Mary Piotrowski[1]; Brendon Kapinos[1]; John Janiszewski[1]; Hui Zhang[1]; Wayne Lootsma[2]; Steve Ainley[2]

[1]Pfizer, Groton, CT; [2]Sound Analytics, Niantic, CT

OVERVIEW:

▪ High-throughput LC-MS bioanalysis is used routinely for in vitro screening applications in drug discovery. Quantification of small endogenous biological substrates (90-200 MW) in cell based matrices can be challenging when using nominal mass instruments due to increases in chemical noise, isobaric interferences and non selective fragmentation. While LC gradient separations can help address these issues, the tradeoff is throughput.

▪ We investigated the application of high resolution mass spectrometry (HR-MS) for high-throughput bioanalysis of biological substrates encountered during biological target validation and screening studies. Stable label Choline (tri-methyl D9) and related metabolites from cell lysate samples were used for the evaluation.

▪ We evaluated throughput in a semi-automated approaches to facilitate batch creation and data review. We report on our current status in terms of throughput, bioanalytical sensitivity, limitations and strengths of the system.

METHODS & PROCEDURES:

LC-HRMS:

• LC methods were developed to optimize performance and throughput. An Apricot Design Dual Arm (ADDA) Autosampler was used for sample delivery. A Sciex Triple TOF 6600 high resolution mass spectrometer and Agilent 1290 pump were used for LC/MS analysis.

• High throughput batch preparation and submission for analysis to the HRMS was performed using ADDA Complete Analysis software. Multiply-injected files were reviewed and processed using Sound Review.

• Analytes were separated on a Waters Atlantis HILIC (50 x 2 mm) column using a linear gradient at a flow rate of 1mL/minute with a 1 minute cycle time. LC mobile phase consisted of A: 10 mM ammonium formate in water and B: acetonitrile with 0.1% formic acid.

• Samples from cell preparations were diluted 10fold with methanol/acetonitrile/water (2:2:1). Stable label compounds were used where available. Nadolol was used as a internal standard until a stable label compound could be obtained.HR-MS data was acquired as a full scan (75-750 m/z). An accumulation time of 0.275 seconds was used (> 15 scans per peak).

• Cell preparations were dosed D9 choline as a surrogate to track choline uptake. D9 choline was measured in cell extracts. Conversion of D9 choline to corresponding metabolites was also examined.

Figure 1. Structure of Metabolites

acetylcholine		betaine aldehyde	
trimethyl glycine		phosphocholine	
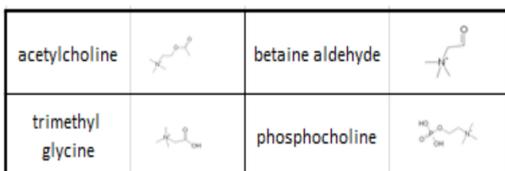
RESULTS:

Figure 2. Representative spectrum's from a blank cell lysate sample (Top) and a cell lysate sample containing ~ 10pmol D9-trimethyl-choline (Bottom).
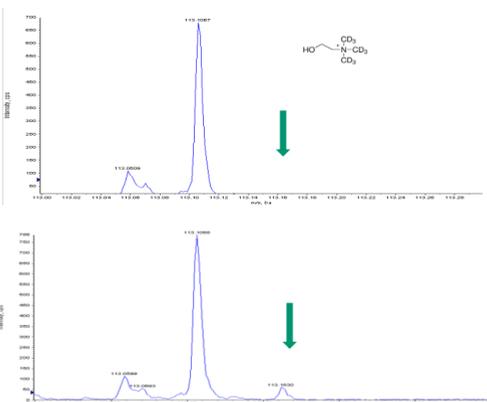
Calibration curves of D9 choline (related metabolite work in progress) had acceptable accuracy (+/-20%) and linearity (0.1-1000nM). LLOQ of 0.1nM (1 pmol, S/N 8) (Figure 3). Related metabolite LLOQ's are expected to be similar with the exception of phosphocholine. Due to poor chromatography and ionization, phosphocholine is 10fold less sensitive. However, early estimates of cell concentrations of phosphocholine are high and it should be easily detectable

Figure 3. Representative chromatogram's from a blank cell lysate sample (Top) and a cell lysate standard containing 1pmol D9 choline (Bottom).
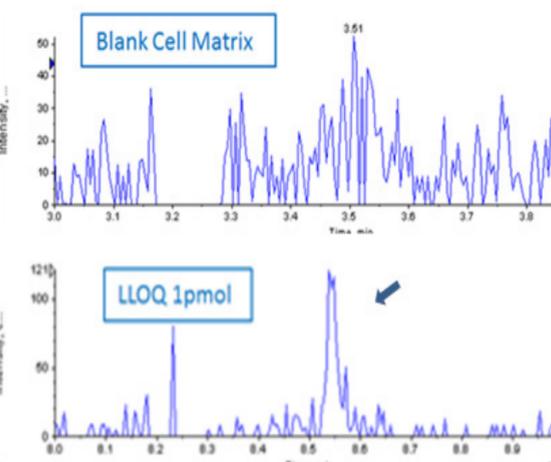
Data Acquisition and Processing using ADDA-C

STEP 1. Create Run Import List in Excel

Samples Run lists were created in Excel containing information necessary to run and process data.

This file contains sample name, well/plate position, analyte names, corresponding exact molecular weights, sample type, nominal concentrations and dilution factor.

Samples are run in multiple injections per data file. Set name is the subwiff data file name. This file is imported to run the samples. Additional analytes can be added sequentially in the file.

