



# Predizioni del metabolismo *in silico* e *in vitro*. Uno studio pilota

*In silico* and *in vitro* metabolism prediction. A pilot study.

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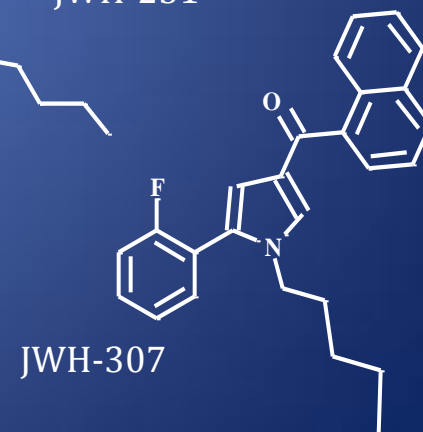
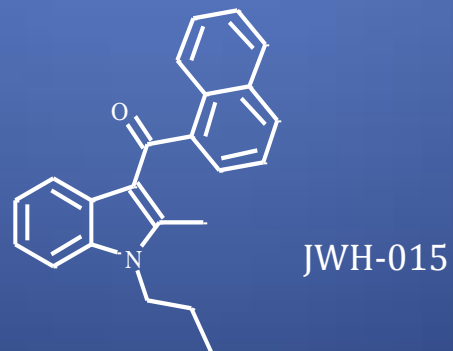
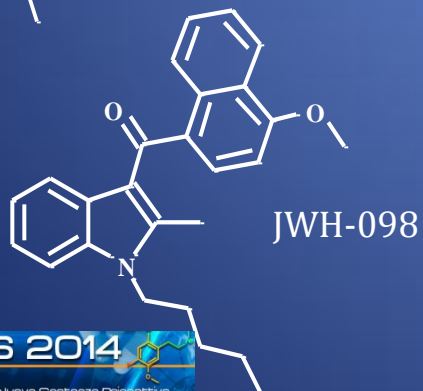
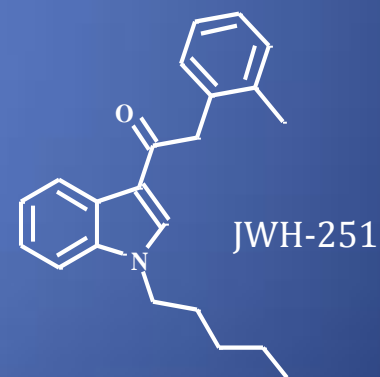
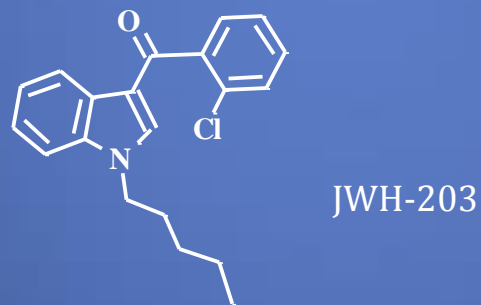
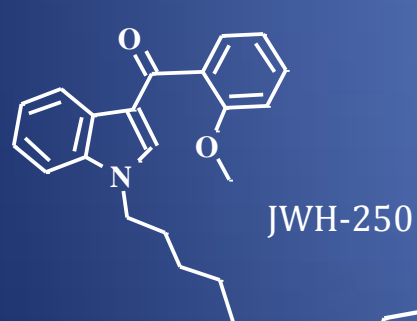
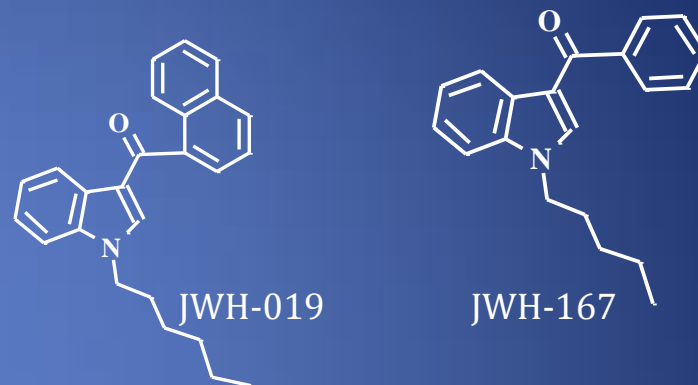
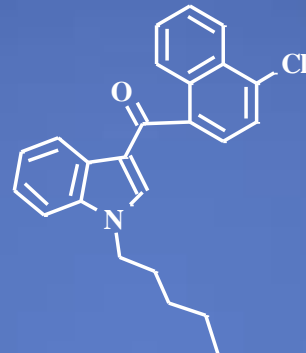
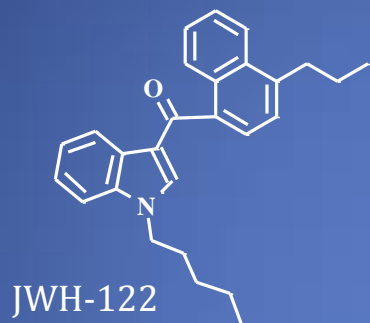
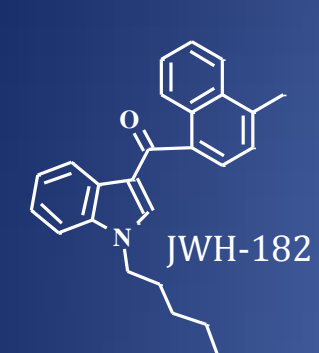
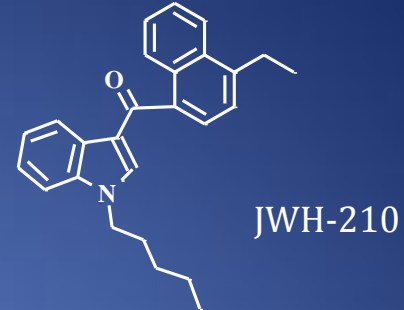
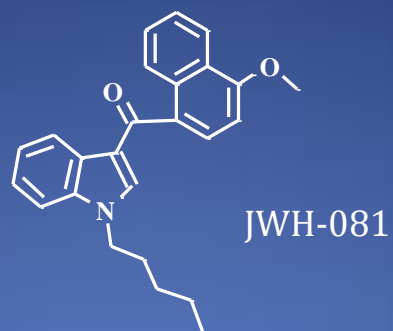
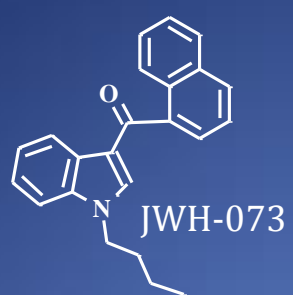
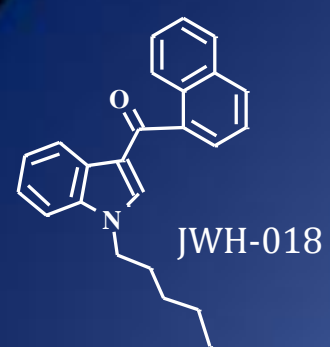
PRESIDENZA DEL CONSIGLIO DEI MINISTRI  
Dipartimento Politiche Antidroga

SISTEMA NAZIONALE DI ALLERTA PRECOCE  
NATIONAL EARLY WARNING SYSTEM - N.E.W.S.

## NEW DRUGS 2014

Congresso Internazionale  
Aggiornamento tecnico-scientifico sulle Nuove Sostanze Psicoattive

In collaborazione con:  **UNODC**  
United Nations Office on Drugs and Crime





# Biological samples

Blood



Parent drug +  
metabolites

Urine



Metabolites >>  
Parent drug

Alternative  
matrices



Parent drug >>  
Metabolites

# How to study drugs metabolism?

- Human studies
- Animal studies
- *In vitro* studies
- *In silico* studies



# Human "Experimentations"



- Ethical Problems
- Difficulties autorisation from ethical committee
- Difficulties in enrolling volunteers and clinical studies set-up
- Safety
- Designer drugs, without authorisation to commerce, without previous clinical studies

# Animal Experimentation

- Animal house
- Authorisation ethical committee
- Metabolism may be different from human



# THE CHIMERIC MOUSE

Mouse “humanized” with human hepatic tissue

- Transplant primary hepatocytes to mice with immunodeficiency (urokinase plasminogen activator-severe combined immunodeficiency)
  - ~ 90% human hepatocytes





# Metabolism studies on anabolic steroids ...and on NPSs

Steroid metabolism in chimeric mice with humanized liver. Lootens et al. *Drug Test Anal.*, 2009, 11-12, 531-537.

The uPA(-/-)-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of **4-Androstene-3,17-dione**. Lootens et al., *Drug Metab. Dispos.*, 2009, 37(12), 2367-2374.

uPA+/+-SCID Mouse with Humanized Liver as a Model for In Vivo Metabolism of Exogenous Steroids: **Methandienone** as a Case Study. Lootens et al., *Clin. Chem.*, 2009, 55(10), 1783-1793.

