# Quantification of Polar Metabolites in Urine using Automated Parallel Derivatization and LC-SWATH-MS

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## Overview

- Automated parallel derivatization combined with column switching improves reaction kinetics control and reproducibility for quantitative analysis.
- QUAL/QUANT SWATH/MS data analysis using $^{13}C$ labelled derivatization reagents in a single LC-MS run.
- Single point calibration using $^{12}C/^{13}C$-DnsCl/DnsHz differential isotope labelling for quantitation at precursor or fragment level.

## Introduction

Quantitative metabolomics bottleneck lies in the availability of isotopic labelled standard for accurate quantification. In addition to improved MS sensitivity and LC retention, chemical derivatization with $^{13}C$ labelled derivatives has proven to be suitable workflow to address this issue. LC-SWATH-MS acquisition allows quantification and identification that generate valuable additional sample information of precursor, fragment ions and chemically related analytes within the same analysis. Despite multiple derivatization methods were reported for LC-MS, no derivatization reagents are available for profiling multifunctional metabolites in a single analytical run. We described a parallel derivatization $^{12}C/^{13}C$ labelled approach to quantify polar metabolites including amines, phenols, ketones and aldehydes in human urine using LC-SWATH/MS. These compounds could be derivatized in less than 15 minutes with gain in sensitivity of up to 100 times due to the increased hydrophobicity and presence of basic heteroatoms.

Automation was found to be essential to allow reproducible derivatization and overlapping the sample preparation with the LC run time to increased the throughput. In addition, column-switching using a C18 trapping column was found to be critical to remove the excess of reagents and salts which can negatively affect the ionization efficiency and chromatographic performance. Quantification is highlighted in the use of analogous $^{13}C_2$-labeled Dansyl-Cl/Dansyl-Hz for derivatization. LC- SWATH/MS, collects all information on precursor ions and fragments in a single run, which enables to take advantage of chemical tagging at MS2 level. The $^{13}C$ labelling enabled quantification for almost any analyte by selecting proper product ions from specific SWATH windows post-acquisition. Selective screening of specific tag-ion fragments ($^{12}C$ *m/z* 171 and $^{13}C$ *m/z* 173) for all derivatized analytes can be performed. The method could be applied for the quantification of 40 different metabolites in human urine from a clinical investigation.

