Customer application note | 003698

Food and beverage

Amino acid analysis in food, beverages, and fertilizers by automated in-needle OPA/FMOC derivatization

Authors

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Keywords

Amino acids, derivatization, automation, OPA, FMOC, custom injection program, Vanquish Core HPLC, Vanquish Flex UHPLC, Accucore column

Application benefits

- Fully automized workflow for the OPA/FMOC derivatization of amino acids within the Thermo Scientific™ Vanquish™ Split Samplers and subsequent liquid chromatographic (LC) analyses.
- Robust and reproducible performance of the workflow for sample matrices of medium and high complexity (beverages, food, fertilizers).
- Low costs per sample due to inexpensive reagents and column stability over several hundred injections.

Goal

Showcase the robust performance of a method for amino acid analysis realized by automated in-needle derivatization in fertilizers and other matrices.

Introduction

As fundamental building blocks of living matter, amino acids (AA) are ubiquitously present in our environment. They play critical roles in metabolic pathways and physiological processes and thus are subject to chemical analysis in various industrial

and scientific fields. Although more than 500 AAs (molecules with amino and carboxylic acid functional groups) are known in nature, the analytical focus in most cases is predominantly on the 20 proteinogenic α-amino acids. Approaches commonly rely on liquid chromatographic (LC) separations like reversed-phase (RP), hydrophilic interaction (HILIC),[1] or ion-exchange (IEX) chromatography. Due to the lack of inherent chromophores in most AAs, different detection techniques have been applied, ranging from amperometric, charged-aerosol (CAD), or mass spectrometric (MS) detection in native state to ultraviolet (UV) or fluorescence (FLD) optical detection after pre- or post-column derivatization of the molecules. One well-established reaction protocol is the pre-column derivatization of primary AAs with ortho-phthaldialdehyde (OPA) followed by the derivatization of secondary AAs with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) facilitating the formation of highly fluorescent derivatives suitable for sensitive and precise analysis (Figure 1). As manual sample preparation can be laborious and error-prone and handling of chemicals can pose health hazards to lab staff, automated workflows become increasingly popular, particularly in routine analytics due to their efficiency, robustness, and throughput capabilities. While the automation of complex sample treatments may require sophisticated lab equipment, some straightforward liquid handling tasks can already be realized by default LC instrumentation with built-in liquid handling capabilities. In a previous technical note[2] the implementation of the automated pre-column OPA/FMOC derivatization of AAs via in-needle sample preparation by the autosampler of the LC system has been showcased.

Now in this application note, the protocol is updated to recent column and instrument hardware and applied for the analysis of AAs from different matrices. The robustness and stability of the method based on standard injections as well as the analysis of beverage and food samples of medium complexity at our in-house sites is shown. Finally, the routine application of the protocol for the quality control (QC) of highly complex fertilizers in a manufacturer's QC and service laboratory (Mundeco by Agronova Biotech, Spain) is presented.

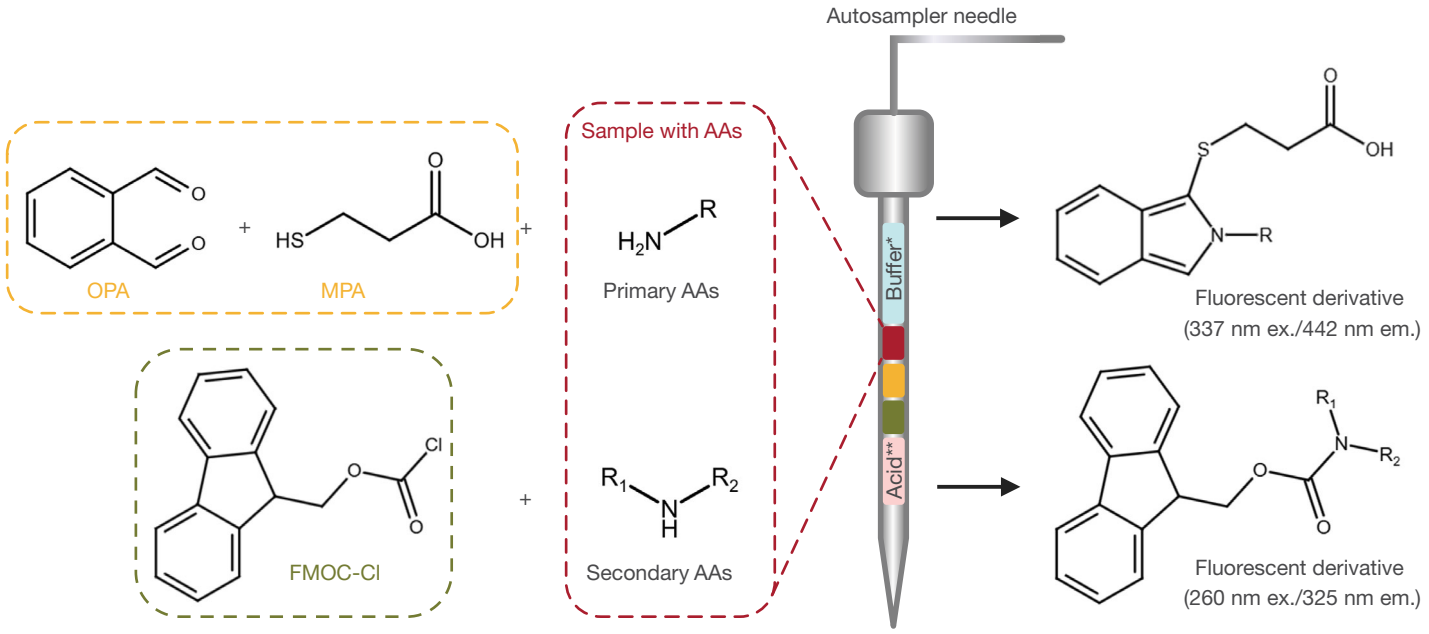


Figure 1. In-needle derivatization of primary and secondary AAs with OPA/MPA and FMOC-Cl reagents for subsequent LC analysis of the fluorescent derivatives. *Borate buffer pH 10, **acetic acid for quenching.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (P/N 50136149)
- Fisher Chemical™ Optima™ LC/MS grade Acetonitrile (P/N A955)
- Fisher Chemical™ Optima™ LC/MS grade Methanol (P/N A456)
- Fisher Chemical™ di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) (P/N S/4520/53)
- Thermo Scientific™ Chemicals Sodium tetraborate decahydrate ($Na_2B_4O_7$ x 10 H_2O) (P/N A16176.36)
- Fisher Chemical™ Sodium hydroxide (NaOH) solution (50%, w/w) (P/N USSS254500)
- Thermo Scientific™ Chemicals Phthaldialdehyde (OPA), 98% (P/N A13299.09)
- Thermo Scientific™ Chemicals 3-Mercaptopropionic acid (MPA), 99+% (P/N 125531000)
- Thermo Scientific™ Chemicals 9-fluorenylmethyl chloroformate (FMOC-Cl), 98+% (P/N A11683.06)
- Fisher Chemical™ Hydrochloric acid (HCl), 37% (P/N H/1200/PB15)
- Fisher Chemical™ Optima™ LC/MS grade Acetic acid (P/N A11350)
- Thermo Scientific™ Amino Acid Standard H (mixture of 18 AAs) (P/N 20088)
- Thermo Scientific™ Chemicals L(-)-Tryptophane, 99% (P/N 140590050)
- Thermo Scientific™ Chemicals L(+)-Glutamine (P/N 386030500)
- Thermo Scientific™ Chemicals L(+)- Asparagine, 99% (P/N B21473.22)
- Thermo Scientific™ Chemicals trans-4-Hydroxy-L-proline, 99+% (P/N A11851.09)
- Gibco™ MEM Non-Essential Amino Acids Solution 100X (P/N 11140035)
- Gibco™ MEM Amino Acids Solution 50X (P/N 11130036)