**In vivo and in vitro metabolism of the synthetic cannabinoid**

Detection and Characterization of a New Metabolite of **17-Methyltestosterone**. Pozo et al., *Drug Metab. Dispos.*, 2009, 37(11), 2115-2126.

**JWH-200**. De Brabanter et al., *Rapid Commun. Mass Spectrom.*, 2013, 27, 2115-2126.

Detection and structural investigation of metabolites of **stanozolol** in human urine by liquid chromatography tandem mass spectrometry. Pozo et al., *Steroids*, 2009, 74, 837-852.

**In vitro and in vivo metabolism of JWH-122**. De Brabanter et al., *Forensic toxicol*, 2013, DOI 10.1007/s11419-013-0179-4





# *In Vitro* Experiments

*In vitro* = 'within the glass'

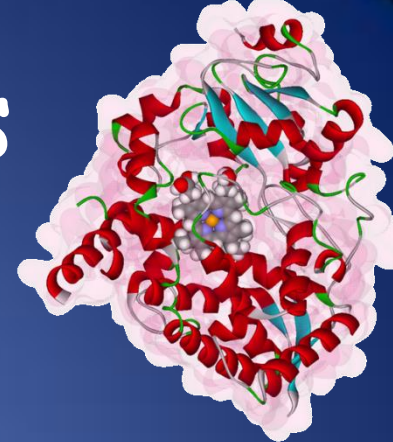
## Cellular Fractions

- Human tissues/cells/organelles obtained by ultracentrifugation
- Commercially available, inexpensive, easy
- From pooled donors (mean of genetic polymorphisms )

## Inherent limitations

- Very different conditions than in a whole organism
- May give misleading results which do not correspond to the situation in a living organism

# cDNA-Expressed Enzymes



- Protein expressed in host cell

Bacteria, yeast, mammal, baculovirus

Harvest and isolate protein and use for metabolism studies

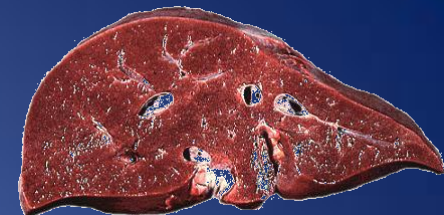
## Pros

- Commercially available
- High-throughput, fast
- One enzyme at a time

## Cons

- One enzyme at a time
- As far from physiological conditions as you can get

# Liver Slices



- Utilize slice of animal organ cut very thin on microtome

## Pros

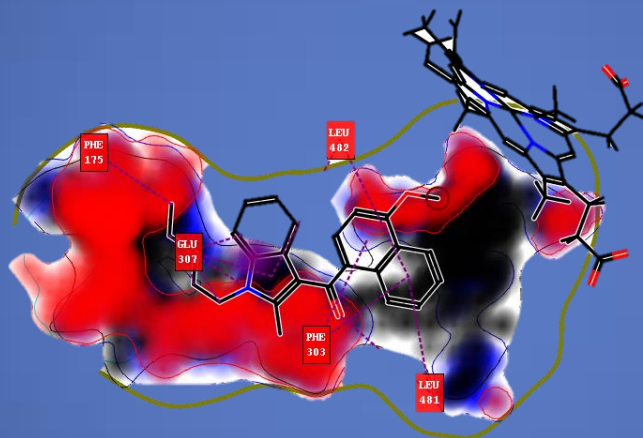
- Contains full complement of metabolizing enzymes, substrates, and **cell-to-cell connections**
- Most closely resembles in vivo situation

## Cons

- Difficult to obtain
- Difficult to cultivate and care for

# *In silico* studies

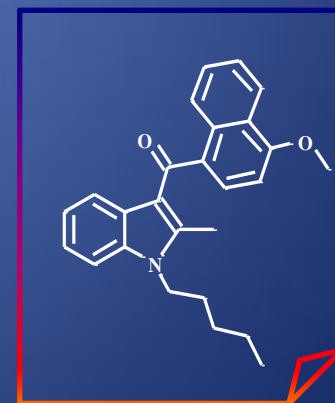
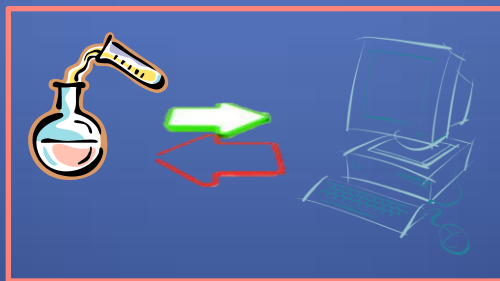
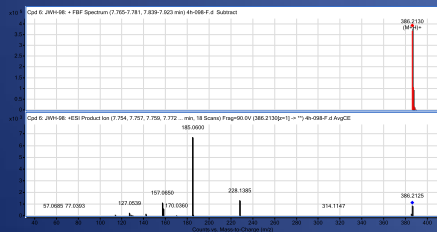
- **MetaSite** is a computational procedure that predicts metabolic transformations related to cytochrome-mediated reactions in phase I metabolism.



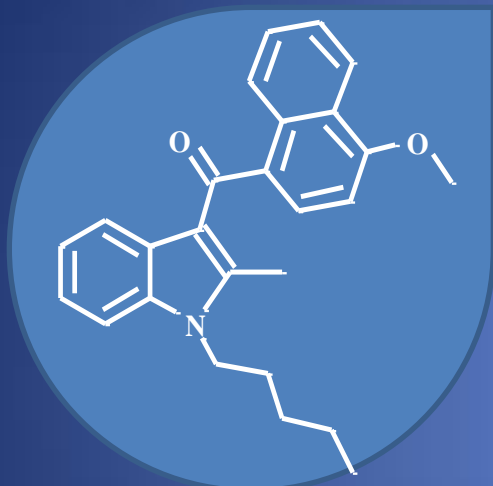
The software attributes the more probable sites of metabolism (SoM), depending on the molecular structure (on the reactivity of its sites), on its possible interaction with the enzyme (on its steric hindrance), and predicts main metabolites that can therefore be formed in various tissues (liver, skin, brain, lungs).

# Combination *in vitro* / *in silico* studies

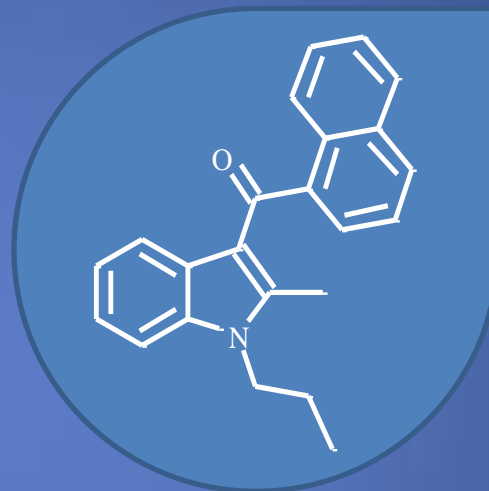
- Mass-MetaSite** is a new approach for the automatic identification of metabolites from LC-HRMS data. The program is able to assign chemical structures to each automatically detected chromatographic peak based on the exact MS and MS/MS fragmentation pattern of the substrates and metabolites. It reduces manual analysis from several hours to only a few minutes per compound.



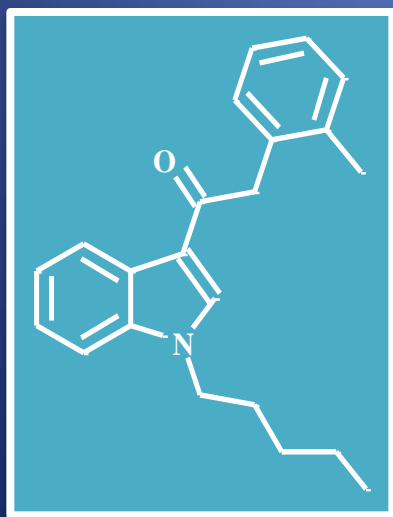
# Pilot Study



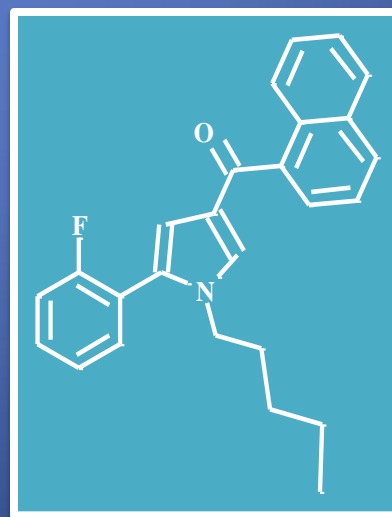
JWH-098



JWH-015



JWH-251



JWH-307



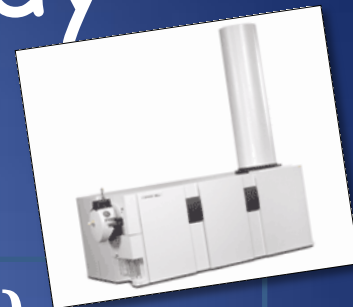
# AIMS

- ✓ evaluate the *in silico* metabolism prediction by comparing the MetaSite data with experimental findings, in order to apply the *in silico* approach to predict the main JWHs metabolites, also in **absence of experimental data**
- ✓ investigate the **metabolic pathway** of four synthetic cannabinoids *in vitro*
- ✓ ...to set-up LC-MS/MS methods for the screening of new JWHs metabolites in biological samples





