UNIVERSITÄTS KLINIKUM FRANKFURT
GOETHE-UNIVERSITÄT

Coping with requirements in forensic toxicology: combination of routine laboratory and forensic science

Stefan W. Toennes, Legal Medicine, University of Frankfurt/Main, Germany

For Forensic Use

Rechtsmedizin Frankfurt am Main

German Legal Medicine & Forensic Toxicology

… between University and Hospital (& some private labs)

… but not associated with the police !

Institute of Legal Medicine, Frankfurt, Germany

University Hospital in Frankfurt/Main

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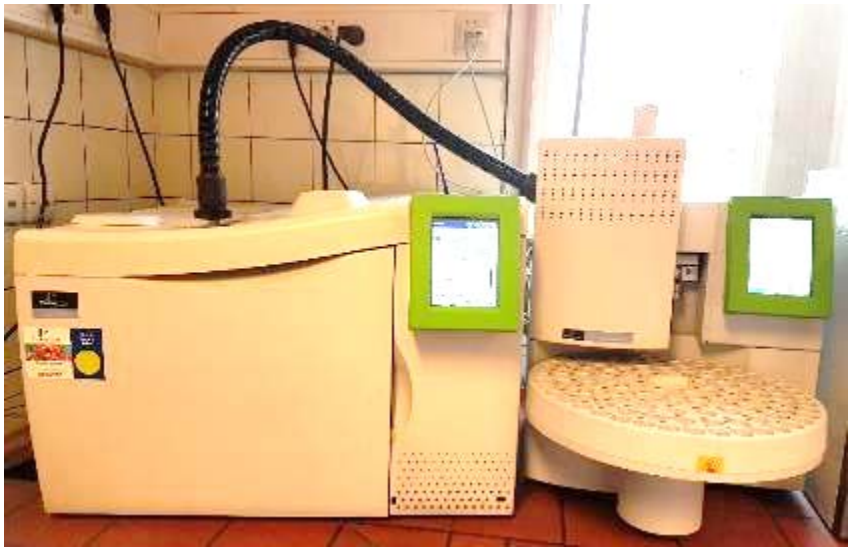
What we (are expected to) do …

- **Analyses**
  - Blood, urine, hair, post-mortem specimens, etc.
- **Expert opinions** (in writing and in court)
  - Driving under the influence of alcohol/drugs
  - Criminal responsibility
  - Any other pharmacological / toxicological aspect…

- **Teaching** (university students, police officers, …)
- Forensic **scientific studies**

Driving under influence of **alcohol**

- Blood alcohol
  - German guideline requires 2 different analyses
  - Typical: headspace-GC-FID and ADH assay



Driving under influence of alcohol or **drugs**

- Blood alcohol
- Drugs: regionally different requirements (federal)

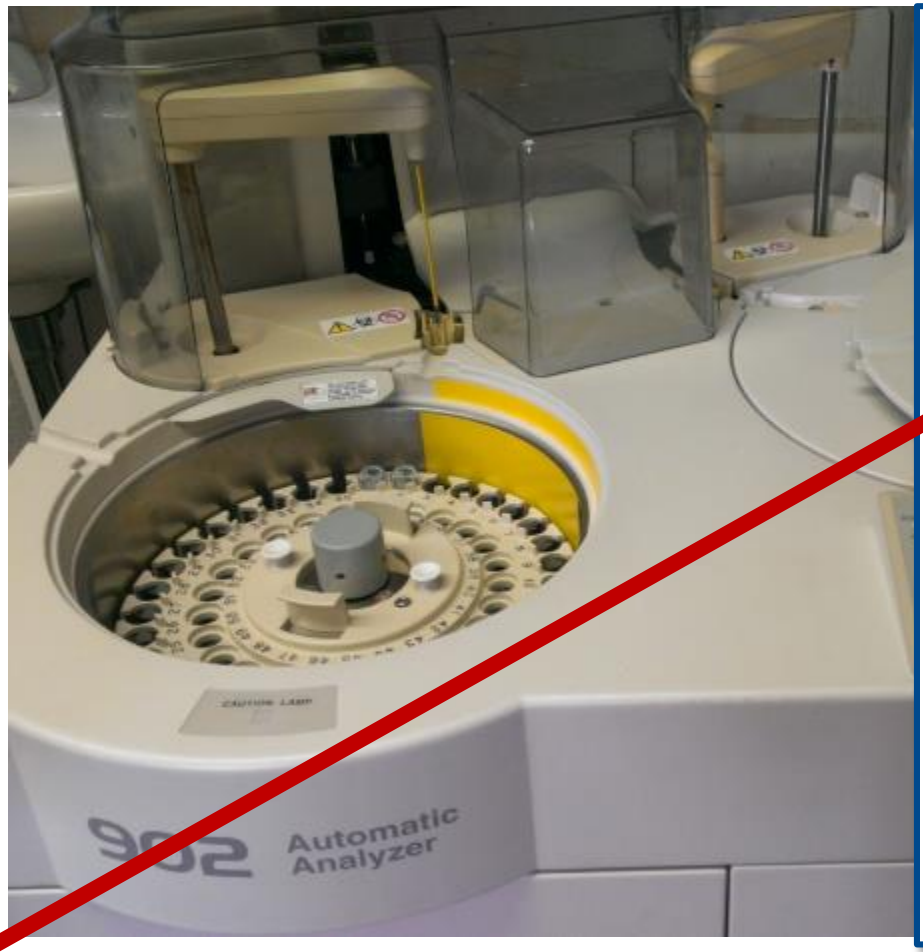
  □ Predefined selection of abused drugs
  (i.e. cannabis, cocaine, amphetamines, opiates)

