     |

**Table 2.** Chromatographic conditions for analysis of caffeine.

| Parameter   | Value   |            |    |    |   |    |   |      |    |    |     |    |    |
|-------------|---|------------|----|----|---|----|---|------|----|----|-----|----|----|
| Column      | Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 μm   |            |    |    |   |    |   |      |    |    |     |    |    |
| Solvent     | A) Water<br>B) Acetonitrile   |            |    |    |   |    |   |      |    |    |     |    |    |
| Gradient    | <table border="1"> <thead> <tr> <th>Time (min)</th> <th>%A</th> <th>%B</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>95</td> <td>5</td> </tr> <tr> <td>3.00</td> <td>50</td> <td>50</td> </tr> <tr> <td>3.1</td> <td>10</td> <td>90</td> </tr> </tbody> </table> <p>Stop time: 4 min<br/>Post time: 3 min</p>   | Time (min) | %A | %B | 0 | 95 | 5 | 3.00 | 50 | 50 | 3.1 | 10 | 90 |
| Time (min)  | %A  | %B         |    |    |   |    |   |      |    |    |     |    |    |
| 0           | 95  | 5          |    |    |   |    |   |      |    |    |     |    |    |
| 3.00        | 50  | 50         |    |    |   |    |   |      |    |    |     |    |    |
| 3.1         | 10  | 90         |    |    |   |    |   |      |    |    |     |    |    |
| Flow Rate   | 0.500 mL/min  |            |    |    |   |    |   |      |    |    |     |    |    |
| Temperature | 40 °C   |            |    |    |   |    |   |      |    |    |     |    |    |
| Detection   | VWD: 273 nm, 20 Hz  |            |    |    |   |    |   |      |    |    |     |    |    |
| Injection   | <p><b>Agilent 1260 Infinity III Vialsampler with 100 μL analytical head and 100 μL loop</b></p> <ul style="list-style-type: none"> <li>– Injection volume: 5 μL</li> <li>– Needle wash: 3 s with acetonitrile:water (50:50; v:v)</li> <li>– Sampling speeds: draw speed: 200 μL/min; eject speed: 400 μL/min</li> <li>– Needle height position: offset 0.0 mm</li> <li>– Sample temperature: 8 °C</li> </ul> <p><b>Agilent 1260 Infinity III Multisampler with 100 μL analytical head and 100 μL loop</b></p> <ul style="list-style-type: none"> <li>– Injection volume: 5 μL</li> <li>– Needle wash: 3 s with acetonitrile:water (50:50; v:v)</li> <li>– Sampling speeds: draw speed: 100 μL/min; eject speed: 400 μL/min</li> <li>– Needle height position: offset –3.0 mm</li> <li>– Sample temperature: 8 °C</li> </ul> |            |    |    |   |    |   |      |    |    |     |    |    |

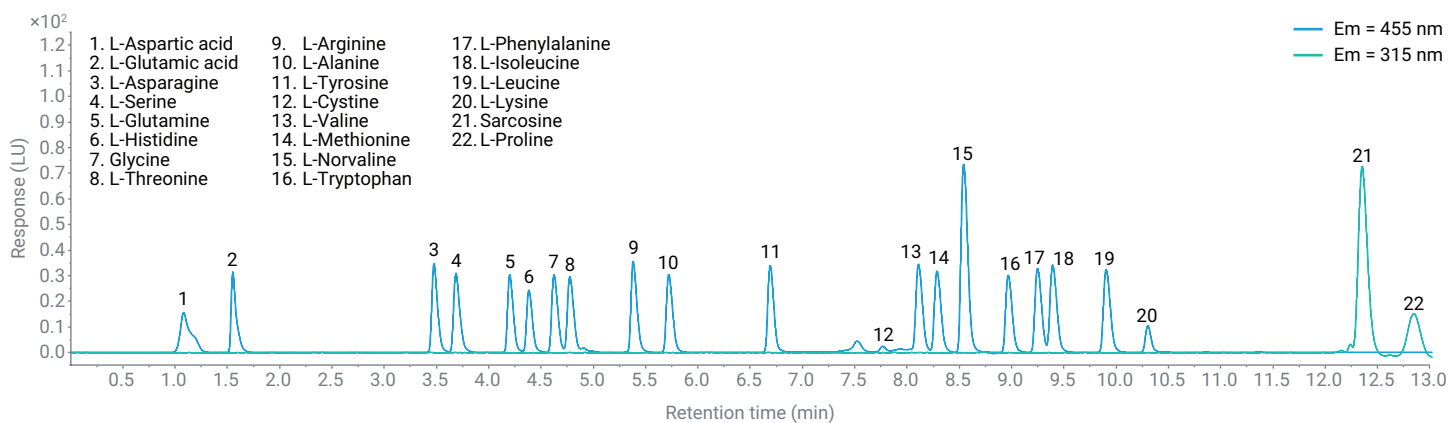
## Results and discussion

To show the automation possibilities of injector workflows, a full amino acid derivatization workflow with calibration standards and a calibration standards dilution workflow with serial dilution of caffeine solution was created. The results for the amino acid derivatization and dilution workflow with caffeine are shown to illustrate that the injector workflow delivers similar results compared to the conventional injector program. The results and performance of the conventional injector program have already been published.<sup>1,2</sup>