# Experimental study



## In vitro metabolism study

- Rat liver slices
- 5 mg/ml of JWHs
- Incubation for 4 hours
- Homogenation of tissue and medium
- Proteins precipitation

## LC-HRMS (QTOF)

- UHPLC: Agilent 1290 Infinity
- HRMS: Agilent 6540 QTOF
- Acquisition in full scan in autoMS/MS mode, no fragmentation and at CE of 20, 30 and 40 V of a preferred list of metabolites



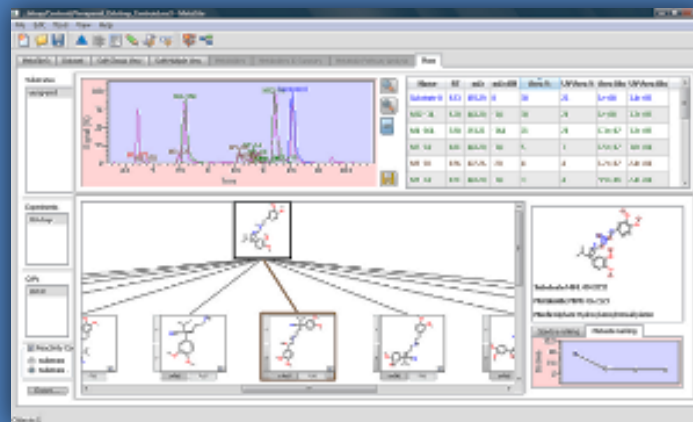
# Data revision: MassMetaSite

Identification  
of the  
accurate  $MH^+$   
of postulated  
metabolites



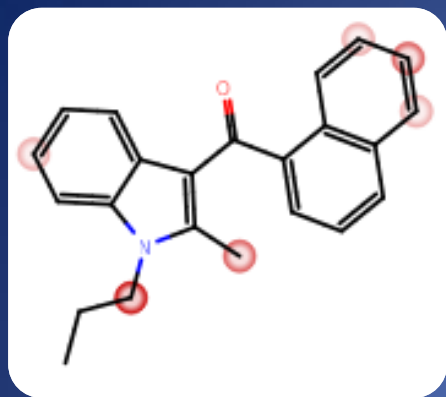
Attribution of  
metabolites

Accurate  
masses of the  
characteristic  
fragments

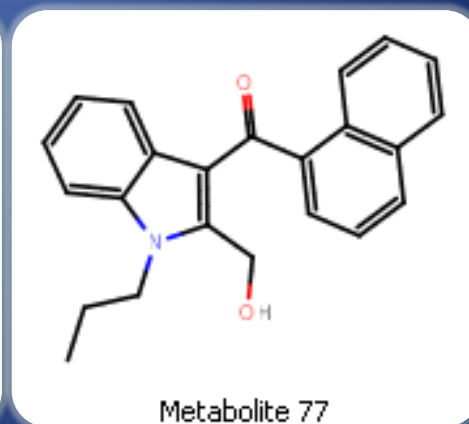
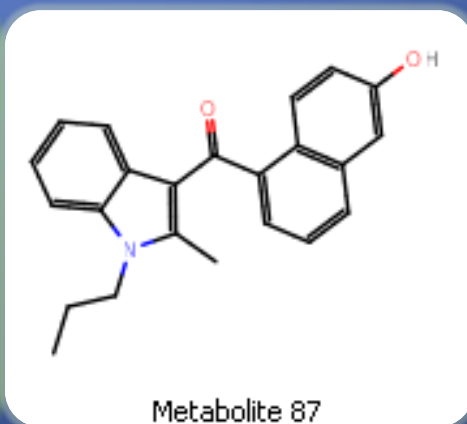
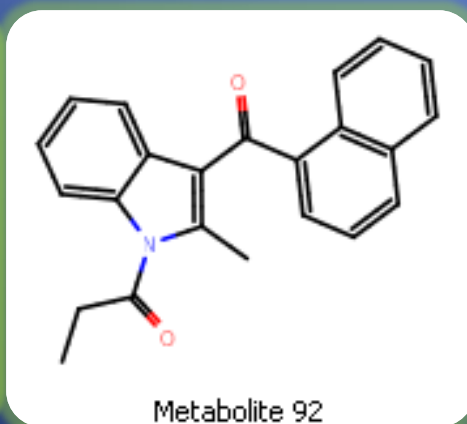
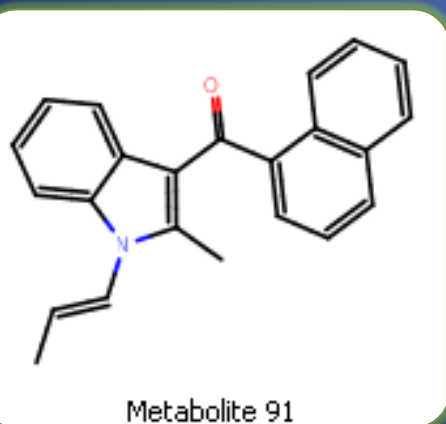
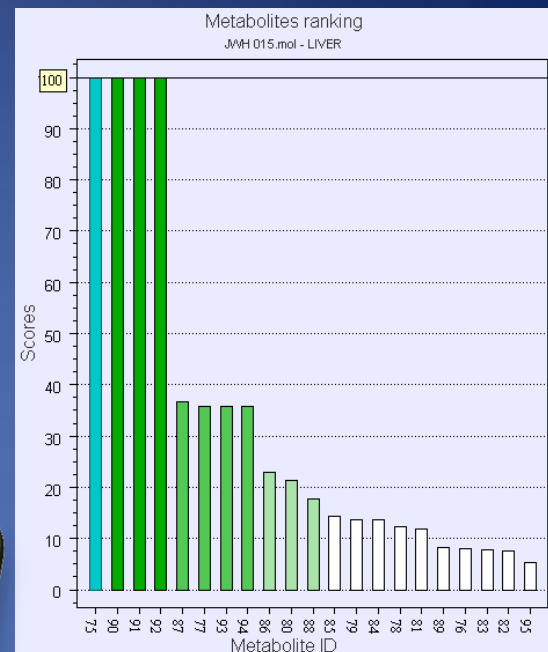
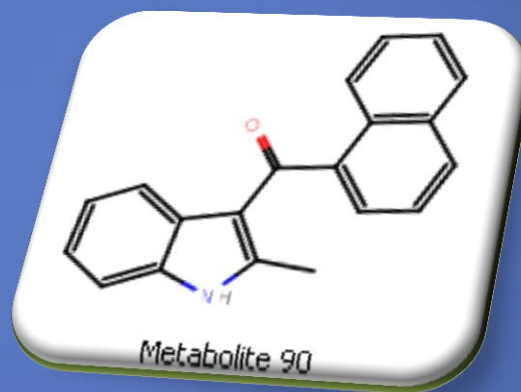
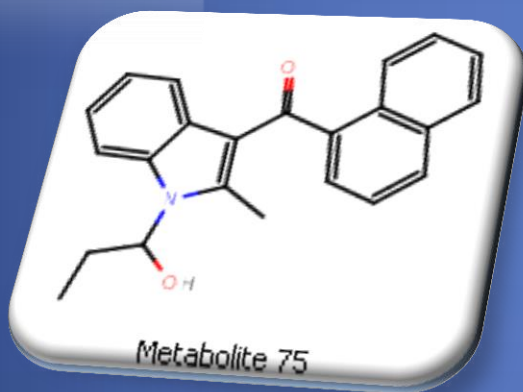


