Automated Parallel Derivatization Strategy with Broad Metabolite Coverage Coupled to SWATH/MS Data Acquisition for Qualitative and Quantitative Analysis

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Overview

- Parallel derivatization with Dansyl-Cl and Dansyl-N_2H_3 covers broad range of polar metabolites
- Improved retention on reverse phase LC by ~10 min for a total run time of 25 minutes
- Enhanced signal to noise (S/N) by a factor of 10 to 200
- Automated derivatization with on-line C18 SPE based fractionation improves reaction kinetics control for quantitative analysis
- QUAL/QUANT SWATH/MS data analysis using (^{13}C) labelled derivatization agents

Introduction

Due to the vast variety of metabolite physicochemical properties there is no single workflow which can provide a large scale metabolomic coverage in particular for polar metabolites. With LC-MS several limitations have to be addressed including: poor MS response, chromatographic retention and MS/MS fragmentation. Among different sample preparation approaches, chemical derivatization has proven to be a suitable workflow to address these issues.

We propose a parallel derivatization approach based on two well established procedures covering a broad range of metabolites with respect to the different functional groups. By derivatization of the metabolites, highly polar analytes are converted to more hydrophobic products, thus improving retention on reverse phase LC by ~10 min for a total run time of 25 minutes. Due to the presence of a basic heteroatom on Dansyl-Cl and Dansyl-N_2H_3, increased chromatographic retention and enhanced signal of analytes with a normally very low ESI response, the S/N can be improved by a factor of 10 to 200 times. Dansyl-Cl derivatizes primary alcohols, phenols, primary and secondary amines while Dansyl-N_2H_3 transforms ketones and aldehydes to hydrazones. To cover both classes of analytes two independent sample preparation batches would be required. With the automated parallel derivatization all analytes could be analyzed in a single run with sufficient detection limits. Automation was found to be essential to allow reproducible derivatization as the reaction kinetics of individual analytes are highly variable within a short time scale (15 min). Using labelled (^{13}C) Dansyl-Cl and Dansyl-N_2H_3 for derivatization of a mix of suitable internal standards with a parallel non-labelled derivatization of the sample (pooled urine sample) can facilitate SWATH data processing by following derivatization-specific *tag-ion fragments* (^{12}C m/z 170 and ^{13}C m/z 172) for all derivatized analytes.

An on-line SPE based fractionation using a C18 guard column was used to remove the excess of reagents and highly lipophilic background compounds which can significantly affect the ionization efficiency and chromatographic performance.

The mix of heavy (^{13}C) derivatized standards can be used for quantitative analysis of metabolites as individual internal standards. Due to the high dynamic range of different metabolites, SWATH/MS data acqusition allows the quatification on both precursor and product ion level including flexibility of the quantifier choice.