Sample handling

- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100–1,000 µL (P/N 4641100N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 10–100 µL (P/N 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 1–10 µL (P/N 4641030N)
- Thermo Scientific™ Orion 3 Star™ pH Benchtop Meter (P/N 13-644-928)
- Thermo Scientific™ SureSTART™ 2 mL Amber Glass Short Thread Screw Top Vials, 100/pack, Level 2 (P/N 6ASV9-2P)
- Thermo Scientific™ SureSTART™ Blue Polypropylene 9 mm AVCS™ Screw Caps with Soft Blue Silicone/Clear PTFE Septa, 100/pack, Level 3 (P/N 6PSC9ST101)

Instrumentation

Module Vanquish Core HPLC system (in-house)

Thermo Scientific™ Vanquish™ Core HPLC system consisting of:

- Vanquish Core System Base (P/N VC-S01-A-02)
- Vanquish Quaternary Pump C (P/N VC-P20-A-01)
- Vanquish Split Sampler CT (P/N VC-A12-A-02)
- Sample loop, stainless steel, 100 µL (P/N 6851.1950)
- Vanquish Column Compartment C (P/N VC-C10-A-03), including passive pre-heater, 3 µL 0.18 × 530 mm, stainless steel (P/N 6732.0170). Optional: passive pre-heater, 5 µL, 0.25 × 580 mm, stainless steel (P/N 6732.0180)
- Vanquish Fluorescence Detector F, D-PMT (P/N VF-D51-A)
- Standard flow cell, 8 µL, biocompatible (P/N 6079.4230)

Module Vanquish Flex UHPLC system (in-house)

Thermo Scientific™ Vanquish™ Flex HPLC system consisting of:

- Vanquish Flex System Base (P/N VF-S01-A-02)
- Vanquish Binary Pump F (P/N VF-P10-A-01)
- Vanquish Split Sampler FT (P/N VF-A10-A-02)
- Sample loop, MP35N, 100 µL (P/N 6850.1913)
- Vanquish Column Compartment H (P/N VH-C10-A-03)
- Passive pre-heater, 3 µL, 0.18 × 530 mm, stainless steel (P/N 6732.0170) or passive pre-heater, 5 µL, 0.25 × 580 mm, stainless steel (P/N 6732.0180)
- Vanquish Fluorescence Detector F (P/N VF-D50-A)
- Standard flow cell, 8 µL, biocompatible (P/N 6079.4230)

Module Vanquish Core HPLC System (Mundeco by Agronova Biotech, Spain)

Vanquish Core HPLC system consisting of:

- Vanquish Core System Base (P/N VC-S01-A-02)
- Vanquish Quaternary Pump C (P/N VC-P20-A-01)
- Vanquish Split Sampler CT (P/N VC-A12-A-02)
- Sample loop, stainless steel, 100 µL (P/N 6851.1950)
- Vanquish Column Compartment C with passive preheater (P/N VC-C10-A-03)
- Vanquish Diode Array Detector CG (P/N VC-D11-A-01)
- Standard flow cell SST (P/N 6083.0510)
- Vanquish Fluorescence Detector F (P/N VF-D50-A)
- Standard flow cell, biocompatible (P/N 6079.4230)

Chromatography Data System

For data acquisition and analysis, the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) was used (versions 7.3 at Mundeco and 7.3.2 MUc at in-house sites).

Mobile phase preparation

Mobile phase A: 1.42 g Na_2HPO_4 and 3.81 g $Na_2B_4O_7$ x 10 H_20 were dissolved in 1 L of water, adjusted to pH 7.8 with HCl, and vacuum filtered (0.45 µm).

Mobile phase B: 450 mL methanol, 450 mL acetonitrile, and 100 mL water were mixed and ultrasonicated.

Reagent preparation

Preparation, storage, and autosampler position data are listed in Table 1.

Standard preparation

The mixed AA standard contained 18 AAs at a concentration of 2.5 mM in 0.1 N HCl solution. Stock solutions of the individual AAs L-Tryptophane, L-Glutamine, and L-Asparagine were prepared in 0.1 N HCl at a concentration level of approximately 4 mM. The calibration stock solution was prepared by mixing the appropriate volumes of mixed AA standard, individual AA stock solutions, and 0.1 N HCl to obtain a concentration of 500 µM for each AA.

Calibration standards were prepared by diluting the calibration stock solution with 0.1 N HCl to concentrations of 1 / 25 / 50 / 75 / 100 µM.

The in-house standards contained the AAs:
Ala, Arg, Asn, Asp, cystine, Gln, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val

The standards at Mundeco by Agronova Biotech contained the AAs:
Ala, Arg, Asp, cystine, Glu, Gly, His, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, and norvaline and sarcosine as internal standards (ISTDs) for primary and secondary AAs

Table 1. Reagent preparation details

Reagent	Preparation	Storage	Position in autosampler
100 mM borate buffer, pH 10	38.137 g/L $Na_2B_4O_7$ x 10 H_2O in water, adjust to pH 10 with NaOH solution	Stable at room temperature for 6 months	R:A1 (2 mL vial)
OPA-reagent	50 mg/mL *o*-phthaldialdehyde in methanol	Stable at -20 °C for 1 month	
OPA/MPA-reagent	980 µL borate buffer + 20 µL OPA-reagent + 20 µL MPA	Should be prepared every day	R:A2 (2 mL vial)
FMOC-reagent	2.5 mg/mL FMOC-Cl in acetonitrile	Stable at -20 °C for 1 month	R:A3 (2 mL vial)
1 M acetic acid	2.862 mL acetic acid filled to 50 mL with water	Stable at 5 °C for 1 month	R:A4 (2mL vial)
0.1 N HCl		Stable at room temperature for 12 months	

Sample preparation

In-house, all samples were diluted with 0.1 N HCl and injected with no further sample treatment. Food and beverage samples were purchased in a local supermarket: apple juice (diluted 1:10), beer (diluted 1:10), white wine (diluted 1:10), red wine (diluted 1:10), orange juice (diluted 1:100), lime juice (diluted 1:100), soy sauce (diluted 1:1000), honey (diluted 0.1 g/mL).