...and manual revision by the operator

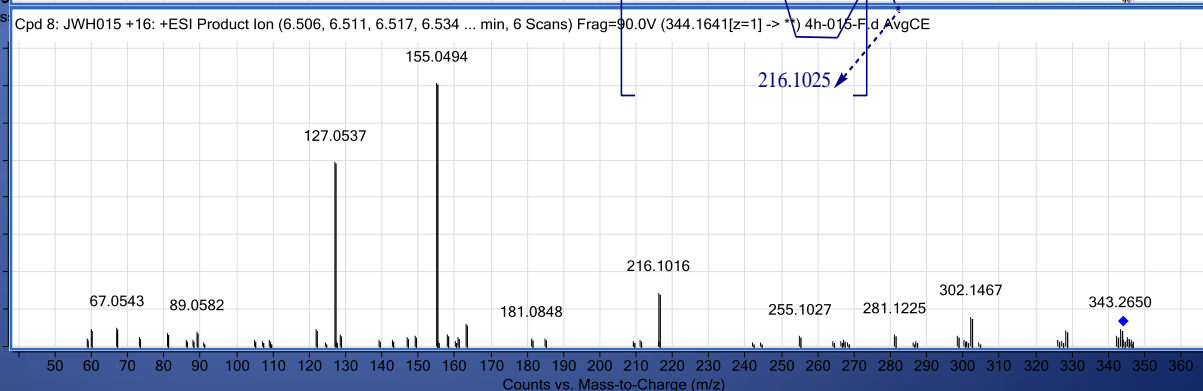
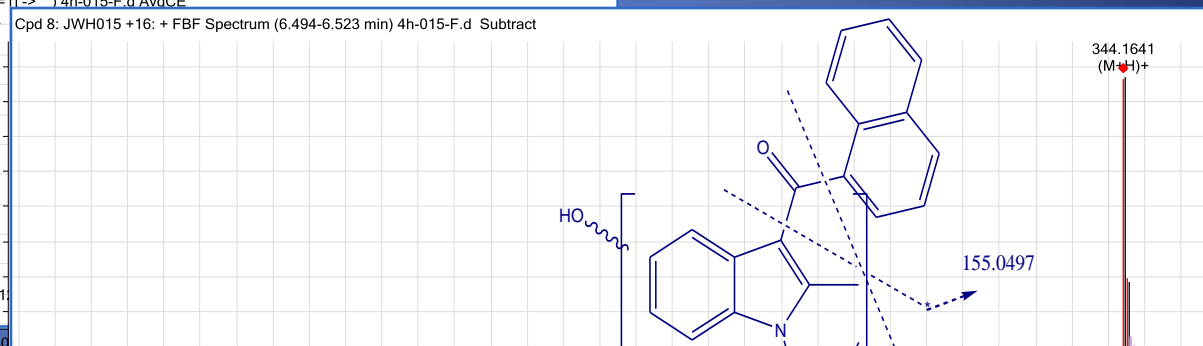
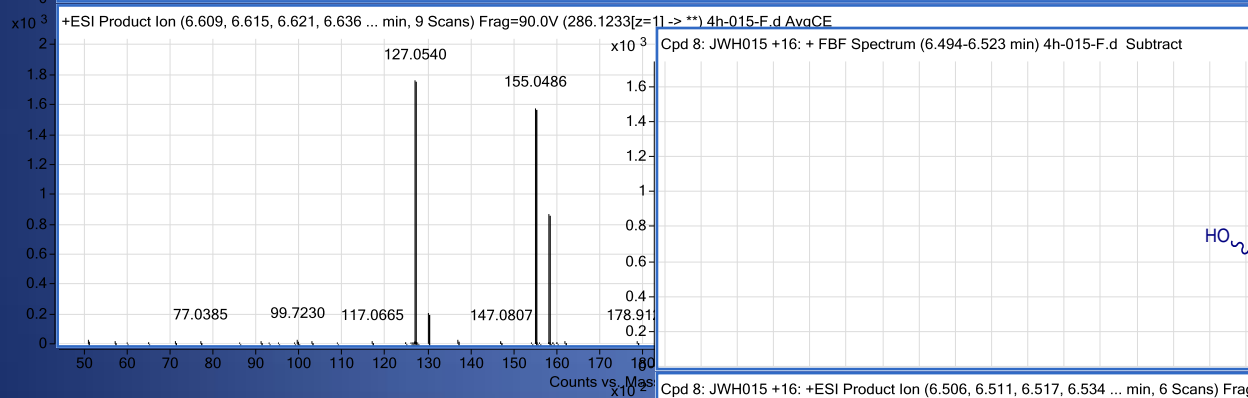
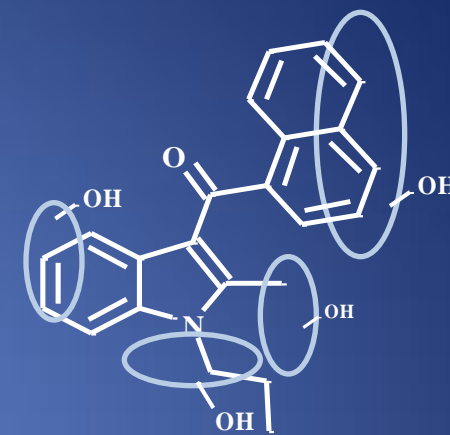
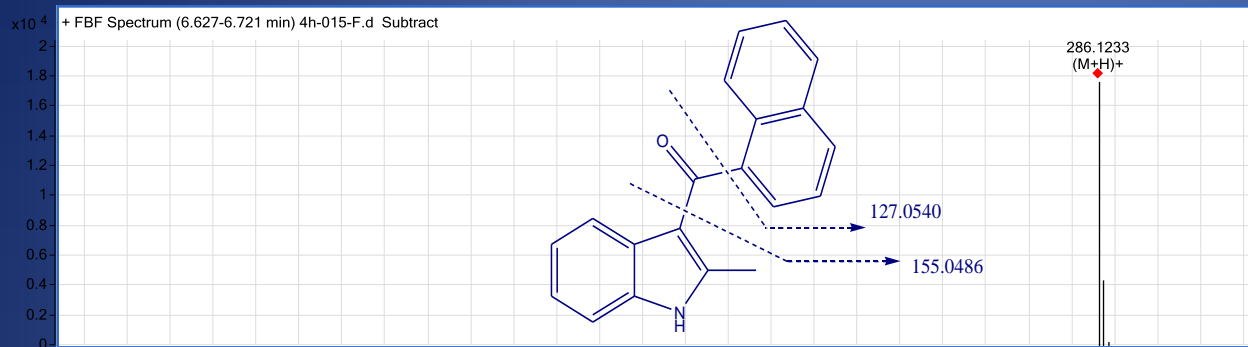
# More probable sites of metabolism: JWH 015