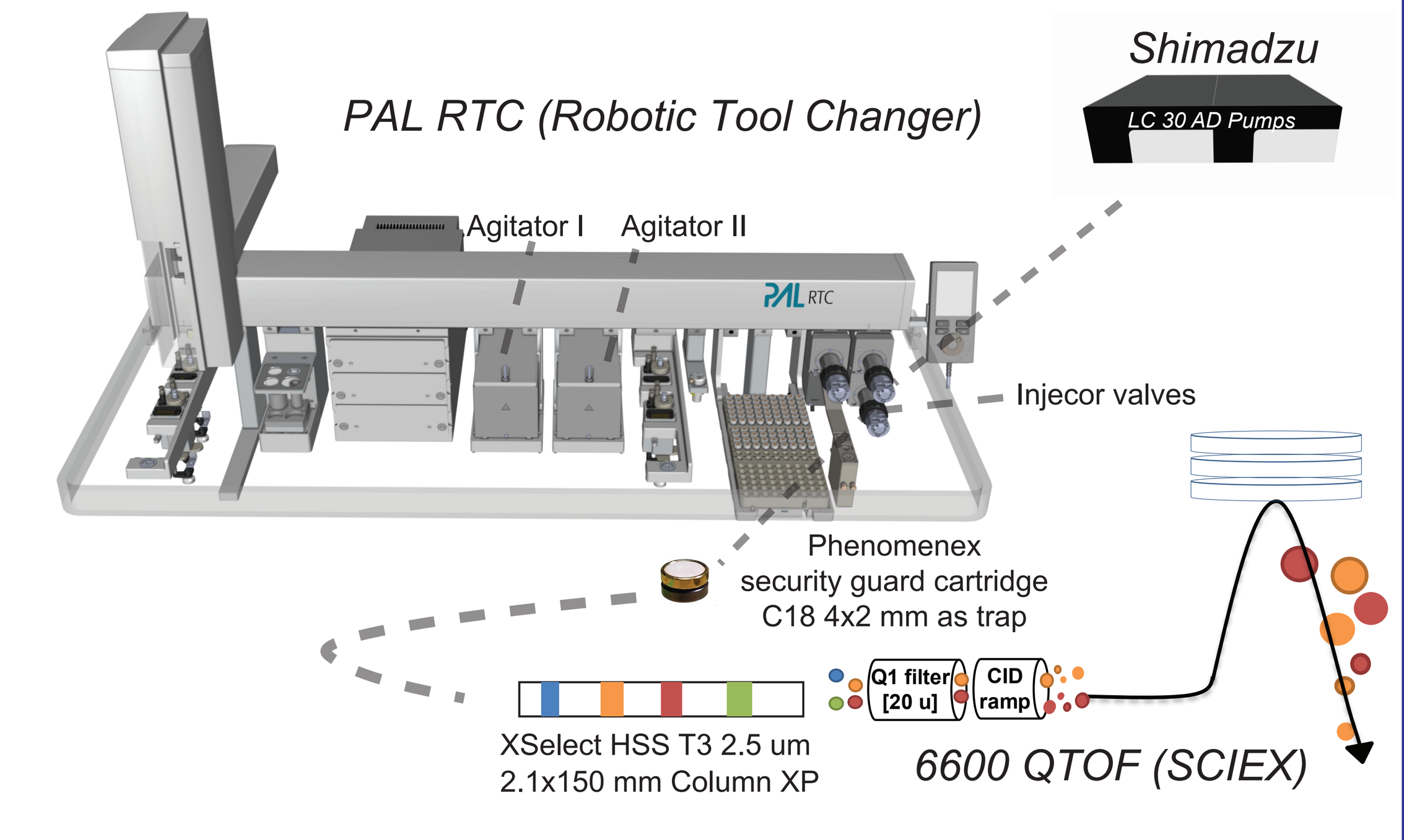


Figure 1. Schematics of the PAL RTC autosampler for automated parallel derivatization with hyphenation to two quaternary LPG pumps and MS QTOF instrument.