The calibration stock solution was used to prepare spiked sample dilutions, adding 10 µM of all AAs.

Two commercially available amino acid solutions for cell culture supplementation were used as control samples and diluted 1:500 before analysis.

At Mundeco, plant fertilizers were diluted with buffer according to their expected AA concentration (no dilution, 5-fold, 10-fold, 20-fold, or 50-fold dilution).

Chromatographic conditions

Chromatographic conditions are listed in Table 2.

The in-needle derivatization of the AAs was realized by a *Custom Injection Program* (CIP) set up within the instrument method wizard of Chromeleon CDS. First the CIP mode was selected as *Replace normal injection*. Afterwards the individual commands were entered into the table as given in Table 3. As the full derivatization program takes approximately 7.2 min, its processing was initiated at -5.0 min run time by the *PrepareThisInjection* command in the Chromeleon CDS instrument method script, to run in parallel to the column re-equilibration.

Analytical in-house sequences typically consisted of 44–55 injections, including blank runs, calibration standards, samples, and spiked samples, to represent a daily analytical routine. Each sequence was concluded by a rinsing method of the column with 100% acetonitrile for 30 min to flush off residual reaction products and matrices.

Table 2. Chromatographic conditions

	In-house	Mundeco
Column	Thermo Scientific™ Accucore™ C18 (3 × 150 mm, 2.6 µm, P/N 17126-153030)	
Guard column	Thermo Scientific™ Accucore™ C18 guard cartridge (3 × 10 mm, 2.6 µm, P/N 17126-013005) with Thermo Scientific™ Unigard™ Direct-Connection Guard Cartridge Holder (P/N 852-00)	
Mobile phase A	10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$ in water, pH 7.8	
Mobile phase B	Acetonitrile/methanol/water (45/45/10, v/v/v)	
Gradient	Time (min) % B -5.0 2 0.0 2 0.4 10 5.0 20 16.0 80 16.1 100 19.0 100 19.5 2 22.0 2	Time (min) % B 0 2 0.4 10 12 34.5 22 100 25 100 25.5 2 30 2
Flow rate	0.6 mL/min	
Column temp.	40 °C (still air) with passive pre-heating	
Autosampler temp.	10 °C	
Needle wash solution	Methanol/water (10/90, v/v)	
Needle wash mode	No wash (wash steps included in CIP)	
Injection volume	0.5 µL sample (defined in CIP)	
Fluorescence detection	Temperature: 40 °C Lamp mode: standard Sensitivity: 1 Emission_1 wavelength switching: 337 nm ex./442 nm em. (0.0–11.7 min), 260 nm ex./325 nm em. (11.7–14.5 min)	Multi-channel performance: fast Sensitivity: 2 337 nm ex./442 nm em. (Emission_1), 260 nm ex./325 nm em. (Emission_2)

Table 3. Custom Injection Program (CIP)

#	Command	Parameters	Comment
1	UDP_PrepareLiquidHandling	Volume=8 [µL]	Sampler prepares for processing (incl. switch injection valve to bypass)
2	UDP_Draw	Position=Air, Volume=0.5 [µL], Speed=1 [µL/s]	Draw air gap to mobile phase
3	UDP_Draw	Position=R:A1, Volume=3.50 [µL], Speed=1 [µL/s], NeedleHeight=2000 [µm]	Draw buffer
4	UDP_NeedleWash	Duration=5 [s]	Outside needle wash
5	UDP_Draw	Volume=0.5 [µL], Speed=0.1 [µL/s], NeedleHeight=2000 [µm]	Draw sample, fixed volume of 0.5 µL, sample position defined in sequence table
6	UDP_NeedleWash	Duration=5 [s]	Outside needle wash
7	UDP_Draw	Position=Air, Volume=0.1 [µL], Speed=0.1 [µL/s,	Draw air gap
8	UDP_InNeedleMix	Volume=4 [µL], DrawSpeed=15 [µL/s], DispenseSpeed=15 [µL/s], Cycles=10	Mixing buffer and sample
9	UDP_Wait	60 [s]	
10	UDP_Draw	Position=R:A2, Volume=0.5 [µL], Speed=0.1 [µL/s], NeedleHeight=2000 [µm]	Draw OPA/MPA reagent
11	UDP_Draw	Position=Air, Volume=0.1 [µL], Speed=1 [µL/s]	Draw air gap
12	UDP_InNeedleMix	Volume=4.5 [µL], DrawSpeed=15 [µL/s], DispenseSpeed=15 [µL/s], Cycles=20	Mixing sample, buffer and reagent
13	UDP_Wait	60 [s]	Reaction time
14	UDP_NeedleWash	Duration=5 [s]	Outside needle wash
15	UDP_Draw	Position=R:A3, Volume=0.5 [µL], Speed=1 [µL/s], NeedleHeight=2000 [µm]	Draw FMOC reagent
16	UDP_Draw	Position=Air, Volume=0.1 [µL], Speed=1 [µL/s]	Draw air gap
17	UDP_InNeedleMix	Volume=5 [µL], DrawSpeed=15 [µL/s], DispenseSpeed=15 [µL/s], Cycles=20	Mixing sample, buffer and reagent
18	UDP_Wait	60 [s]	Reaction time
19	UDP_NeedleWash	Duration=5 [s]	Outside needle wash
20	UDP_Draw	Position=R:A4, Volume=2 [µL], Speed=0.5 [µL/s], NeedleHeight=2000 [µm]	Draw acetic acid to quench reaction
21	UDP_Draw	Position=Air, Volume=0.1 [µL], Speed=1 [µL/s]	Draw air gap
22	UDP_InNeedleMix	Volume=7 [µL], DrawSpeed=15 [µL/s], DispenseSpeed=15 [µL/s], Cycles=10	Mixing reaction mix and quencher
23	UDP_Wait	30 [s]	Reaction time
24	UDP_NeedleWash	Duration=5 [s]	Outside needle wash
25	UDP_PrepareInject		Injection

Results and discussion

The in-needle-OPA/FMOC-derivatization protocol for AAs eliminates the need for laborious and time-consuming manual lab work and provides robust and reproducible results in AA analysis. Furthermore, the use of inexpensive reagents negates the need for costly AA quantification kits, offering a cost-effective and convenient solution. This application note showcases its implementation at in-house (Thermo Fisher Scientific, Germany) and external (Mundeco by Agronova Biotech, Spain) laboratories.

The Accucore C18 column is highly suitable for the resolution of the AA derivatives under moderate backpressure, and the exact gradient profile can be modified according to the AAs in scope. The method was developed for 20 AAs in food and beverages on a Vanquish Core HPLC system with a quaternary pump and later applied with few modifications at a QC lab for fertilizers. In addition, the transfer to a Vanquish Flex UHPLC system with a binary pump is showcased.