Along with a run import list, an ADDA template controls the autosampler functions. The injection volume, injector rinses, number of injections and mass spec calibration requirements are designated in the ADDA template. The template also contains an Analyst starter methods to control general acquisition parameters used to acquire data.

Figure 4. Excel Run Import List for one Analyte and Internal Standard, containing Blanks, Standard Curve, QC's and Unknown Samples.

STEP 2. Load Run Import File in ADDA-C.
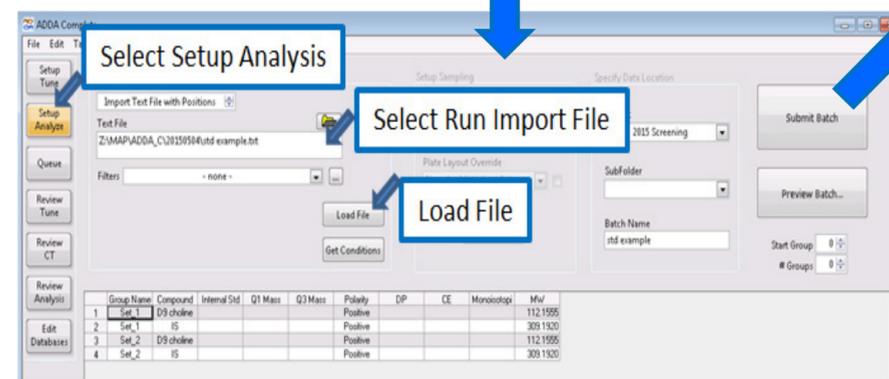
Figure 5. Example of Loading Run Import List in ADDA-C.

STEP 3. Processing Data in ADDA-C/Sound Review

The Batch Data File (BDF) is a metadata file that contains information needed for loading and reviewing wiffs. The BDF is automatically created when the samples are run. The Mass extraction window (MEW) can be chosen in Da or ppm.
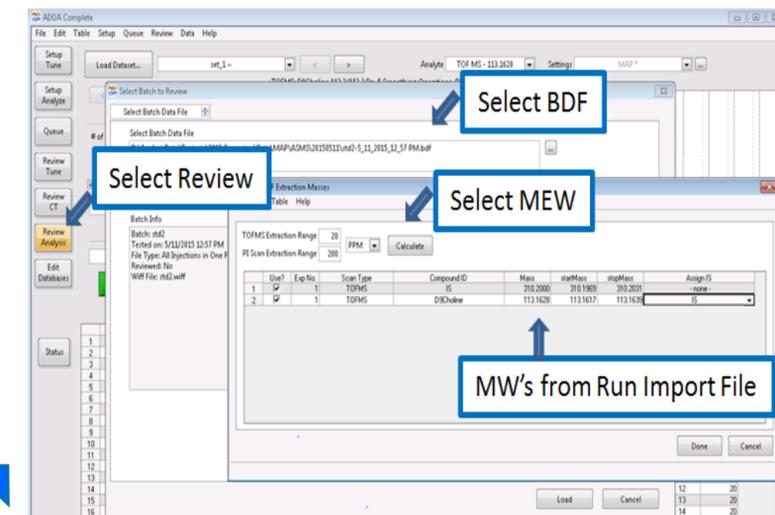
Figure 6. Example of Processing Data in SoundReview.

STEP 4. Review Data in ADDA-C/Sound Review

Samples are automatically quantified using information from the Excel Run Import List from Step 1.
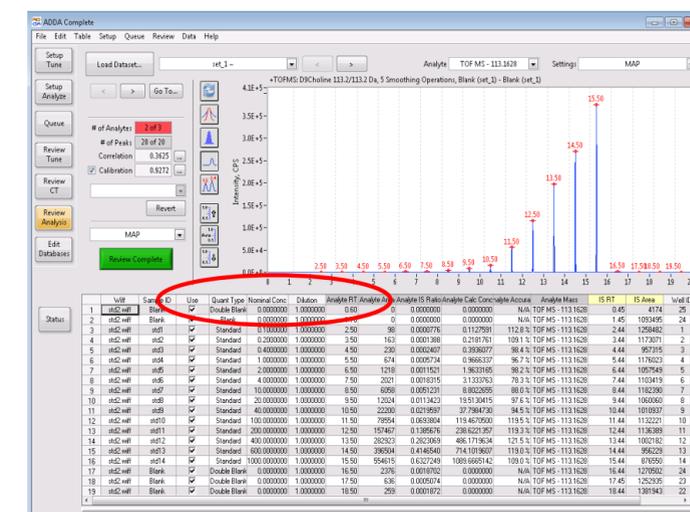
Figure 7. Example of Reviewing Data in SoundReview.

CONCLUSIONS:

▪ HR-MS has gained popularity as an alternative to nominal mass analysis (MS/MS) when increases in chemical noise, isobaric interferences or non selective fragmentation occurs. Here we show an example of HR-MS using a Sciex Triple TOF 6600 that provided sensitivity and resolution needed for choline and related metabolites in cell preparations.

▪ Throughput of HR-MS for quantitation has previously been a limiting factor. The combination of a 1 minute cycle time for the analysis of choline and related metabolites, the fast capability of the ADDA autosampler and ADDA-C software has improved efficiency.

▪ The ADDA-C software used for data acquisition and data processing has increased throughput by greatly simplifying the process. All sample information is supplied once, at the beginning of the process in Step 1.

▪ The current system has limitation due to file size.

▪ In addition to predetermined analytes, after the data is acquired TOF scans can also be probed for additional analytes.

▪ Also, Product Ion Scans can be acquired using the MW from the Run List in an automated process (data not shown).