### Amino acid analysis

Figure 6 shows a chromatogram obtained from the analysis of the amino acid calibration standards containing 22.5 pmol/μL of amino acids and 50 pmol/μL of ISTD, which were derivatized using the 1260 Infinity III Multisampler and injector workflows. The chromatogram shows a successful separation of 20 amino acids and two ISTDs within 17 minutes.

To determine sensitivity, the calibration was performed from 90 to 0.45 pmol/μL. To show repeatability, 10 consecutive injections of calibration standard containing 22.5 pmol/μL of amino acids and 50 pmol/μL of ISTD were measured. Tables 3 and 4 show the results for repeatability, sensitivity, and calibration during analysis.



**Figure 6.** Chromatogram from an analysis of 22.5 pmol/ $\mu$ L amino acid mixture and 50 pmol/ $\mu$ L ISTD.

**Table 3.** Calibration linearity, sensitivity, and repeatability of amino acid analysis using injector workflows on an Agilent 1260 Infinity III Multisampler. Ten consecutive injections of the amino acid mixture with a final concentration of 22.5 pmol/ $\mu$ L for each amino acid and 50 pmol/ $\mu$ L ISTDs were used for relative standard deviation (RSD) calculations. The limit of detection (LOD) was determined by using a threshold of 3x the signal-to-noise (S/N) value.

| Peak No. | Compound        | RT (min) | RT RSD (%) | Area RSD (%) | LOD (pmol/ $\mu$ L) | Calibration Range (pmol/ $\mu$ L) | Calibration Type | R <sup>2</sup> |
|----------|-----------------|----------|------------|--------------|---------------------|-----------------------------------|------------------|----------------|
| 1        | L-Aspartic acid | 1.07     | 0.61       | 0.34         | 0.227               | 0.45 to 90                        | Linear           | 0.99999        |
| 2        | L-Glutamic acid | 1.56     | 0.60       | 0.36         | 0.117               | 0.45 to 90                        | Linear           | 0.99997        |
| 3        | L-Asparagine    | 3.47     | 0.07       | 0.25         | 0.112               | 0.45 to 90                        | Linear           | 0.99990        |
| 4        | L-Serine        | 3.68     | 0.06       | 0.29         | 0.068               | 0.45 to 90                        | Linear           | 0.99992        |
| 5        | L-Glutamine     | 4.20     | 0.04       | 0.23         | 0.121               | 0.45 to 90                        | Linear           | 0.99986        |
| 6        | L-Histidine     | 4.38     | 0.03       | 0.98         | 0.143               | 0.45 to 90                        | Quadratic        | 0.99999        |
| 7        | Glycine         | 4.62     | 0.03       | 0.32         | 0.090               | 0.45 to 90                        | Linear           | 0.99997        |
| 8        | L-Threonine     | 4.77     | 0.03       | 0.20         | 0.122               | 0.45 to 90                        | Linear           | 0.99997        |
| 9        | L-Arginine      | 5.38     | 0.02       | 0.21         | 0.107               | 0.45 to 90                        | Linear           | 0.99998        |
| 10       | L-Alanine       | 5.72     | 0.02       | 0.21         | 0.110               | 0.45 to 90                        | Linear           | 0.99998        |
| 11       | L-Tyrosine      | 6.69     | 0.02       | 0.30         | 0.110               | 0.45 to 90                        | Linear           | 0.99998        |
| 12       | L-Cystine       | 7.76     | 0.04       | 1.71         | 1.399               | 2.25 to 90                        | Quadratic        | 0.99994        |
| 13       | L-Valine        | 8.11     | 0.03       | 0.23         | 0.109               | 0.45 to 90                        | Linear           | 0.99997        |
| 14       | L-Methionine    | 8.28     | 0.03       | 0.91         | 0.142               | 0.45 to 90                        | Linear           | 0.99998        |
| 15       | L-Norvaline*    | 8.54     | 0.03       | 0.36         | NA                  | NA                                | NA               | NA             |
| 16       | L-Tryptophan    | 8.96     | 0.03       | 0.49         | 0.133               | 0.45 to 90                        | Linear           | 0.99996        |
| 17       | L-Phenylalanine | 9.24     | 0.02       | 0.21         | 0.126               | 0.45 to 90                        | Linear           | 0.99998        |
| 18       | L-Isoleucine    | 9.39     | 0.02       | 0.33         | 0.116               | 0.45 to 90                        | Linear           | 0.99999        |
| 19       | L-Leucine       | 9.90     | 0.02       | 0.34         | 0.121               | 0.45 to 90                        | Linear           | 0.99999        |
| 20       | L-Lysine        | 10.30    | 0.02       | 2.32         | 0.39                | 0.45 to 90                        | Quadratic        | 0.99993        |
| 21       | Sarcosine*      | 12.35    | 0.02       | 3.29         | NA                  | NA                                | NA               | NA             |
| 22       | L-Proline       | 12.84    | 0.02       | 3.18         | 1.606               | 2.25 to 90                        | Quadratic        | 0.99943        |

