# A HTS METHODOLOGY FOR METABOLITE DETECTION AND IDENTIFICATION WITHOUT PRIOR KNOWLEDGE OF METABOLITES USING A DATA INDEPENDENT STRATEGY WITH A Q-TOF INSTRUMENT

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*Jose Castro-Perez[1], John Shockcor[1], Kate Yu[1], Jane Kirby[2]*
*[1]Waters Corporation, 34 Maple St, Milford, MA, USA; [2]Waters MS Technologies, Floats Road, Wythenshawe, Manchester, UK*

## INTRODUCTION

**Finding and identifying metabolites in cold in vivo studies by LC-MS is extremely challenging, as metabolites are often present at such low levels that chromatographically they are indistinguishable from the background endogenous materials. In practice this means several LC-MS-(MS) experiments are usually performed for each sample to fully explain the metabolic fate of the compound. In this work we describe a novel approach that enables all of the key metabolite information to be collected in a single LC/MS run.**

**Data was acquired using a Waters QTof Premier mass spectrometer (Figure 1). The separation was performed on a Waters ACQUITY UPLC™ system.**

**The data was acquired using two alternating scan functions[1] with the first quadrupole using a wide band mode rf. The first scan function collected information about the intact (5 eV) metabolites and the second scan function used a collision energy ramp to collect fragment ions (25eV - 40eV). This approach provided a great deal of information, such as metabolite masses, precursor, product ions, and neutral losses. Data visualization and alignment between high and low energy scans was accomplished through a new software tool MetaboLynx MS$^E$ designed to mine both data sets simultaneously. We illustrate this approach using Buspirone and its metabolites spiked in bile.**

**By the use of this approach we were able to determine all the metabolites expected and unexpected from the low energy acquisition. Moreover, we were able to obtain important common product ion information from the high energy scan which allowed spectral correlation between drug and its metabolites. Neutral loss chromatograms were also generated from the data using exact mass differences between the precursor and fragment ions. Since the data was acquired with no preconceptions on the likely routes of metabolism or precursor ion information, this approach has the potential to be truly comprehensive and universal in its use for *in vivo* and *in vitro* metabolite identification.**

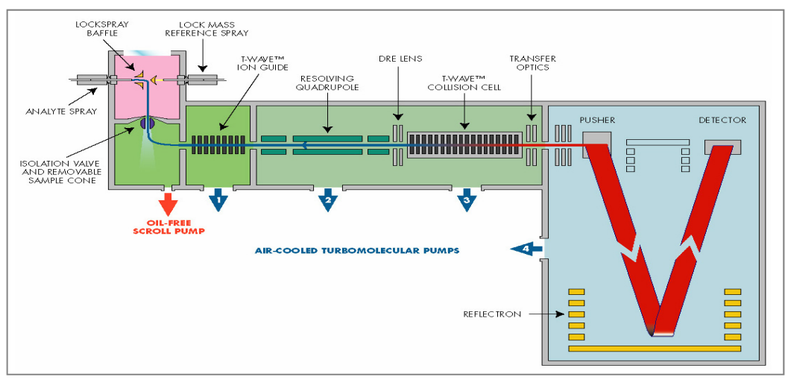


*Figure 1. Schematic of QTof Premier.*

## METHODS

### Samples

Buspirone was incubated with rat liver microsomes at 100 μ M at 37 ° C, in a solution of 50 mM potassium phosphate adjusted to pH 7.4 containing the appropriate cofactors. The reaction was terminated after 60 minute with 2 volumes of cold acetonitrile to 1 volume of sample. Then, the sample was diluted 1/10 with Water and spiked into a 1/4 dilution of rat bile. Finally, the samples were centrifuged and the supernatant was injected directly to the UPLC-TOF-MS system for analysis.

