Application Note

Pharma/Biopharma

Automated Amino Acid Derivatization and Sample Handling Workflow

Using an Agilent 1260 Infinity III diode array detector WR and an Agilent 1260 Infinity III fluorescence detector spectra with a Waters Empower Chromatography Data System

Authors

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Abstract

The need for automated laboratory workflows is increasing as the number of samples continues to grow. However, setting up full automation systems can be complex, time-consuming, and expensive. The Agilent drivers for Waters Empower Chromatography Data System enable injector workflow automation for sample preparation and analysis. Integration of these drivers allows users to define and execute automated injector-based procedures directly within the Waters Empower Chromatography Data System (CDS), reducing the need for manual intervention during routine LC operations. This application note presents a simple and practical alternative for automating sample preparation using injector workflows with the Agilent 1260 Infinity III multisampler and the Agilent 1290 Infinity III multisampler, together with the Empower CDS software. The approach allows key steps such as amino acid derivatization, sample dilution, and preparation of calibration standards to be automated directly within the injection sequence. As a result, it reduces manual work and improves overall laboratory efficiency. It also minimizes variation caused by different operators, ensuring more consistent results. The Agilent 1260 Infinity III LC system with the Agilent 1260 Infinity III diode array detector WR and the Agilent 1260 Infinity III fluorescence detector spectra delivers highly reproducible performance with excellent precision, repeatability, and sensitivity across all tested applications.

Introduction

Manual sample preparation steps such as amino acid derivatization, sample dilution, and preparation of calibration standards are time-consuming and require significant operator effort. In addition, results can vary depending on user experience, leading to inconsistencies between analyses. While laboratory automation can significantly improve reproducibility and reduce manual workload, conventional liquid handling systems are often complex, costly, and challenging to implement in routine laboratory environments.

The Agilent drivers for Waters Empower Chromatography Data System enable injector workflow automation for sample preparation and analysis. Integration of these drivers allows users to define and execute automated injector-based procedures directly within the Empower Chromatography Data System (CDS), reducing the need for manual intervention during routine LC operations. Sample preparation steps such as dilution, calibration standard preparation, and derivatization can be incorporated into a single automated sequence. As a result, workflows become more efficient, reproducible, and less dependent on operator experience, while maintaining full control and traceability within the Empower CDS environment.

Agilent autosamplers—including the 1260 Infinity III multisampler—in combination with injector workflows in Empower CDS, provide a simple and efficient approach to automating sample preparation directly within the LC sequence. This approach enables streamlined workflow setup and improved reproducibility without the need for additional external automation hardware.