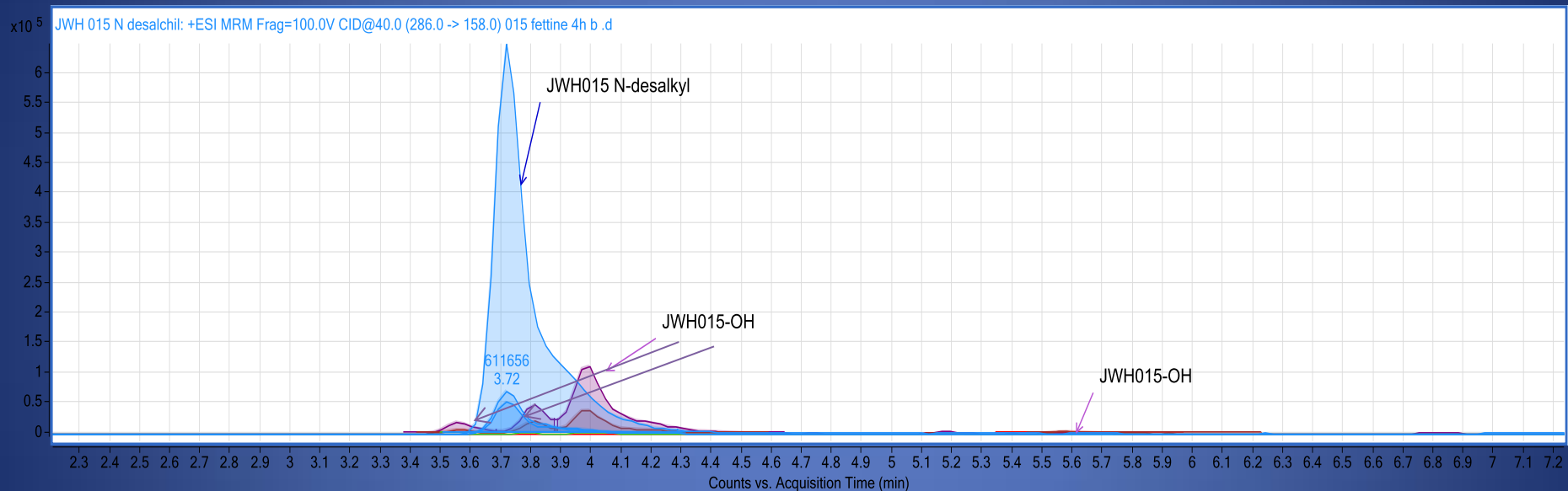
...and **main metabolites** predicted



# JWH015 main metabolites detected

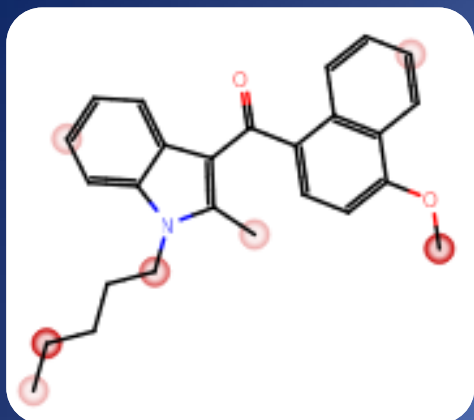


# LC-MS/MS: ionic chromatogram of main metabolites JWH015

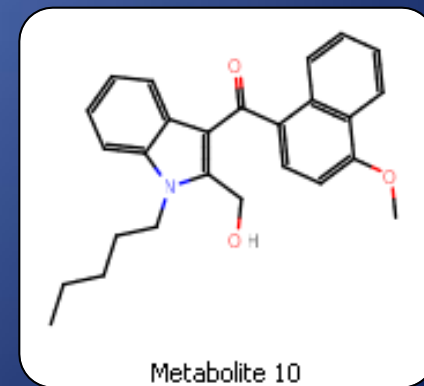
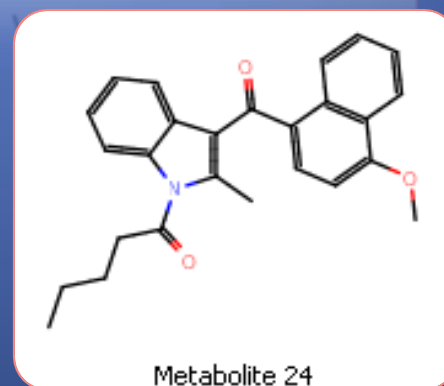
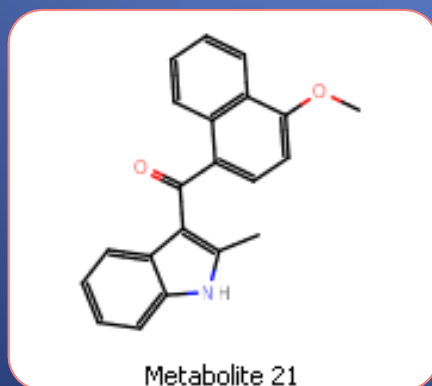
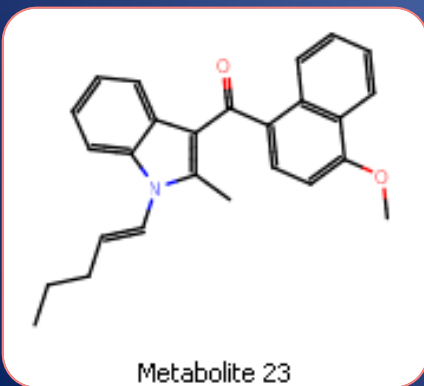
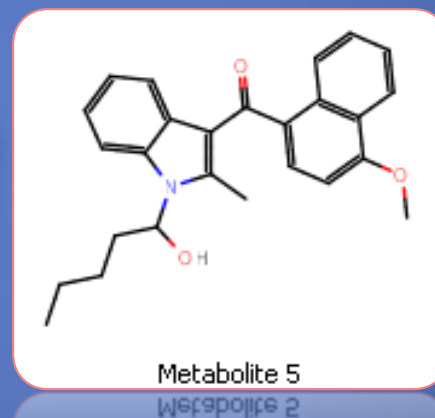
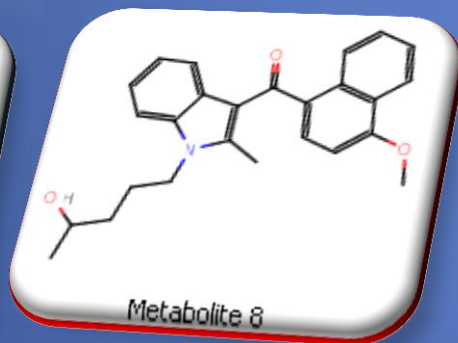
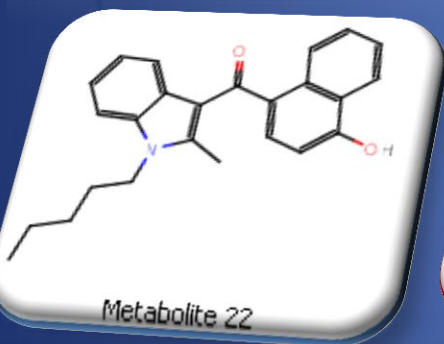
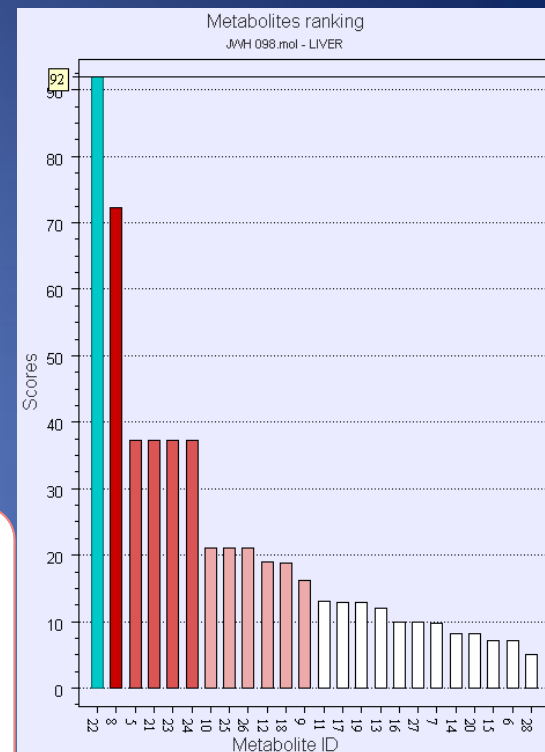