  □ All possibly impairing abused or medical drugs

⇒ Different analytical strategies

Driving under influence of alcohol or **drugs**

Drugs of abuse „screening“: immunochemistry



Limitation by kit availability

- Cannabis
- Amphetamine
- Cocaine(metabolite)
- Opiates
- Methadone
- Benzodiazepines
- Buprenorphin
- Tricyclic antidepressants
- Barbiturates

„Target compound screening“ (LC-MS/MS)

Immunochemistry	vs. chromatography (LC-MS/MS, targeted)
Cannabis	THC, THC-carboxylic acid
Amphetamines	Amphetamine, methamphetamine, MDMA, MDA, MDE, ephedrine, phenylpropanolamine, norephedrine, norpseudoephedrine, kath
Cocaine	Benzoylecgonine
Opiates	Morphine, codeine, 6-acetylmorphine, dihydrocodeine, oxycodone, hydromorphone
Methadone	Methadone
Benzodiazepines	Diazepam, nordiazepam, temazepam, oxazepam, clonazepam, bromazepam, tetrazepam, midazolam, flurazepam, phenazepam, nitrazepam, flunitrazepam, alprazolam, brotizolam, lorazepam, lormetazepam, diclazepam
	Zolpidem, zopiclon, diphenhydramine, doxylamine
	Venlafaxine, risperidone, paliperidone, citalopram, escitalopram, doxepin, amitriptyline, nortriptyline, quetiapine, mirtazapine, chlorphenamine, fentanyl, tramadol, tilidine
	LSD, ketamine, methylphenidate, dextromethorphan

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	LSD, **ketamine**, methylphenidate, dextromethorphan

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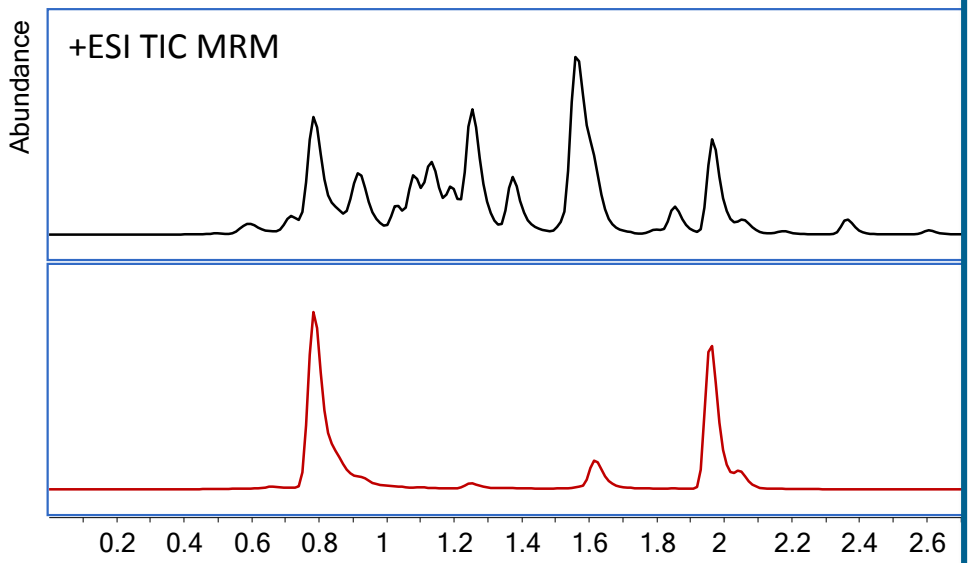
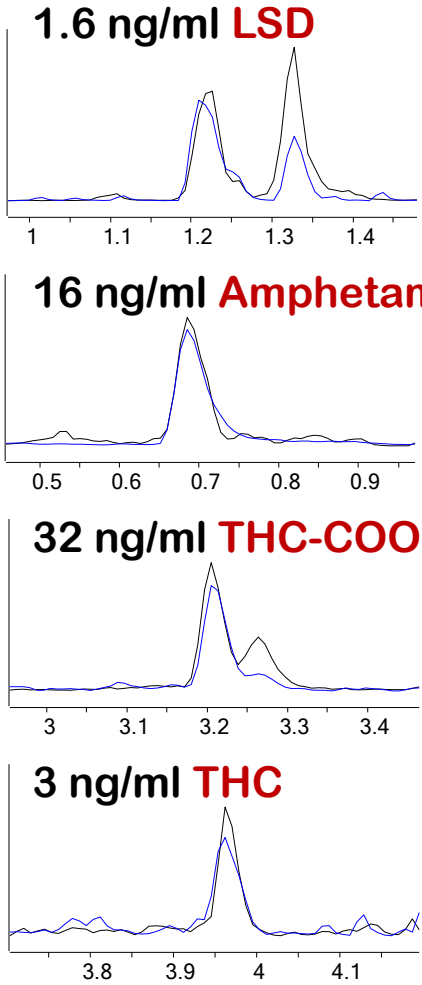
„Target compound screening“ (LC-MS/MS)

