

## An Intelligent UPLC/TOF-MS Workflow for Metabolite Identification using a Software Tool Including C-Heteroatom Cleavages and Automatic Generation of Mass Defect Filters

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### Abstract

This application note presents the integration of this algorithm into a fully-featured metabolite identification workflow, utilizing the ACQUITY UPLC System for chromatographic separation and the SYNAPT HDMS System for ion-mobility mass spectrometry along with MetaboLynx and MassFragment Software for automated data processing.

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### Introduction

The major bottleneck in metabolite identification is data processing, because it is still mainly a manual process. The biggest challenge for automating data processing is managing the large number of false positives that may be generated.

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A powerful software tool to help with the removal of these false positives is the Mass Defect Filter (MDF).<sup>1,2</sup> Optimizing the MDF on a compound-specific basis is an important step, since different compounds will give rise to different metabolic cleavages and may not exhibit a predictable fixed linear range of mass defect, such as drugs containing S, Cl, or Br.

We have previously presented a proof of concept approach that uses a simple algorithm that can quickly generate an intelligent compound-specific mass defect filter.<sup>3</sup>

In this work, we present the integration of this algorithm into a fully-featured metabolite identification workflow, utilizing the ACQUITY UPLC System for chromatographic separation and the SYNAPT HDMS System for ion-mobility mass spectrometry along with MetaboLynx and MassFragment software for automated data processing (Figure 1). This allows us to query a particular drug up front to generate a list of C-heteroatom and heteroatom-heteroatom cleavages. This information is used to generate an automatic filter with adjustable limits depending upon whether Phase 1 or 2 metabolites are selected.

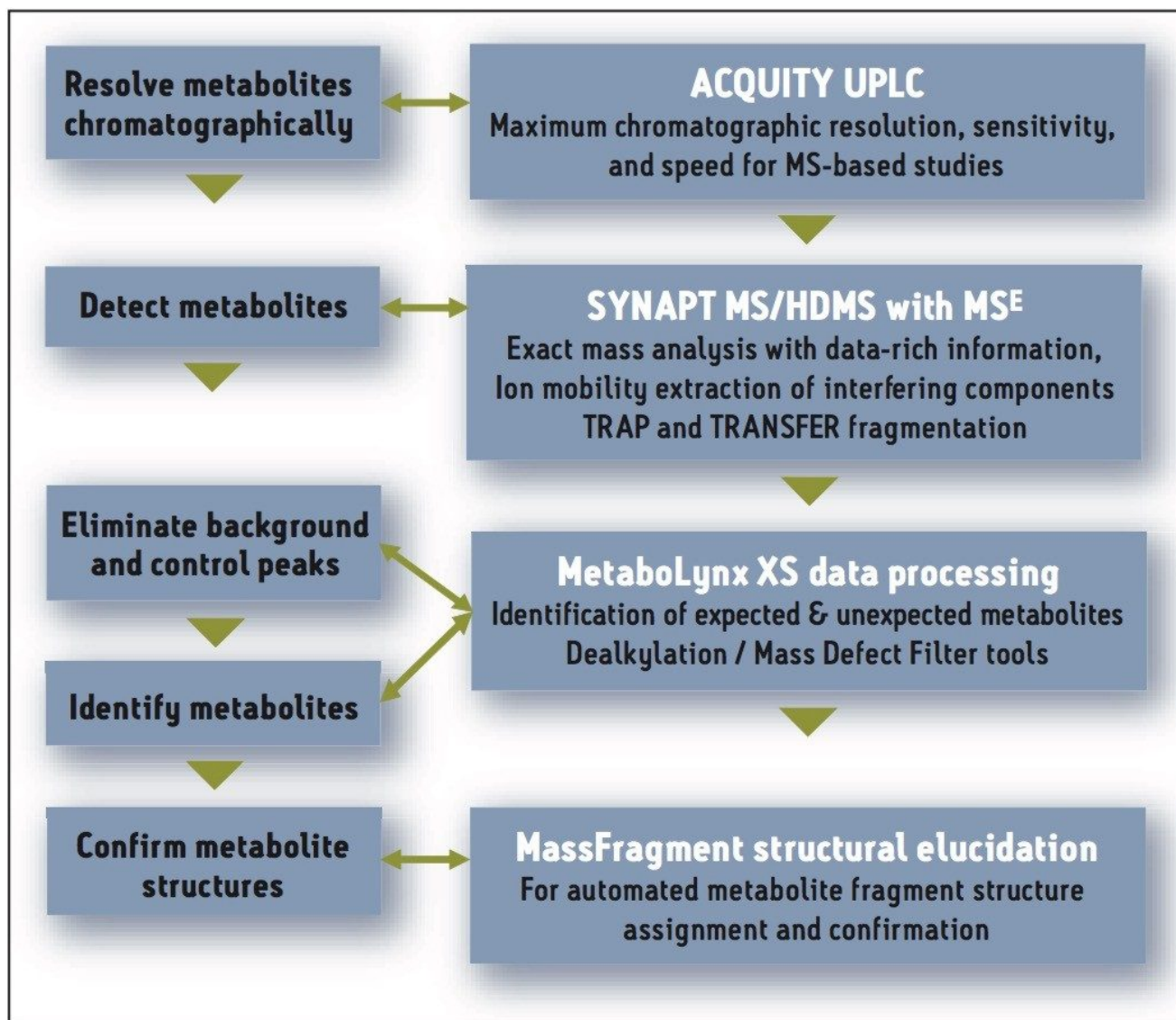


Figure 1. UPLC-MS<sup>E</sup> metabolite identification workflow.

