ANALYSIS OF HUMAN SALIVA BY INTACT PROTEIN LC/MS: REPRODUCIBILITY, VARIABILITY, AND PTM CHARACTERIZATION

Waters

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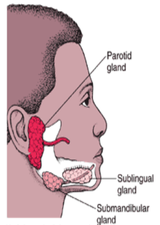
OVERVIEW

- **The large range of protein concentration in serum and plasma has furthered the desire to find alternative, low-invasive samples for the identification and monitoring of protein biomarkers.**
- **Human saliva is easily obtained in significant quantity, and can readily be processed for analysis by the combination of chromatographic separations and mass detection.**
- **We have chosen to address fundamental questions about the utility of saliva for proteomic and biomarker studies: the analytical, day-day and person-person variability inherent in characterization of salivary proteins.**
- **Whole saliva was collected for consecutive days from several from five healthy volunteers, processed (filtration, acidification, and centrifugation), and analyzed using LC ESI-TOF MS.**
- **Automated processing of the resulting intact protein LC/MS data sets was accomplished using novel software (Automated Maximum Entropy, AutoME) developed by our group.**
- **Our results show that excellent analytical reproducibility can be obtained from such analyes, that individuals can produce similar salivary protein profiles day-to-day, but significant differences can be observed in the protein pattern between individuals.**

SALIVA



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Three pairs of major salivary glands (parotid, sublingual, and submandibular) and many minor salivary glands are distributed throughout the oral cavity. Together, these glands produce whole saliva: a mixture of water, electrolytes, mucus, proteins and enzymes. These various species collaborate to break down food, remineralize teeth, regulate oral flora and viral activity, and provide general oral lubrication.

METHODS

Sample Collection: Whole human saliva was collected from four healthy volunteers between 1130h - 1200h on three consecutive days. A fifth sample was collected on a single day to serve as an analytical control for reproducibility over the course of LC/MS analysis. One hour prior to collection, volunteers were requested to rinse with Listerine™ for one minute to minimize protein contributions from food and oral microbes.

Sample Processing: Samples (~3 ml) were microcentrifuged (12,000 g, 30 min., 4 °C) and filtered through a 0.1 micron centrifugal filter to remove mucus and particulates. Aliquots were stored at -80 °C. Samples were mixed 1:1 with 1% formic acid in 10% acetonitrile, recentrifuged, and kept at 4 °C prior to LC/MS analysis, which occurred within that day.

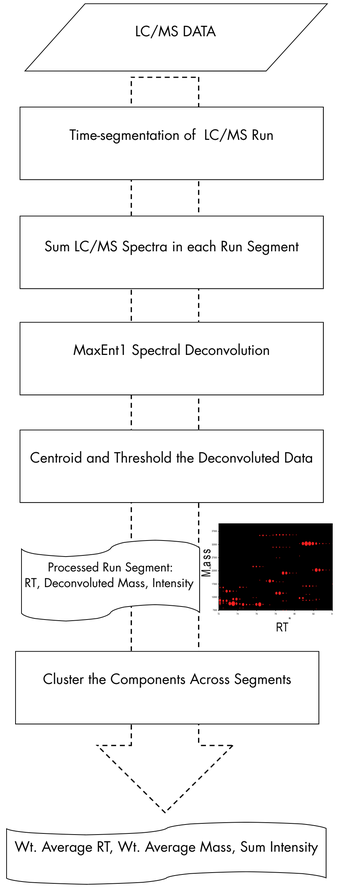
LC/MS Analysis: Samples were randomized and subjected to a single round of analysis over the course of three days. A control saliva sample was run at the beginning and end of each block of samples.

- LC System: Waters 2796 Alliance® Bioseparations Module
- MS System: Waters LCT Premier™ ESI-TOF MS, ESI+ V-mode(6,000 Resolution), 1 Hz scan (650-2990 m/z)
- Chemistry: Waters 2.1 x 100 mm Symmetry® 300 C4 3.5µ
- Gradient: 60 min, 5-60% Acetonitrile, 0.5% Formic
- Flowrate: 250 µl/min (1:5 post-column split to MS)
- Sample: 10 µl of processed Saliva

Data Processing: Automated processing of Saliva LC/MS Data was accomplished by AutoME (Automated Maximum Entropy), a Visual Basic Macro functioning within the MassLynx 4.0 Software package.