- Sample 0.2 ml serum/plasm
-
-
-
-
-
-
-

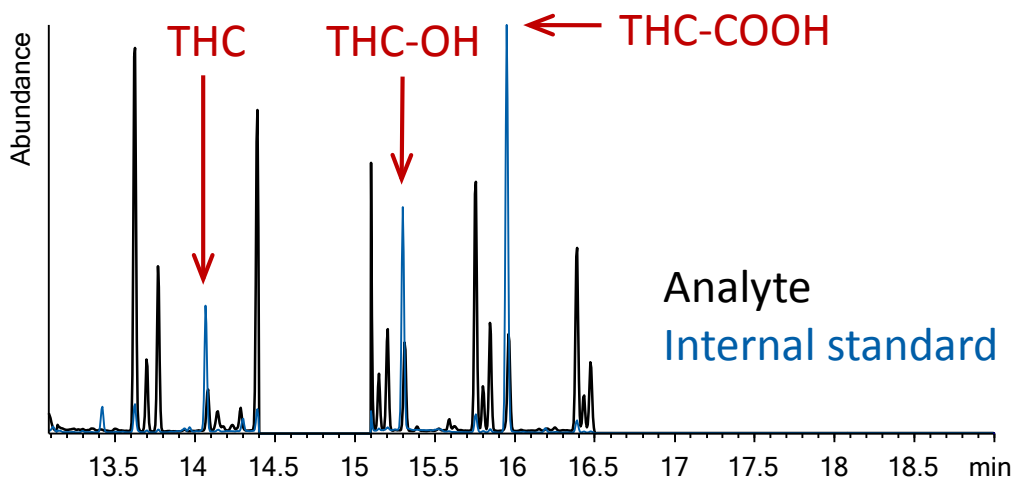
Quality control for pos/neg decision





„Historical“ GC-MS SIM

- 1 ml serum, C18 SPE,
  Trimethylsilylation (40 µL MSTFA),
  Agilent 30 m VF-1ms, GC-EI MS SIM
- Sample2Sample: 22-23 min



- LLOQs

THC	**1.0 ng/ml**
THC-OH	0.3 ng/ml
THC-COOH	3.0 ng/ml

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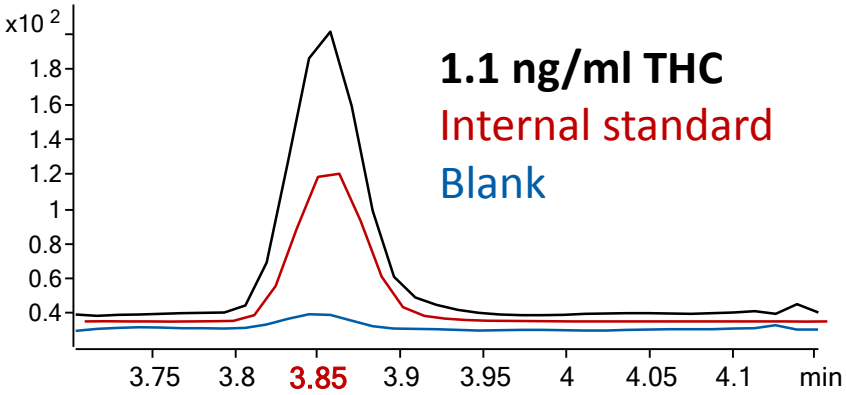
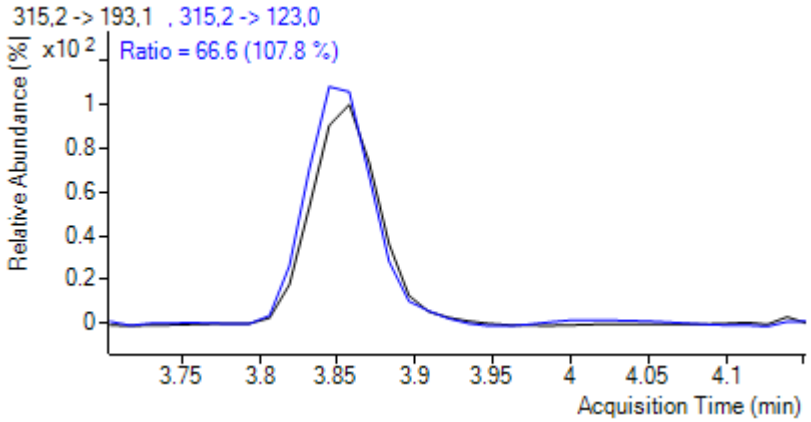
LC-MS/MS