# More probable sites of metabolism:

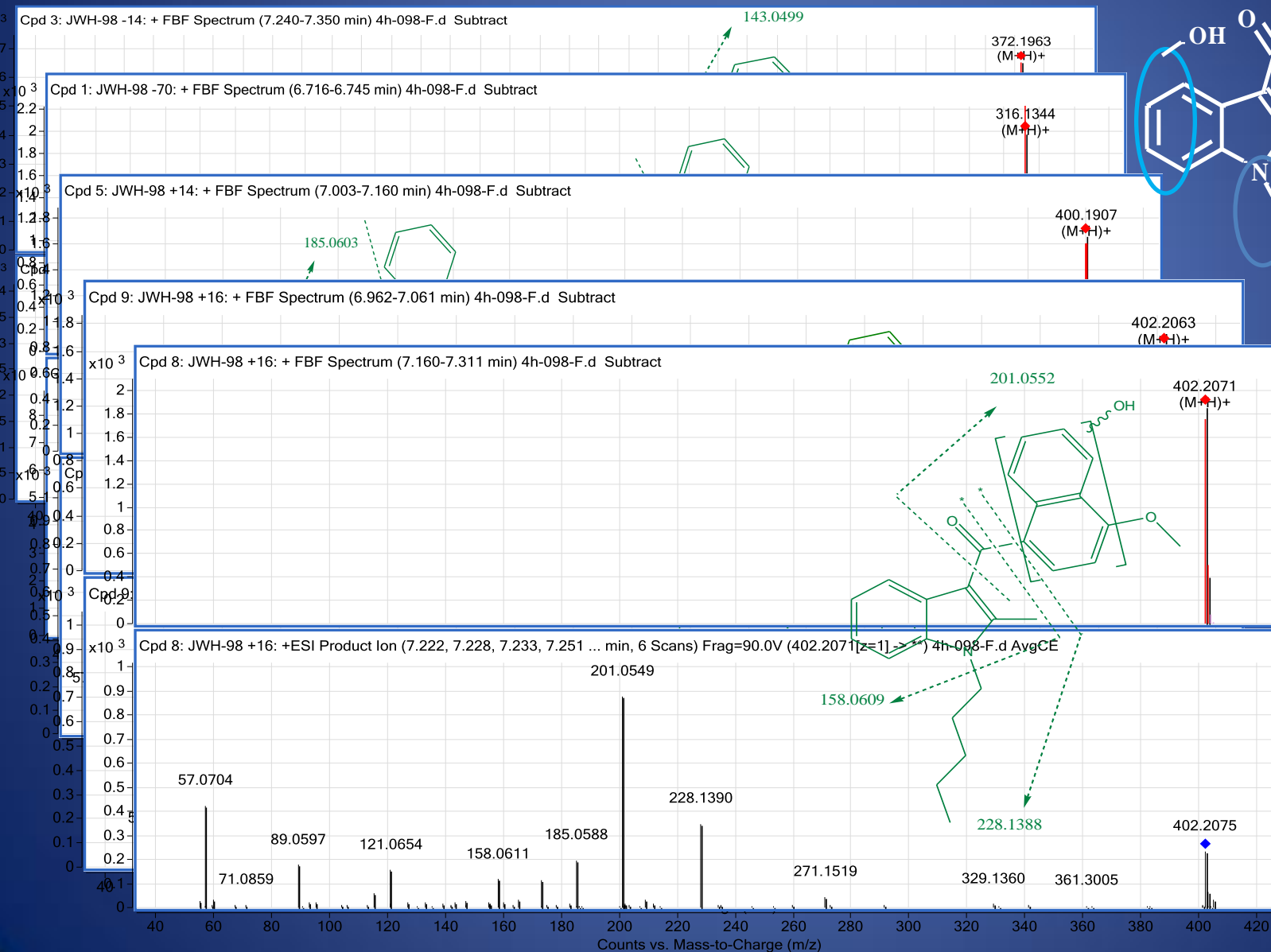
## JWH 098



...and **main metabolites** predicted



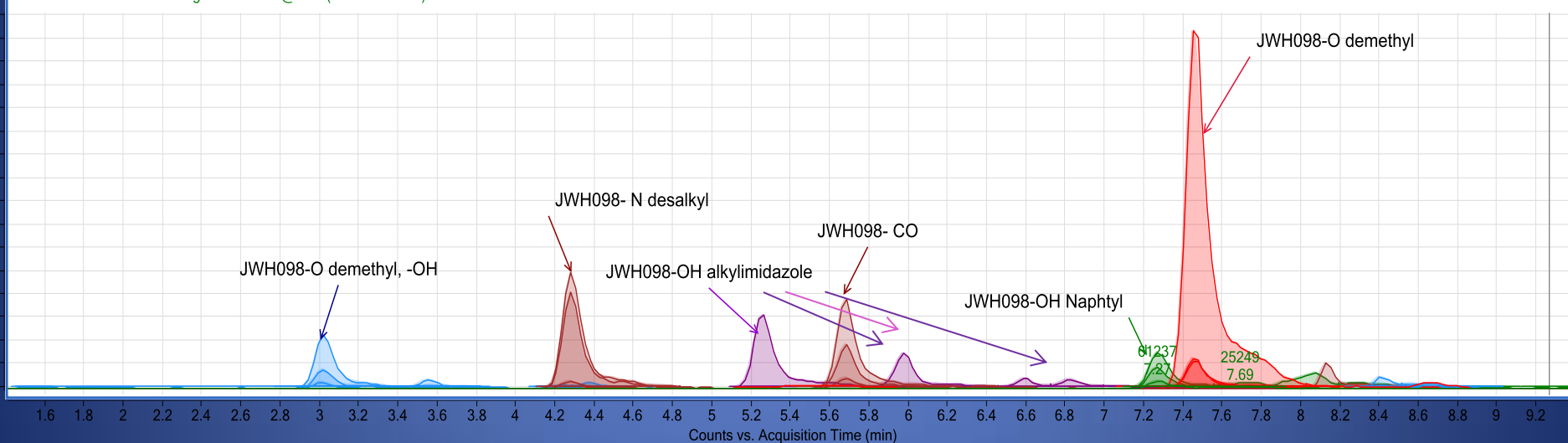
# JWH098 main metabolites detected



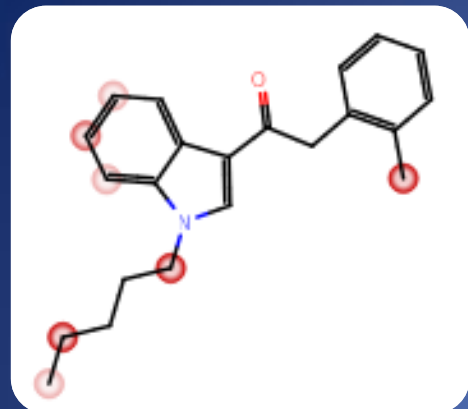


# LC-MS/MS: ionic chromatogram of main metabolites JWH 098

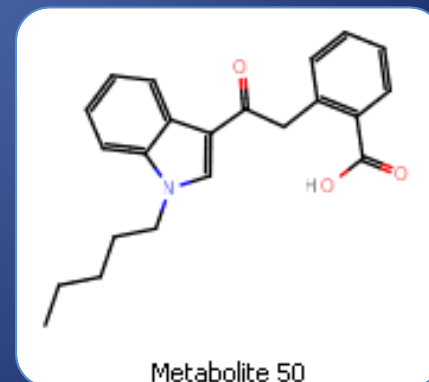
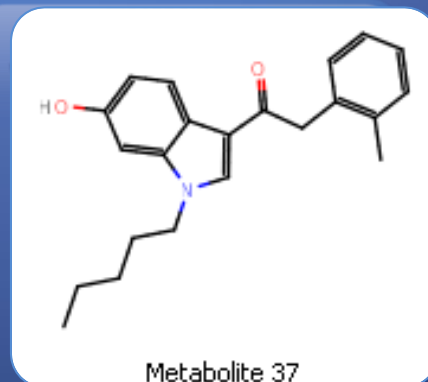
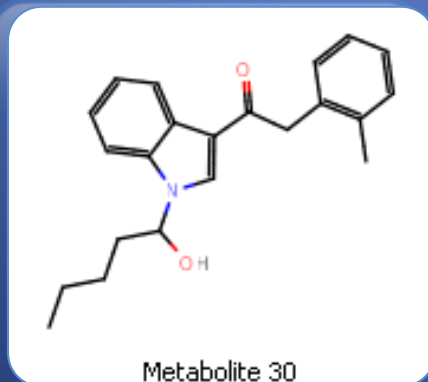
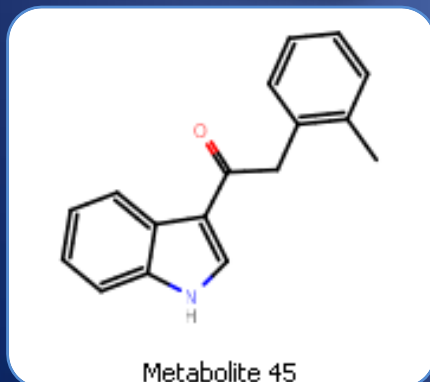
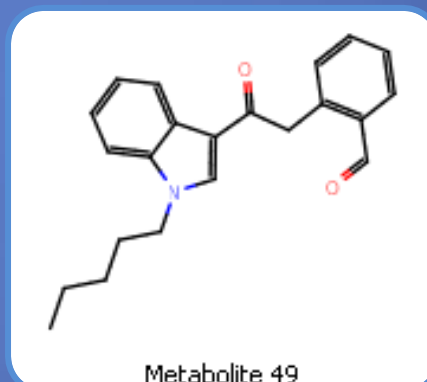
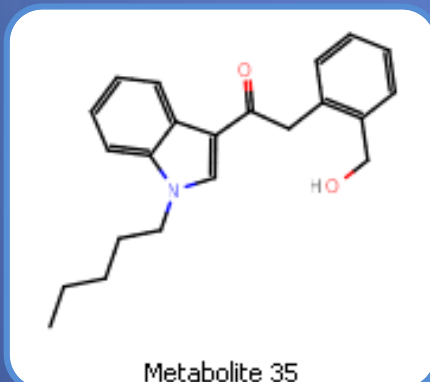
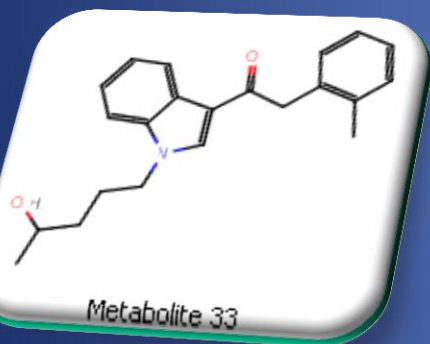
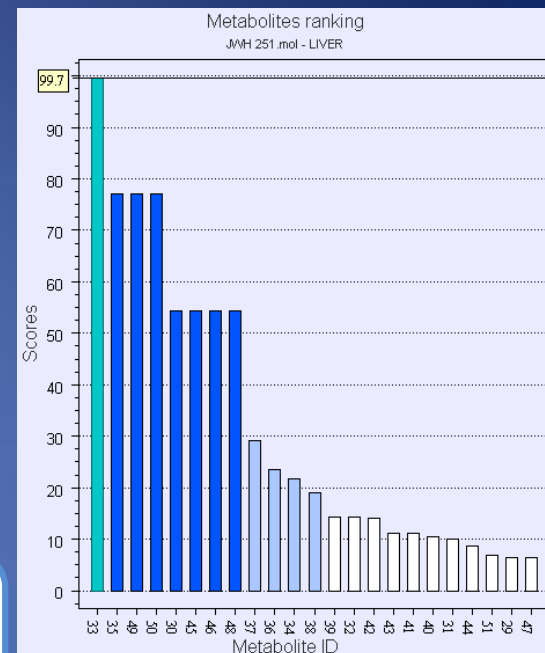
JWH 098 OH: +ESI MRM Frag=100.0V CID@40.0 (402.0 -> 228.0) 098 fettine 4h b.d