Acknowledgements

Methods

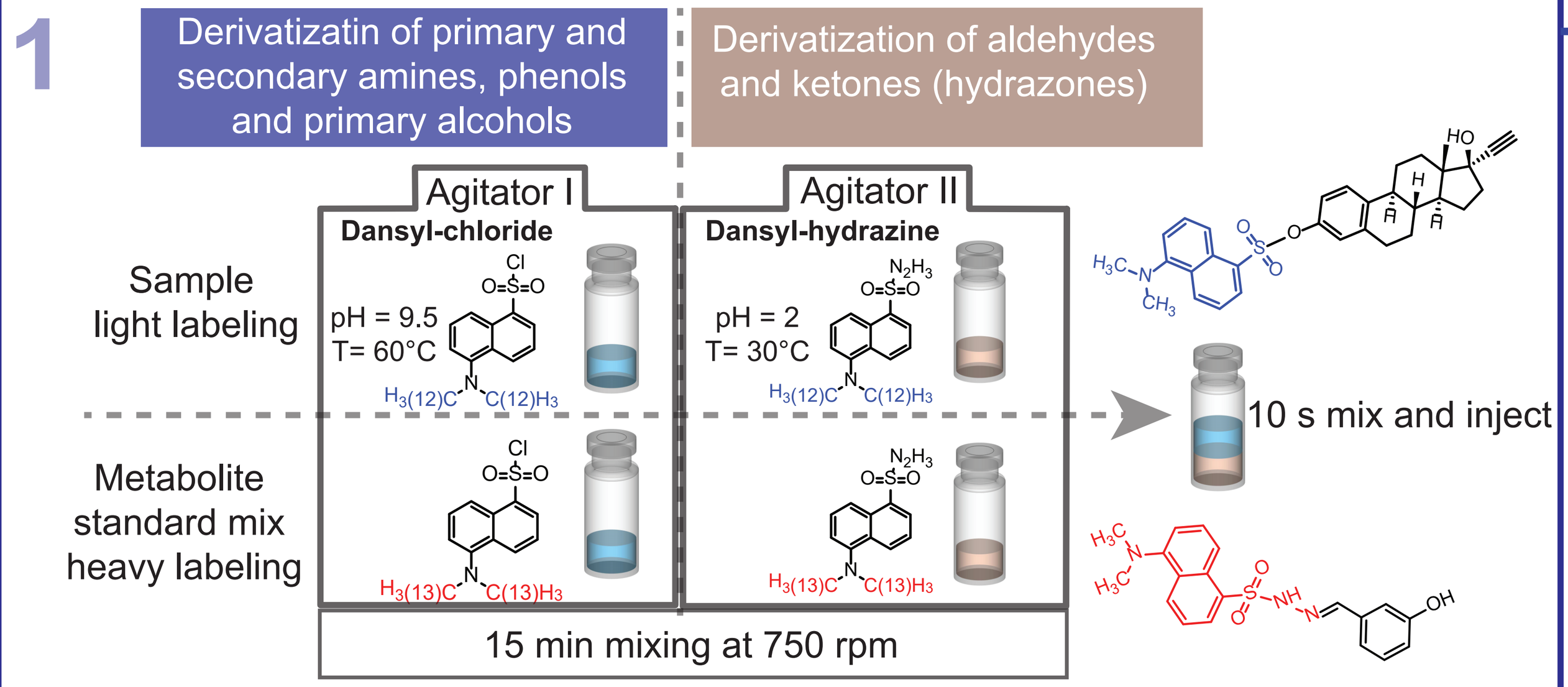


Figure 2. Schematics of the parallel derivatization of the sample with Dansyl-Cl and Dansyl-N_2H_3 and the metabolite standard mix with (^{13}C) labelled Dansyl-Cl and Dansyl-N_2H_3. The incubation is performed in separate thermally stable compartments. Automated sample preparation is stopping both reactions by having an exact control of the reaction time between injections.