## Analytes

| No | Compound | Molecular Weight (g/mol) | Derivative | Precursor ion of derivative (Da) | Tag-fragment ion of derivative (Da) | Select fragment of derivative (Da) |
|---|---|---|---|---|---|---|
| 1 | L-Glycine | 75.07 | Dansyl-Cl | 309.0901 | 171.1023 | 294.0672 |
| 2 | Pyruvic acid | 88.06 | Dansyl-Hz | 336.1011 | 171.1023 | 240.2319 |
| 3 | L-Alanine | 89.09 | Dansyl-Cl | 323.1063 | 171.1023 | 308.0833 |
| 4 | L-Tyrosine | 181.2 | Dansyl-Cl | 415.1313 | 171.1023 | 247.0540 |
| 5 | 2-aminobutyric acid | 103.06 | Dansyl-Cl | 337.1211 | 171.1023 | 334.1224 |
| 6 | L-valine | 117.2 | Dansyl-Cl | 351.1377 | 171.1023 | 303.1166 |
| 7 | L-threonine | 119.1 | Dansyl-Cl | 353.1165 | 171.1023 | 338.0923 |
| 8 | L-tryptophan | 204.23 | Dansyl-Cl | 438.1475 | 171.1023 | 130.0650 |
| 9 | L-phenylalanine | 165.2 | Dansyl-Cl | 399.1369 | 171.1023 | 120.0806 |
| 10 | S-phenyl-L-cysteine | 197.05 | Dansyl-Cl | 431.1094 | 171.1023 | 354.0789 |
| 11 | 5-aminolevulinic acid | 131.06 | Dansyl-Hz | 365.1530 | 171.1023 | 325.1792 |
| 12 | Trans-4-hydroxy-L-proline | 131.06 | Dansyl-Cl | 365.1166 | 171.1023 | 343.1904 |
| 13 | L-asparagine | 132.05 | Dansyl-Cl | 366.1120 | 171.1023 | 319.1475 |
| 14 | L-carnosine | 226.23 | Dansyl-Cl | 460.1653 | 171.1023 | 303.1162 |
| 15 | Acetaminophen | 151.17 | Dansyl-Cl | 385.1217 | 171.1023 | 321.1600 |
| 16 | 2,4-Dihydroxybenzoic acid | 154.03 | Dansyl-Cl | 388.0849 | 171.1023 | 264.1248 |
| 17 | 4-hydroxybenzoic acid | 138.03 | Dansyl-Cl | 372.0898 | 171.1023 | 289.1001 |
| 18 | L-lysine | 146.20 | Dansyl-Cl | 613.2125 | 171.1023 | 317.1316 |
| 19 | Vanillic acid | 168.04 | Dansyl-Cl | 402.0999 | 171.1023 | 338.1385 |
| 20 | 4-hydroxyphenylpyruvic acid | 180.16 | Dansyl-Hz | 414.1006 | 171.1023 | 383.1822 |
| 21 | L-histidine | 155.20 | Dansyl-Cl | 389.1278 | 171.1023 | 343.1216 |
| 22 | L-arginine | 174.20 | Dansyl-Cl | 408.1940 | 171.1023 | 234.0557 |
| 23 | Oxoglutaric acid | 147.02 | Dansyl-Hz | 394.1071 | 171.1023 | 217.1338 |
| 24 | L-Glutamic acid | 147.10 | Dansyl-Cl | 381.1108 | 171.1023 | 293.1041 |
| 25 | Taurine | 125.01 | Dansyl-Cl | 359.0731 | 171.1023 | 344.0498 |
| 26 | Caffeic acid | 180.04 | Dansyl-Cl | 648.1812 | 171.1023 | 369.1273 |
| 27 | L-methionine | 149.20 | Dansyl-Cl | 383.1091 | 171.1023 | 371.1407 |
| 28 | L-serine | 105.10 | Dansyl-Cl | 339.1006 | 171.1023 | 324.0784 |
| 29 | Citrulline | 175.10 | Dansyl-Cl | 409.1541 | 171.1023 | 338.1383 |
| 30 | L-Aspartic acid | 133.10 | Dansyl-Cl | 367.1216 | 171.1023 | 325.1794 |
| 31 | L-proline | 115.10 | Dansyl-Cl | 349.1219 | 171.1023 | 303.1166 |
| 32 | Tyramine | 137.18 | Dansyl-Cl | 604.1914 | 171.1023 | 370.1336 |
| 33 | α-ketoisovaleric acid | 116.05 | Dansyl-Hz | 364.1325 | 171.1023 | 121.0648 |
| 34 | Daidzein | 254.06 | Dansyl-Cl | 721.0873 | 171.1023 | 441.2958 |
| 35 | Estradiol | 272.38 | Dansyl-Cl | 506.2756 | 171.1023 | 427.2484 |
| 36 | Ethinyl estradiol | 296.41 | Dansyl-Cl | 530.3666 | 171.1023 | 452.2530 |
| 37 | Estrone | 270.37 | Dansyl-Cl | 504.2302 | 171.1023 | 426.2681 |
| 38 | Testosterone | 288.21 | Dansyl-Hz | 535.2294 | 171.1023 | 409.2354 |
| 39 | L-Cystine | 240.30 | Dansyl-Cl | 474.1132 | 171.1023 | 390.1002 |
| 40 | Androstenedione | 286.19 | Dansyl-Hz | 781.5202 | 171.1023 | 468.2788 |

## Methods

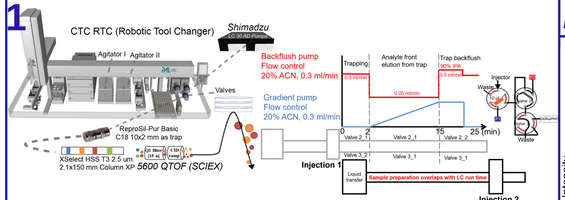



**Figure 1.** Schematic representation of the Robotic Tool Changer (RTC) autosampler with hyphenation to two quaternary LPG pumps and MS QTOF instrument used for the automated parallel derivatization in combination with a column switching setup. Derivatization procedure and sample clean up (C18 trap column) overlapped with the LC run time. Pump P2 is used for analytical column conditioning and for backflush the trap after 15 min of the LC run. Pump P1 is the analytical pump running in gradient.