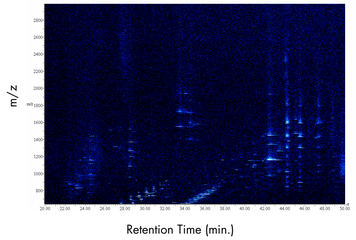
- LC/MS Data Processing Segment: 10 scans (10 seconds)
- MaxEnt1 Processing (3,500-60,000 output mass range at 1 Da resolution with up to 20 MaxEnt1 iterations, Peak width of 0.7 Da),
- Deconvoluted spectral peaks were centroided (Top 80%), spectral harmonics were removed with a 2.5 Da tolerance, and the Top 30 most intense peaks were recorded per processed LC/MS run segment.
- Individual components were recognized by clustering across segments (min= 3), using a moving weighted average mass window with a tolerance of +/- 2 Da, and a maximum gap of two processed segments within a cluster.

DATA PROCESSING (AutoME)

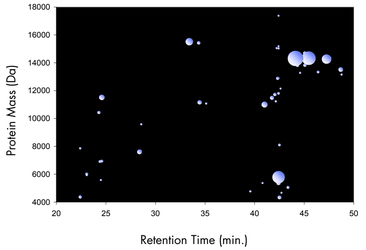


SALIVA DATA

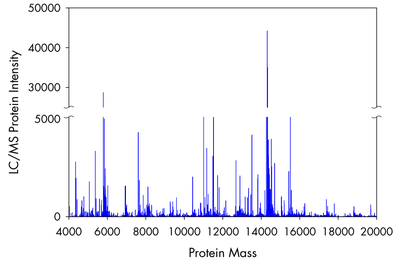
Raw LC/MS (m/z) Data for Saliva QC Run 1



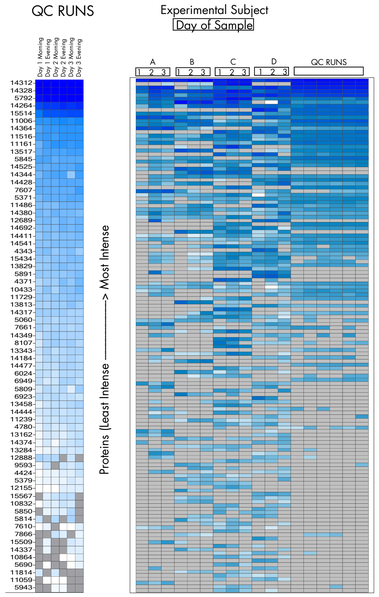
AutoME Processed LC/MS Data for Saliva QC Run1



LC/MS Protein Fingerprint for Saliva QC Run1 (Protein Mass vs. Intensity)



RESULTS OVERVIEW



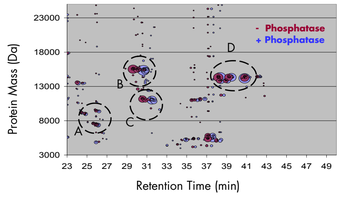
HEAT MAPS OF PROTEIN INTENSIITY IN QC RUNS (LEFT) and ALL SALIVA ANALYSIS (RIGHT)

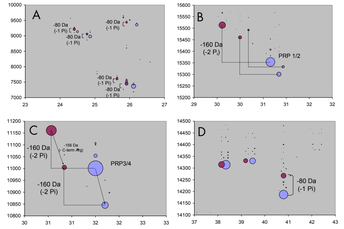
KEY:
Column= 1 LC/MS Run (Sample)
Row= Protein (Unique RT, Mass Pair)
Maps are arranged by average protein intensity across all samples.
Most Intense = DARK BLUE
Least Intense = WHITE
Not Detected= GREY

1) The six QC runs (LEFT SIDE) over three experimental days produced reproducible protein identifications (ID = Nominal mass as listed), and comparable run-run component intensities.
2) Patterns for individual subjects were largely reproducible day-day, although component intensity differences were apparent between days.
3) Much greater variation was observed between individual subjects than day-day variation within a subject.

ID OF PHOSPHOPROTEINS

LC/MS of Saliva -/+ Alkaline Phosphatase Treatment





CONCLUSIONS

- Intact protein LC/MS analysis can yield analytically reproducible results for a given saliva sample.
- Phosphoproteins could be easily identified by treatment with alkaline phosphatase.
- Individuals were observed to have a similar salivary protein pattern over the course of several days, although intensities of the observed proteins were variable.
- More significant differences in protein pattern were observed between individual subjects.
- The lack of strong salivary homeostatic mechanisms may explain the observed differences between samples, and could complicate proteomic or biomarker investigations using saliva samples.
- The need for larger and better controlled studies of intra-subject and inter-subject saliva protein variation are indicated.

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