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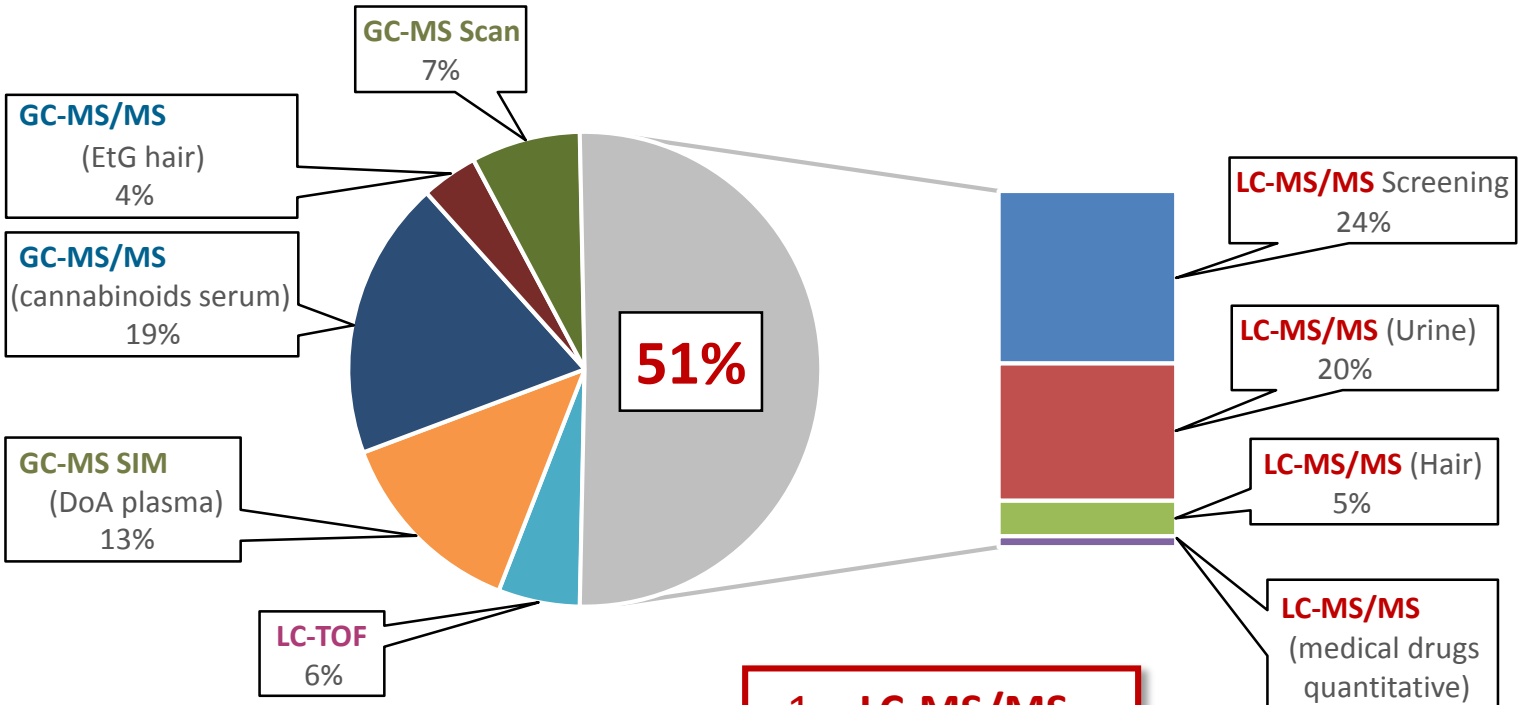
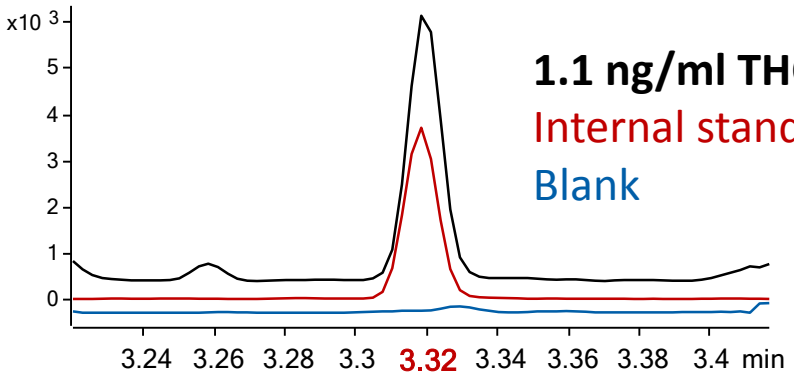
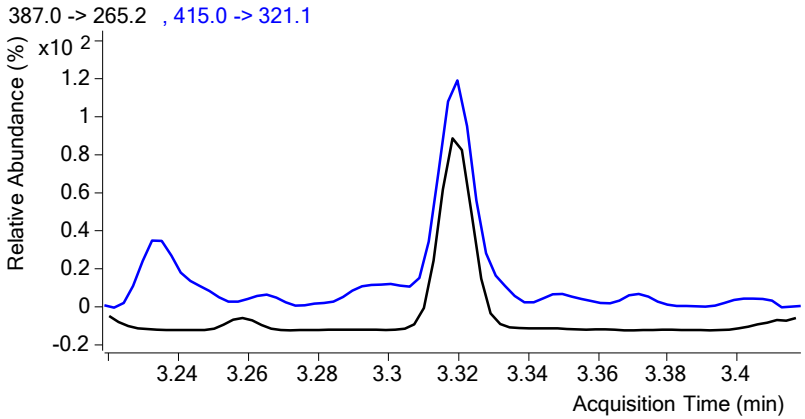
How we manage our routine analyses…



1	LC-MS/MS
1	GC-MS/MS
2	GC-MS
1	LC-TOF MS

GC-MS/MS





LTM column
(low thermal mass)