To ensure stable method performance, it is highly recommended to have either the passive pre-heater, 3 µL, 0.18 × 530 mm (P/N 6732.0170; default with the Vanquish Core HPLC system) or the passive pre-heater, 5 µL, 0.25 × 580 mm (P/N 6732.0180) installed, independent of which Vanquish system is used. The larger diameter capillaries in front of the column facilitate a proper dilution of the aggressive reagents by the mobile phase before arrival at the column, which improves method stability and column lifetime. The installation of the 100 µL sample loop at the autosampler ensures a thorough mixing of the sample and all reagents during the derivatization step.

In-house method setup

Figure 2 depicts the AA separation of the method, developed in-house with a Vanquish Core HPLC system with quaternary pump, and its transfer to a Vanquish Flex UHPLC system with binary pump without any modification, except for the move of the fluorescence wavelength switching time. Good separation of the 20 AAs is achieved at both systems, with a resolution of the most critical peak pair Phe/Ile being 1.75 and 1.97, respectively. The differences in retention times and elution pattern resulted mainly from the different pumps in use, which differ in mixing principle and gradient delay volume (GDV). The lower GDV of the binary pump caused an earlier arrival of the gradient at the column and thus earlier elution. As the Pro peak was only partially separated from an interfering reaction peak, a slight adaptation of the gradient might be considered for the binary pump.

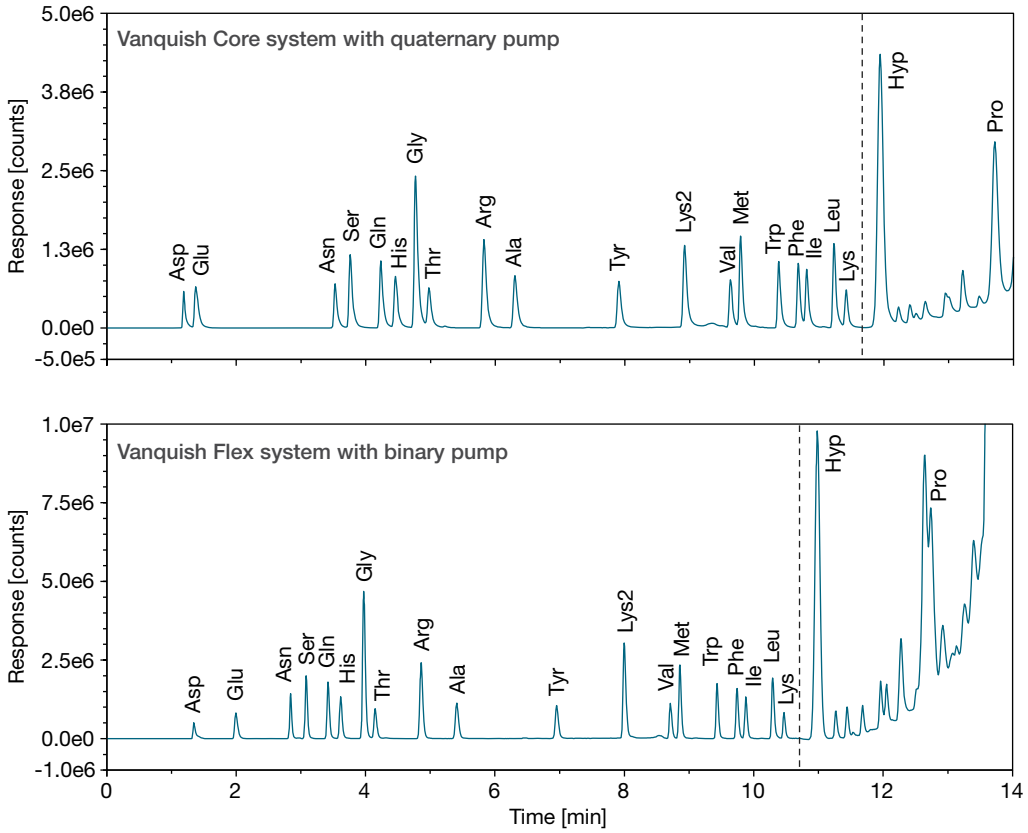


Figure 2. AA separation with the in-house gradient run on a Vanquish Core HPLC system with quaternary pump and a Vanquish Flex UHPLC system with binary pump. Switching of extinction/emission wavelengths is indicated as dotted lines.

With the method protocol applied, cystine was not detected, while Lys, the only AA with two amino groups, eluted in two peaks, which indicated an incomplete derivatization process. To shift the Lys peak area ratio towards the later eluting one (and increase overall peak areas and sensitivity), the OPA concentration of the reagent might be increased (tenfold or more). Potential detrimental effects of increased OPA concentrations on the column lifetime were not evaluated here. Instead, the protocol applying the low concentration of the toxic derivatization agent achieved stable and reproducible results, as shown in the following.

The reproducibility of the separation and the in-needle derivatization process is illustrated by Table 4 for six consecutive injections of the 50 µM calibration standard with the Vanquish Core HPLC system. The standard deviation (SD) of the retention times (t_R) was <0.005 min for most AAs, except for the early eluting Asp and Glu (<0.02 min). The relative standard deviations (RSD) of the peak areas were usually <2%, except for the secondary AAs Pro and Hyp (5.5 %). Quadratic fits with offset were enabled for the external amount calibration of all AAs. Usually, the determination coefficients (r^2) were >0.999 and RSDs of the calibration points as a measure of the relative error in the calibration were <3%. As an example, typical representative calibration parameters of one daily sequence with the Vanquish Core HPLC system are given in Table 4.

As commercial amino acid solutions for cell culture nutrition come with well-defined AA contents, they are well suited for an accuracy check of the method. Figure 3 shows the amount results on four representative days of measurement for one of these products. In most cases the deviation of the determined amount from the amount specified in the product description was ≤2 µM, or ≤10%. His results depended a lot on the age of the calibration standards and samples, as His seemed to degrade quickly over time. For Thr, a consistent deviation of approximately 6 µM from the expected amount was found, which may point to an error in the product description. (Note: Dilution or weighing errors of the calibration standards can be excluded as Thr was added by a certified mixed standard together with the other AAs, which achieved expected results.)