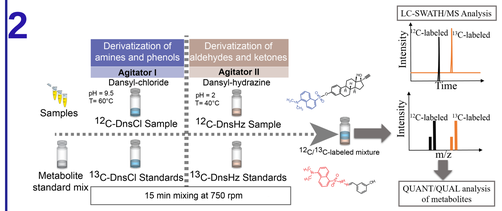



**Figure 2.** Workflow of the parallel derivatization of samples with Dansyl-Cl/Dansyl-Hz and the metabolite standard mix with ($^{13}C$) labelled Dansyl-Cl/ Dansyl-Hz for quantification.

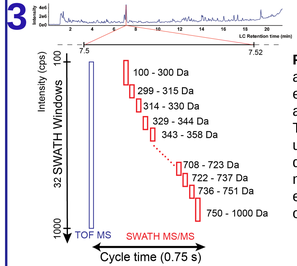



**Figure 3.** SWATH data independent acquisition method. A TOF MS experiment from 100-1000 Da in addition to 32 Q1 SWATH MS windows. The windows size were optimized to 15 units each for a medium mass range due to the mass shift resulting from metabolite derivatization. The collision energy of 40 eV with an energy spread of 30 eV was used.

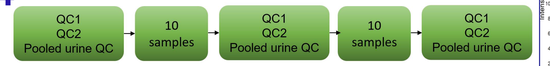



**Figure 4.** Sequence of analysis of urine samples from prostate cancer patients. Samples were derivatized and analyzed on triplicate using $^{12}C$-Dansyl-Cl/Dansyl-Hz and a mix of 40 metabolite standards with $^{13}C_2$-Dansyl-Cl/ Dansyl-Hz for quantification.

## Results

### Method performance

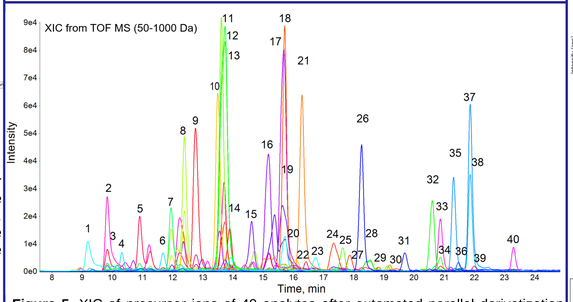



**Figure 5.** XIC of precursor ions of 40 analytes after automated parallel derivatization using $^{12}C/^{13}C$-Dansyl-Cl/Dansyl-Hz of a pooled urine sample for QUAL/QUANT analysis.

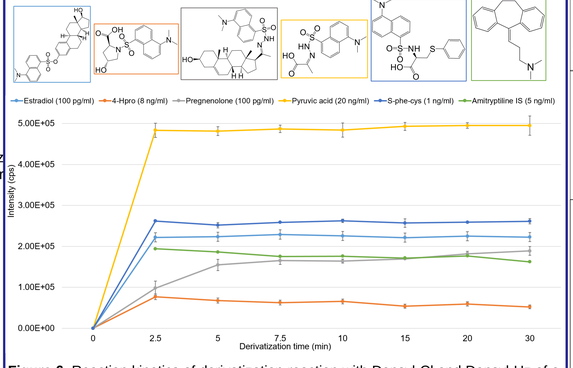
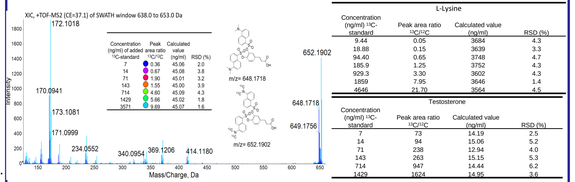



**Figure 6.** Reaction kinetics of derivatization reaction with Dansyl-Cl and Dansyl-Hz of a standard mix of 5 compounds at endogenous levels and amitriptyline as internal standard. Data show that both reaction are completed at 5 minutes and are stable across the 30 minutes of reaction.




