

SiTrap: rapid and high throughput clinical multiomics

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1) Introduction and method

- Sample processing in omics analyses is obligatory and has traditionally been the largest source of variability in proteomics and metabolomics data (Fig. 1). Resultantly, many biomarker “discoveries” are subsequently traced to batch or run order effects. Such errors unfortunately continue to the present day, thus compelling for clinical deployment of omics technologies first serious improvements in reproducibility especially in sample processing.
- Second, for widespread use, sample throughput should be as high as possible, necessitating extension to an automated platform. Moreover, to enable eventual point of care (POC) deployment of omics techniques, sample processing time should be as rapid as possible.
- Third, as we do not necessarily have a priori knowledge of which biomolecule will prove to be useful as diagnostic, prognostic, therapeutic or predictive signals for a given biological condition, sample processing should ideally produce from one sample different fractions for multiple omics interrogations.
- Fourth, such a solution should be affordable enough to be accessible to the majority of labs.

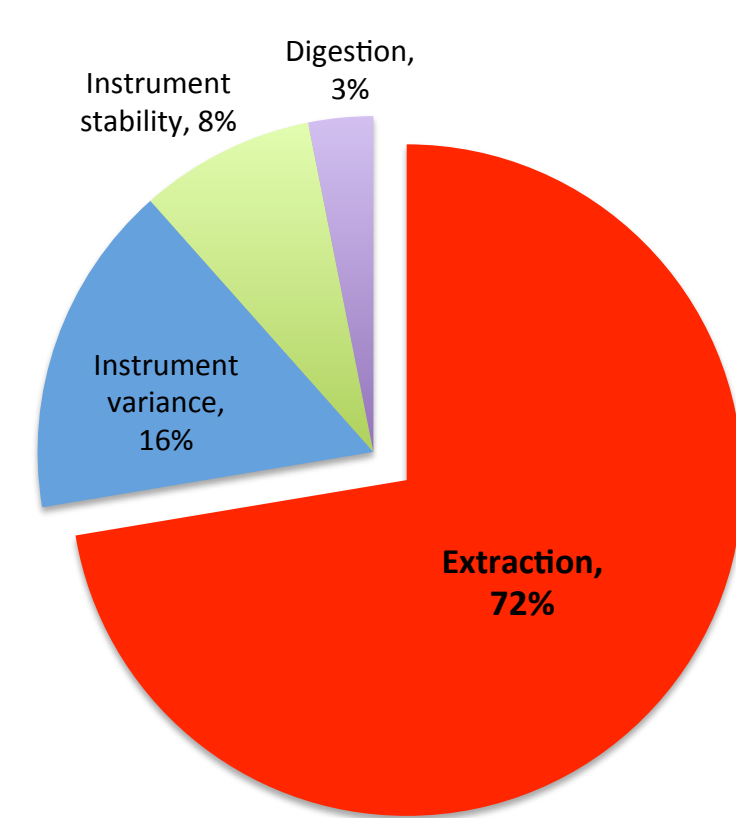


Fig. 1: The vast majority of variability in proteomics data arises during protein extraction. Percent of total variability. Data from reference 1.

Here, we present SiTrap, representing **S**imultaneous **T**rapping, a new high-throughput solution to address all necessities for clinical implementation of omics analyses with application to clear cell renal carcinoma. A new development over the original S-Trap[2-5] sample processing platform, SiTrap yields from the same sample both metabolomic and proteomic fractions with extraordinary inter- and intrarun reproducibility over time. It requires only minutes of processing time per sample and can be executed either in loose spin columns or in an automated 96-well format (4 - 96 samples simultaneously). Importantly, automation and its commensurate high reproducibility can be achieved on an inexpensive Tecan A200 positive pressure workstation, a general automation platform accessible – due to its low cost – to the majority of research and clinical labs. We anticipate that SiTrap will become an essential omics tool in laboratory and clinical settings and will enable novel discoveries, thereby helping to usher in a new era of clinical proteomics.