\* ISTD

**Table 4.** Calibration linearity, sensitivity, and repeatability of amino acid analysis using injector workflows on an Agilent 1260 Infinity III Vialsampler. Ten consecutive injections of the amino acid mixture with a final concentration of 22.5 pmol/μL for each amino acid and 50 pmol/μL ISTDs were used for RSD calculations. The LOD was determined by using a threshold of 3x the S/N value.

| Peak No. | Compound        | RT (min) | RT RSD (%) | Area RSD (%) | LOD (pmol/μL) | Calibration Range (pmol/μL) | Calibration Type | R <sup>2</sup> |
|----------|-----------------|----------|------------|--------------|---------------|-----------------------------|------------------|----------------|
| 1        | L-Aspartic acid | 1.194    | 0.338      | 0.469        | 0.167         | 0.45 to 90                  | Linear           | 0.99994        |
| 2        | L-Glutamic acid | 1.808    | 0.456      | 0.632        | 0.267         | 0.45 to 90                  | Linear           | 0.99995        |
| 3        | L-Asparagine    | 3.657    | 0.139      | 0.512        | 0.137         | 0.45 to 90                  | Linear           | 0.99980        |
| 4        | L-Serine        | 3.875    | 0.110      | 0.563        | 0.137         | 0.45 to 90                  | Linear           | 0.99997        |
| 5        | L-Glutamine     | 4.389    | 0.081      | 0.623        | 0.141         | 0.45 to 90                  | Linear           | 0.99967        |
| 6        | L-Histidine     | 4.573    | 0.079      | 1.313        | 0.254         | 0.45 to 90                  | Quadratic        | 0.99972        |
| 7        | Glycine         | 4.829    | 0.079      | 0.559        | 0.166         | 0.45 to 90                  | Linear           | 0.99996        |
| 8        | L-Threonine     | 4.966    | 0.075      | 0.541        | 0.213         | 0.45 to 90                  | Linear           | 0.99984        |
| 9        | L-Arginine      | 5.569    | 0.070      | 0.588        | 0.184         | 0.45 to 90                  | Linear           | 0.99998        |
| 10       | L-Alanine       | 5.933    | 0.050      | 0.639        | 0.186         | 0.45 to 90                  | Linear           | 0.99997        |
| 11       | L-Tyrosine      | 6.912    | 0.078      | 0.643        | 0.163         | 0.45 to 90                  | Linear           | 0.99998        |
| 12       | L-Cysteine      | 7.991    | 0.048      | 1.181        | 0.886         | 0.90 to 90                  | Quadratic        | 0.99973        |
| 13       | L-Valine        | 8.326    | 0.044      | 0.676        | 0.149         | 0.45 to 90                  | Linear           | 0.99998        |
| 14       | L-Methionine    | 8.517    | 0.037      | 2.892        | 0.563         | 0.90 to 90                  | Linear           | 0.99962        |
| 15       | L-Norvaline*    | 8.764    | 0.034      | 0.573        | NA            | NA                          | NA               | NA             |
| 16       | L-Tryptophan    | 9.209    | 0.026      | 0.653        | 0.156         | 0.45 to 90                  | Linear           | 0.99981        |
| 17       | L-Phenylalanine | 9.479    | 0.025      | 0.675        | 0.187         | 0.45 to 90                  | Linear           | 0.99999        |
| 18       | L-Isoleucine    | 9.615    | 0.025      | 0.525        | 0.169         | 0.45 to 90                  | Linear           | 0.99997        |
| 19       | L-Leucine       | 10.137   | 0.017      | 0.581        | 0.189         | 0.45 to 90                  | Linear           | 0.99997        |
| 20       | L-Lysine        | 10.535   | 0.012      | 1.287        | 0.564         | 0.45 to 90                  | Quadratic        | 0.99999        |
| 21       | Sarcosine*      | 12.578   | 0.016      | 3.521        | NA            | NA                          | NA               | NA             |
| 22       | L-Proline       | 13.067   | 0.013      | 2.961        | 0.477         | 0.90 to 90                  | Quadratic        | 0.99965        |

