

# Ultra-fast amino acid analysis using the SCIEX Triple Quad™ 4500 System and the SCIEX MicroLC 200 System with aTRAQ™ reagent technology

*Maximizing speed, sample throughput and cost effectiveness of established assays*

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Micro flow chromatography with column diameters ≤1mm is an exciting approach for sensitive, high-throughput LC-MS/MS within the clinical research arena. High-throughput workflows, such as the analysis of amino acids from physiological fluids, require fast, high performance separations, as well as low carryover and fast injection-to-injection cycle times. Such performance is particularly critical within the field of amino acid analysis, due to the large number of structurally similar, isobaric compounds present which can demand extended chromatography in order to achieve the required separation for quantitation.

The MicroLC 200 System is a dedicated microflow UHPLC system that has been designed with these considerations in mind, for optimal performance in the microflow regime, including a new autosampler injection system with modifications for very small volume sample handling with minimal sample waste as well as very low carryover.

## Key Features of the SCIEX MicroLC 200 System

- High performance pumping system
- Microfluidic Flow Control™ (MFC) for accurate rapid gradients with exceptional accuracy and reproducibility
- Robust UHPLC performance with operating pressures up to 10,000 PSI
- Fast reproducible sample injections
- Small volume injections with minimal sample waste
- Very low carry-over
- Low delay volumes enable ultrafast gradient separations for LC-MS applications
- Green LC with smaller ID columns to reduce mobile phase consumption by over 95%, providing significant cost savings
- Robust integration with SCIEX hardware and software

## Robust and simple sample preparation using aTRAQ technology

Sample preparation for amino acid analysis using the SCIEX MicroLC 200 System is identical to the approach used for the application running with conventional chromatography. No modifications or additional steps are needed.

Current dedicated amino acid analyzer systems have long runtimes, are prone to interferences from buffers, matrices, and other co-eluting amino acids, and can be difficult to maintain. An LC-MS/MS method for the analysis of amino acids in physiological fluids using amine-reactive isotope-coded tags (aTRAQ reagents) has been established. Because only compounds with the same mass require chromatographic separation, the analysis time can be significantly reduced versus traditional methods. The combination of LC-MS/MS with aTRAQ reagents also provides better sensitivity (therefore lower detection limits), a wider dynamic range, and the ability to use labeled internal standards for more accurate and robust quantitation.

## Experimental

Analysis was carried out by first precipitating any proteins out of the sample with sulfosalycylic acid, followed by derivatization with the reagents supplied in the aTRAQ Kit for Amino Acid Analysis. The samples labeled with aTRAQ reagent Δ8 are mixed with the internal standards pre-labeled with aTRAQ reagent Δ0. This mixture is then injected for LC-MS/MS analysis using either a triple quadrupole or QTRAP® system operating in MRM mode. The use of the *Scheduled* MRM™ Algorithm maximizes dwell time while monitoring of large numbers of MRM transitions, resulting in optimum data quality and reproducibility. Quantitation is performed using Analyst® Software or Cliquid® Software for amino acid analysis. An illustration of analyte labeling and amino acid quantitation is shown in Figure 1.

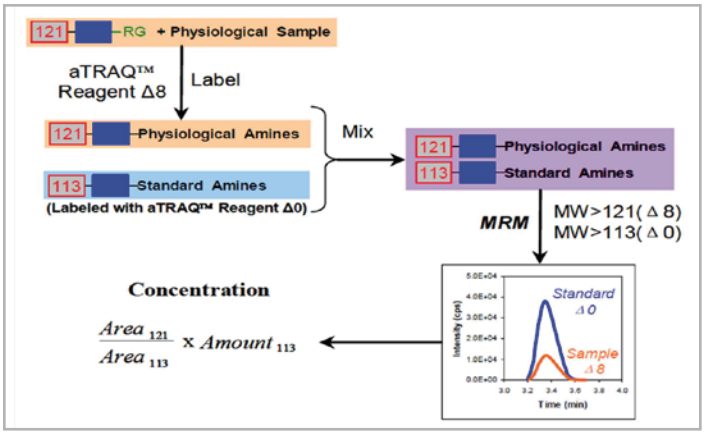


**Figure 1: Illustration of aTRAQ reagent labeling. The sample is labeled with the Δ8 reagent and the internal standard (IS) is prelabeled with the Δ0 reagent. The sample and IS are mixed and injected for LC-MS/MS analysis. Quantification of the individual amino acids is calculated using a ratio of the sample to internal standard peak areas.**

Under the conventional method, chromatographic analysis was carried out using a standard pressure HPLC system and an SCIEX AAA C18 column. Mobile phases were prepared as described in the aTRAQ reagent kit documentation. For analysis using the SCIEX MicroLC 200, a 0.5 x100mm C18 HALO column was used. Again, mobile phases were as documented in the aTRAQ reagent kit. Sample preparation in both cases was as per the kit documentation; injection volume was 2µL for conventional HPLC and 200nL for microflow LC. Data shown was collected on the SCIEX 4000 QTRAP® Mass Spectrometer (conventional HPLC analysis) or the SCIEX QTRAP® 4500 Mass Spectrometer (microflow LC analysis).