### LC-MS Methodology

| | |
|---|---|
| Mass Spectrometer: | Q-Tof Premier™ |
| MS Scan Range: | 50-800 Da |
| Mode of Operation: | + ion mode ESI<br>V-mode, pDRE (dynamic range enhancement) |
| Lock Mass: | Leucine Enkephalin at 200pg/mL |

### MS$^E$ Methodology

The QTof Premier was operated in a parallel data acquisition mode with a wide band RF mode in Q1 (Figure 2). Thus, allowing all ions in the collision cell. This resulted in one single injection in which data was collected under one single data file with two functions. These were;

Function 1) Low energy acquisition (5eV) which contained the unfragmented compounds

Function 2) High energy or MS$^E$ acquisition (25eV-40eV ramp) which contained all of the fragmented ions

### LC-conditions

Acquity UPLC™

Acquity BEH C18 Column 100x2.1mm id, 1.7μm

| | |
|---|---|
| Mobile Phase: | A: 0.1 % formic acid; B: Acetonitrile |
| Flow Rate: | 0.6 mL/min |
| Gradient: | 0 min 98% A, 0-4.7 min 30% A, 4.7-5.1 min 0% A, 5.1-8 min 98%A |
| Injection Volume: | 5 μL |

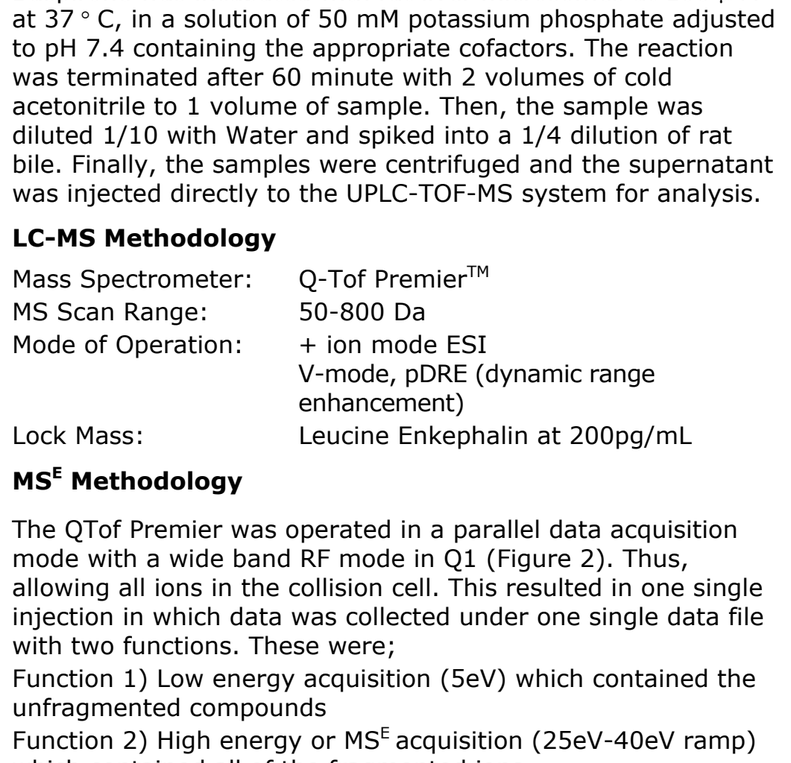


*Figure 2. MS$^E$ data acquisition mode with the QTof Premier.*

### Description of software algorithm for data processing - MetaboLynx MS$^E$ - How does it work?

MetaboLynx is a software application manager, which automatically detects putative metabolites and present results in a data browser format.

It operates by comparing and contrasting each metabolised sample with a control sample—although unexpected metabolite searching may still be performed in the absence of a suitable control. Samples from *in vitro* incubations or *in vivo* dosing experiments can be quickly analysed by LC/MS, followed by a multi-dimensional data search which correlates retention time, m/z value, intensity and components from alternative detection technologies (e.g. diode array UV or radiochemical monitoring). Comparison of analyte data with the control sample allows filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks.

MS$^E$ (Figure 3) can be processed with this software algorithm and will align the data from the low energy and MS$^E$ functions based upon retention times and exact mass alignment.