L-Lysine

| Concentration (ng/ml) $^{12}C$-standard | Peak area ratio $^{12}C/^{13}C$ | Calculated value (ng/ml) | RSD (%) |
|---|---|---|---|
| 9.44 | 0.05 | 3684 | 4.3 |
| 18.88 | 0.15 | 3639 | 3.3 |
| 94.40 | 0.65 | 3748 | 4.7 |
| 185.9 | 1.25 | 3752 | 4.3 |
| 929.3 | 3.30 | 3602 | 4.3 |
| 1859 | 7.95 | 3646 | 1.4 |
| 4646 | 21.70 | 3564 | 4.5 |

Testosterone

| Concentration (ng/ml) $^{12}C$-standard | Peak area ratio $^{12}C/^{13}C$ | Calculated value (ng/ml) | RSD (%) |
|---|---|---|---|
| 7 | 73 | 14.19 | 2.5 |
| 14 | 94 | 15.06 | 5.2 |
| 71 | 238 | 12.94 | 4.0 |
| 143 | 263 | 15.15 | 5.3 |
| 714 | 947 | 14.44 | 6.2 |
| 1429 | 1621 | 14.95 | 3.6 |

**Figure 7.** An example of HR-SRM/MS spectrum for illustrate the linear response of different endogenous metabolites in urine to the addition of increasing concentration of $^{13}C$-standards metabolites for further quantification using single point calibration at MS1 or MS2 level.

### Analysis of Clinical samples

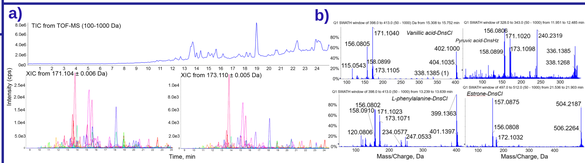
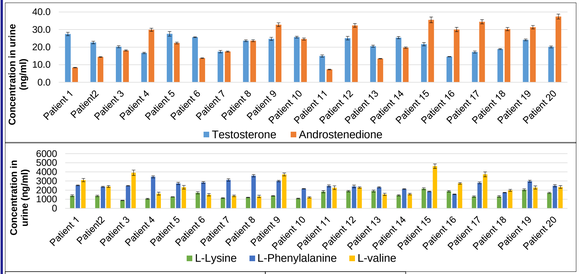
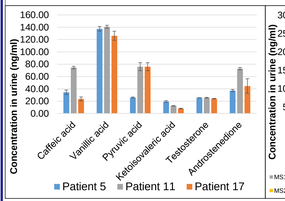



**Figure 8.** Derivatized urine sample from prostate cancer patients using $^{12}C/^{13}C$-Dansyl-Cl/Dansyl-Hz. (a)TIC and overlayed XICs from all SWATH Q1 windows of tag-fragment *m/z* 171 (left) and *m/z* 173 (right) that represent common fragments of derivatized metabolites that belong to same chemical group located in same SWATH windows and retention what eases SWATH data processing and metabolite profiling. (b) HR-SRM/MS spectrum of derivatized vanillic acid, pyruvic acid, L-phenylalanine and estrone identified and quantified in the sample using differential isotope labelling $^{12}C/^{13}C$ of precursor ions (MS1 level) or fragments ions (MS2 level).



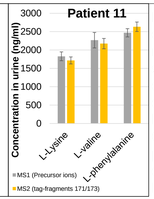




**Figure 9.** Quantification of some endogenous metabolites in urine samples from prostate cancer patients using differential isotope labelling $^{12}C/^{13}C$-Dansyl-Cl/DnsHz using single point calibration and quantified at MS1 or MS2 level.

## Conclusions

**Automated parallel derivatization combined with SWATH/MS acquisition enables:**

Reproducible quantification of multifunctional metabolites (aldehydes, ketones, amines and phenols) during a single LC run time.

QUAL/QUANT analysis of metabolites in biological samples using analogous $^{13}C$ labeled derivatization reagents and MS1 and MS2 level.

To generate valuable additional sample information of precursor, fragment ions and chemically related analytes within same batch.

To facilitate post-acquisition identification of metabolites chemically related with the help of tag fragment ions.

## References


1. Guo, K. and L. Li, *Differential 12C-/13C-Isotope Dansylation Labeling and Fast Liquid Chromatography/Mass Spectrometry for Absolute and Relative Quantification of the Metabolome.* Anal. Chem., 2009. **81**(10): p. 3919-3932.
2. Zhao, S., et al., *Development of High-Performance Chemical Isotope Labeling LC–MS for Profiling the Carbonyl Submetabolome.* Anal. Chem., 2017. **89**(12): p. 6758-6765.