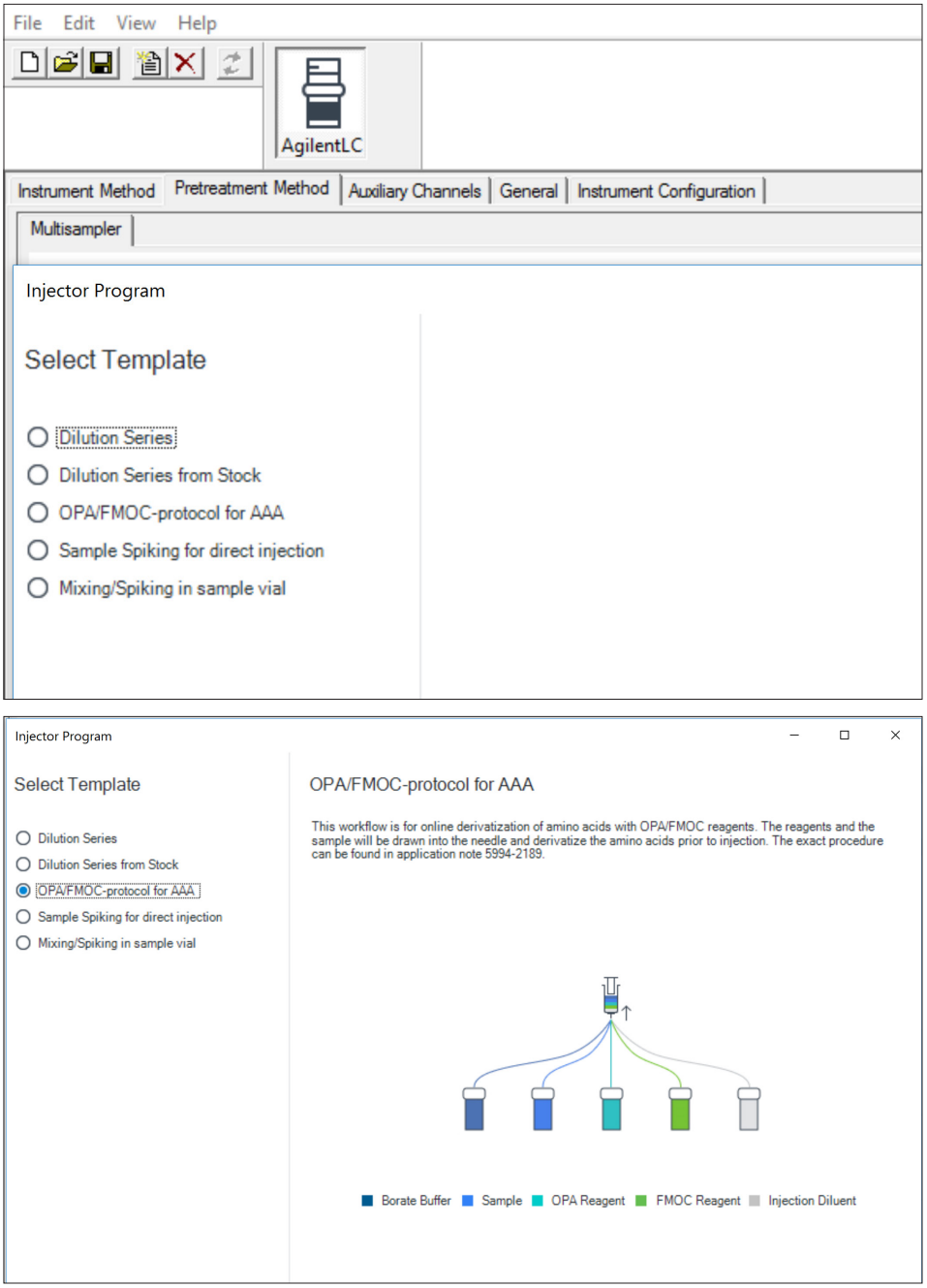


Figure 1. Selection interface with different workflow templates in Empower CDS with Agilent InfinityLab LC instruments.

Furthermore, with reagents, standards, columns, and application support, the approach enables fast, sensitive, and fully automated amino acid analysis using the latest Agilent InfinityLab LC instruments and column technology. The Agilent AdvanceBio AAA LC columns provide high efficiency and resolution comparable to sub-2 µm particle columns while operating at 50% lower backpressure, reducing the risk of column clogging and extending column lifetime. The AdvanceBio AAA LC column is based on well-established ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) derivatization chemistry. Together with the 1260 Infinity III multisampler, the 1290 Infinity III multisampler, and the AdvanceBio AAA LC columns and standards, it enables robust qualitative and quantitative amino acid analysis with high sensitivity, speed, and reliability. When used according to the recommended protocol, the solution provides efficient separation of amino acids commonly found in protein and peptide hydrolysates.

Experimental

Equipment

The Agilent 1260 Infinity III LC system comprised the following modules:

- Agilent 1260 Infinity III Quaternary Pump (G5654A)
- Agilent 1260 Infinity III Multisampler (G5668A) with 100 µL analytical head and 100 µL sample loop
- Agilent 1260 Infinity III Multicolumn Thermostat (G7116B)
- Agilent 1260 Infinity III Diode Array Detector (DAD) WR (G7115A), equipped with a 10 mm bio-inert flow cell
- Agilent 1260 Infinity III Fluorescence Detector Spectra (FLD) (G7121B), equipped with an 8 µL bio-inert FLD flow cell (G1321-60005)
- Agilent InfinityLab Assist (G7180A)

Software

Waters Empower CDS 3 WICF 4.0. Agilent LC instruments were controlled through Empower CDS using the Waters Instrument Control Framework (WICF), which enables seamless communication and injector workflow automation.

Columns

Agilent AdvanceBio AAA LC column 4.6 × 100 mm, 2.7 µm (p/n 655950-802) with Agilent AdvanceBio AAA guard column 4.6 × 5 mm, 2.7 µm, 3/pk (p/n 820750-931)

Prepare HPLC mobile phases

Mobile phase A: 10 mM Na_2HPO_4 and 10 M $Na_2B_4O_7$, pH 8.2

- To prepare 1 L, weigh out 1.4 g of anhydrous Na_2HPO_4 and 3.8 g of $Na_2B_4O_7 \cdot 10H_2O$ in 1 L water.
- Adjust to approximately pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid and adjust to a final pH of 8.2.
- Allow stirring time for complete dissolution of borate crystals before adjusting pH.
- Filter through 0.45 µm regenerated cellulose membranes (p/n 3150-0576).

Mobile phase B:
Acetonitrile:methanol:water (45:45:10, v:v:v)

All mobile-phase solvents are HPLC grade. Mobile phase A is consumed at a faster rate than mobile phase B. Therefore, preparing 2 L of mobile phase A for every 1 L of mobile phase B is recommended.