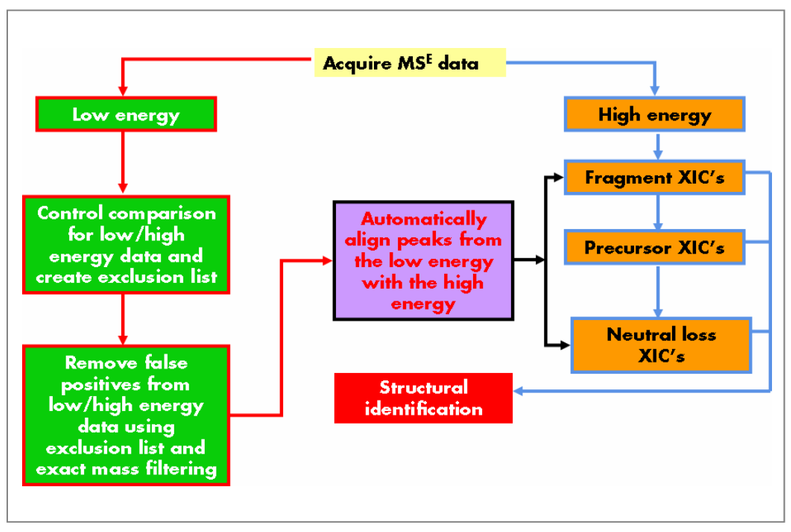


*Figure 3. MS$^E$ data acquisition and processing schematics with MetaboLynx.*

### Exact mass data filtering (EMDF) and MetaboLynx MS$^E$ - How does it work?

- It is based on exact mass and mass deficiencies, which are specific to each parent compound[2].
- Each parent drug has a specific number of elements ( C,H,N,O, …) which is known by the chemist.
- Depending on the number of each one of the elements mentioned, the drug of interest will have a very specific mass deficiency. For example in the case of Buspirone, it contains the following elements; $C_{21}H_{31}N_5O_2$.
- Metabolism of buspirone to the 2-piperazine-1-pyrimidine (1-PP) results in a fractional mass shift of - 141.6 mDa (Figure 4).

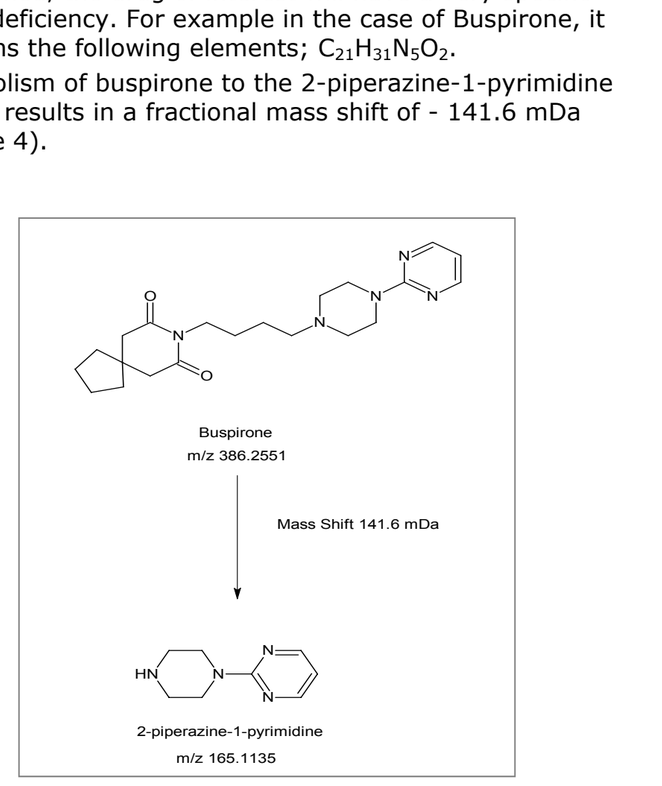


*Figure 4. Example for the use of mass defect filtering for Buspirone and one of its metabolites.*

- Therefore, if we were use an EMDF specific for this loss with a narrow window at either side of the 1-PP metabolite then we can see the metabolite very clearly (Figure 5).