Gradient profile for conventional HPLC was as suggested in the aTRAQ reagent kit. Total run time was 18 minutes including column re-equilibration time, at a flow rate of 800µL/min. Gradient profile for microflow LC follows a similar profile, and is as shown in Table 1, however total runtime was shortened to 8 minutes including column re-equilibration time, and the flow rate was optimized at 20µL/min.

| Time (min) | % A | % B | Flow Rate (µl/min) |
|---|---|---|---|
| 0.0 | 98 | 2 | 20 |
| 0.3 | 98 | 2 | 20 |
| 2.0 | 73 | 27 | 20 |
| 4.5 | 73 | 27 | 20 |
| 5.0 | 10 | 90 | 20 |
| 6.0 | 10 | 90 | 20 |
| 6.5 | 98 | 2 | 20 |
| 8.0 | 98 | 2 | 20 |

**Table 1: Microflow LC gradient profile**

## Results and Discussion

Control Plasma samples (control plasma used was provided with the aTRAQ reagent 50 assay starter kit for physiological samples) was processed as per the aTRAQ procedure and injected onto the appropriate conventional- or micro-LC systems. Figure 2 shows the comparison in speed of analysis for the two separation techniques

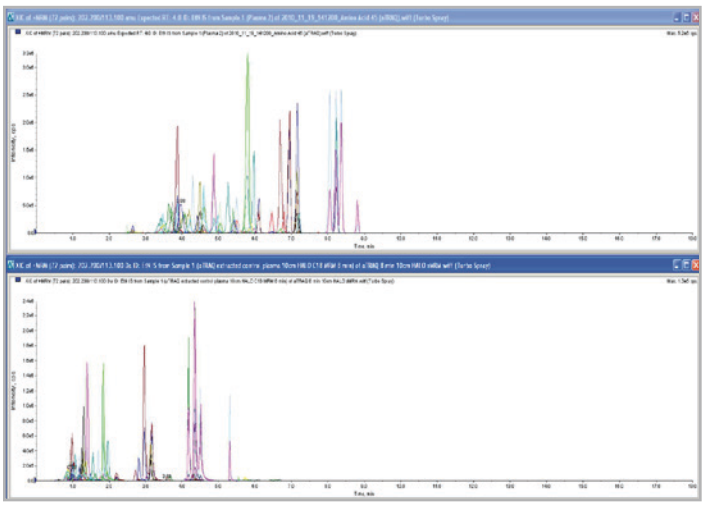

**Figure 2: Comparison of conventional (top) and microflow LC (bottom) for aTRAQ analysis. Both chromatograms are scaled to the runtime of conventional LC (18 min)**

Separation of the isobaric amino acids is critical and one of the reasons that a long conventional column is used in the traditional aTRAQ™ kit. In developing the microflow LC application care was taken to ensure the separation was maintained for these compounds. The properties of the HALO column used for this application allow similar separation of isobarics across a much shorter timescale, as is seen in figures 3 and 4.

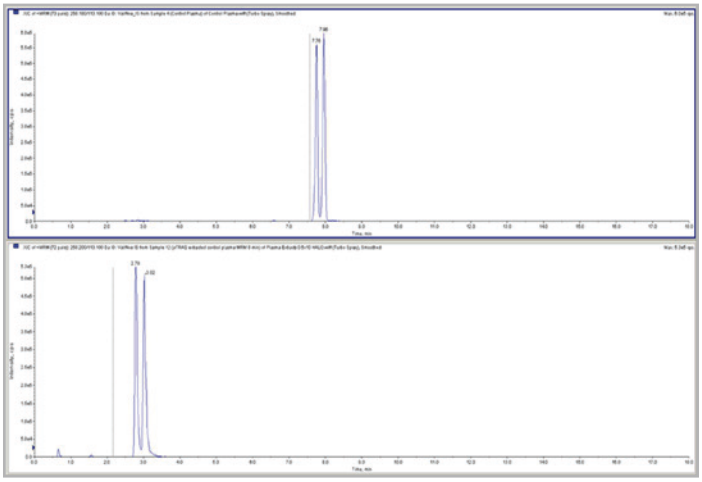

**Figure 3: Separation of Norvaline and Valine with conventional (top) and microflow LC (bottom). Both chromatograms are scaled to the runtime of conventional LC (18 min)**