Injection diluent

The injection diluent is 100 mL of mobile phase A and 0.4 mL concentrated H_3PO_4. This solution is prepared in a 100 mL bottle that should be stored at 4 °C.

0.1 N HCl

Extended amino acid and internal standard stock solutions are prepared in 0.1 N HCl solution. To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) to a 500 mL volumetric flask that is partially filled with water. Mix and fill up to the mark with water. Store at 4 °C.

Derivatization reagents

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent. Simply transfer these reagents from their container into an autosampler vial.

Recommended precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, OPA is potent for about 7 to 10 days. Therefore, it is recommended to transfer 100 µL aliquots of OPA to microvial inserts and store them in a refrigerator. Replace the OPA autosampler microvial daily.
- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred to microvial inserts in 100 µL aliquots and stored in a refrigerator. Like OPA, an open FMOC ampoule transferred to 10 microvial inserts can stay potent for approximately 7 to 10 days.
- Borate buffer can be transferred to a 1.5 mL autosampler vial without a vial insert. Replace it every three days.

Prepare amino acid standards

Agilent provides solutions of 17 amino acids (AA) in five concentrations (10 pmol/µL to 1 nmol/µL) for calibration curves. Store solutions at 4 °C. To make the extended amino acid (EAA) stock solution, weigh:

- 59.45 mg asparagine
- 59.00 mg hydroxyproline
- 65.77 mg glutamine
- 91.95 mg tryptophan

Add the weighed out amino acids to a 25 mL volumetric flask. Fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Then, fill up to the mark with water for a total concentration of 18 nmol/µL of EAA. Keep them frozen and discard at first signs of reduced intensity.

Prepare internal standard (ISTD) stock solution

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill halfway with 0.1 N HCl and shake or sonicate until dissolved. Finally fill up to the mark with water for a final concentration of 10 nmol for each ISTD amino acid/µL (standard sensitivity). Store at 4 °C.

Preparation of final standard

1. Take 5 mL of 18 nmol EAA into 20 mL volumetric flask and dilute with water and mix well.
2. Take 5 mL of above diluted EAA into a 10 mL volumetric flask and dilute with 10 nmol ISTD solution and mix well.
3. Take 100 µL of above EAA-ISTD mix and add 900 µL of 250 pmol Agilent AA standard, 250 pmol/µL (p/n 5061-3331). Final AA solution with EAA 225 and 500 pmol/µL ISTD.

Figure 2. Ready-to-use Agilent AdvanceBio Amino Acid Analysis (AAA) standard and Agilent AdvanceBio amino acid reagents kit.

Instrument conditions

Parameter	Value
Column	Agilent AdvanceBio AAA LC column, 4.6 × 100 mm, 2.7 µm (p/n 655950-802) with Agilent AdvanceBio AAA guard columns, 4.6 × 5 mm, 2.7 µm, 3/pk (p/n 820750-931)
Fow Rate	1.5 mL/min
Sample Volume	1 µL
Total Run Time	18 min
Gradient	Time (min) %MP-A %MP-B 0.0 98 2 0.35 98 2 13.4 43 57 13.5 0 100 15.7 0 100 15.8 98 2 18.0 98 2
Column Temperature	40 °C
DAD	Signal-A: wavelength 338 nm; BW-10; reference wavelength 390 nm; BW-20 Signal-B: wavelength 262 nm; BW-16; reference wavelength 324 nm; BW-8 To detect both OPA- and FMOC-derivatized amino acids in a single chromatogram, it is necessary to switch detector wavelength between the last eluting OPA-derivatized amino acid, lysine (peak 20 in the standard), and before the first eluting FMOC-derivatized amino acid, hydroxyproline (peak 21 in the standard). Determining the appropriate transition point using the DAD is possible by initially collecting two channels (Signal A 338 nm, to detect OPA-derivatized amino acids and Signal B 262 nm, to detect FMOC-derivatized amino acids). This will determine the ideal point at which to switch the wavelength during the run. Subsequent runs can be made using a single channel with the detector timetable function used to program a wavelength switch from 338 to 262 nm at the appropriate time. Between the elution of OPA-lysine and FMOC-hydroxyproline, allow time for both OPA- and FMOC-derivatized amino acids to be detected in a single chromatogram. **Timetable signal** Time (min) Function Parameter 0.0 Change signal Wavelength 338 nm; BW-10; reference wavelength 390 nm; BW-20 10.60 Change signal Wavelength 262 nm; BW-16; reference wavelength 324 nm; BW-8
Peak Width	> 0.013 min (0.25 s response time) (20 Hz)
FLD	Excitation 340 nm; emission 450 nm; filter 390 nm **Timetable signal** 0.00 min excitation 340 nm, emission 450 nm; gain (as needed) 10.6 min excitation 260 nm, emission 325 nm; PMT gain 10 (as needed; transition between lysine and hydroxyproline)