## Experimental

### Samples

Nefazodone and indinavir were incubated with rat liver microsomes at 10  $\mu$ M at 37 °C, in a solution of 50 mM Tris

buffer adjusted to pH 7.4 containing NADPH regenerating system and GSH at 5  $\mu$ M. The reaction was terminated after 90 min with two volumes of cold acetonitrile to one volume of sample. Then the sample was centrifuged at 13,000 RPM for 15 min and the supernatant was diluted 1/2 with water + 0.1 % formic acid. Finally, the supernatant was injected directly to the UPLC-MS/MS system for analysis.

## LC-MS Methodology

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C <sub>18</sub> Column 2.1 x 100 mm I.D., 1.7 $\mu$ m
Column temp.:	45 °C
Mobile phase A:	0.1 % Formic acid
Mobile phase B:	Acetonitrile
Flow rate:	0.6 mL/min
Gradient:	98% A to 40% A in 8 min, ramp to 40 to 0% A in 1.5 min before returning to 98% A for re- equilibration
Injection volume:	5 $\mu$ L
MS system:	Waters SYNAPT HDMS System
MS acquisition:	50 to 900 Da
Mode of operation:	Positive ion mode ESI
Lock mass:	Leucine Enkephalin at 200 pg/mL

## Processing Software

The MetaboLynx Application Manager, part of MassLynx Software was used for MS<sup>E</sup> data mining<sup>4,5</sup> and peak detection of putative metabolites (Figure 2). MassFragment was used for structure elucidation.