2) Materials and methods

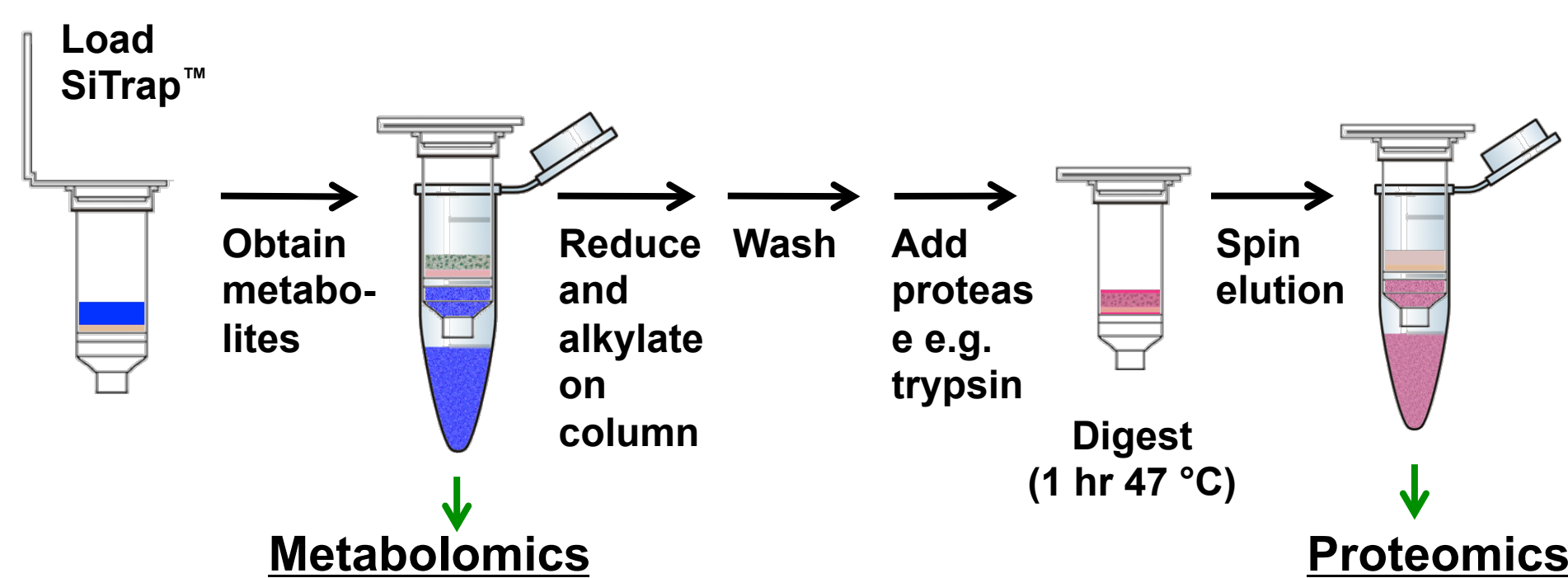


Fig. 2: The SiTrap™ method. Biological samples are dissolved either in a native dissolution buffer or a denaturing buffer to which methanolic binding buffer is added. The proteins are captured on a derivatized protein trap while metabolites flow through. Reduction and alkylation is on column, as is digestion, and samples are ready for proteomics analysis after proteolytic treatment.

MDA-MB-231 cell pellets were lysed by probe sonication on ice in 250 µl of lysis solutions or alternatively 3% SDS or 3% P407. The extracts were clarified by centrifugation at 11,000 g for 2 min at 18 °C. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo). 30 µg of protein was processed in six replicates in each case. For native extraction by SiTrap processing, four volumes of binding buffer were added to the lysate. For denaturing extraction by SiTrap, binding solution 1 was followed by the addition of two volumes of wash/bind buffer. The samples were loaded into SiTraps which were centrifuged at 2000 g. Captured proteins were washed by adding 80 µl of binding/wash buffer. Captured proteins were further denatured, reduced and alkylated in situ by adding 60 mM triethylammonium bicarbonate (TEAB), 10 mM tris(2-carboxyethyl)phosphine (TCEP), 25 mM chloroacetamide (CAA) solution to the tips followed by heating at 80 °C for 30 min (the reduction/alkylation solution should be prepared prior to the start of experiment and thoroughly vortexed right before use). After a wash with 80 µl of wash buffer 2 at 2500 g for 30 sec, the traps were removed and placed into fresh 1.5-ml sample tubes. 20 µl of Sequencing Grade trypsin (Promega) in 100 mM ammonium bicarbonate at a concentration of 0.07 µg/µl was added to the tips. Tryptic digestion was done by incubation at 47 °C for 1 hour. Post-digest elution was performed consecutively with 70 µl 300 mM ammonium bicarbonate and 70 µl of 3% formic acid. The peptides were concentrated using C8 Stage tips for the downstream analysis by mass spectrometry. For SDS processing, 30 µg of protein was processed with trypsin after SDS removal. The digestion, peptide elution and concentration were the same as for SiTrap.

Renal tissues. Frozen renal tissue from three matched clear cell renal carcinoma (G2 pT3a, G2 pT1b, G1 pT2) and adjacent normal sample pairs were obtained from The Leeds Multidisciplinary Research Tissue Bank. Approximately 1 cm² sections with a thickness of 10 µm were cut for each sample and placed into 1.5 ml sample tube. 80 µl of denaturing solubilization buffer was added to the tube and the tissue was lysed by probe sonication. The tube was centrifuged at 11,000 g for 2 min at 18 °C to remove the debris. The supernatant was removed for further processing. The SiTrap load was normalized by protein concentration. 50 µg of protein was loaded into the SiTraps as described above. The collected flow-through fraction, devoid of proteins, was dried down using a Speed-Vac for targeted metabolomics analysis. The captured protein fraction, in turn, was digested as described above, the resulting peptides were concentrated for proteomics analysis.