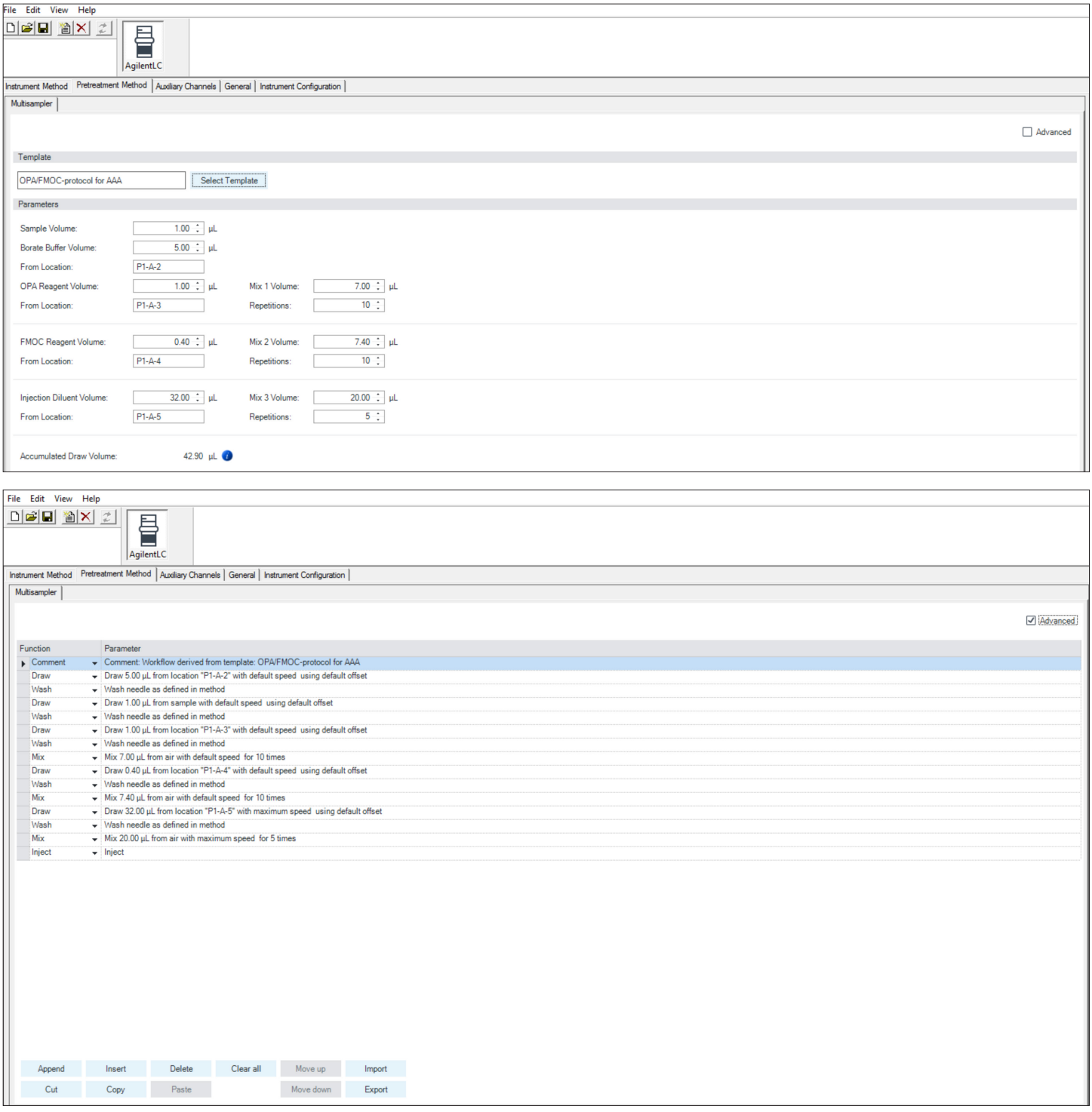


Figure 3. Workflow template for precolumn OPA/FMOC derivatization of amino acids in Empower CDS with Agilent LC instruments.