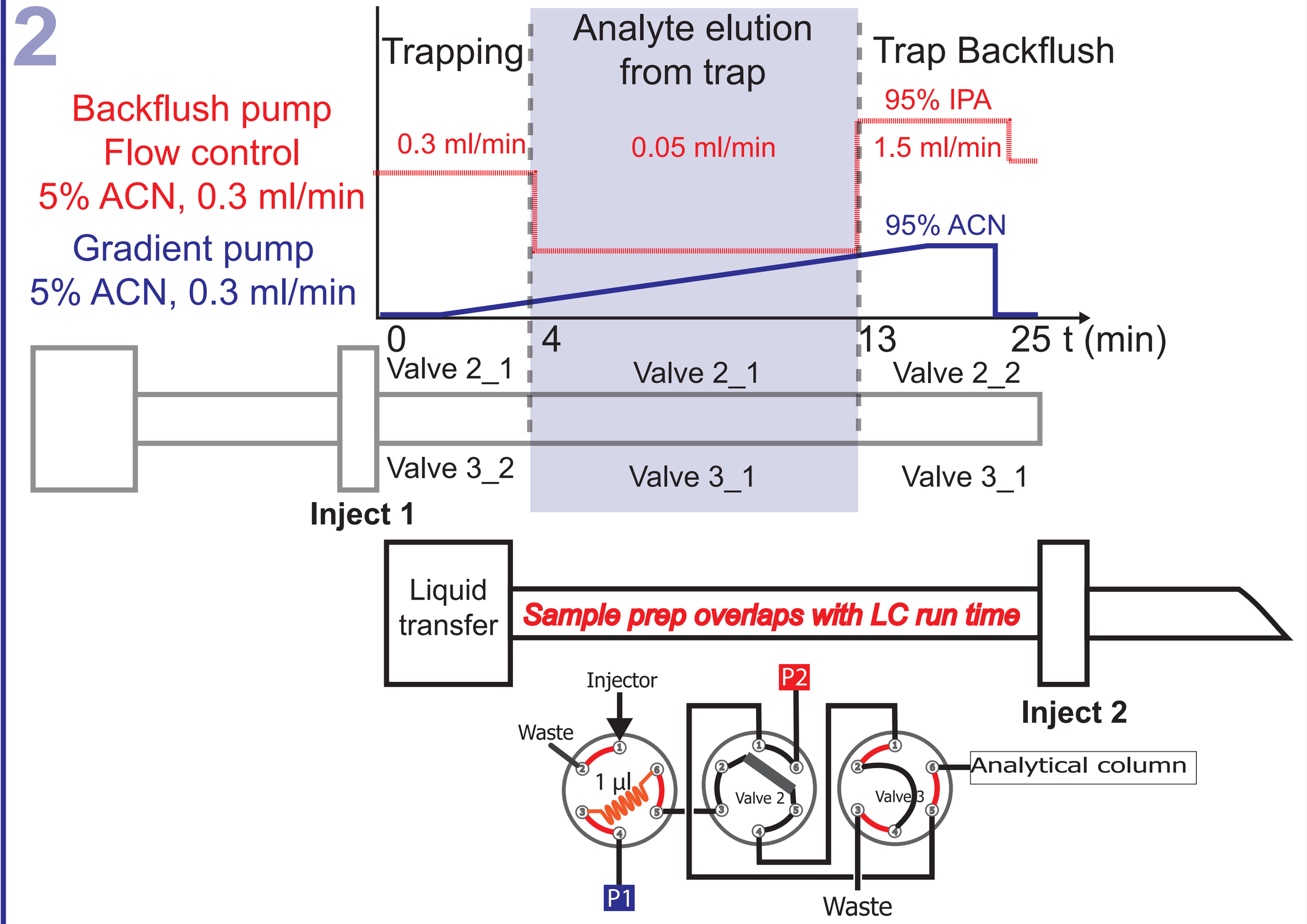


Figure 3. Time frame for the automatized sample derivatization and on-line SPE based sample clean up overlapped with the LC run time. At the start, Pump P2 is used for analytical column conditioning and for backflushing the trap after 13 min of the LC run. Pump P1 is gradient pump and at the start, it is trapping and washing out salt from the trap. All events are controlled with valve positions depicted on the graph.

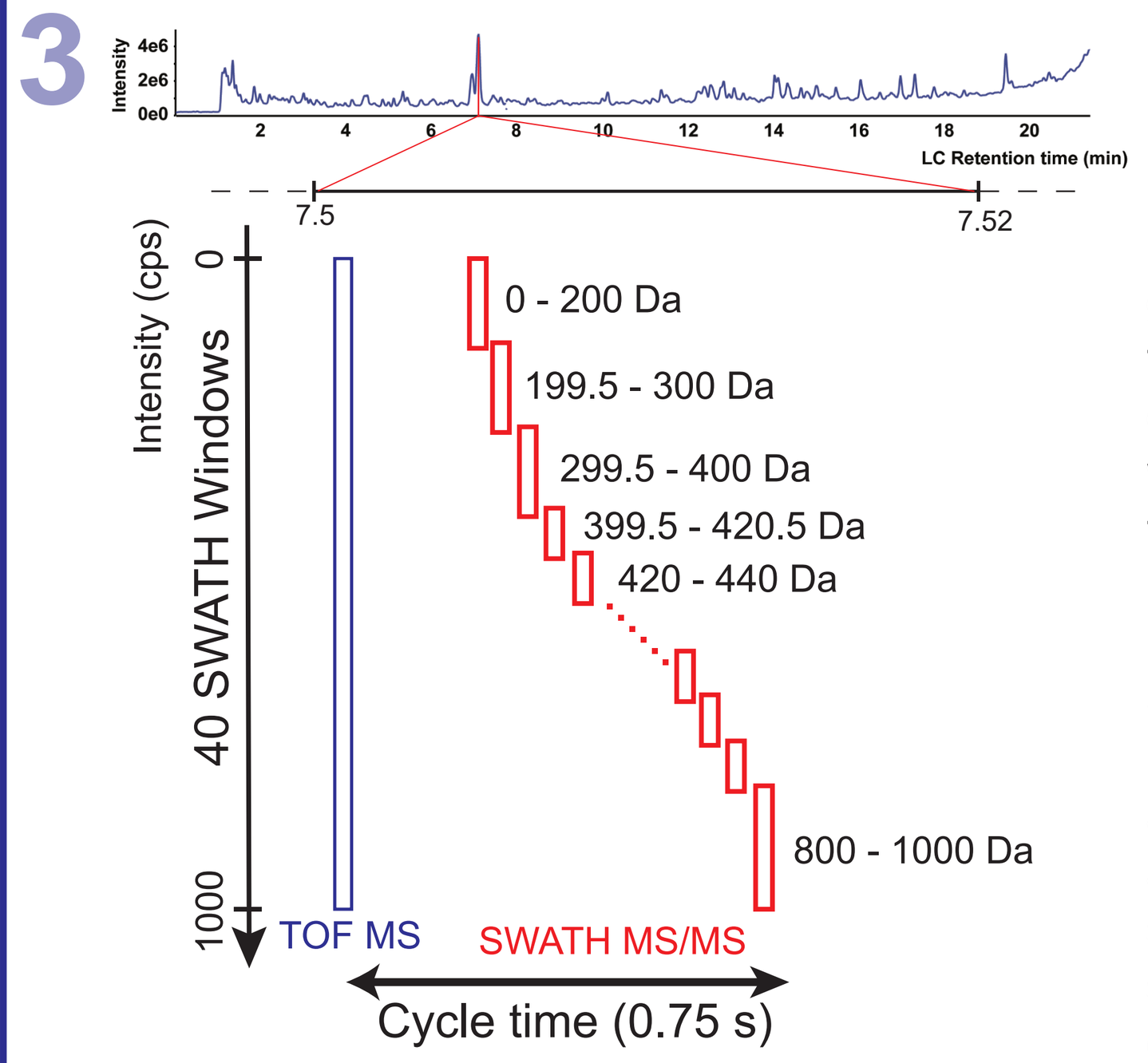


Figure 4. SWATH data acquisition.