Proteomics. Peptides were separated online by reversed-phase capillary liquid chromatography using an EASY-nLC 1000 system (Proxeon) connected to a custom-made 30-cm capillary emitter column (inner diameter 75 µm, packed with 3 µm Reprosil-Pur 120 C18 media, Dr. Maisch). The chromatography system was hyphenated with a linear quadrupole ion trap - orbitrap (LTQ-Orbitrap) Velos mass spectrometer (Thermo). The total acquisition time was 100 min for cellular and 140 min for tissue analyses; the major part of the chromatographic gradient was 3% - 22% acetonitrile in 0.1% formic acid. Survey MS scans (scan range of 305–1350 amu) were acquired in the orbitrap with the resolution set to 60,000. Up to 20 most intense ions per scan were fragmented and analyzed in the linear trap. Data were processed against a Uniprot human protein sequence database (October, 2018) with MaxQuant 1.5.2.8 software package (www.maxquant.org) [3]. Carbamidomethylation of cysteine was set as a fixed modification, with protein N-terminal acetylation and oxidation of methionine as variable modifications. Up to three missed cleavages and at least one unique peptide for valid protein identification were chosen. The maximum protein and peptide false discovery rates were set to 0.01. Analysis of Gene Ontology (GO) features was undertaken with Panther 14.0 (www.pantherdb.org) [4]. Perseus software package 1.6.2.3 (https://maxquant.net/perseus/) [5] was used for volcano plot significance analysis – the mean LFQ intensities of proteins were log₂-transformed and their differences plotted against the corresponding p values from t-test, the significance cut-offs were set to 0.05 for FDR and 0.01 for S0. For data comparison only proteins identified with at least two peptides and one unique peptide were used.

Metabolomics. Targeted metabolomic LC-MS analysis of acyl-carnitines, free fatty acids and bile acids. A solution of 10 µM palmitoyl-L-carnitine (N-methyl-d3) (Sigma), 10 µM palmitic acid-d31 (Sigma) and 10 µM deoxycholic acid-d6 (Sigma) in LC-MS grade methanol was prepared as internal standard spiking solution (ISSS). Samples were reconstituted in 100 µl LC-MS grade water and 100 µl ISSS, vortex mixed and sonicated for 30 min before being transferred to LC vials. Chromatography was performed using an ACQUITY UPLC system (Waters) equipped with a CORTECS T3 2.7 µm (2.1 X 30 mm) column, which was kept at 60 °C. The ACQUITY UPLC system was coupled to a Xevo TQ-XS mass spectrometer (Waters Corporation). The binary solvent system used was solvent A comprising LC-MS grade water, 0.2 mM ammonium formate and 0.01% formic acid, and solvent B comprising analytical grade acetonitrile/isopropanol 1:1, 0.2 mM ammonium formate, and 0.01% formic acid. For all analyses a 10 µl injection was used and mobile phase was set at a flow rate of 1.3 ml/min. For acylcarnitine analysis, the column mobile phase was held at 2% solvent B for 0.1 min followed by an increase from 2% to 98% solvent B over 1.2 min. The mobile phase was then held at 98% solvent B for 0.9 min. The mobile phase was then returned to 2% solvent B held for 0.1 min to re-equilibrate the column. For free fatty acid analysis, the column mobile phase was increased from 50% to 98% solvent B over 0.7 min. The mobile phase was then held at 98% solvent B for 0.5 min. The mobile phase was then returned to 50% solvent B held for 0.1 min to re-equilibrate the column. For bile acid analysis, the column mobile phase was held at 20% solvent B for 0.1 min followed by an increase from 20% to 55% solvent B over 0.7 min. The mobile phase was increased to 98% solvent B and held for 0.9 min. The mobile phase was then returned to 20% solvent B held for 0.1 min to re-equilibrate the column. Analyses were performed using multiple reaction monitoring (MRM). Transitions and ionization conditions are given in tables 1, 2 and 3. For acylcarnitine analyses the Xevo TQ-XS was operated in positive electrospray ionization (ESI) mode. For free fatty acid and bile acid analyses the Xevo TQ-XS was operated in negative ESI mode. A cone gas flow rate of 50 ml/hr and desolvation temperature of 650 °C was used. Data were processed and peak integration performed using the Waters Targetlynx application (Waters Corporation).