Results and discussion

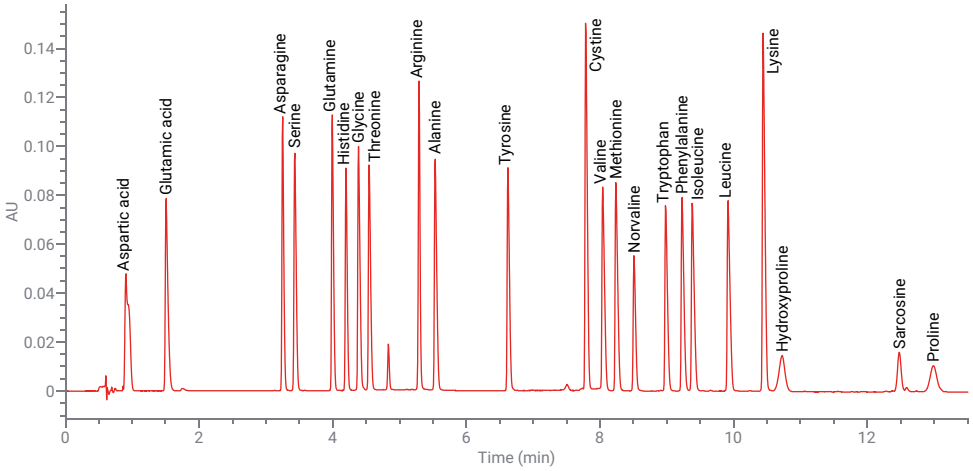
To show the automation possibilities of injector workflows, a full amino acid derivatization workflow solution was created. The results for the amino acid derivatization were shown to illustrate that the injector workflow delivers similar results compared to the conventional injector program. The results and performance of the conventional injector program have already been published.[1,2,4,5,6]

The chromatograms in Figures 4 and 6 illustrate typical routine standard sensitivity in high-throughput applications that can be obtained using the AdvanceBio AAA columns. These separations were produced using the 1260 Infinity III LC system with an AdvanceBio AAA, 100 mm, 2.7 µm column, the 1260 Infinity III diode array detector WR and the 1260 Infinity III fluorescence detector spectra. A single run can be completed in under 20 minutes (including re-equilibration) with adequate resolution. The primary amino acids (1-20, OPA-derivatized) were monitored at 338 nm, while the secondary amino acids (21-23, FMOC-derivatized) were monitored at 262 nm.

To determine the transition point needed with fluorescence detection (FLD), it is necessary to perform two separate runs: the first using excitation 340 nm, emission 450 nm to detect the OPA-derivatized amino acids and the second using excitation 260 nm, emission 325 nm to detect the FMOC-derivatized amino acids. Both OPA- and FMOC-derivatized amino acids can be detected in a single chromatogram, using the detector timetable function. This function programs a wavelength switch at the appropriate point after the last eluting OPA-derivatized amino acid, lysine (peak 20 in the standard), and before the first eluting FMOC-derivatized amino acid, hydroxyproline (peak 21 in the standard).

The first 20 amino acids in Figure 4—the primary amino acids—are derivatized with OPA. The last three—hydroxyproline, sarcosine, and proline—are derivatized with FMOC. A programmable wavelength switch from 338 to 265 nm takes place after lysine (peak 20) elutes and before hydroxyproline (peak 21) elutes.

The European Pharmacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe. Leucine (Leu) is a branched-chain α-amino acid, produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of not less than 1.5.3 The resolution between leucine and isoleucine is 6.7.



Peak Results

	Name	RT	Area	% Area	Height	USP Tailing	USP Resolution	USP Plate Count
1	Aspartic acid	0.905	119400	2.51	47829			1625
2	Glutamic acid	1.507	215727	4.53	78605	2.0	0.1	6698
3	Asparagine	3.250	211855	4.45	111849	1.6	27.8	63553
4	Serine	3.432	220008	4.62	96823	1.6	3.2	47668
5	Glutamine	3.993	222863	4.68	112503	1.6	9.6	86042
6	Histidine	4.199	179100	3.76	90676	1.5	3.8	96747
7	Glycine	4.386	225646	4.74	99501	1.7	3.2	78436
8	Threonine	4.545	209075	4.39	91859	1.7	2.5	83098
9	Arginine	5.293	220976	4.64	125714	1.2	10.0	203527
10	Alanine	5.532	224796	4.72	94121	1.8	4.2	110686
11	Tyrosine	6.622	209738	4.41	90884	1.7	16.7	171801
12	Cystine	7.786	344995	7.25	149610	1.8	3.7	234527
13	Valine	8.040	216371	4.55	82648	1.9	3.7	193288
14	Methionine	8.237	225010	4.73	85009	1.9	2.7	196704
15	Norvaline	8.509	132742	2.79	55052	1.6	3.9	263541
16	Tryptophan	8.982	197498	4.15	75690	1.9	6.8	245324
17	Phenylalanine	9.227	211550	4.45	78978	1.9	3.3	241299
18	Isoleucine	9.380	215798	4.53	76390	2.0	2.0	222617
19	Leucine	9.914	222697	4.68	77580	2.0	6.7	238799
20	Lysine	10.440	379770	7.98	146065	1.9	6.8	325397
21	Hydroxyproline	10.725	88648	1.86	14442	1.0	2.4	64997
22	Sarcosine	12.478	62024	1.30	16114	1.1	13.0	242192
23	Proline	12.988	76429	1.61	10671	1.1	3.5	75461

