

## ABSTRACT No. 1

### The Analysis of Halogenated Flame Retardants in Environmental Samples by LC-MS/MS

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Thousands of halogenated organic chemicals (HOCs) are currently used in industry and commerce in a wide variety of applications such as flame retardants, fire suppressants, heat transfer agents, surfactants and pesticides, mainly because of their chemical inertness and stability. Many of them are persistent, toxic and/or bioaccumulative. Until recently, gas chromatography, mass spectrometry (GC-MS) has been the method of choice for the analysis of most HOCs because it provided significantly greater chromatographic resolution and sensitivity than liquid chromatography – tandem mass spectrometry (LC-MS/MS). Newer liquid chromatographs can operate at higher pressures and significantly greater resolution than previous ones. Tandem mass spectrometers are now significantly more sensitive and it is possible to meet the required data quality objectives for many HOCs using LC-MS/MS.

Both atmospheric pressure photo ionization (APPI) and atmospheric pressure chemical ionization (APCI) LC-MS/MS can be used for the analysis of PBDEs and a variety of other halogenated flame retardants (HFRs). APPI is the more sensitive technique, but it also is not as common and requires the use of a dopant for ionization. The method exhibits a linear range of about 4 orders of magnitude and similar sensitivity to GC-HRMS. This method can analyze temperature sensitive compounds like HBCD as well other ubiquitous HFRs like PBDEs, PBBs, DP in the same analytical run which cannot be done using GC-HRMS.

## ABSTRACT No. 2

### *In-vivo* Solid-Phase Microextraction for Targeted Pharmacokinetic and Global Metabolomics Studies in Mice

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**Novel aspect:** This is the first successful use of *in vivo* solid-phase microextraction (SPME) for untargeted LC-MS metabolomics studies and an important step towards the development of sampling methodology that can properly capture the true metabolome at the time of sampling.

**Introduction:** In metabolomics studies of biofluids, the efficiency of metabolism quenching and stability of analytes in selected biofluid dictate how accurately the analytical results represent true metabolome composition at the time of sampling. However, complete quenching of metabolism is not easily accomplished and/or changes due to poorly stable compounds can occur, so the processes of sampling and sample preparation can significantly affect metabolome's composition. The use of SPME for direct *in vivo* sampling of drugs and metabolites in the bloodstream of freely moving animals eliminates the need for blood withdrawal in order to generate pharmacokinetic (PK) or metabolomic profiles in support of pharmaceutical drug discovery studies. This is particularly important for small rodents with a limited blood volume such as mice because it enables the use of a single animal to construct an entire profile or conduct

longitudinal studies. The aim of the current research was to apply SPME for in vivo sampling in mice for the first time. Furthermore, we investigate the use of in vivo SPME as an effective sample preparation method for both targeted pharmacokinetic and untargeted LC-MS metabolomics studies.

**Method:** In vivo SPME sampling procedure suitable for repeated sampling of mice without the need for blood withdrawal was developed. Subsequently, this procedure was validated versus discrete terminal sampling and automated serial sampling approaches within the context of PK study after single intravenous 2 mg/kg dose of carbamazepine. A subsequent study using the same in vivo sampling procedure was performed using global metabolomics workflow developed recently within our lab. This workflow utilizes two complementary LC-MS (reverse-phase using pentafluorophenyl column and HILIC using unmodified silica column) methods run in both positive and negative ESI modes using high-resolution benchtop Orbitrap instrument in order to achieve broad metabolite coverage. Final SPME extract was directly compatible for the analysis by both methods. Data processing was performed using SIEVE software followed by principal component analysis using SIMCA P software. The performance of SPME method was also compared to plasma protein precipitation and ultrafiltration in terms of metabolite coverage, matrix effects and method precision for untargeted metabolomics study of plasma.

**Results:** The use of biocompatible SPME coating enables the extraction of small molecules while large macromolecules are excluded, thus ensuring an accurate representation of the metabolome at the time of sampling. This was demonstrated by comparing the results of in vivo SPME versus ex vivo SPME extraction (n=4 mice). In vivo SPME captured a significant number of metabolites that could not be observed with ex vivo SPME or traditional methods based on blood withdrawal such as  $\beta$ -NAD. The use of short extraction times (2 min) provided good metabolite coverage with ~ 1500-2000 features detected for mixed-mode coating. In comparison to traditional methods, SPME reduced ionization suppression effects throughout the entire chromatographic space due to its non-exhaustive nature. In positive ESI mode, the best metabolite coverage was found for plasma protein precipitation with methanol/ethanol (3245 features), whereas in negative ESI mode, the best metabolite coverage was achieved by SPME (3320 features). Method precision achieved by SPME was similar or better than traditional methods with median RSD of 11 and 17% (n=7 extractions) in positive and negative ESI mode respectively. To obtain free concentration information, SPME provided significantly better performance than ultrafiltration in terms of method precision and more balanced coverage of hydrophilic and hydrophobic species. Plasma precipitation methods were found to perform poorly for the analysis of many polar species due to very significant matrix effects and/or solubility issues. In addition to excellent method precision, metabolite coverage and reduction of ionization suppression effects, additional benefits of SPME for metabolomics studies include (i) high sample throughput (ii) reduction in overall number of sample preparation steps which can help minimize inadvertent sample losses (iii) ability to perform simultaneous in vivo sampling of biofluids and tissues and/or longitudinal sampling (iv) elimination of need to quench metabolism and (v) good metabolite stability in coating.