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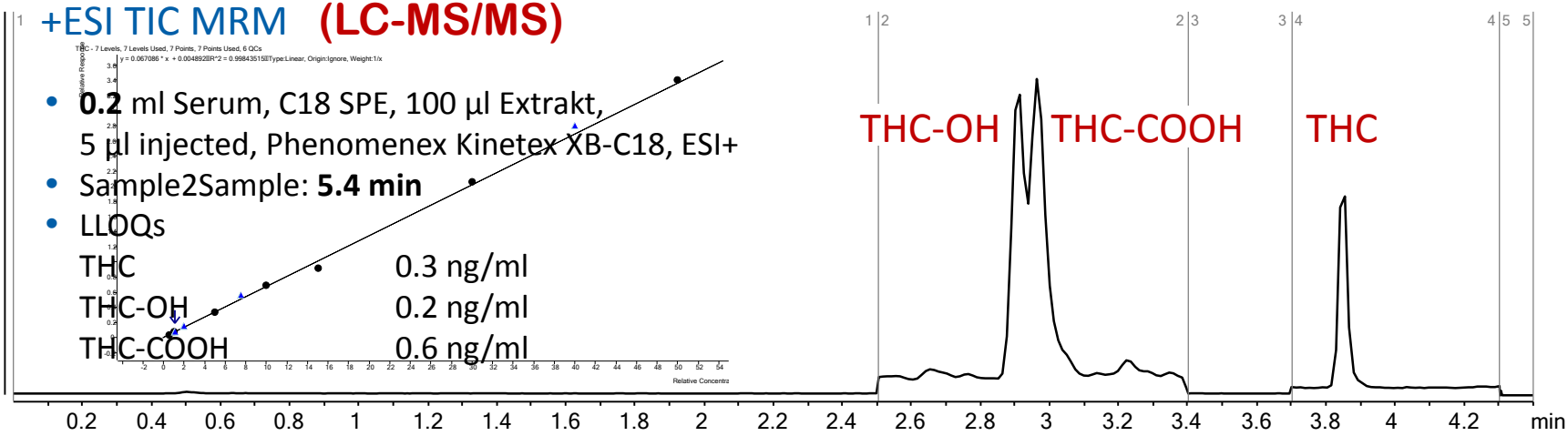
GC-MS/MS complementary to LC-MS/MS

+ESI TIC MRM **(LC-MS/MS)**

- **0.2** ml Serum, C18 SPE, 100 µl Extrakt, 5 µl injected, Phenomenex Kinetex XB-C18, ESI+
- Sample2Sample: **5.4 min**
- LLOQs

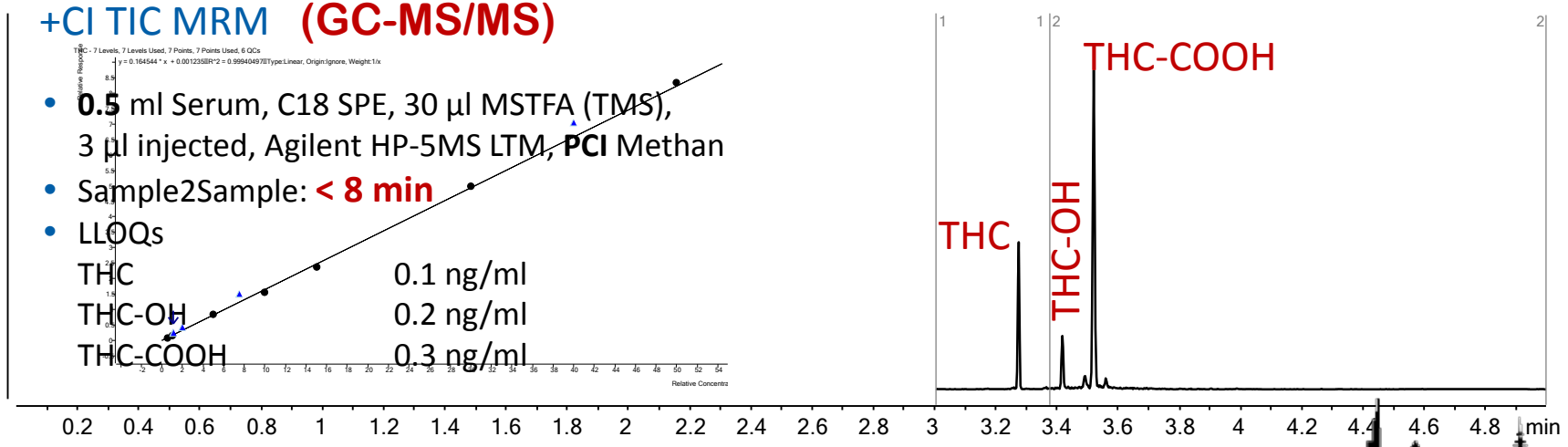
THC	0.3 ng/ml
THC-OH	0.2 ng/ml
THC-COOH	0.6 ng/ml



+CI TIC MRM **(GC-MS/MS)**

- **0.5** ml Serum, C18 SPE, 30 µl MSTFA (TMS), 3 µl injected, Agilent HP-5MS LTM, **PCI** Methan
- Sample2Sample: **< 8 min**
- LLOQs