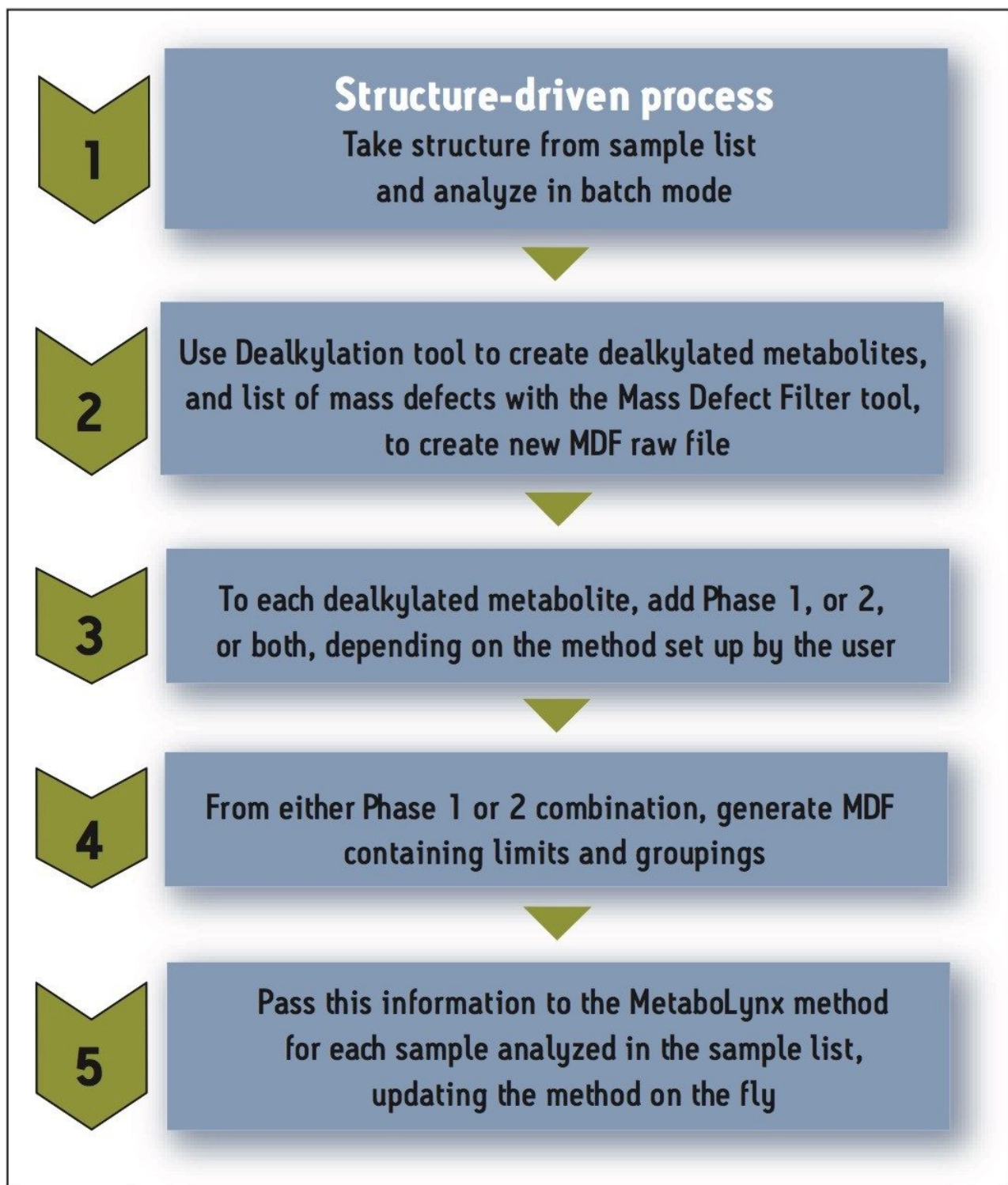


Figure 2. MetaboLynx's chemically-intelligent workflow.

## Mass Detect Filter

The Mass Defect Filter (MDF) can be utilized to remove false positives and improve the metabolite identification workflow when combined with prior knowledge of the mass and its decimal places (elemental composition). The mass defect can either be positive (larger than the nominal mass) or negative (smaller than the nominal mass).

For example, the mass defect for H is +0.0078, with 1.0078 as its exact mass. The mass defect for OH is -0.0051, with 15.9949 as its exact mass.

This software applies a post-acquisition data filtering technique that is set based upon the mass defect of the parent drug and its metabolites. The rationale and assumption supporting MDF are that the majority of metabolites only have small shifts in their mass defect when comparing with the exact mass of the parent drug.

Applying the MDF technique allows users to capture and identify metabolites from complex matrices in a rapid and effective manner.

However, applying the MDF can be challenging and very much compound-specific. First, the mass defect may have rather large significant shifts when a dealkylation occurs for the parent drug prior to the formation of metabolites (Figure 3). Second, the mass defect shifts are not linear to their respective integer masses (Figure 4).

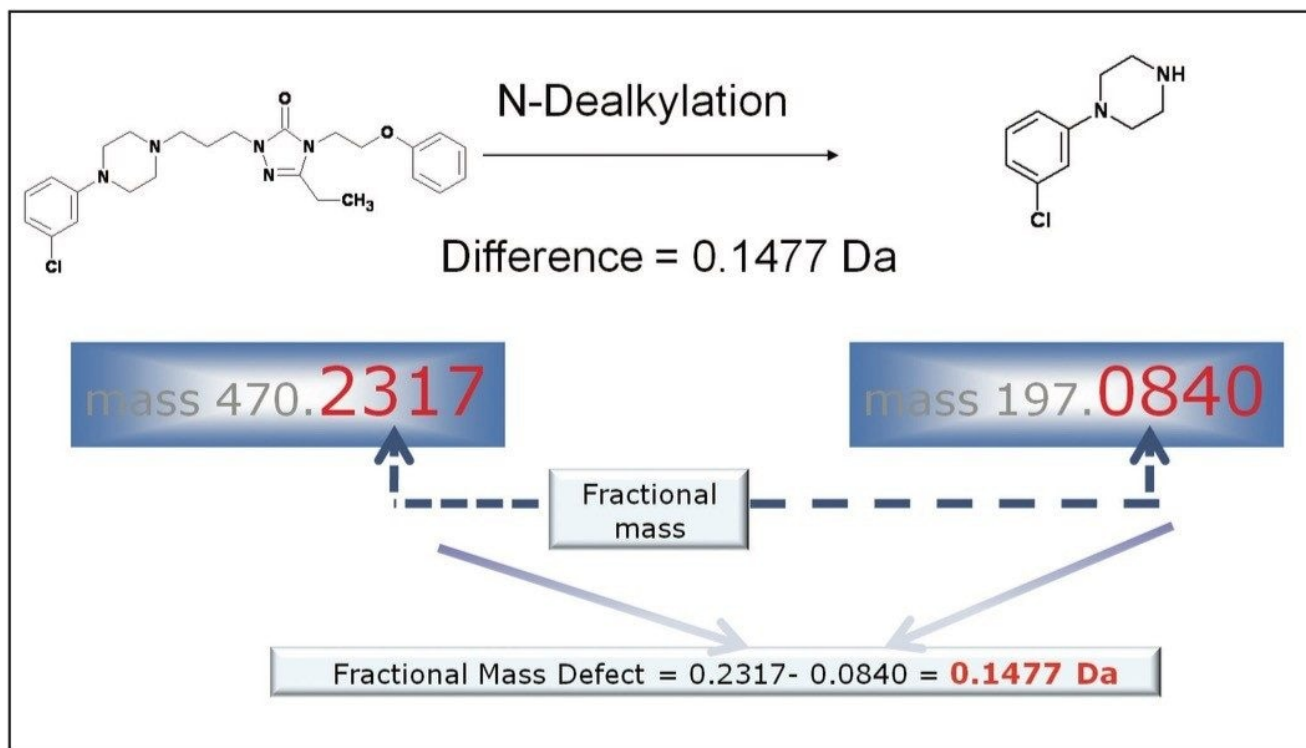


Figure 3. Mass defect shift after N-dealkylation occurred for nefazodone.