An independent data acquisition that gives information of precursors and fragments within selected Q1 mass windows illustrated as a red frame. The window size and position is optimized for a medium mass range due to the mass shift resulting from metabolite derivatization. The cycle time of 0.75 s (40 SWATH windows including TOF MS (100-1000 Da)) gives about 15 points across the chromatographic peak suitable for quantitative analysis. The collision energy of 50 eV with an energy spread of 40 eV allows an optimum compromise between sensitivity and fragmentation efficiency.

Results

SWATH QUAL ANALYSIS

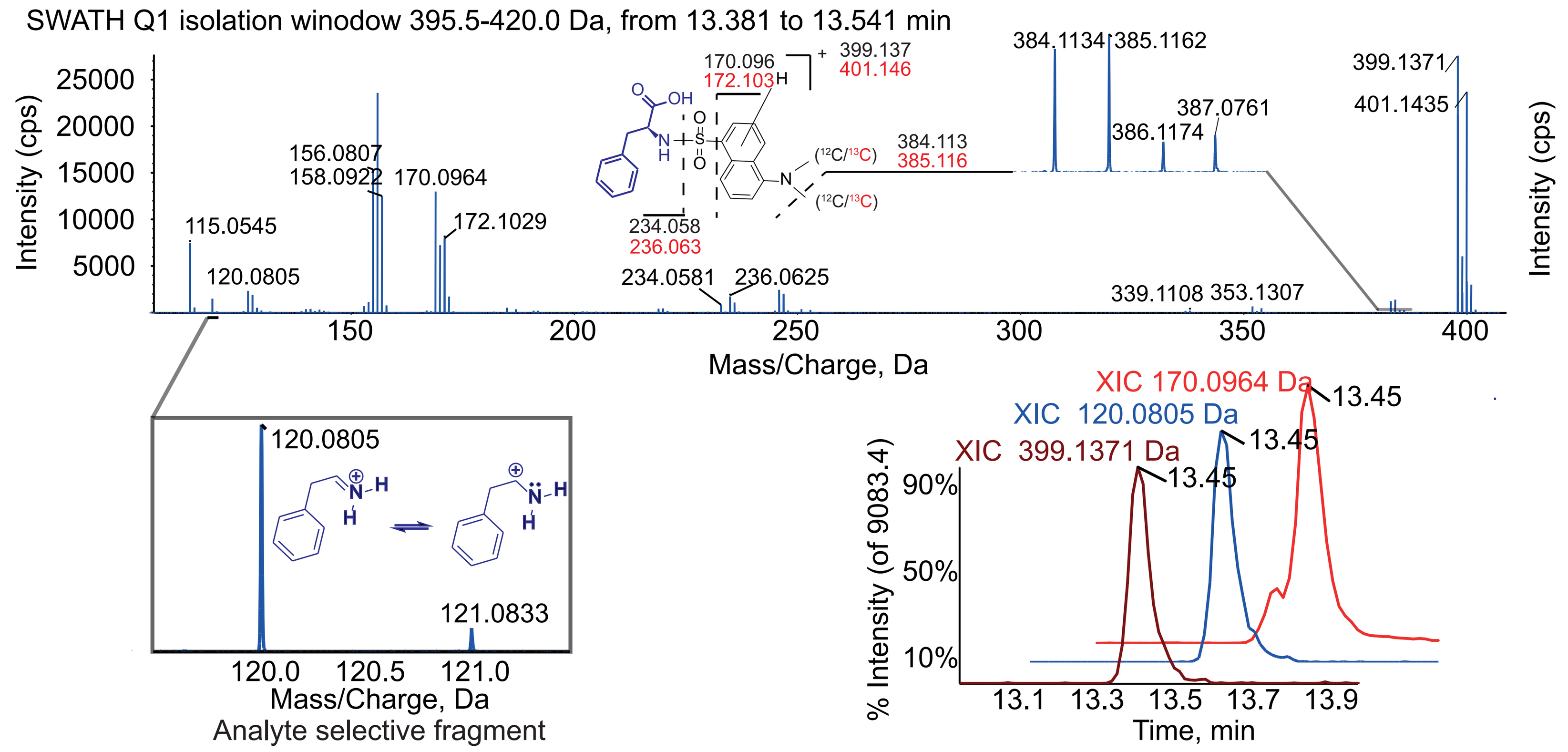


Figure 5. An example of fragmentation pattern of ($^{12}C/^{13}C$) derivatized L-phenylalanine in urine sample. In the spectrum on the top, three *tag-fragments* that represent common fragments of derivatized metabolites that belong to same chemical group containing amino, phenol or primarily alcohol group can be seen. The parallel derivatization with heavy/light isotope labelling helps the assignment of fragments that are related to *tagging* molecule and the identification of analyte-selective fragments. XIC offset traces of the precursor ion *m/z* = 399.1371, *the tag-fragment m/z* = 170.0964 and the analyte-selective fragment *m/z* = 120.0805 demonstrate that the *tag-fragment* is not a selective fragment and can therefore not be used as a quantifier.