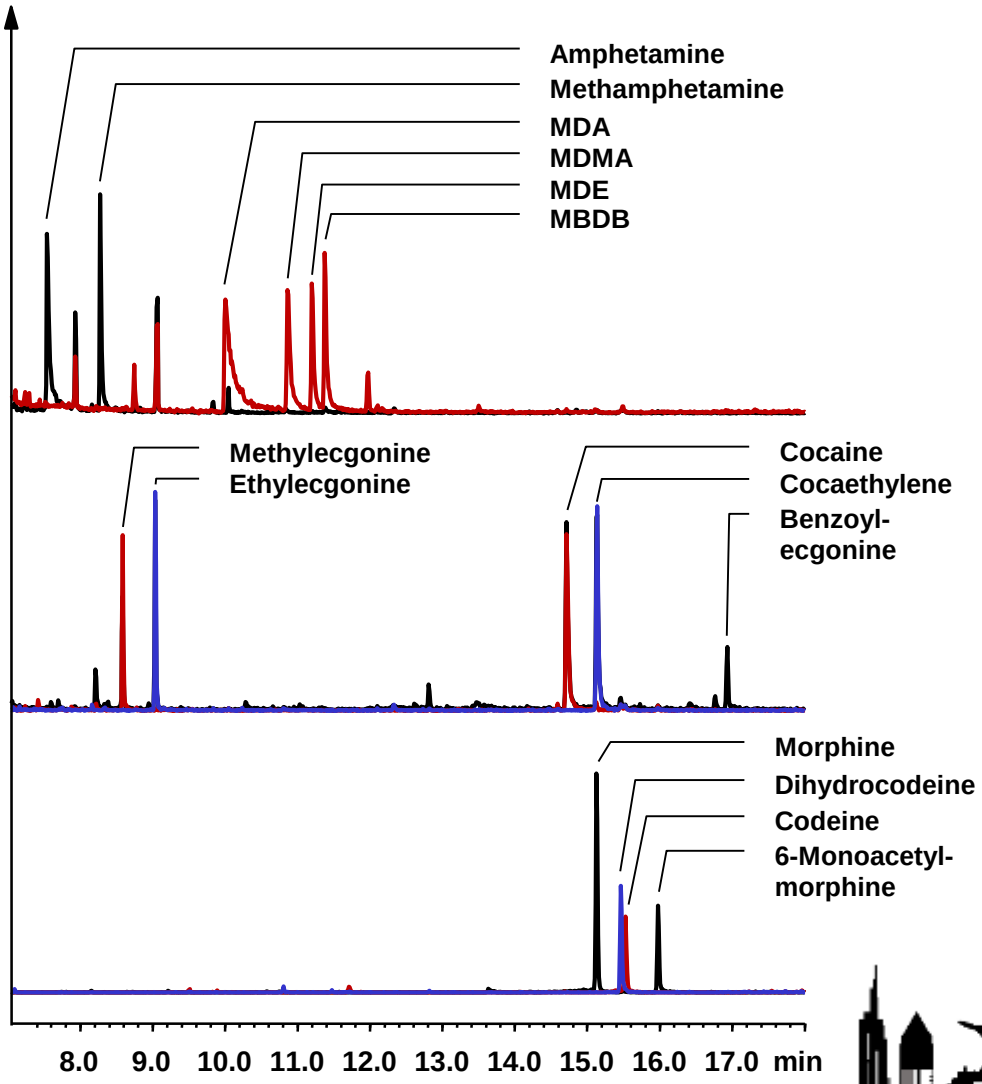
THC	0.1 ng/ml
THC-OH	0.2 ng/ml
THC-COOH	0.3 ng/ml



Analysis of basic drugs with GC-MS SIM

- 0.5 ml serum, Certify HF
- 40 µl **MBTFA** +
  40 µl **MBDSTFA**
- Agilent 30 m VF-1ms,
  GC-EI MS SIM

- Sample2Sample: ca. 23 min
- LLOQs (16 analytes)
  7 < 2 ng/ml
  5 < 5 ng/ml
  4 < 10 ng/ml



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Driving under influence of alcohol or drugs

„All possibly impairing abused or medical drugs“

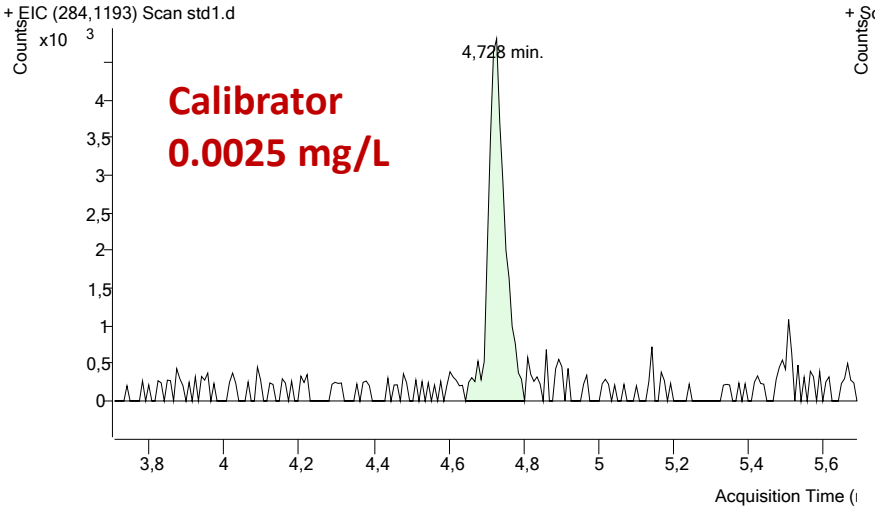
- Sensitive *targeted screening* sufficient in most cases
- Prior to reporting: evaluation of case documentation
  (statements of suspect, police observations,
  medical officer observations)
  Medication? Other drugs? Illnesses? Symptoms?
- Further (specialized) analyses (e.g. NPS)
- Additional *untargeted screening*, e.g. in accidents cases