3) Results

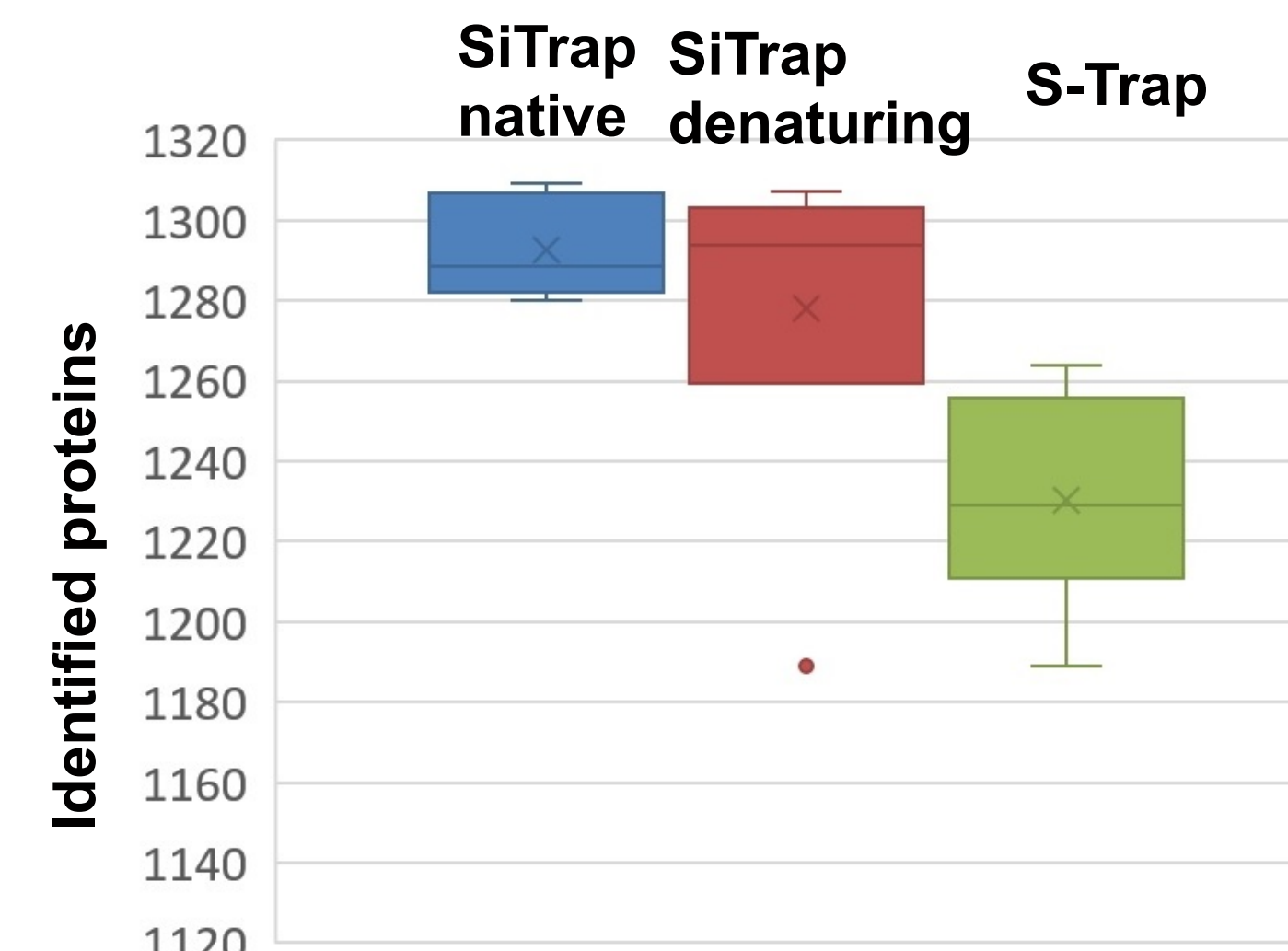


Fig. 3: SiTrap and S-Trap methods achieved similar protein depth and coverage.