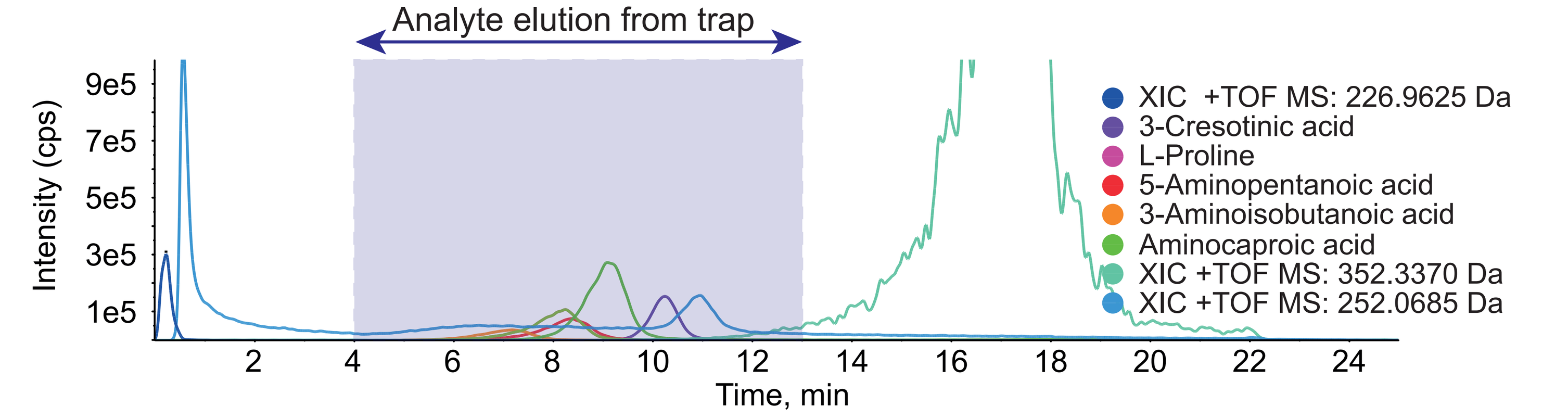


Figure 6. The graph represents the chromatography obtained from the trap without column switching. On-line SPE based sample fractionation and analyte are illustrated by the blue frame. XIC of the most abundant background components are on the left and right side of the chromatogram. The *m/z* = 226 and *m/z* = 252 are reagent related fragments (1 mg/ml was used for derivatization). The *m/z* = 352, on the right side represents one of the highly lipophilic background components that is removed by the trap column backflush. Analytes from blue region (4 – 13 min) are eluted on the analytical column and significantly improving the chromatographic performance and column lifetime.