Table 4. Reproducibility of peak parameters measured with standard injections (50 µM, n=6) and typical representative calibration parameters with the Vanquish Core HPLC system

	Peak parameter reproducibility		Calibration parameters	
	SD(t_R) [min]	RSD (area)	r^2	Calibration RSD [%]
Asp	0.013	1.41%	0.99975	1.67
Glu	0.017	1.24%	0.99984	1.36
Asn	0.003	1.33%	0.99984	1.36
Ser	0.003	1.04%	0.99984	1.39
Gln	0.004	1.05%	0.99986	1.29
His	0.003	1.68%	0.99943	2.70
Gly	0.003	1.09%	0.99991	1.00
Thr	0.003	0.91%	0.99988	1.19
Arg	0.002	1.06%	0.99987	1.24
Ala	0.002	1.09%	0.99980	1.52
Tyr	0.002	1.15%	0.99984	1.35
Val	0.002	1.32%	0.99981	1.47
Met	0.002	1.29%	0.99983	1.41
Trp	0.002	1.04%	0.99985	1.31
Phe	0.001	1.03%	0.99985	1.32
Ile	0.001	1.10%	0.99981	1.50
Leu	0.001	1.03%	0.99984	1.35
Lys	0.001	1.76%	0.99985	1.32
Hyp	0.001	5.47%	0.99983	1.39
Pro	0.001	5.55%	0.99982	1.40

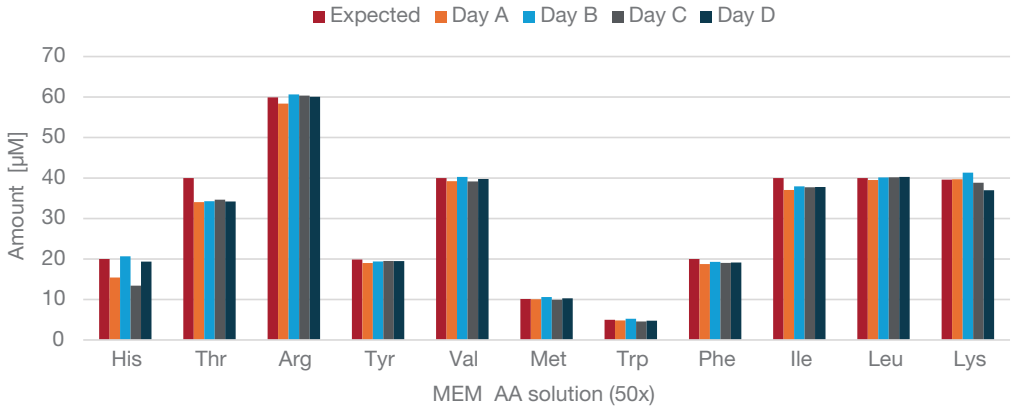


Figure 3. AA amount results of the "MEM AA solution (50x)" with the Vanquish Core HPLC system compared to the amount specified in the product description. Representative data are shown for four different days of measurement.

Table 5 gives an overview of amount results of the different food and beverage samples, measured on six different days (distributed over three weeks). Standard deviations (SDs) were usually well below 5 µM (10 µM for higher amounts), showcasing very good inter-day reproducibility of the method. In addition, samples spiked with 10 µM of each AA were added to each analytical sequence. The recovery of the spiked AA amount was usually between 80% and 120%. An example is given in Table 6 for the orange juice sample.

Application for QC of fertilizers

AAs significantly contribute to plant growth and health. Depending on the specific needs of different crops and the environmental conditions, the AA profile for optimal fertilization may differ widely. The close control of the respective product formulations brought into the ground is crucial in the balancing of optimized crop yield, economic efficiency, and environmental footprint. Thus, careful product monitoring is key for manufacturers.

Mundeco by Agronova Biotech (Spain) is an official service laboratory for the agroindustrial and environmental sector, accredited under ISO 17025 standard. In addition, it covers internal QC and research and development purposes in fertilizer and crop solution production. As this, the handling of complex matrices, such as fertilizers, animal feed, wheat, fruit flours, or microalgae hydrolysates, is daily routine in this lab.

As mentioned before, the above protocol for AA analysis might be altered according to the analytical needs. Some modifications that were introduced by Mundeco for the QC of fertilizers included changes of the AAs in scope, the gradient profile and run time, a switch to ISTD calibration, and multi-channel fluorescence recording instead of wavelength switching. Typical chromatograms for calibration standards and fertilizer samples are depicted in Figure 4.

Table 5. Amount results [µM] and SDs of food and beverage samples averaged over six days of measurements with the Vanquish Core HPLC system (n.d.= not detected, << = below calibrated range, >> = above calibrated range)

	Orange juice (1:100)		Soy sauce (1:1000)		Apple juice (1:10)		Lemon juice (1:100)		White wine (1:10)		Red wine (1:10)		Beer (1:10)		Honey (0.1 g/mL)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Asp	28.2	1.2	17.6	1.5	80.5	5.3	40.2	0.9	29.4	1.3	17.1	1.0	5.4	0.5	17.7	1.8
Glu	7.9	0.5	81.1	8.9	15.9	0.8	14.1	0.6	33.7	1.2	19.1	0.8	9.5	0.5	19.1	1.7
Asn	61.0	2.5	<<		>>		19.8	0.8	13.5	0.8	7.9	0.3	2.1	0.4	14.3	0.7
Ser	18.6	0.7	43.2	3.3	16.4	0.4	21.0	0.6	13.3	0.8	7.9	0.3	4.1	0.2	7.6	0.2
Gln	3.8	0.6	0.7	0.3	1.4	0.4	n.d.		n.d.		n.d.		6.1	1.6	16.2	0.4
His	3.3	2.4	12.6	3.9	2.7	1.7	n.d.		7.8	0.9	6.9	3.5	16.1	5.3	3.9	2.2
Gly	2.6	0.3	31.1	2.0	1.4	0.2	1.7	0.2	21.8	1.1	16.6	0.7	26.3	1.0	3.8	0.2
Thr	1.9	0.3	25.0	2.4	2.6	0.2	1.3	0.3	7.7	0.4	7.0	0.9	n.d.		2.6	0.4
Arg	63.4	3.0	32.2	2.2	1.0	0.4	2.3	0.2	66.3	1.9	45.6	1.5	24.9	1.0	4.6	0.3
Ala	11.4	0.5	89.2	9.2	16.6	0.6	14.5	0.5	48.3	2.6	42.5	1.9	79.7	3.7	16.8	0.7
Tyr	0.8	0.3	4.0	0.5	<<		n.d.		8.5	0.4	3.2	0.2	24.6	1.2	14.0	0.7
Val	3.2	0.4	42.7	3.8	4.4	0.4	2.8	0.4	29.4	4.3	24.5	2.0	41.7	2.0	6.1	0.4
Met	<<		8.4	0.7	1.5	0.2	<<		9.5	0.3	4.3	0.3	3.0	0.3	0.6	0.2
Trp	<<		0.7	0.3	n.d.		n.d.		1.3	0.2	1.0	0.2	14.3	0.8	2.4	0.2
Phe	1.7	0.3	24.7	2.0	1.5	0.2	1.1	0.2	13.2	0.5	5.7	0.2	18.3	0.8	90.2	5.2
Ile	<<		33.1	2.8	2.9	0.4	<<		6.4	0.3	3.2	0.3	5.1	0.2	6.2	0.2
Leu	0.6	0.3	52.7	4.6	1.3	0.3	<<		23.7	0.8	8.9	0.6	13.4	2.6	3.5	0.3
Lys	2.3	0.2	33.2	4.0	1.0	0.3	<<		26.3	0.1	10.5	1.3	<<		2.1	0.9
Hyp	<<		<<		<<		<<		4.5	0.7	2.0	0.4	<<		2.7	0.3
Pro	73.7	10.4	31.9	2.7	2.6	0.5	26.6	2.6	>>		>>		>>		>>	