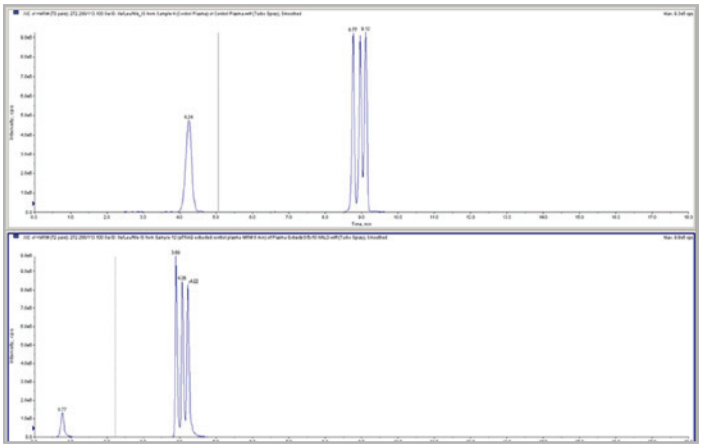

**Figure 4: Separation of Isoleucine, Leucine and Norleucine with conventional (top) and microflow LC (bottom). Both chromatograms are scaled to the runtime of conventional LC (18 min)**

Sensitivity improvements were calculated based on sample injection volume and average peak area of the amino acids analysed. Only 200nL of extract was injected on the SCIEX 4500 Series mass spectrometer coupled to the SCIEX MicroLC 200 System, while 2µL was injected on the SCIEX 4000 Series Mass Spectrometer using conventional HPLC. Peak areas and heights show approximately a 10-fold increase in sensitivity using the SCIEX MicroLC 200 System, based on the amount injected into either system. It must be stressed that this is the combined sensitivity increase of the whole system, with contributions from both the LC system and the mass spectrometer.

Concentrations of amino acids in control plasma (SCIEX, p/n 4386703, lot no A2086) were calculated following analysis by aTRAQ analysis and microflow LC, using reports generated offline using Cliquid® Software with the Amino Acid feature activated. Calculated concentrations were within the accepted range given on the control plasma data sheet. Examples of typical results (mM) achieved across a range of amino acids of retention times across the run are provided in Table 2. "A" signifies the SCIEX Triple Quad™ 4500 + MicroLC 200 system (microflow LC), "B" signifies the API 4000™ + Shimadzu LC System (conventional HPLC).

| Amino Acid | Result A | Result B | Accuracy A | Accuracy B |
|---|---|---|---|---|
| Gly | 200.2 | 186.6 | 117 | 109 |
| Gln | 267.2 | 249.8 | 97 | 91 |
| Asn | 27.3 | 30.2 | 83 | 92 |
| Ala | 305.2 | 400.3 | 88 | 115 |
| Abu | 15.8 | 12.2 | 115 | 89 |
| Pro | 239.9 | 193.0 | 121 | 97 |
| Phe | 88.9 | 69 | 120 | 93 |
| Trp | 53.6 | 41.9 | 121 | 94 |

**Table 2: Retention times**

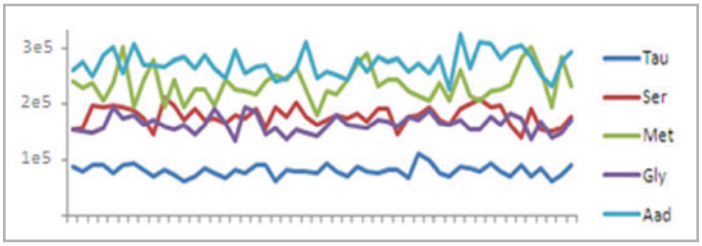


**Figure 5: peak area plot of internal standard peak area vs. injection number for several amino acids throughout the run, analysed using the SCIEX Triple Quad™ 4500 and the SCIEX MicroLC 200 system**

Figure 5 gives example data for a series of 50 injections (approximately 7 hours uninterrupted running) of internal standard, showing peak area of internal standard using the proposed system, demonstrating the injection to injection reproducibility of the microflow LC system. Average %CV across the range of amino acids analysed was 9.7%

## Conclusions

We have presented here an updated approach to the analysis of amino acids using the established aTRAQ™ kit technology. In order to maximize sample throughput, microflow LC has been considered as a way to enhance performance and to achieve robust separation of amino acids, in particular the isobaric compounds, required for reliable quantitation.

The use of the reduced diameter columns allows significantly reduced flow rates and injection volumes, reducing sample consumption, instrument downtime, and cost of ownership.

The SCIEX MicroLC 200 shows potential for the analysis of amino acids in physiological fluids using the aTRAQ reagents. The improvements to the method given by the use of microflow LC allow the quantitation of 45 amino acids in samples within 8 minutes, including column re-equilibration time, compared to 18 minutes using conventional HPLC. Microflow LC allows dramatic savings in solvent consumption and hence cost of ownership. Sample preparation remains unchanged and assay performance, in terms of sensitivity based on injection volume comparisons, is greatly improved.



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