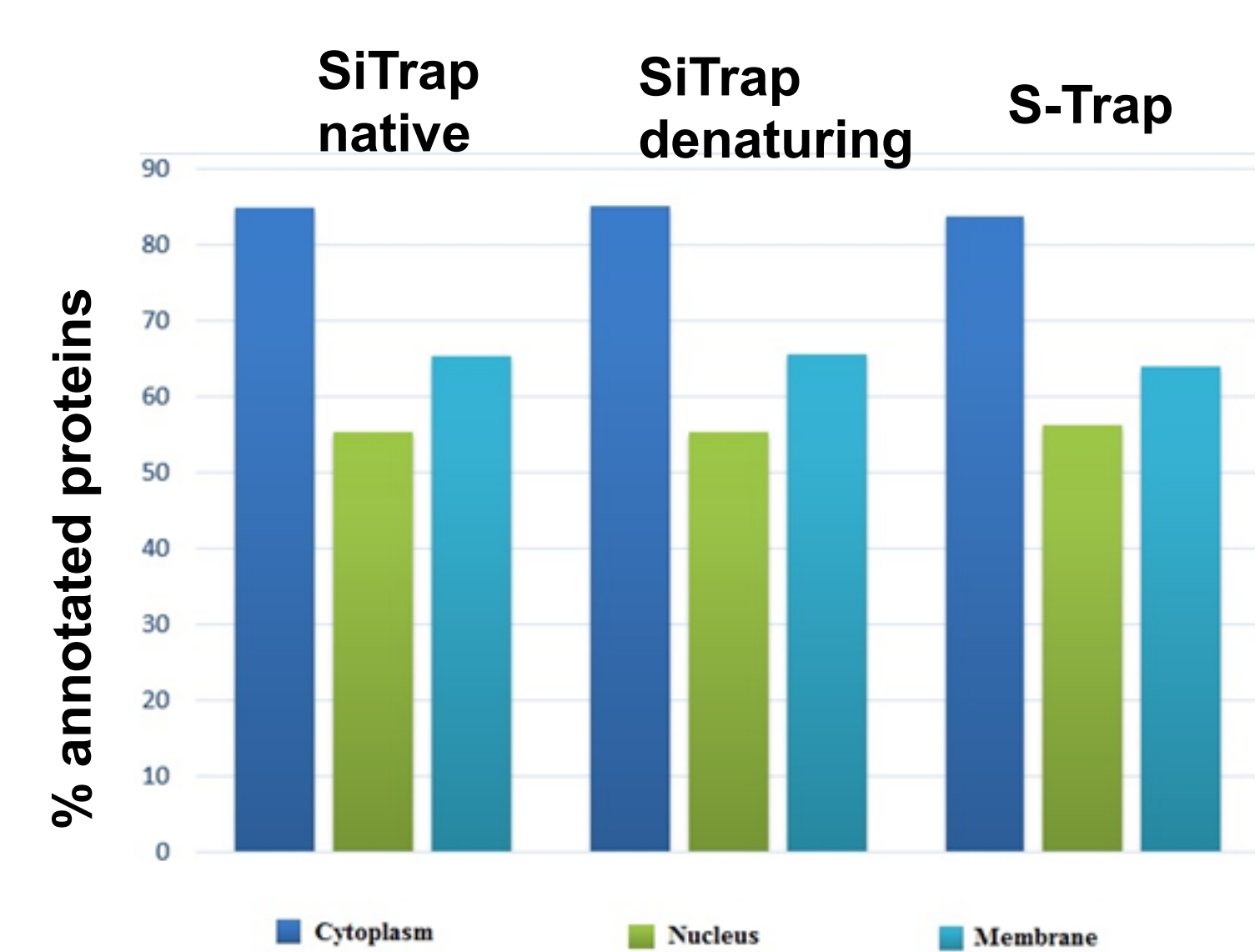


Fig. 4: SiTrap and S-Trap methods reproducibly sampled all cellular components of the cell.