Table 6. Recovery results from an unspiked and spiked orange juice sample (spiked with 10 µM of each AA)

	Orange juice (1:100) [µM]	Orange juice (1:100) spiked with 10 µM [µM]	Δ [µM]	Recovery [%]
Asp	28.3	38.4	10.0	100.0
Glu	7.9	17.6	9.7	97.1
Asn	62.6	73.2	10.6	106.5
Ser	18.8	28.7	9.9	99.0
Gln	4.5	13.9	9.5	94.7
His	4.5	13.8	9.3	92.9
Gly	2.9	12.1	9.2	92.3
Thr	2.1	10.4	8.3	82.7
Arg	64.1	76.0	11.9	119.1
Ala	11.4	22.7	11.3	113.3
Tyr	0.9	10.1	9.1	91.4
Val	3.4	14.2	10.7	107.2
Met	<<	9.9	9.1	90.7
Trp	n.d.	10.4	10.4	104.1
Phe	1.9	11.0	9.2	91.5
Ile	<<	8.9	8.2	81.9
Leu	0.7	9.9	9.2	92.0
Lys	2.2	10.0	7.9	78.5
Hyp	n.d.	8.4	8.4	84.1
Pro	70.4	82.8	12.3	123.1

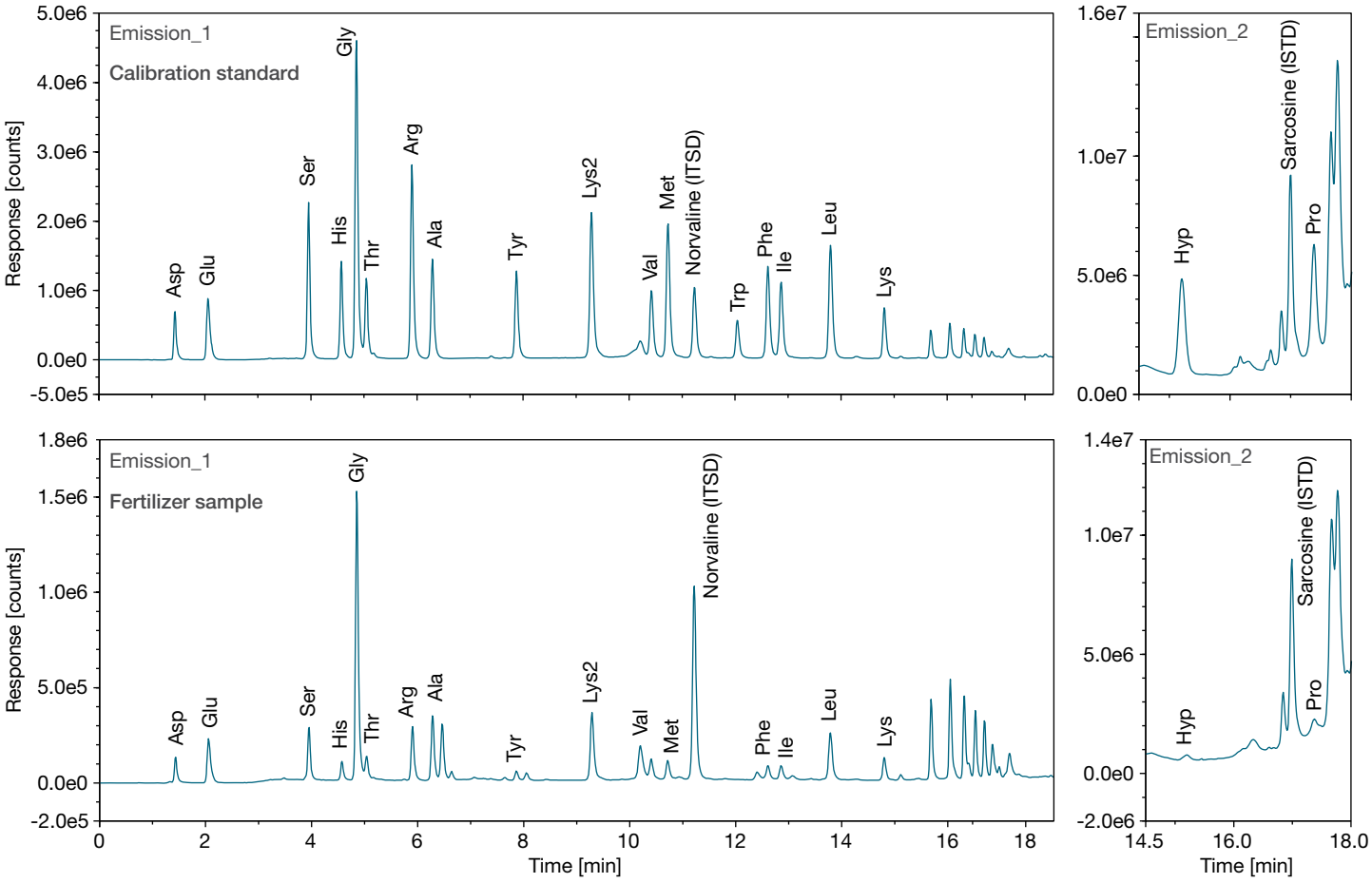


Figure 4. Multichannel chromatograms recorded at Mundeco QC lab for a calibration standard and fertilizer sample

Linear fits with offset were applied for the calibration with ISTDs of all AAs and typically achieved $r^2 > 0.999$ and RSDs <3%, as shown in Figure 5. These were applied for quantification in fertilizers, which contained a broad concentration range of 0.1% w/w up to >10% w/w of single AAs and were diluted accordingly. A reference material, a solid organic fertilizer with a total content of free AAs of 75%, was analyzed on a regular basis for method performance and reproducibility verification. Data were available for eight different days over three months, recorded by different operators. The minimum, maximum, and average amounts determined, and RSDs are indicated in Table 7, while in Figure 6 all data are visualized for some selected AAs. The average sum of AAs was 75.7% with an RSD <4%, and for most individual AAs, reproducibility was very good with RSDs ≤7% with a few exceptions due to column aging (e.g., resolution decline of Gly/Thr).