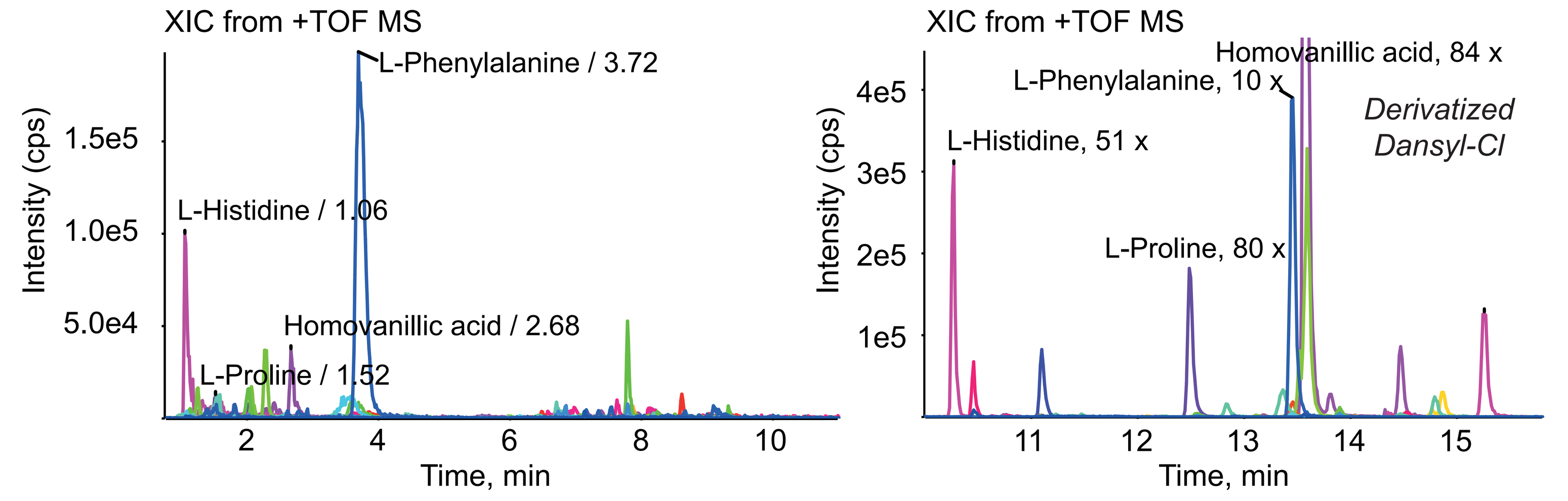


Figure 7. XIC from precursor ions depicted in different colours for each analyte. The derivatized amino acids have a significant retention time shift of ~10 min on the reverse phase LC, moving to a retention time window with less interferences. Whereas the effect of the derivatization on the absolute signal is not significant, the S/N ratio is increased by up to 84 x (labelled on the right chromatogram).

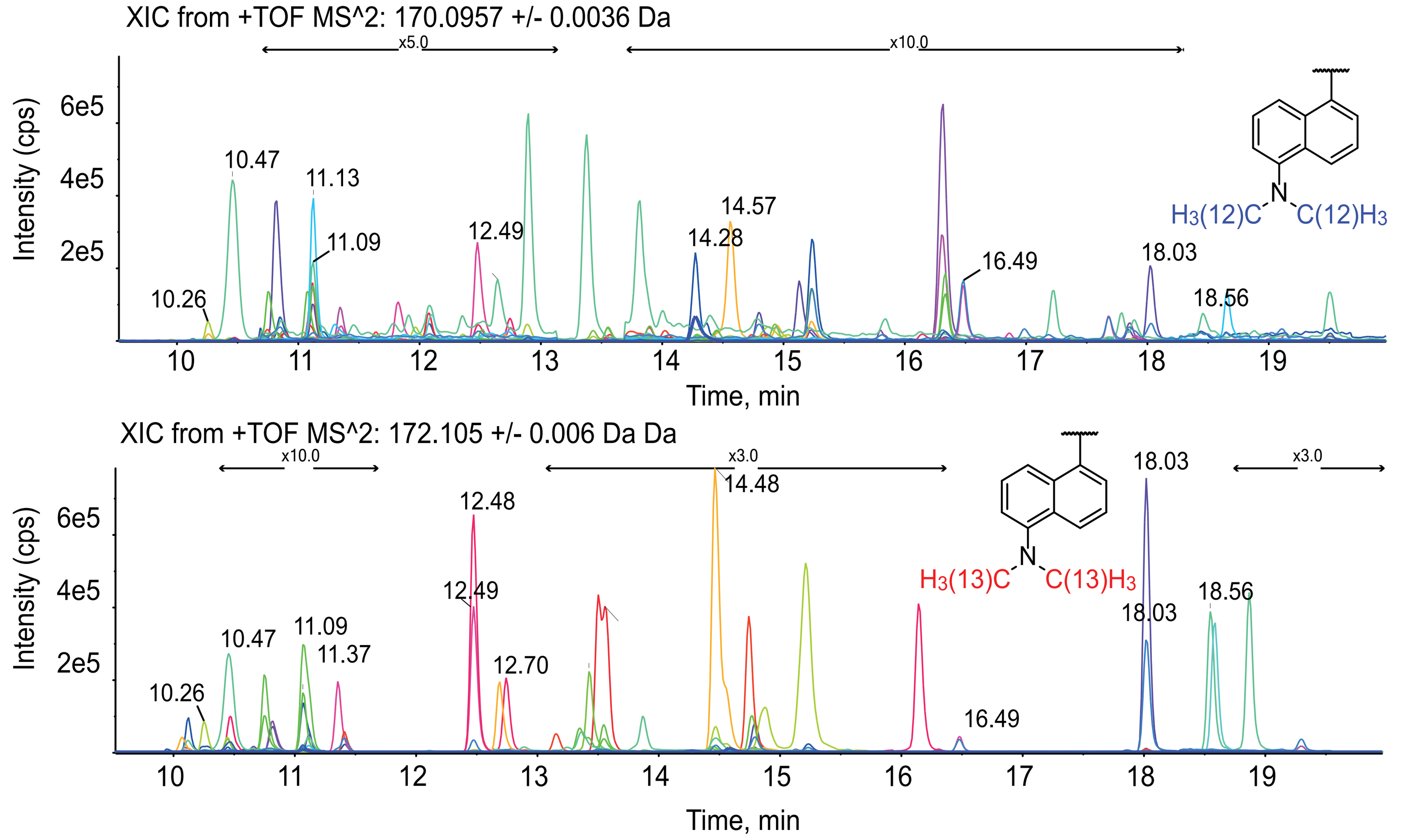
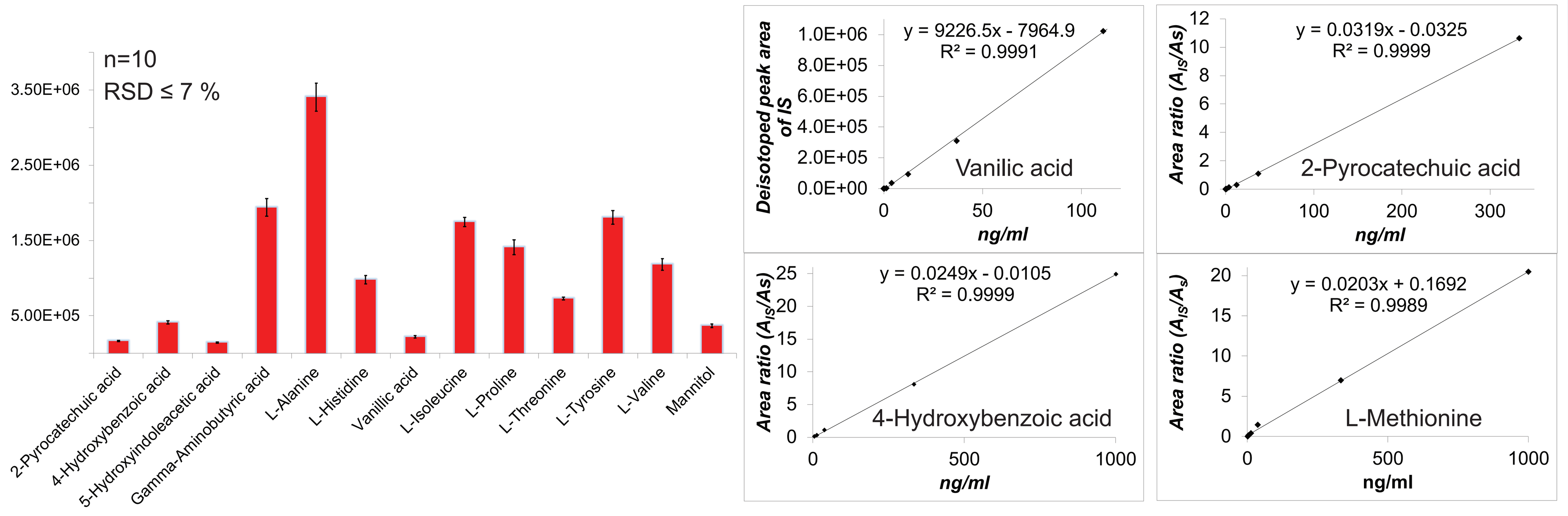