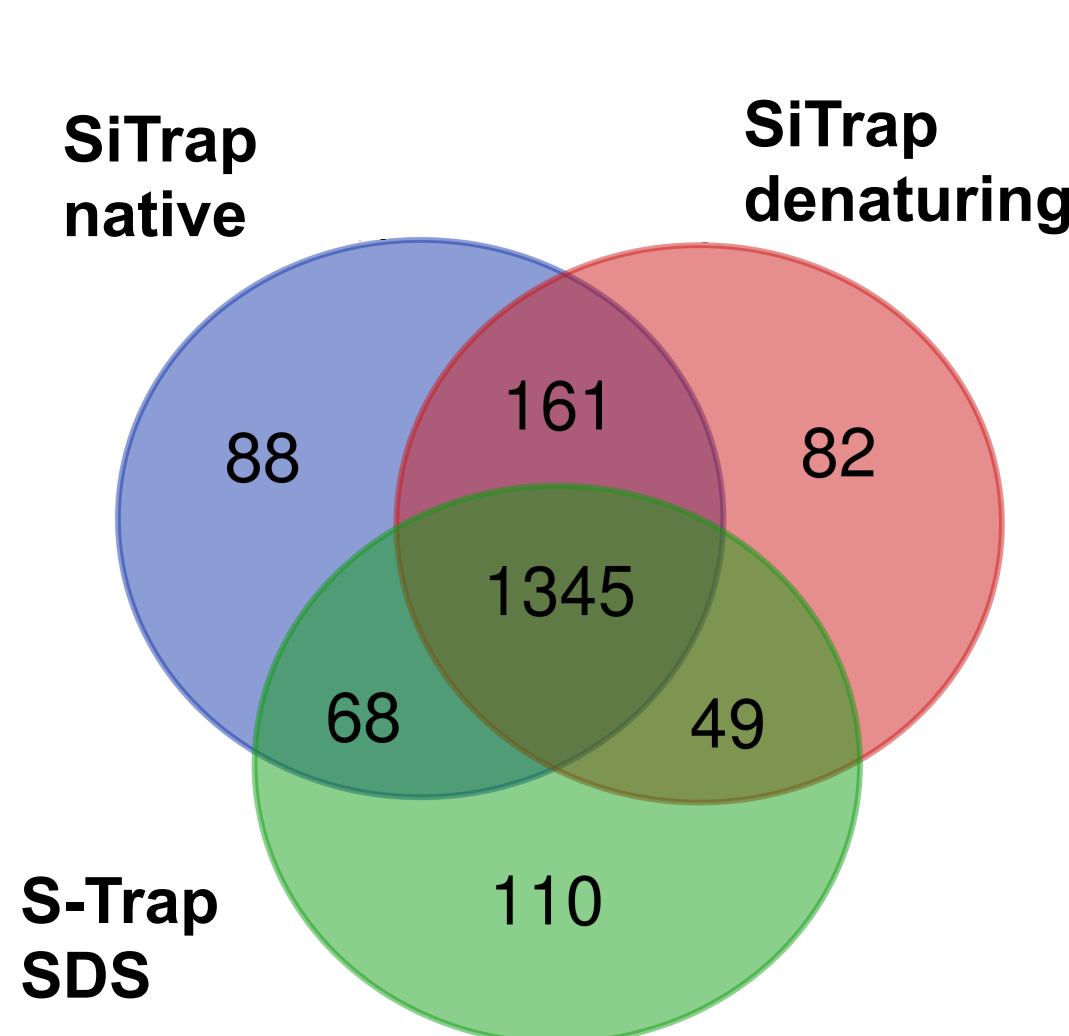


Fig. 5: SiTrap in denaturing and native modes, and S-Trap methods, identified highly similar protein populations.

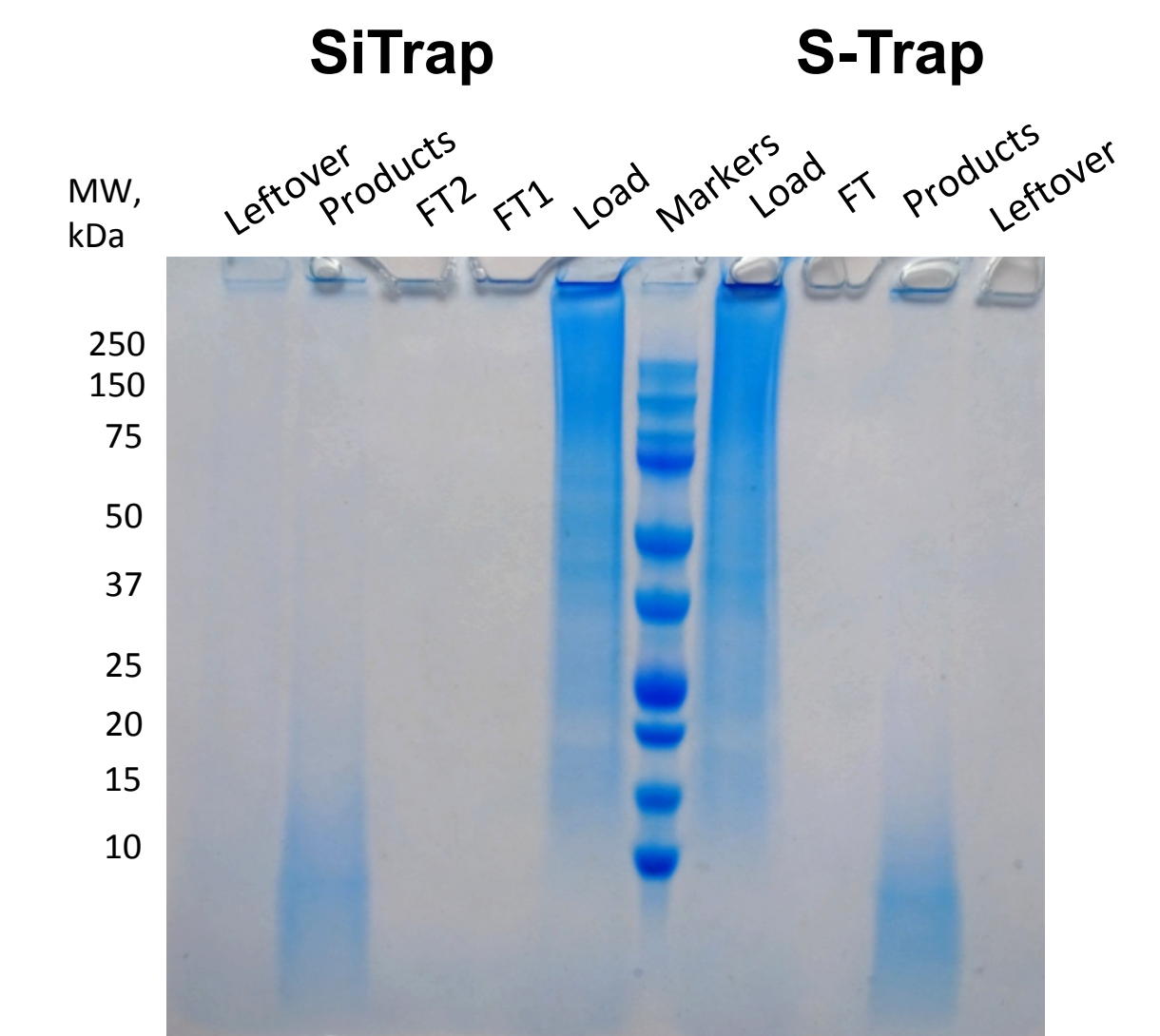


Fig. 6: SiTrap was just as effective as S-Trap in the processing of FFPE tissues.