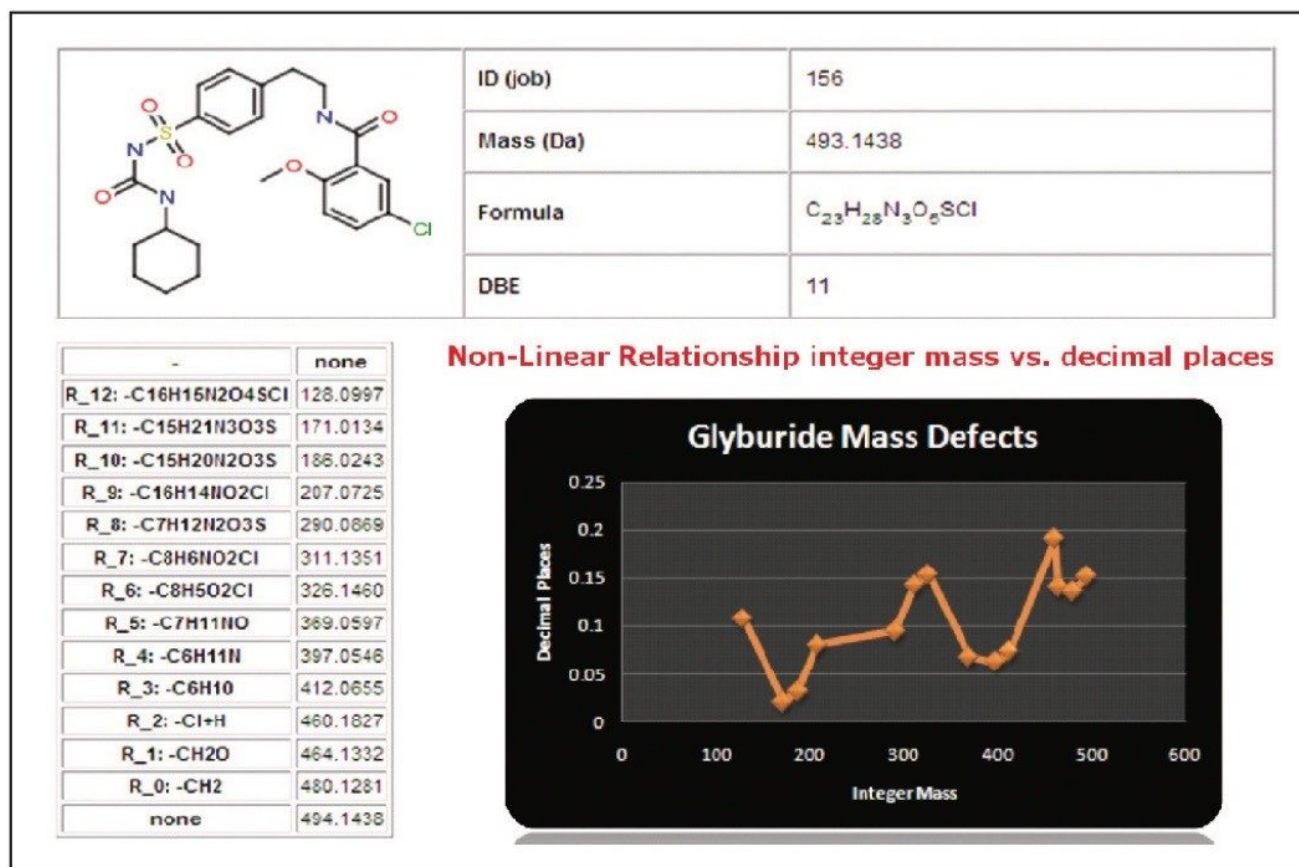


Figure 4. Mass defect shift after dealkylation occurred for glyburide.

## MDF in MetaboLynx

In the UPLC-MS<sup>E</sup> metabolite identification workflow shown in Figure 1, the Mass Defect Filter is applied at the initial stage during the MetaboLynx data processing. Once the user sets up the MetaboLynx method, the parent drug structure is first automatically transferred to the dealkylation tool to identify metabolic cleavages.