Figure 8. Overlayed XICs from all SWATH Q1 isolation windows of tag-fragment *m/z* = 170 (top) and *m/z* = 172 (bottom). The urine sample was derivatized with non-labelled Dansyl-Cl II Dansyl-N_2H_3 whereas the metabolite standard mix (45 compounds) was derivatized with C13 labelled Dansyl-Cl II Dansyl-N_2H_3. The ^{12}C and ^{13}C analogues match in retention time (10.26 min, 10.47 min, 11.09 min, 12.49 min, 16.49 min, 18.03 min) and are located within the same Q1 SWATH isolation windows. Chemically related metabolites are in similar SWATH windows and have similar retention what eases SWATH data processing and metabolite profiling.

SWATH QUANT ANALYSIS

Figure 9. Automatized parallel light/heavy derivatization of sample/metabolite standard mix shows very high accuracy with low LOD and LOQ. All heavy derivatized analytes are individual internal standards for the corresponding identified metabolite. In combination with SWATH data acquisition the method has a high dynamic range due to the possibility for quantification at precursor or fragment level.



Conclusions

Automated parallel light II heavy derivatization of sample II metabolite standard mix with Dansyl-Cl and Dansyl-N_2H_3 with SWATH independent data acquisition allows qualitative and quantitative analysis within same batch.

SWATH data acquisition gives information about precursor and fragment ions of both light and heavy labelled analytes that eases fragmentation path assignments based on isotope pattern.

XIC based on tag-ion fragment facilitate data processing giving information about chemically related analytes in addition to retention time match and accurate mass between heavy/light labelling.

Fragmentation profile of derivatized analytes gives information about compound specific fragments (QUANT) and tag-fragment (QUAL)