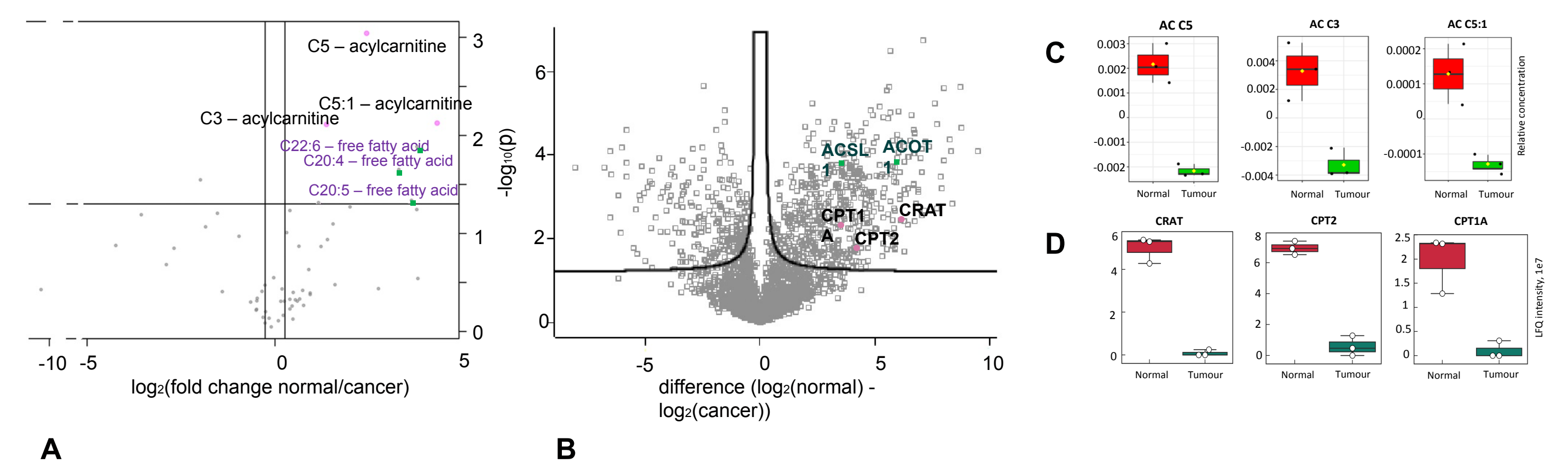


Fig. 7: Volcano plot significance analysis of the metabolomics and proteomics profiling data for normal vs tumor renal sections. The significance cut-offs were set to 0.05 for false discovery rates (FDR). (A, C) The results of the metabolomics analysis indicate a decrease in both short chain acylcarnitines (C5, C5:1 and C3) and in polyunsaturated free fatty acids (C20:5, C20:4, C22:6) in the tumor samples. (B, D) The results of the proteomics analysis indicate downregulation of enzymes in the carnitine pathway, Carnitine O-acetyltransferase (CRAT), Carnitine O-palmitoyltransferase 2 (CPT2) and Carnitine O-palmitoyltransferase 1 (CPT1A) in the tumor samples. Downregulation of enzymes in the polyunsaturated fatty acid pathway, Acyl-CoA Thioesterase 1 (ACOT1) and long chain Fatty acid-CoA ligase (ACSL1), is also observed in the tumor samples.