Figure 4. UV chromatogram from the analysis of 225 pmol/µL amino acid mixture and 500 pmol/µL ISTD in Empower CDS software with Agilent LC instruments.

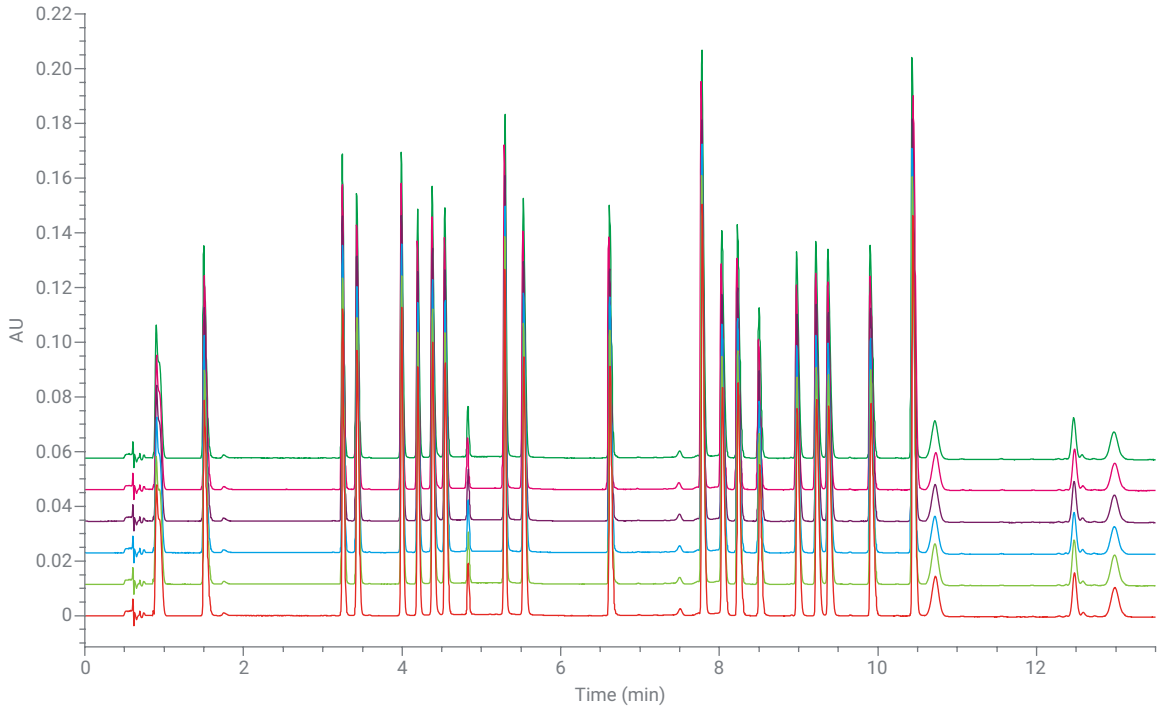
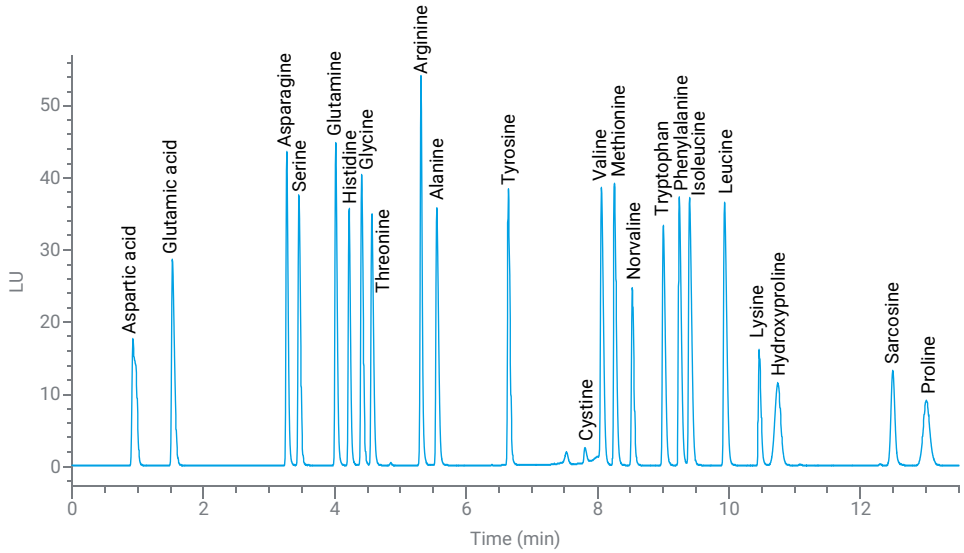