Upon identifying the major dealkylated fragments, their corresponding MDFs are automatically grouped with expected biotransformations. The MDFs are calculated based on their individual metabolic cleavages and biotransformation addition to each metabolic cleavage and parent.

Having done this, MetaboLynx can generate mass defect filtered chromatograms for control and dosed samples (.MDF files). The next step is for MetaboLynx to go through its regular metabolite identification procedure using the filtered chromatograms, with the aim of having a much more reduced list of unexpected metabolites.

## Results and Discussion

Figure 5 shows how the mass defect filters are automatically set in MetaboLynx. Because of the large fluctuation of the mass defect as a result of dealkylation, multiple mass defect filters were automatically set for the metabolite identification.

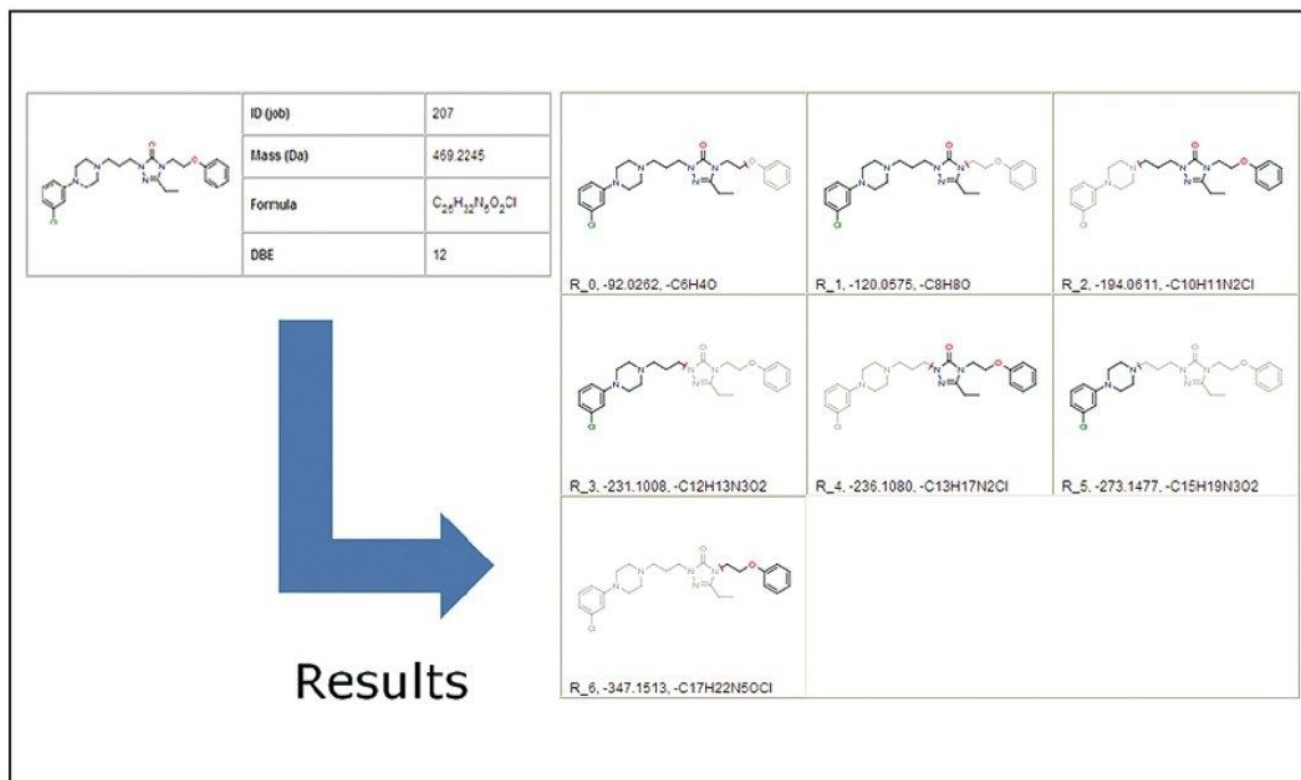


Figure 5. Automatic generation of metabolic cleavages for nefazodone.

Figures 5, 6, and 8 also show the automatic generation of metabolic cleavages for nefazodone and indinavir. Comparisons of the number of nefazodone and indinavir metabolites identified using linear-fixed MDF and intelligent MDF are shown in Figure 7 and Figure 9, respectively.