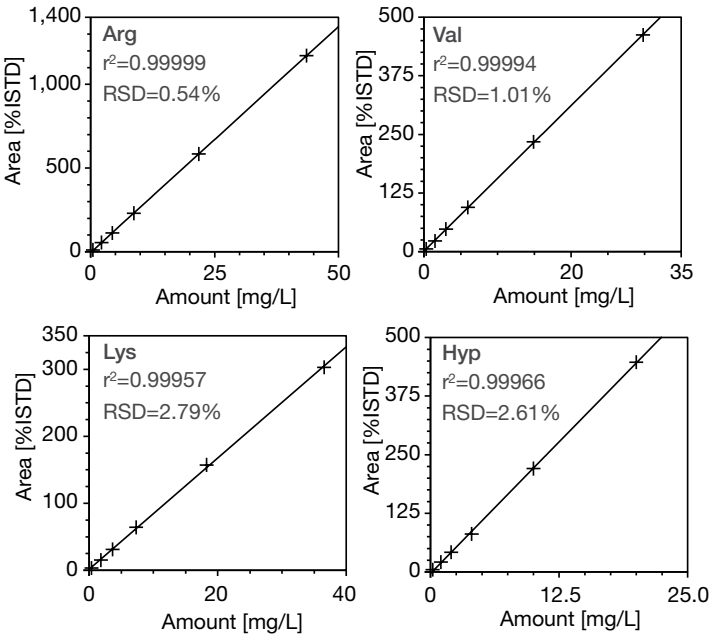


Figure 5. Examples of ISTD calibration curve fits recorded at the Mundeco QC lab

Table 7. Amount results of all AA in [% w/w] of a solid organic fertilizer reference material with total content of 75% of free AAs, analyzed at the Mundeco lab on eight different days over three months

AA	Minimum	Maximum	Average	RSD [%]
Asp	7.38	8.42	7.87	4.59
Glu	7.57	8.32	8.02	3.46
Ser	2.48	2.75	2.63	3.55
His	2.83	3.49	3.19	7.08
Gly	8.62	11.17	9.80	8.89
Thr	4.18	6.25	5.37	13.01
Arg	2.65	2.86	2.75	2.26
Ala	5.28	5.85	5.72	3.41
Tyr	1.76	1.94	1.85	3.79
Val	4.35	5.11	4.90	6.34
Met	0.64	0.80	0.69	6.26
Phe	4.33	4.87	4.57	3.37
Ile	0.46	0.71	0.58	12.24
Leu	9.08	10.72	9.96	4.83
Lys	5.13	5.61	5.43	2.71
Pro	2.11	2.51	2.38	5.67
Sum AAs	**71.22**	**81.19**	**75.69**	**3.93**

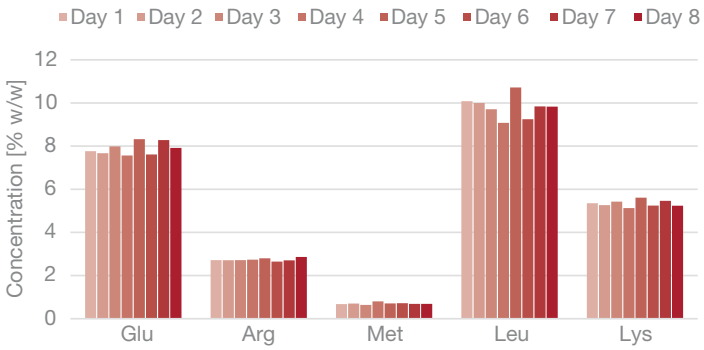


Figure 6. Amount results in [% w/w] for selected AAs of a solid organic fertilizer reference sample analyzed at the Mundeco lab on eight different days over three months

Long-term column endurance

The conditions of the OPA/FMOC AA analysis may pose a special challenge to the stationary column phase, and thus may impact column lifetime, one major contributor to the operating costs. The experienced lifetimes of the columns involved in the current study are summarized in Table 8.

Table 8. Column lifetime at the different sites and application fields

Column #	Operated on	Samples	Injection count	Guard cartridges
1	Vanquish Core HPLC (in-house, site 1)	Standards, food/beverage	640 (ongoing, Figure 7)	1
2	Vanquish Flex UHPLC (in-house, site 1)	Standards, food/beverage	460 (ongoing)	1
3	Vanquish Core HPLC (in-house, site 2)	Standards	530 (ongoing, Figure 8	2 (change after 300 injections)
4	Vanquish Core HPLC (Mundeco)	Standards, fertilizers	450 (to be replaced)	2
5	Vanquish Core HPLC (Mundeco)	Standards, fertilizers	300 (to be replaced)	2

Our in-house tests involved three different Accucore C18 (3 × 150 mm, 2.6 µm) columns from two different lots, which were operated at three instruments at two sites. One column was run on a Vanquish Core HPLC system with standard injections for calibration, and food and beverage samples for analysis. The chromatograms in Figure 7 depict the method stability over more than 600 injections. Slight alterations of peak parameters (decrease in t_R, increase in peak width at half height ($w_{50\%}$)) due to column aging can be expected and some are given as examples in the tables. After several hundred injections, the most critical peak pairs (Phe/Ile, Val/Met) were still sufficiently resolved for proper sample evaluation. After 640 injections, experiments were concluded. Equivalent experiments were performed with another column on a Vanquish Flex UHPLC system. After approximately 460 injections, work was stopped. By then only a slight fronting of the peak shapes was commencing.

At a different in-house site, one column was run on a Vanquish Core HPLC system with standard injections only and no samples. After approximately 300 injections, peak shapes started to deteriorate but fully recovered after changing the guard cartridge (Figure 8). At >530 injections, the performance was still good, and experiments were terminated.

The fertilizer samples analyzed at Mundeco presented a more challenging matrix for the column hardware. One column, which also covered some method development, was used over approximately 450 injections with two guard cartridges being consumed. A second one, which was used in a more routine-like application, had to be replaced after approximately 300 injections due to insufficient peak shapes and resolution.

Method speed-up

For many labs, AA analysis is a daily routine with high sample throughput demands. These labs can significantly profit from automation processes as well as decreased sample run times. With the in-needle-derivatization application, the reaction process cannot be accelerated; however, the run time of the AA separation bears some potential for speed-up. In a proof-of-concept attempt, the translation of our in-house HPLC method into a UHPLC method was tested with the Thermo Scientific™ Accucore™ Vanquish™ C18+ column (1.5 µm, 2.1 × 100 mm, P/N 27101-102130) installed on the Vanquish Flex UHPLC system. The built-in UHPLC speed-up tool of Chromeleon CDS was used for the translation. Only minor method adaptions were applied. The chromatogram of a calibration standard (50 µM) in Figure 9 shows sufficient separation, which could potentially be optimized by the gradient profile. The processing time per sample (including derivatization) decreased to approximately 17.5 min compared to approximately 29 min with the HPLC method, while backpressures of up 850 bar (UHPLC) were generated compared to 320 bar (HPLC). The stability of the method was checked with three typical standard and sample sequences as above.