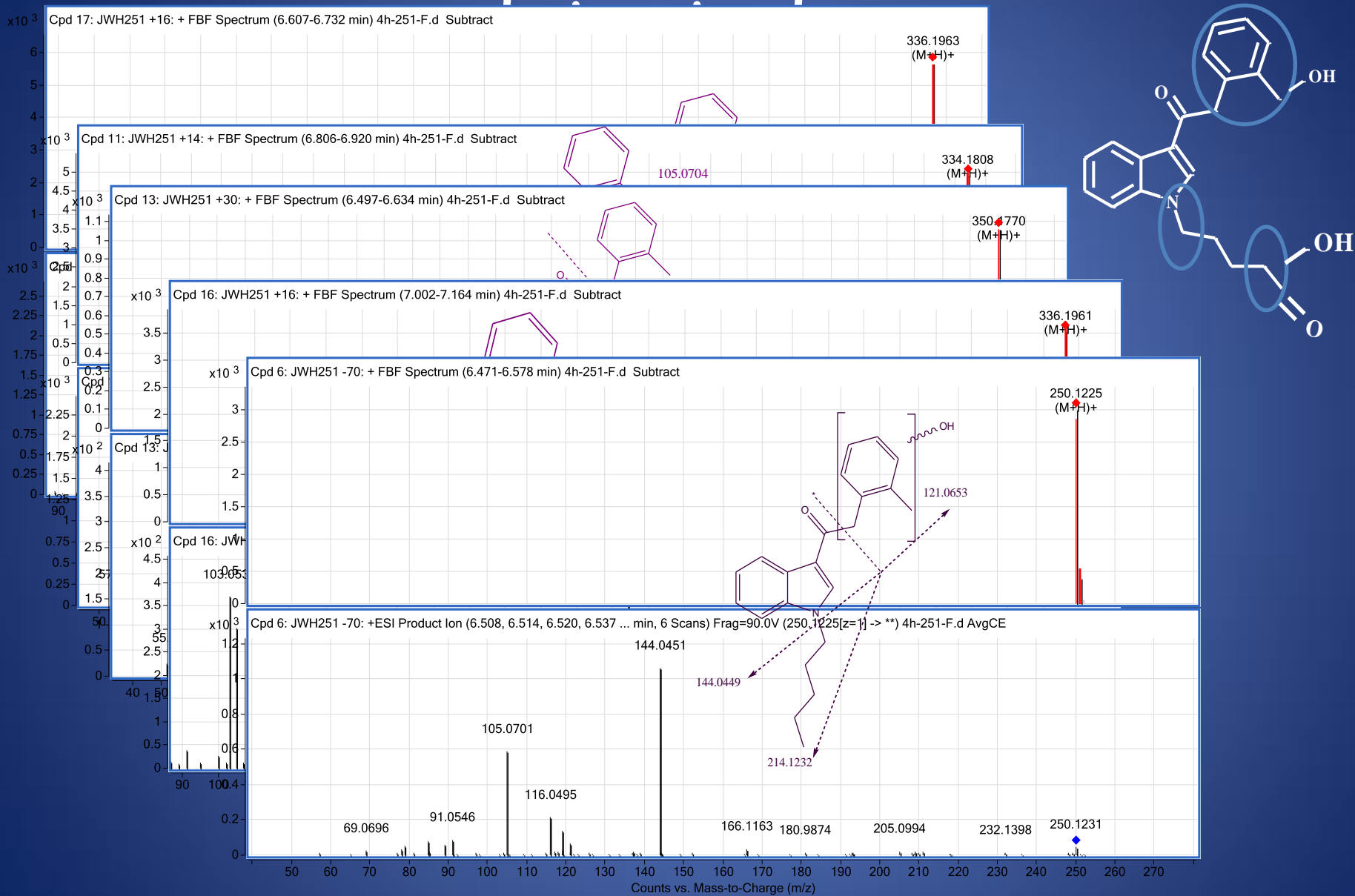
# More probable sites of metabolism: JWH 251



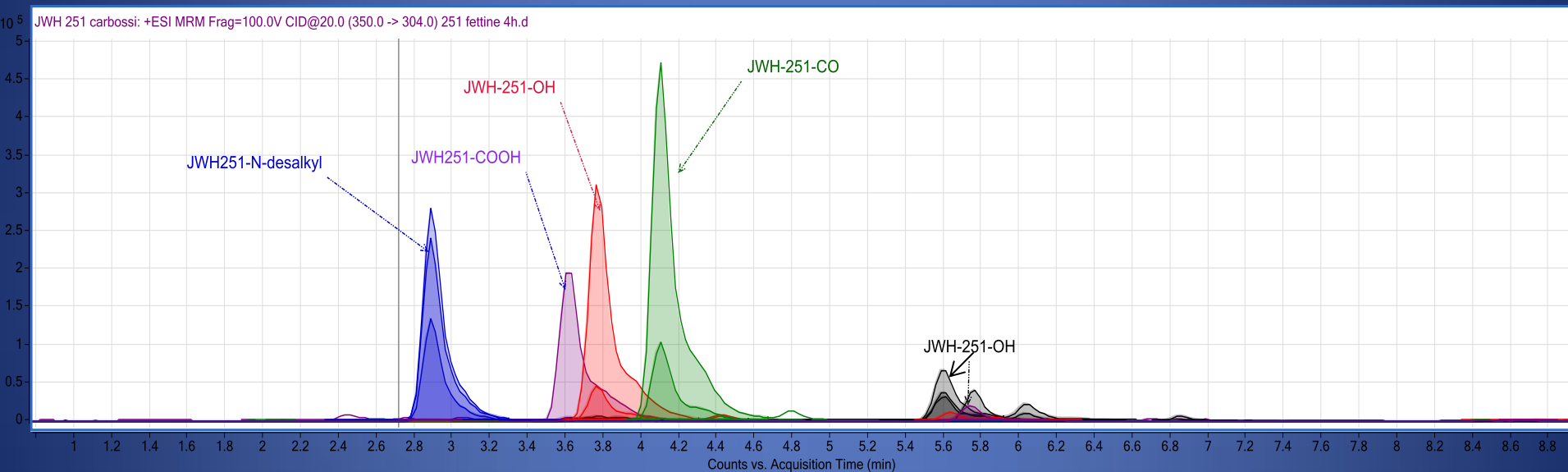
...and **main metabolites** predicted



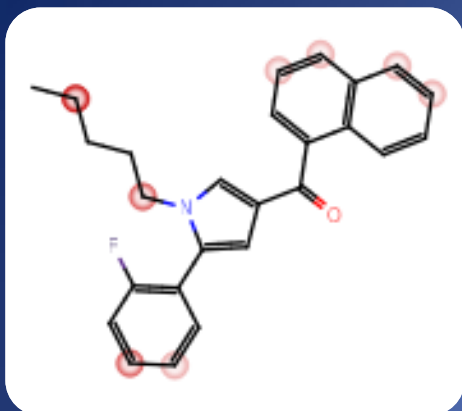
# JWH 251 main metabolites



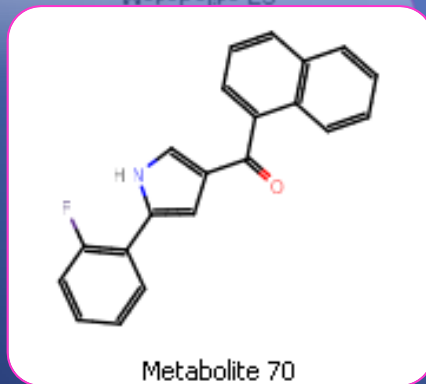
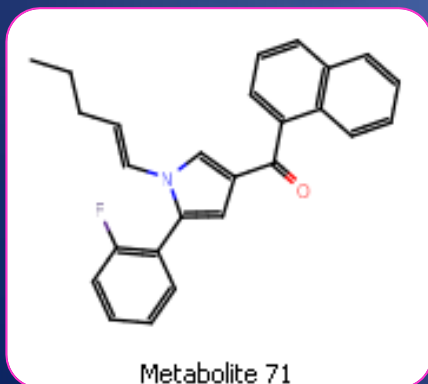
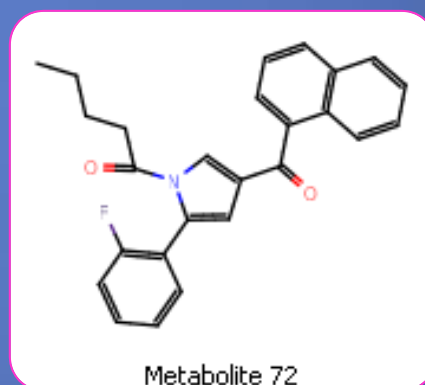
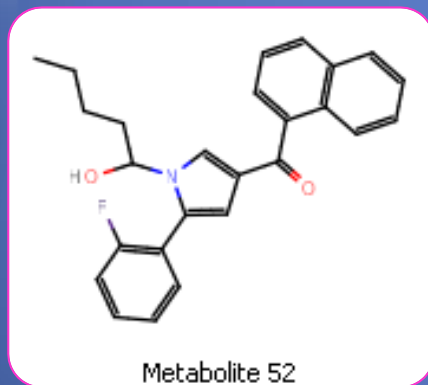
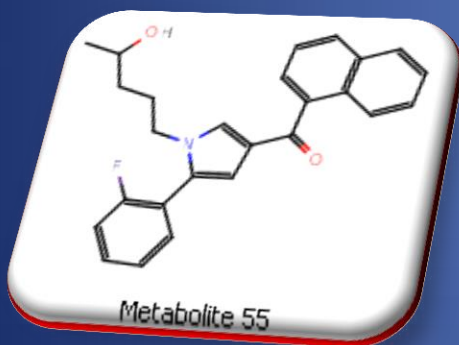
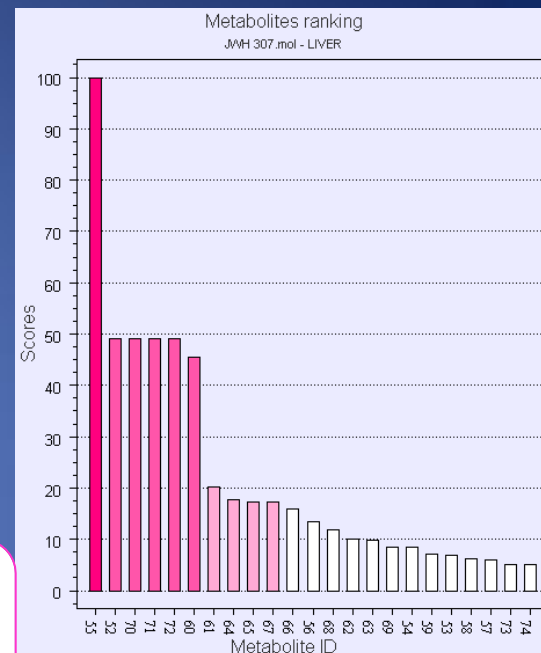
# LC-MS/MS: ionic chromatogram of main metabolites JWH 251