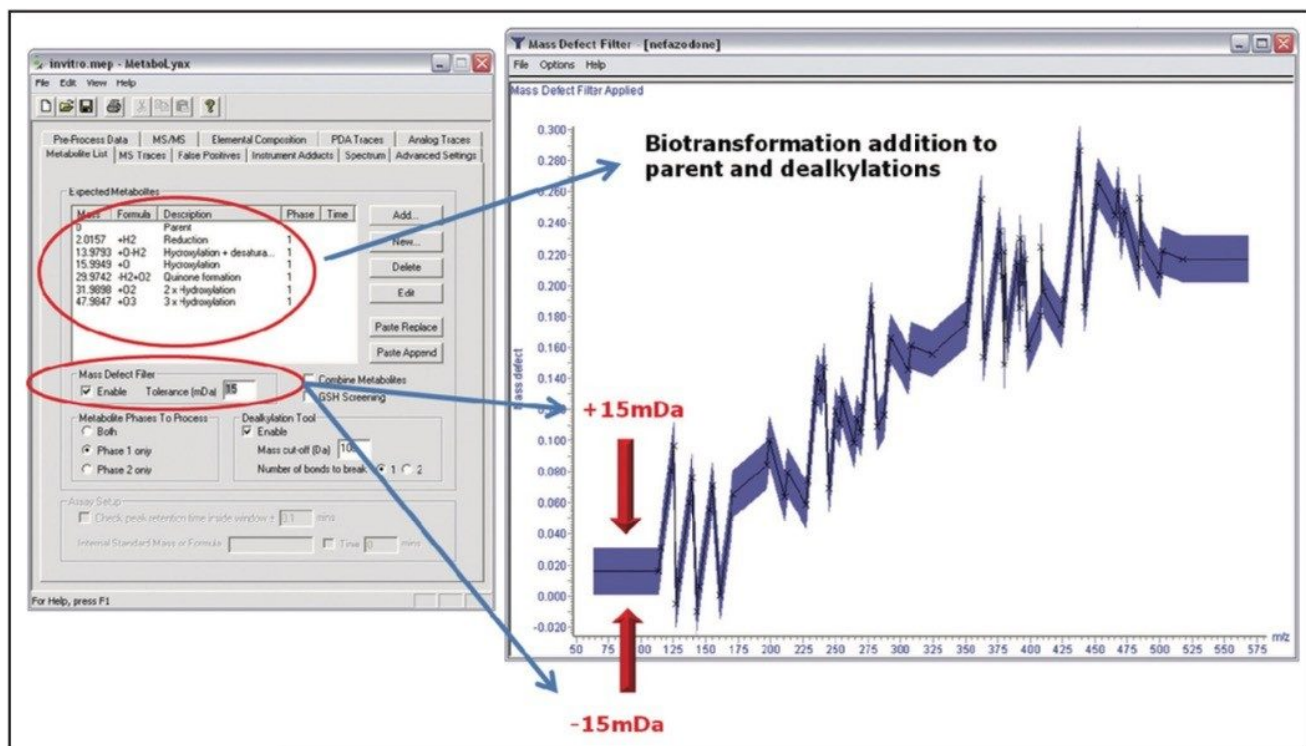


Figure 6. Automatic generation of metabolic cleavages and Mass Defect Filters for nefazodone.