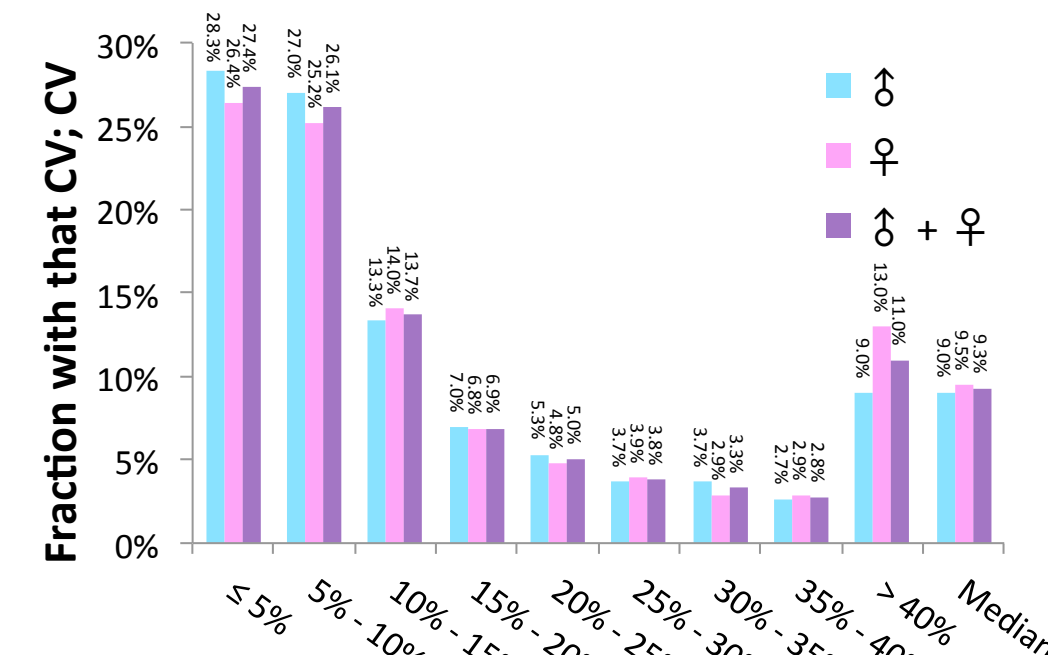


Fig. 8: >50% of CVs for all quantified protein and metabolites analytes fell below 10%. >67% of CVs fell below 15%.

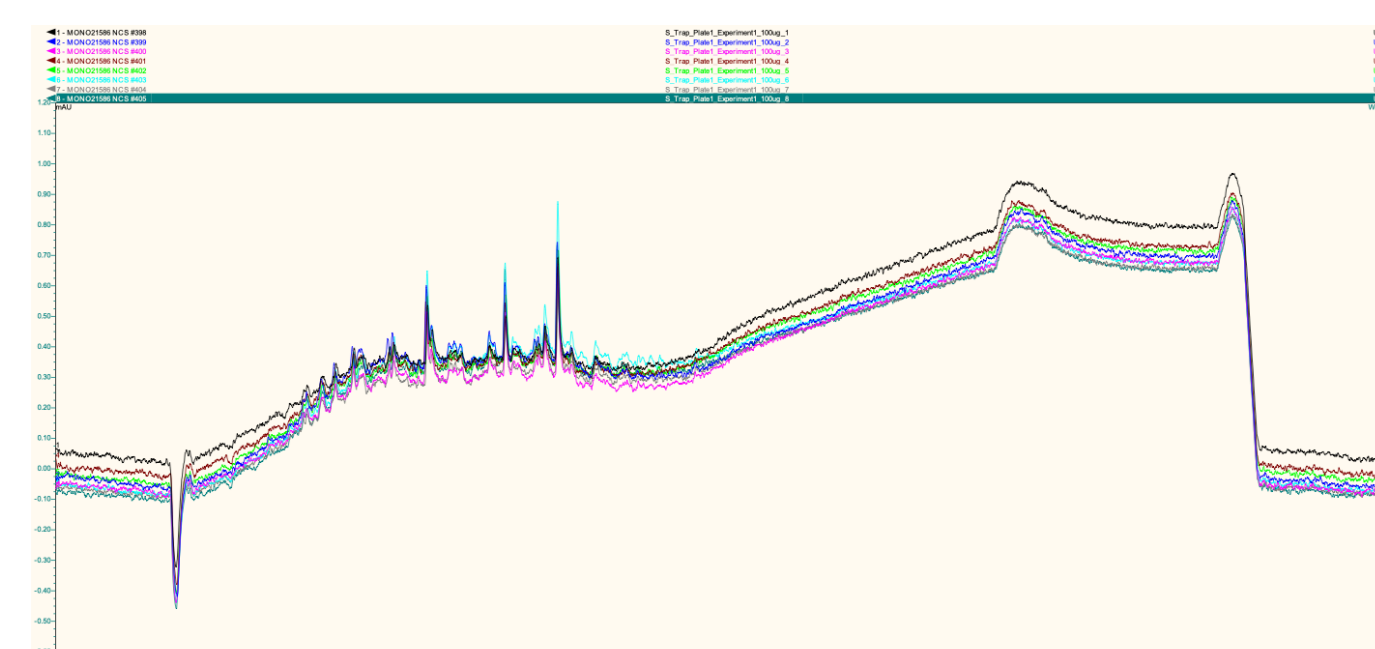


Fig. 9: The Tecan A200 is a low cost automation solution which can process 4 - 96 S-Trap or SiTrap samples simultaneously.



Fig. 10: Analysis of replicate digestions monitored by long-capillary monolithic columns [6] shows very high reproducibility in the extent of digestion and recovery. A200 data.

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