\* ISTD

Excellent retention time (RT) and peak area precision (n = 10) were obtained for most compounds, showing values below 0.1% for RT RSD and below 1% for peak area RSD. In addition, excellent sensitivity with LOD mostly below 0.4 pmol/μL was obtained for all amino acids except L-cysteine and L-proline. The lower sensitivity of L-cysteine can be explained by the fact that the cysteine adduct formed with OPA exhibits lower fluorescence than the adducts of other amino acids.<sup>3</sup>

## Caffeine calibration

Figure 7 shows a calibration curve obtained from analysis of caffeine standards, which were prepared with the 1260 Infinity III Multisampler by using the dilution series template from injector workflows. This workflow involves a serial dilution of the caffeine stock solution within a range of 25 to 0.04 µg/mL and the subsequent measurement.

The calibration function (Table 5) was determined, showing that the 1260 Infinity III Multisampler and 1260 Infinity III Vialsampler provide excellent dilution precision. To evaluate the calibrations, a deviation control sample was prepared manually with a concentration of 12.5 µg/mL. Table 4 shows that calibration with the 1260 Infinity III Multisampler and Vialsampler delivers exceptional results for the control sample.

## Conclusion

Injector workflows, in combination with the Agilent 1260 Infinity III Multisampler or Agilent 1260 Infinity III Vialsampler enable automation of certain sample preparation methods such as derivatization of amino acids, sample dilution, and preparation of calibration standards with excellent precision, repeatability, and sensitivity. The injector workflow differs from the conventional injector program in its ability to set up methods faster and easier without error, saving time and cost.

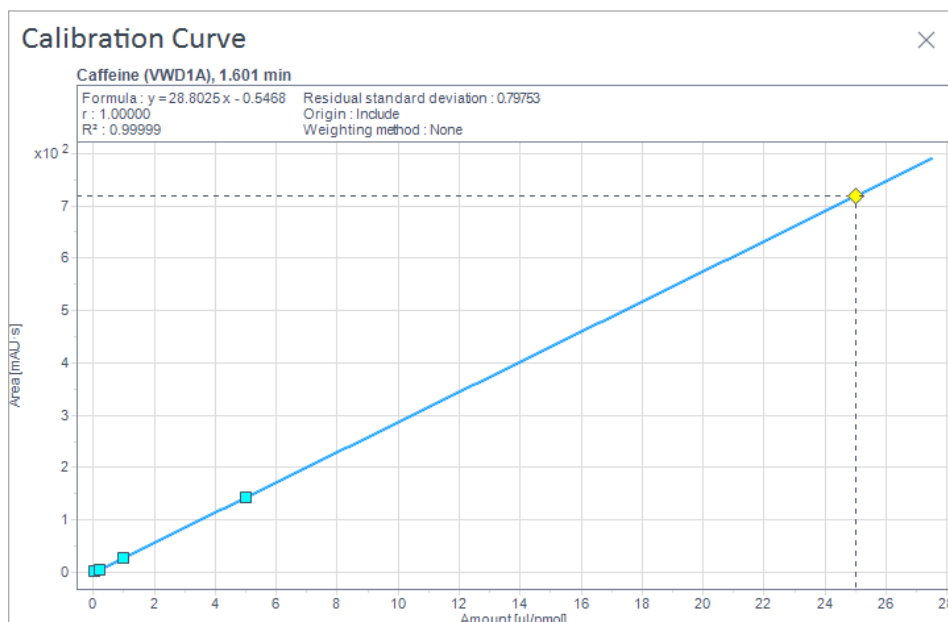


Figure 7. Calibration curve obtained with the Agilent 1260 Infinity III Multisampler.

Table 5. Caffeine calibrations using calibration solutions prepared with the Agilent 1260 Infinity III Multisampler and Agilent 1260 Infinity III Vialsampler.

| Autosampler                           | Calibration Function    | Correlation R <sup>2</sup> | Deviation Control Sample (%) |
|---------------------------------------|-------------------------|----------------------------|------------------------------|
| Agilent 1260 Infinity II Multisampler | $y = 28.8025x - 0.5468$ | R <sup>2</sup> = 0.99999   | 0.59%                        |
| Agilent 1260 Infinity II Vialsampler  | $y = 28.5657x - 0.7107$ | R <sup>2</sup> = 0.99999   | 0.79%                        |

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