### ABSTRACT No.3

#### Quantitative Proteomic: from *Tetrahymena Thermophila* to Cancer Biomarker Discovery

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**Introduction:** *Tetrahymena thermophila* is a ciliated protozoan that contains two distinct nuclei: the somatic macronucleus (MAC) and germline micronucleus (MIC). During conjugation, large scale DNA rearrangements take place in the new developing macronucleus. In an effort to understand these processes, we studied the nuclear protein expression from different developmental stages using the iTRAQ labelling and LC/MS/MS approach. Here will be discussed the analytical methodologies used, and the biologically interesting results that were discovered. I will also cover some methodology development through exclusion list generation using UPLC (Ultra high Performance Liquid Chromatography) and its application in GBM (Glioblastoma multiforme) cancer biomarker discovery.

**Methods:** *T. thermophila* nuclei from different stages of growth and development were purified by differential centrifugation. The four types of nuclei analyzed were: MIC and MAC from vegetative cells, MAC from starved cells, and the newly developing macronucleus (anlagen) at 10 h of conjugation. Proteins from each sample are trypsinized and labeled with the differential tags, iTRAQ. The four labeled samples were pooled and separated, first by strong-cation exchange chromatography (SCX), and then by reverse-

phase on a nano-LC column. Resulting MS/MS analyses of these fractions produced both sequence information and relative abundances. The MS/MS data files were searched against Tetrahymena genome databases using ProteinPilot software for identification and quantification.

**Results:** For *T. thermophila* project, a total of 786 unique proteins were identified in three replicate runs. A number of these proteins such as Pdd1p, Pdd2p, Twi1p and heterochromatin protein 1 are known to be expressed specifically during nuclear development, while overexpression of the other proteins appears to be a novel finding. The iTRAQ data were also correlated with expression microarray analyses of *Tetrahymena thermophila* during different stages of the life cycle. All the 11 proteins (overexpressed in both runs) were also significantly overexpressed by microarray data. The consistent results from both mRNA and protein level suggest that in those instances, differential expression is a result of regulation at the transcriptional level. They also indicate that these proteins might play important roles during genome rearrangement. For cancer biomarker discovery of GBM (Glioblastoma multiforme), we identified 547 unique proteins in total from 4 tissue samples ( 2 cancer and 2 normal) which 50 proteins are characterised as regulated. These include several interesting potential biomarkers and are subjected for further validation using traditional biochemistry methods.

#### **ABSTRACT No.4**

#### **Limitations of Quantitative LC-MS/MS in Clinical Pharmacology: The Macrolide Immunosuppressant Drugs Case Study**

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High performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the technique of choice for measuring macrolide immunosuppressants concentrations and making dosage decisions in transplant recipients. Data in literature show that ligand binding assays display less accuracy than LC-MS/MS assays during the quantification of these drugs at low concentrations. However, although LC-MS/MS is a universal, sensitive, selective and fast technique, there are many limitations to overcome before a robust assay is developed for these compounds. In this lecture, the limitations of LC-MS/MS as a bioanalytical technique for the quantitation of Sirolimus, Tacrolimus and Everolimus in clinical pharmacology studies are described and the solutions on how to overcome them are provided. Indeed, since these compounds are sequestered within erythrocytes, whole blood needs to be extracted in order to obtain valid clinical and pharmacological data. These clinical assays require specific extraction procedures to disrupt red blood cells and to minimize ion suppression. It was noted in method development that the LC-MS/MS signal for these compounds tends to rapidly decrease throughout the injection of an analytical run. In order to optimize and understand the system stabilization, which is very time consuming, a systematic research was conducted to discover the parameters that could influence this behavior. Moreover, to optimize method sensitivity both ESI and APCI sources in positive and negative modes were thoroughly optimized. Because the overall sensitivity of the method was strongly affected by the ionization technique employed, specific method development strategies were used to select the most sensitive ionization and to couple it with chromatographic conditions. All the above experiments were performed systematically during method development in order to successfully overcome LC-MS/MS limitations and to obtain rugged and reliable methods for sample analysis.