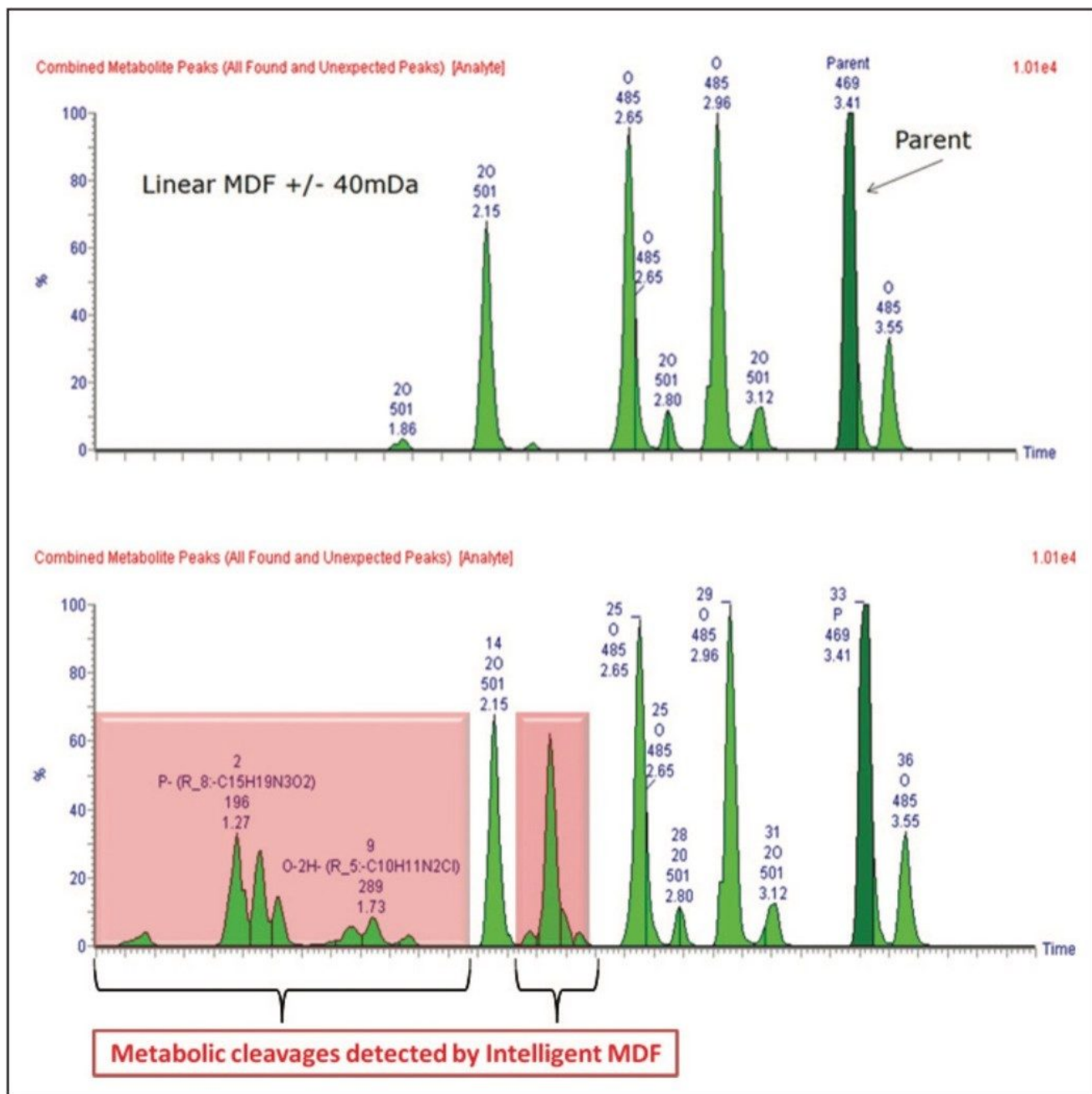


Figure 7. Nefazodone metabolites comparison using linear-fixed MDF (top) and intelligent MDF (bottom).

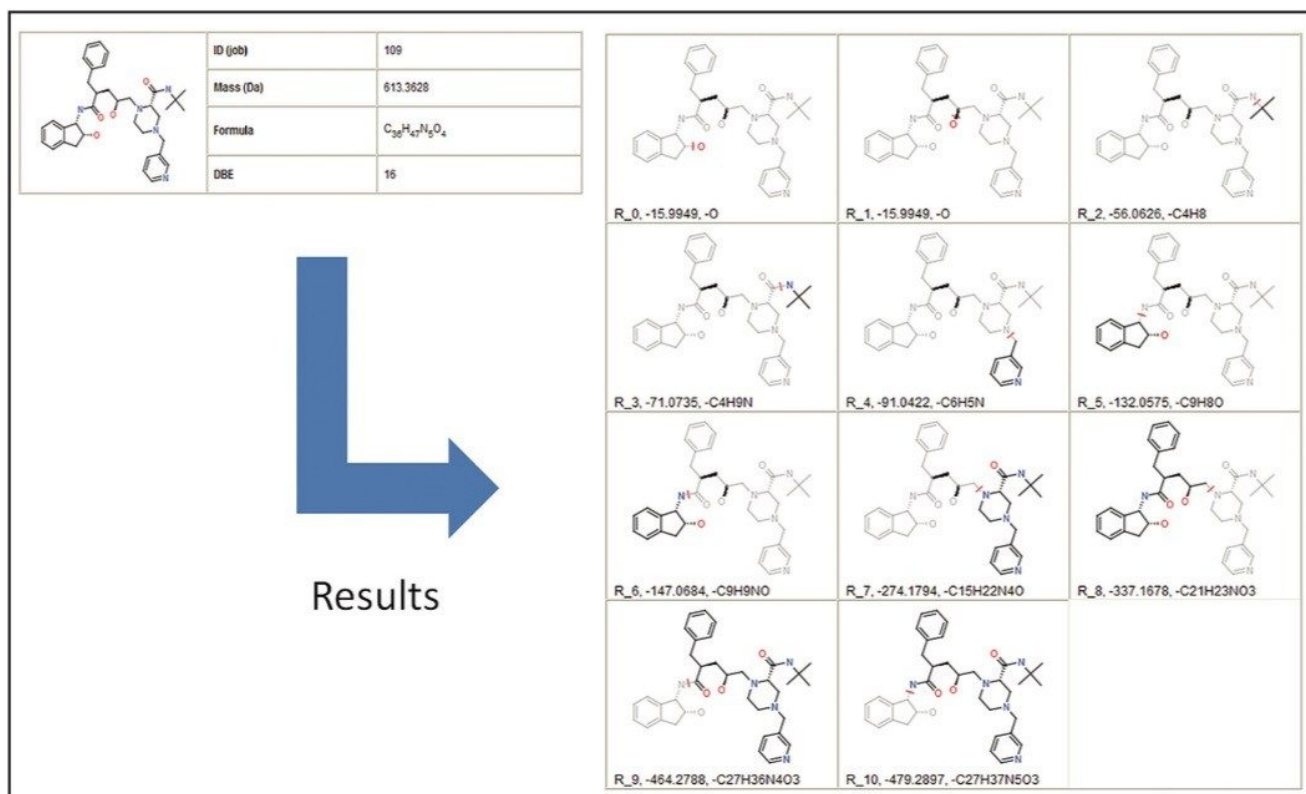


Figure 8. Automatic generation of metabolic cleavages for indinavir.