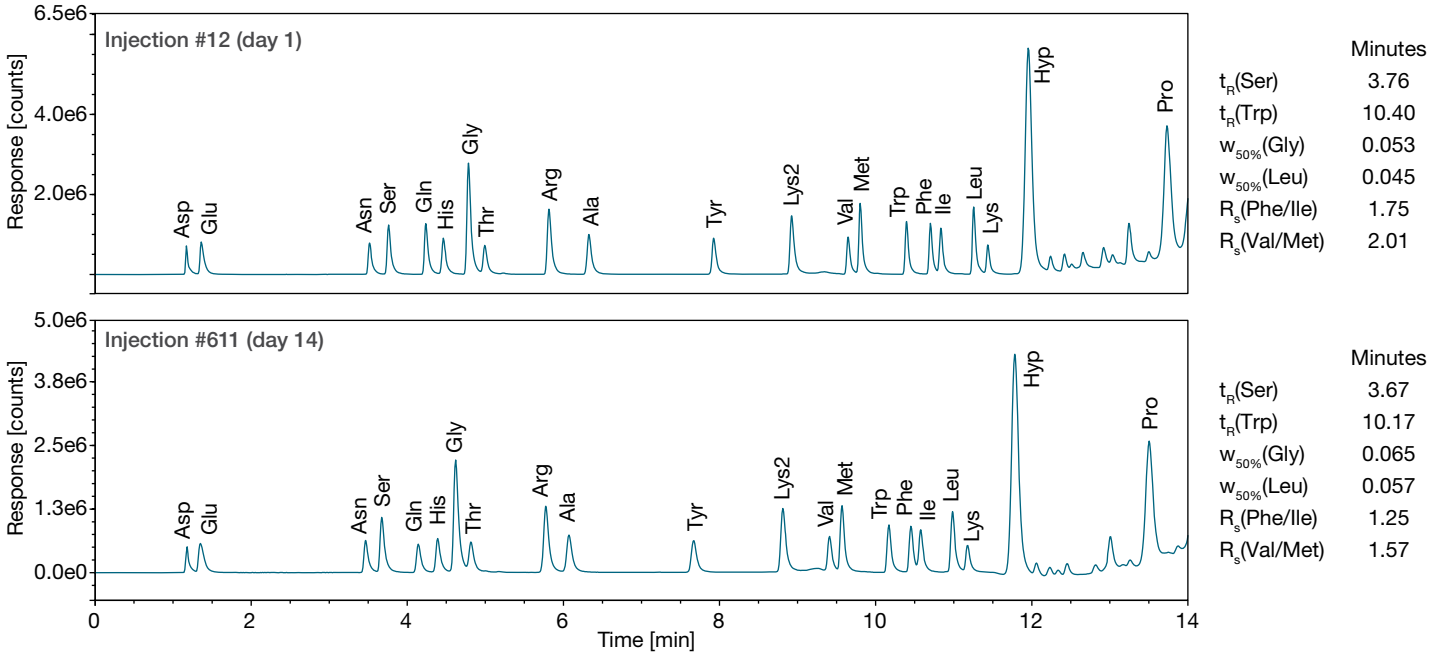


Figure 7. Chromatographic results of calibration standard (50 µM) measured with the Vanquish Core HPLC system, comparing method performance on 1st and 14th day of measurements

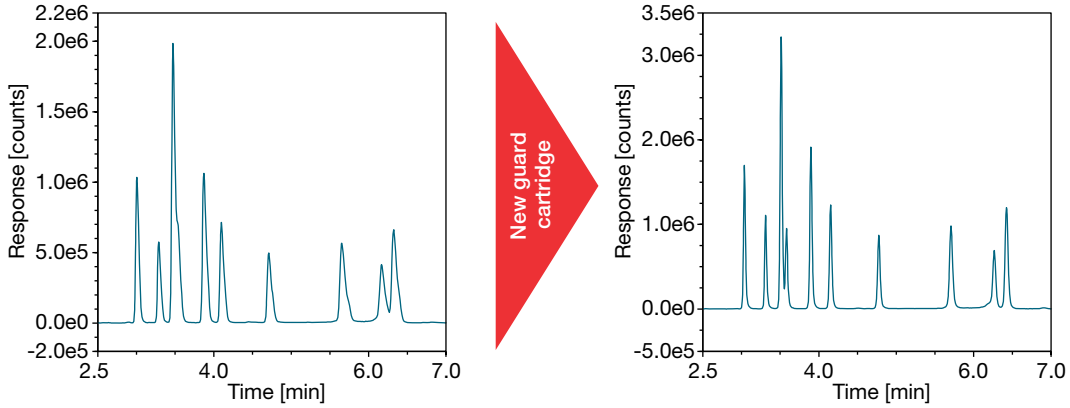
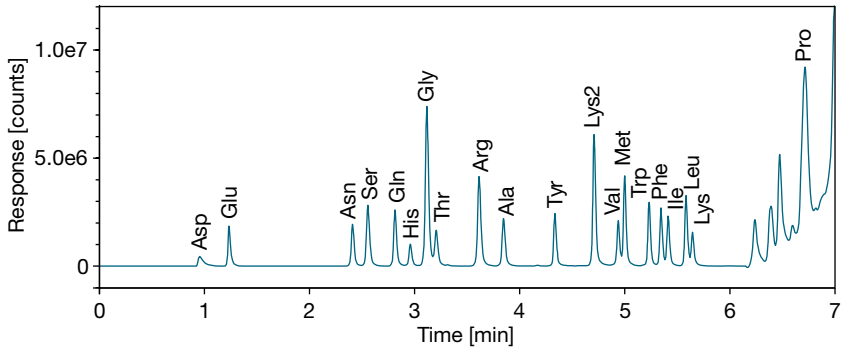


Figure 8. Recovery of column performance by guard cartridge exchange



Time [min]	Flow [mL/min]	%B
-5.0	0.4	2
0.0	0.4	2
0.154	0.4	10
1.923	0.4	20
6.154	0.4	75
6.192	0.4	100
8.4	0.4	100
8.5	0.4	2
10.5	0.4	2

Figure 9. Gradient table and chromatogram of a calibration standard (50 µM) after method translation into a UHPLC method. Hyp not detected due to late wavelength switching.

Conclusion

- The automated in-needle OPA/FMOC derivatization is a time and labor-saving approach for AA analysis that demonstrated robust and reproducible performance at different internal and external analytical laboratories for medium and highly complex matrices.
- Reliable method performance, column stability over several hundred injections, and usage of inexpensive reagents (compared to pricy AA quantification kits) facilitate high sample throughput at low costs per sample for routine laboratories.
- The applicability of the HPLC workflow was highlighted with Vanquish Core and Vanquish Flex systems, as well as its potential for an upgrade to UHPLC performance for increased throughput.

Tips for a robust method performance:

1. Have a 100 µL sample loop (P/N 6851.1950 for Vanquish Core system, P/N 6850.1913 for Vanquish Flex system) installed to ensure thorough and reproducible mixing of the sample and reagents during the derivatization step.
2. Have a passive pre-heater (P/N 6732.0170 or P/N 6732.0180) installed at your Vanquish system, independent of which Vanquish LC tier is used, to facilitate proper dilution of the reagents before the column.
3. Use guard cartridges for your columns and replace them when column performance diminishes. Smart tools like the intelligent run control (IRC) in Chromeleon CDS may be used for automated checks.
4. Replace derivatization reagents in the autosampler daily.
5. Periodically flush the column (e.g., with 100% acetonitrile for 30 min) to remove persistent reaction products and matrices.

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2. Thermo Scientific Technical Note 107: Automated in-needle derivatization applying a user-defined program for the Thermo Scientific Dionex WPS-3000 Split-Loop Autosampler, 2016. https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-107-automated-in-needle-derivatization-lpn2849-en.pdf

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