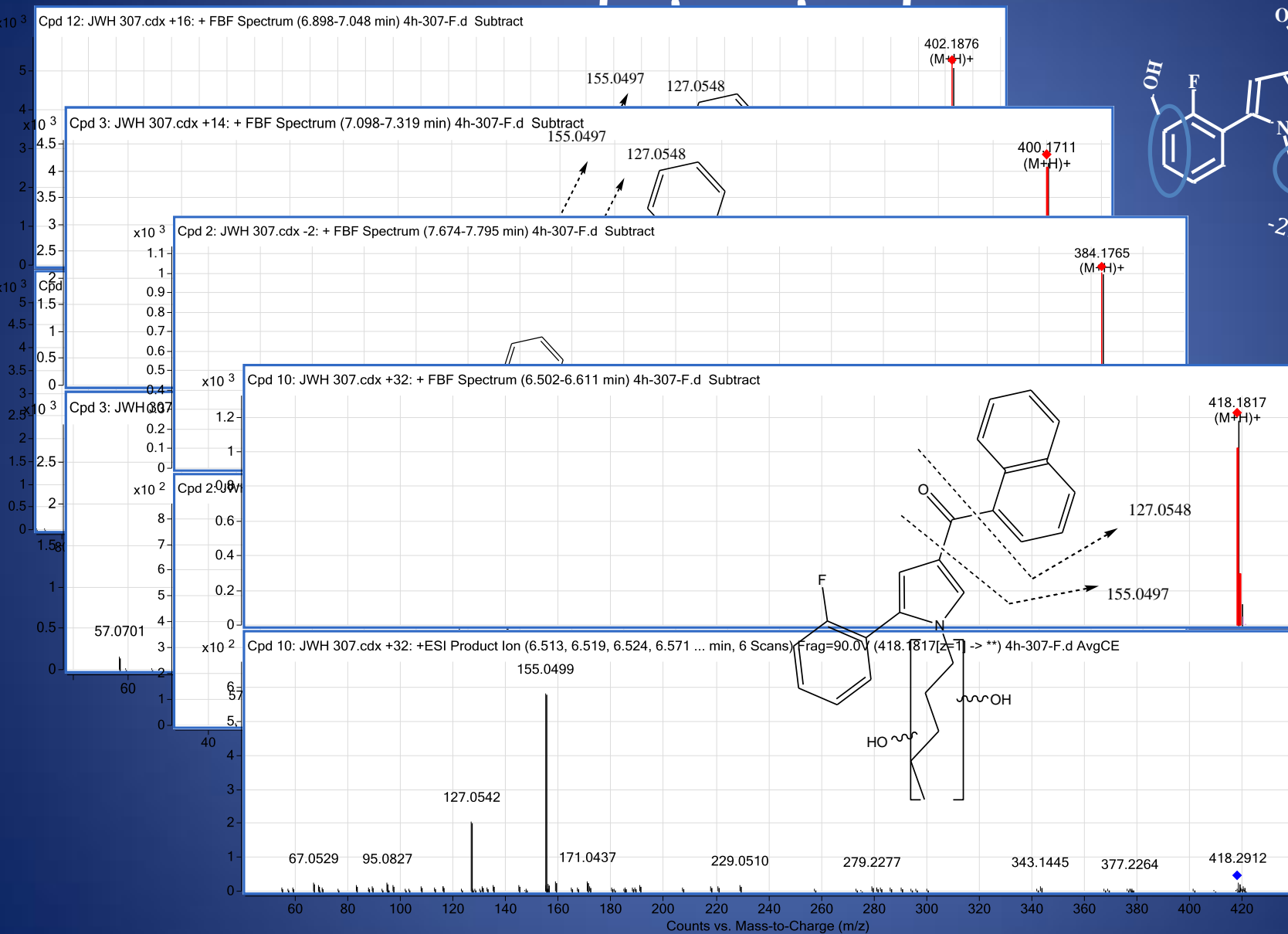
# More probable sites of metabolism: JWH307



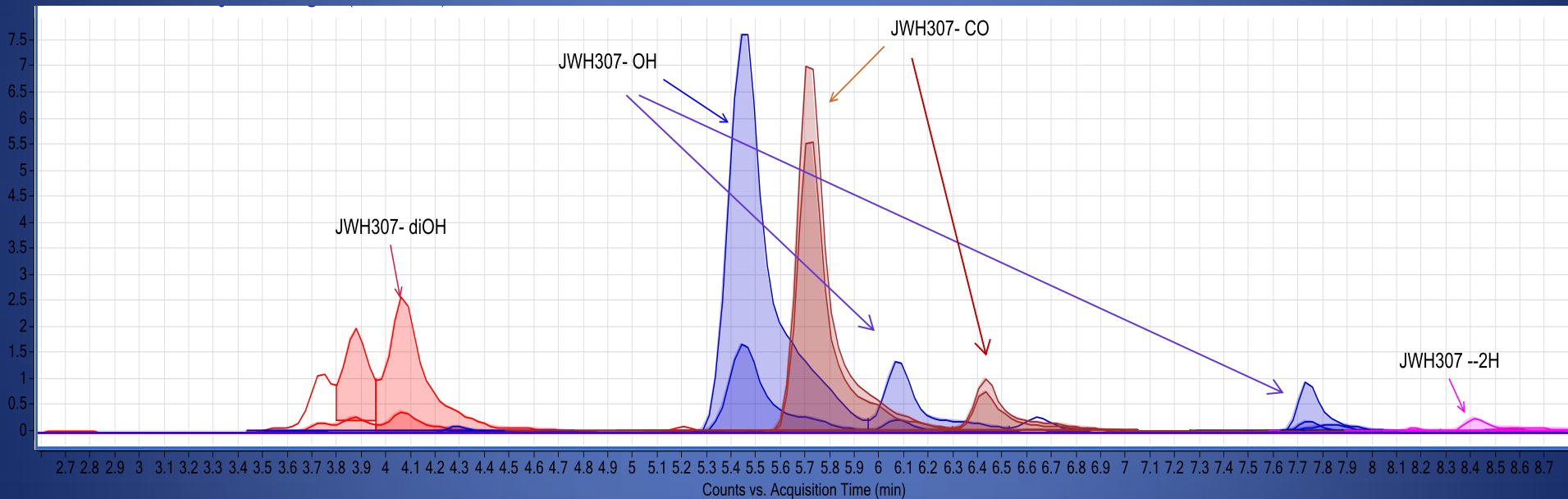
...and **main metabolites** predicted



# JWH 307 main metabolites



# LC-MS/MS: ionic chromatogram of main metabolites JWH 307





# Routine method development: MRM LC-MS/MS

- UHPLC: Agilent 1290 Infinity
- Agilent 6460 triple quadrupole mass spectrometer
- Column: superficially porous C18
- A: 5 mM ammonium formate + 0.05 % formic acid B: methanol/acetonitrile 1:1 + 0.1% of formic acid
- gradient from 50% B to 100% B within 98 min.
- MRM: transitions and CE chosen according to the best conditions in Q-TOF

# CONCLUSIONS



- ✓ Identification of main metabolites of **JWH015**, JWH098, JWH251, JWH307 by QTOF-HRMS
- ✓ The main metabolites predicted by MetaSite software were confirmed in the majority of cases by the studies performed *in vitro* on rat liver slices . The first ranked was always the main metabolite found
- ✓ **Starting point for setting up analytical methods, based on metabolism and typical fragments prediction, also in absence of analytical standards**

Police forces

Emergency care  
units

Clinical/forensic  
toxicologies  
toxicologies

Helthcare  
Institution

EWS



Sharing of  
knowledge



Grazie