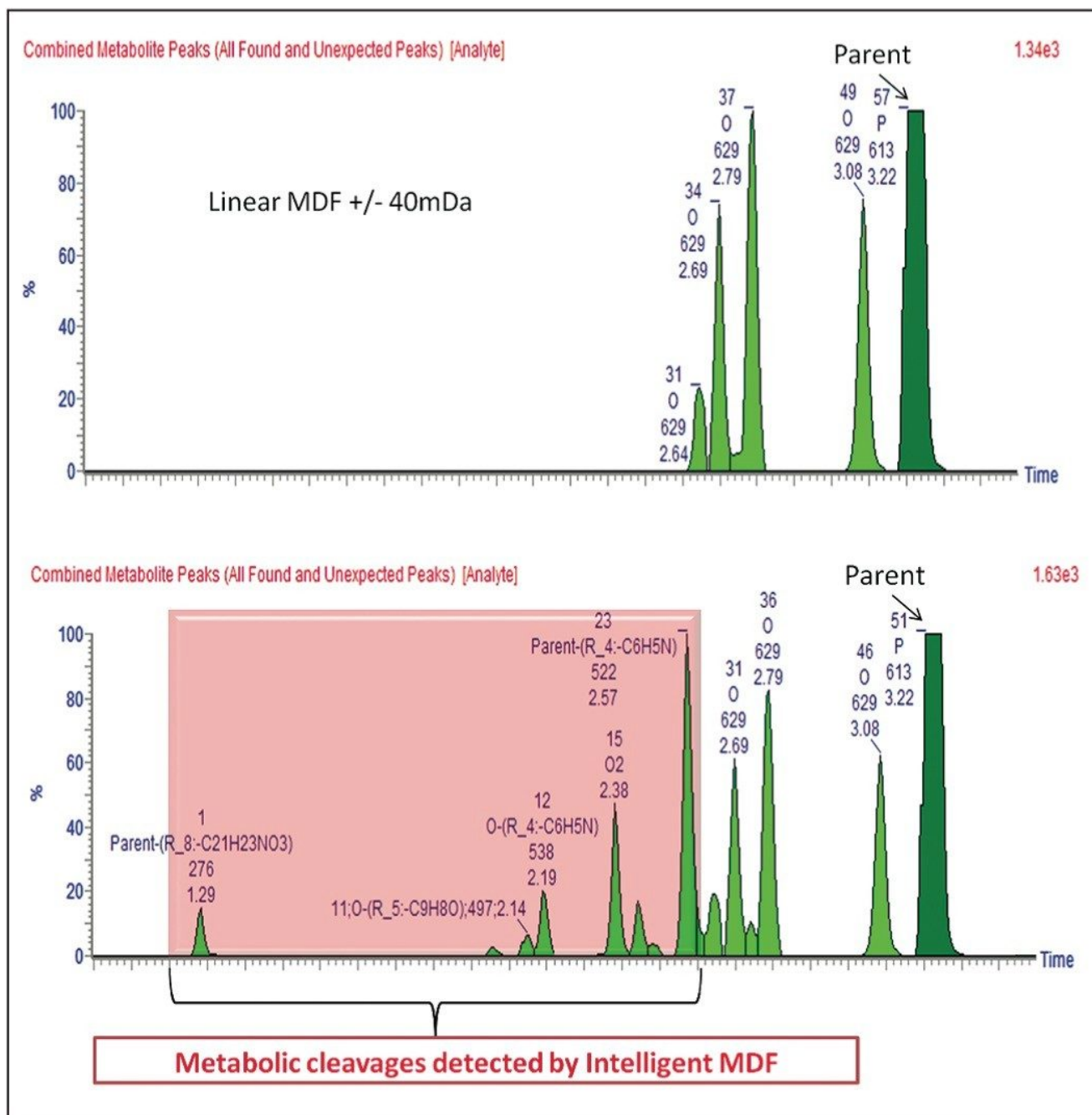


Figure 9. Indinavir metabolites comparison using linear-fixed MDF (top) and intelligent MDF (bottom).

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## Conclusion

With the use of MetaboLynx's dealkylation tool in combination with the Mass Defect Filter, we are able to rationalize the expected and unexpected metabolites more efficiently and minimize the number of false positives.

From this initial step it is possible to generate an extensive expected metabolite list on the fly. The unexpected metabolites, such as ring contractions or formations, are also possible to detect since we allow for a confidence limit window for the MDF, which will cover such biotransformations.

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## References

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