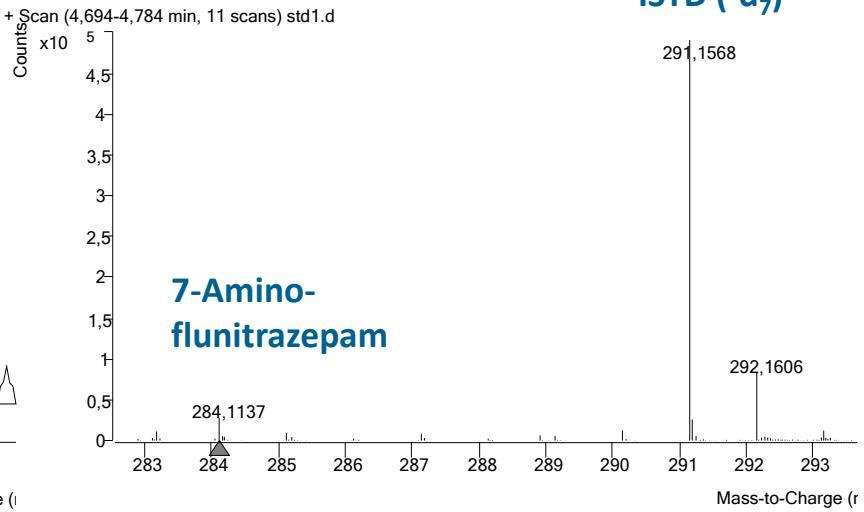
LC-TOF MS

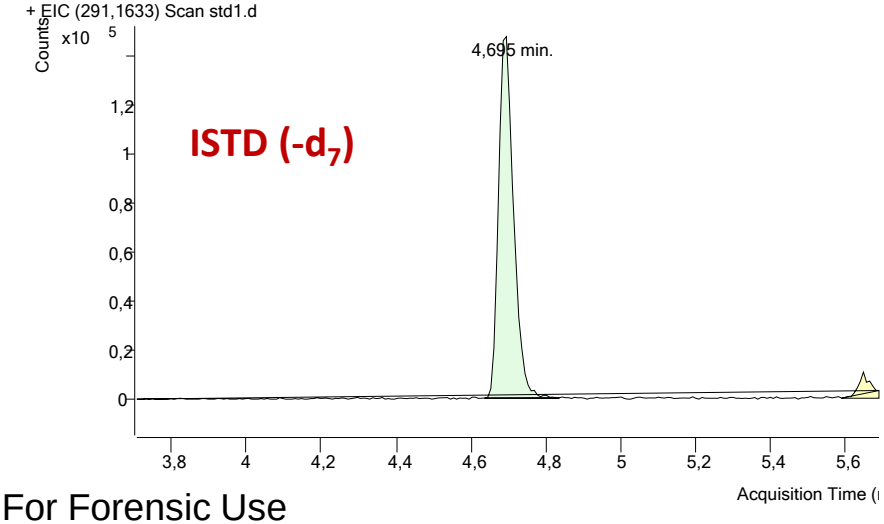
- Single stage
- No fragmentation
  (in-source fragmentation = „All-Ions“ @ 125, 190 and 230 V results in large data files)

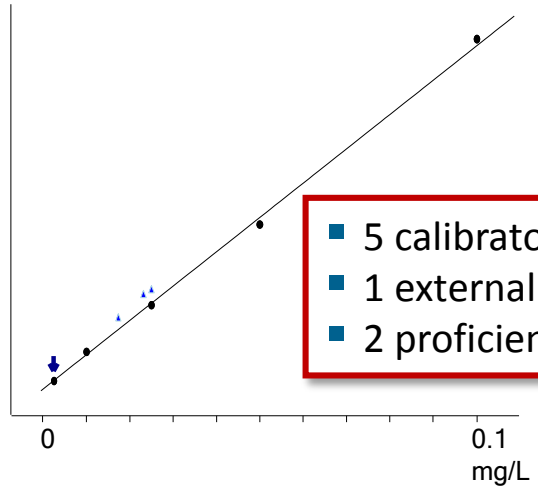
- Screening with in-house database
  (covering psychoactive medical drugs, designer stimulants – benzodiazepines – fentanyls)
- Sensitive detection
- Quantification (confirmed by proficiency tests)