Figure 5. Six injections overlay UV chromatogram from the analysis of 225 pmol/µL amino acid mixture and 500 pmol/µL ISTD.



Peak Results

	Name	RT	Area	% Area	Height	USP Tailing	USP Resolution	USP Plate Count
1	Aspartic acid	0.925	84567424	4.06	17566514	2.1		814
2	Glutamic acid	1.528	88875347	4.26	28502038	1.9	5.6	5345
3	Asparagine	3.271	98007120	4.70	43434151	1.6	24.3	47985
4	Serine	3.453	98042619	4.70	37379998	1.6	2.8	38552
5	Glutamine	4.014	104638886	5.02	44634487	1.6	8.5	66632
6	Histidine	4.220	82471276	3.96	35518329	1.5	3.3	75647
7	Glycine	4.407	105393817	5.06	40214399	1.6	2.8	62505
8	Threonine	4.566	91419763	4.39	34676250	1.7	2.2	66196
9	Arginine	5.312	112497832	5.40	53897533	1.4	11.9	153457
10	Alanine	5.554	98410977	4.72	35580552	1.7	3.7	88052
11	Tyrosine	6.643	102595907	4.92	38188244	1.7	14.8	135512
12	Cystine	7.809	7407884	0.36	2272799		3.2	153105
13	Valine	8.062	121974447	5.85	38256879	1.6	3.1	156526
14	Methionine	8.259	118617602	5.69	38986428	1.8	2.3	128451
15	Norvaline	8.530	68282618	3.28	24584279	1.6	3.3	211768
16	Tryptophan	9.004	99400281	4.77	33171145	1.7	6.1	195547
17	Phenylalanine	9.249	113773300	5.46	37044871	1.8	2.9	187699
18	Isoleucine	9.402	118470888	5.68	36786120	1.8	1.7	166127
19	Leucine	9.937	119791701	5.75	36455238	1.8	5.8	192370
20	Lysine	10.462	47683343	2.29	15988643	1.8	6.1	265872
21	Hydroxyproline	10.744	73698897	3.54	11371107	1.0	2.2	60868
22	Sarcosine	12.499	55608924	2.67	13194289	1.1	12.4	215214
23	Proline	13.010	65938941	3.16	9027967	1.1	3.4	72139

Figure 6. FLD chromatogram from an analysis of 225 pmol/µL amino acid mixture and 500 pmol/µL ISTD in Empower CDS software with Agilent LC instruments.