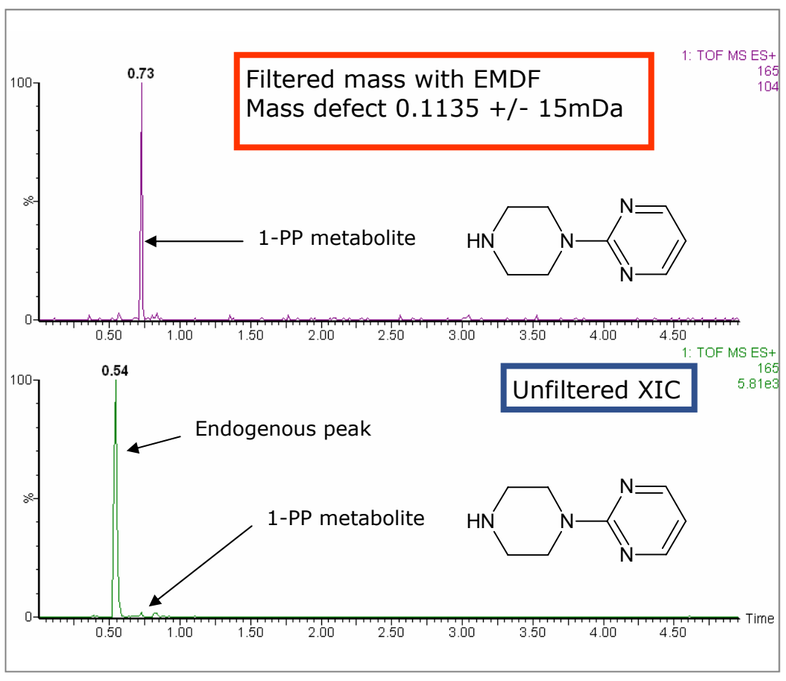


*Figure 4. Comparison between unfiltered XIC vs. filtered XIC for 1-PP metabolite of Buspirone in Bile.*

## DISCUSSION

From Figure 5, it can be observed how the use of mass defect filtering may be used to eliminate false positives.

- Another advantage of this approach is the fact that we can set low thresholds and search for low levels and then let the filtering tools such as the mass defect filter and peak area threshold filter to remove the endogenous peaks which may be present in the unexpected metabolite trace

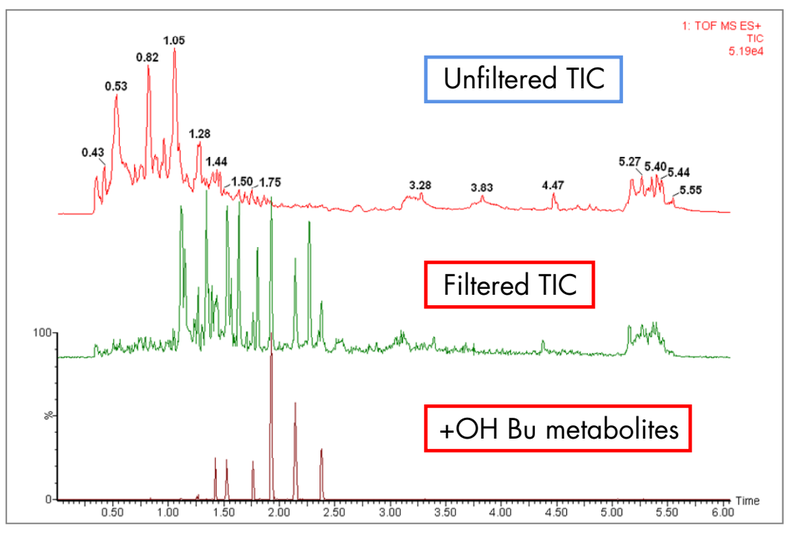


*Figure 5. Comparison of Total Ion Chromatograms for Buspirone hepatocyte incubated sample spiked into rat bile with and without mass defect filtering.*

- MS$^E$ data may also be filtered using exact mass (Figure 6). In this case the mass of each metabolite is recorded and then filtered accordingly with a moving mass filter set up by the user which is applicable to the mass range of interest