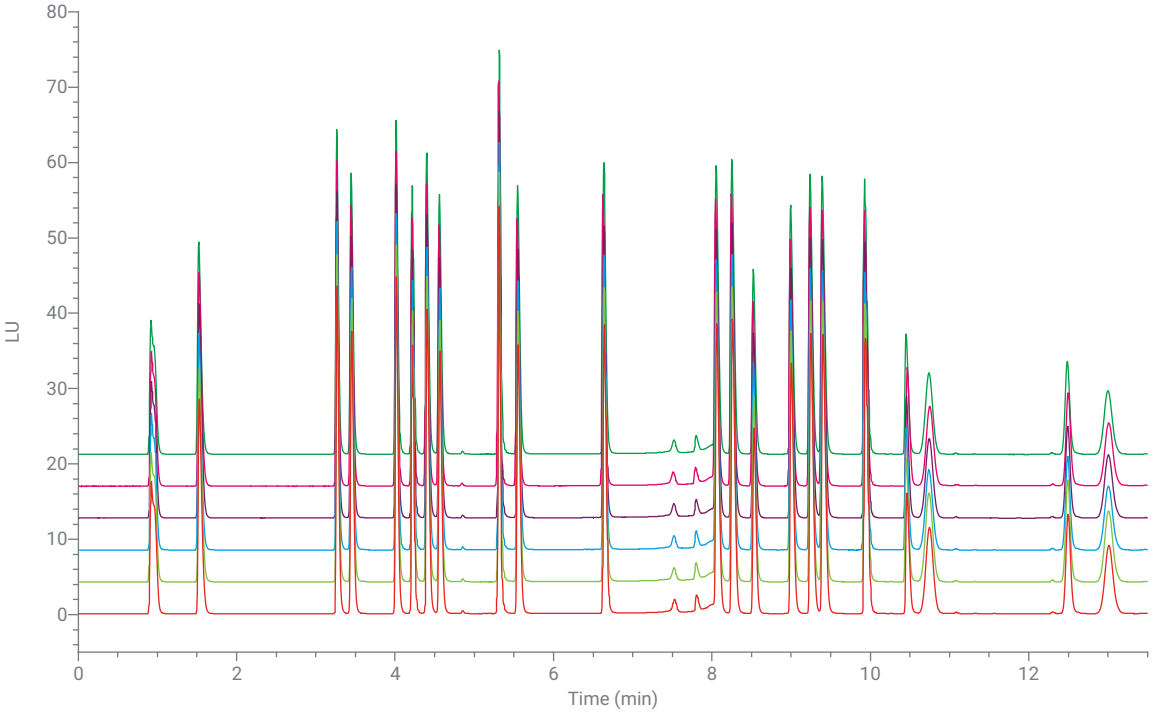


Figure 7. Six injection overlay FLD chromatogram from an analysis of 225 pmol/µL amino acid mixture and 500 pmol/µL ISTD.

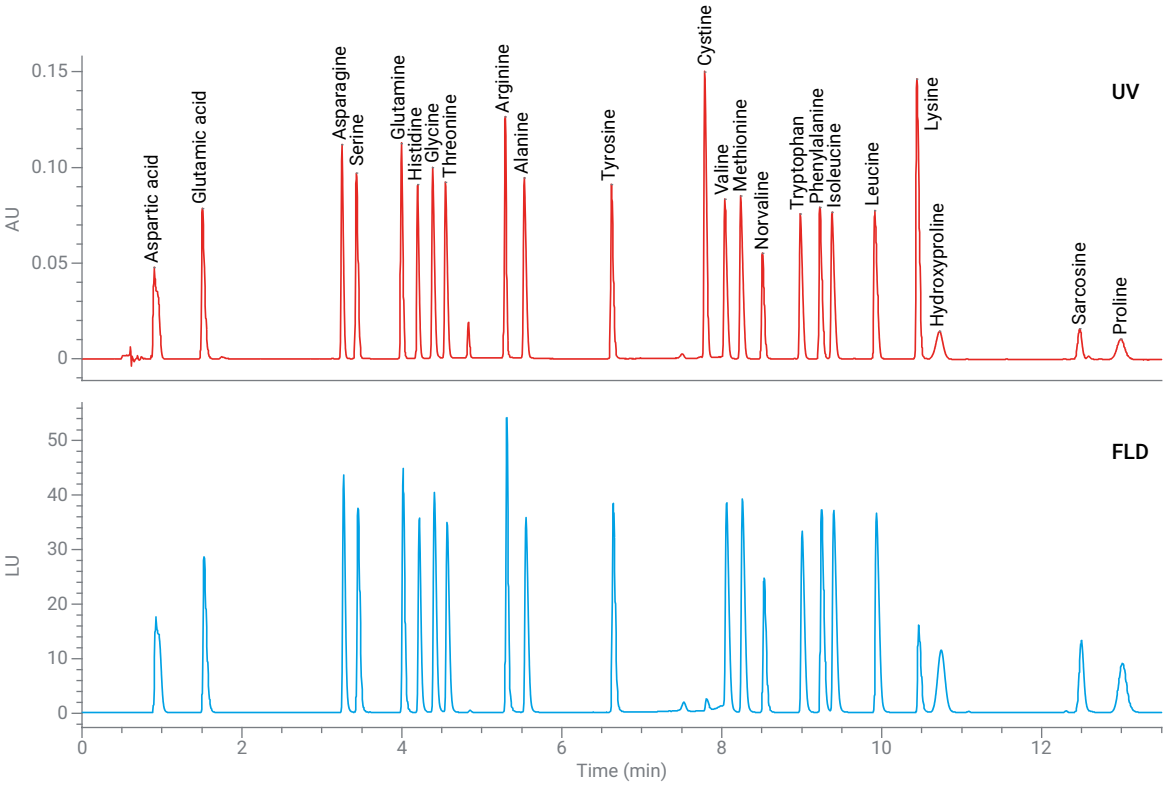


Figure 8. Overlay UV and FLD chromatogram from an analysis of 225 pmol/µL amino acid mixture and 500 pmol/µL ISTD.

Conclusion

Injector workflows, in combination with the Agilent 1260 Infinity III multisampler or Agilent 1260 Infinity III vialsampler enable the 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.

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