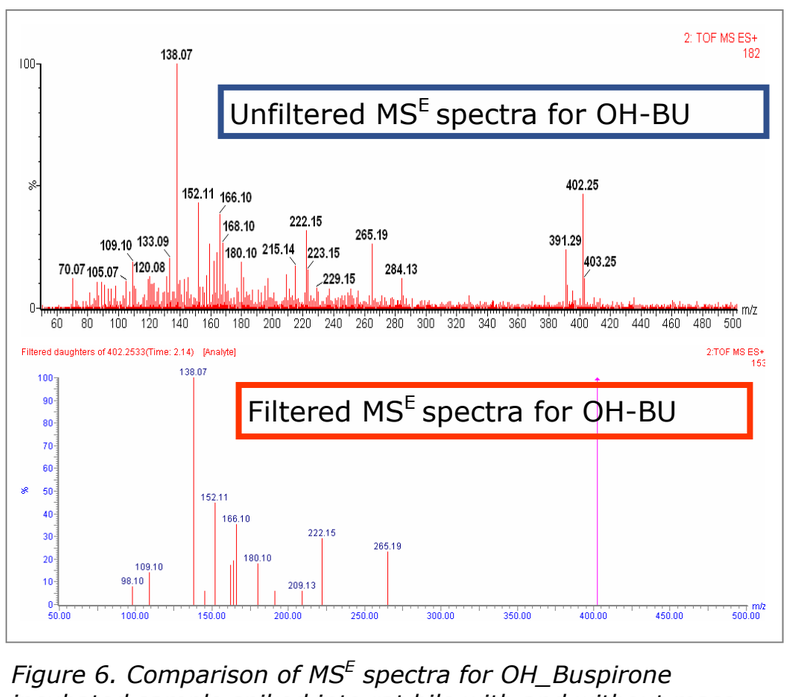


*Figure 6. Comparison of MS$^E$ spectra for OH_Buspirone incubated sample spiked into rat bile with and without mass defect filtering.*

- From this analytical strategy, fragment ion, precursor ion and neutral data was generated in the MS$^E$ function
- Here an example is shown where precursor ion information (Figure 7) is used for the confirmation of metabolites found corresponding to the hydroxylated metabolites m/z 402 giving rise to a common precursor ion with the parent drug at m/z 222

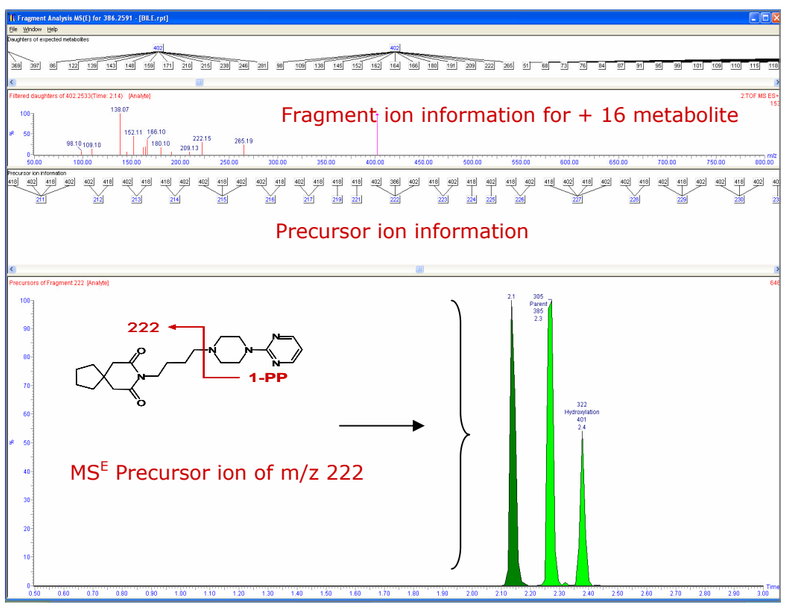


*Figure 7. MS$^E$ Fragment analysis for the metabolites of Buspirone spiked in rat bile.*

## CONCLUSION

- **By this approach we were able to acquire vast amounts of information during the time scale of UPLC**
- **Exact mass data filtering provided us with a very powerful strategy to data mine the samples with great accuracy**
- **The use of exact mass data filtering also allowed us to remove false positives in a much faster manner without the need of re-injection**
- **Methodology easy to set-up without any prior knowledge of metabolites**
- **Generation of fragment, precursor and neutral loss information from a single injection**
- **This approach provides a quick 'snapshot' for fragment ion information**
- **It will also help to decide what further MS/MS experiments to carry out**

### References

1. Mark Wrona, Kevin Bateman, Russell Mortishire-Smith, Desmond O'Connor; Rapid Commun. Mass Spectrom. 2005; 19: 2597–2602.
2. Mingshe Zhu, Li Ma, Donglu Zhang, Kenneth Ray1, Weiping Zhao, W. Griffith Humphreys, Gary Skiles2, Mark Sanders, and Haiying Zhang ; DMD 34:1722-1733, 2006.

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