LC-TOF quantification: 7-aminoflunitrazepam



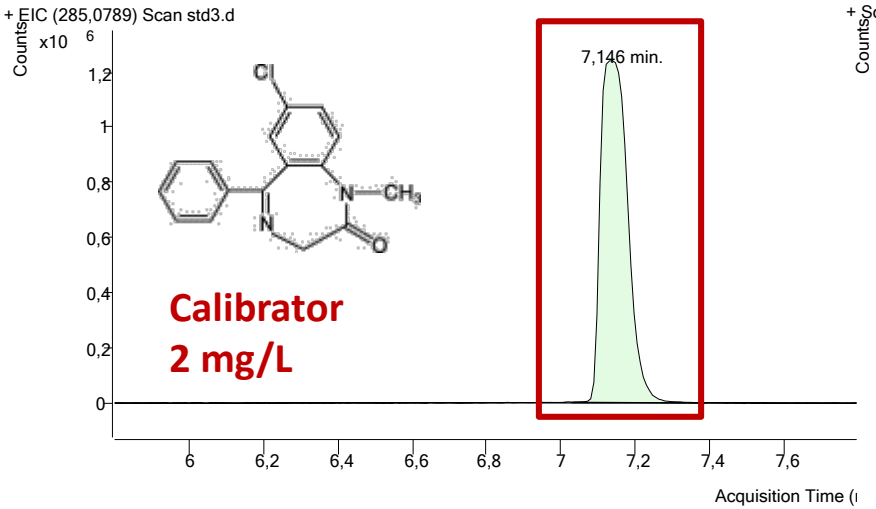


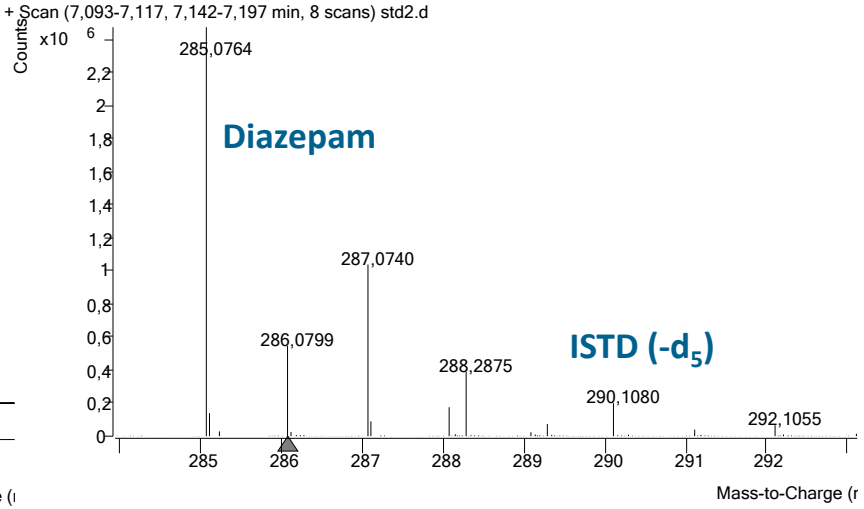


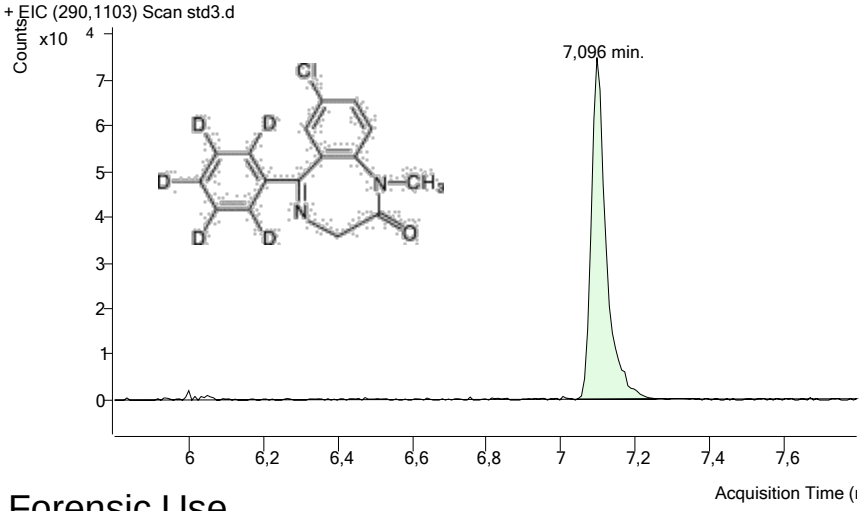


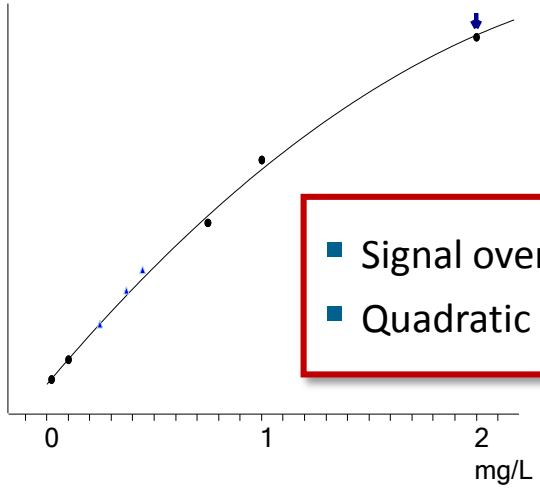
- 5 calibrators
- 1 external accuracy control
- 2 proficiency test samples

LC-TOF quantification: diazepam



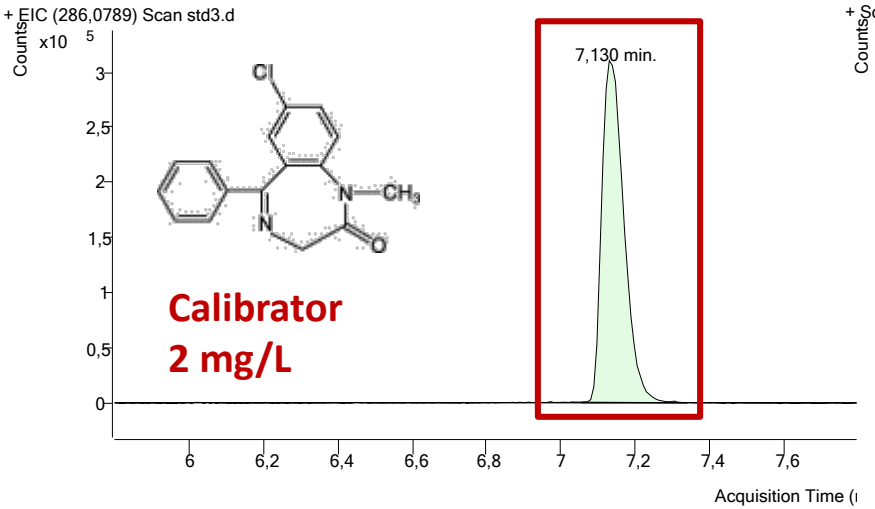


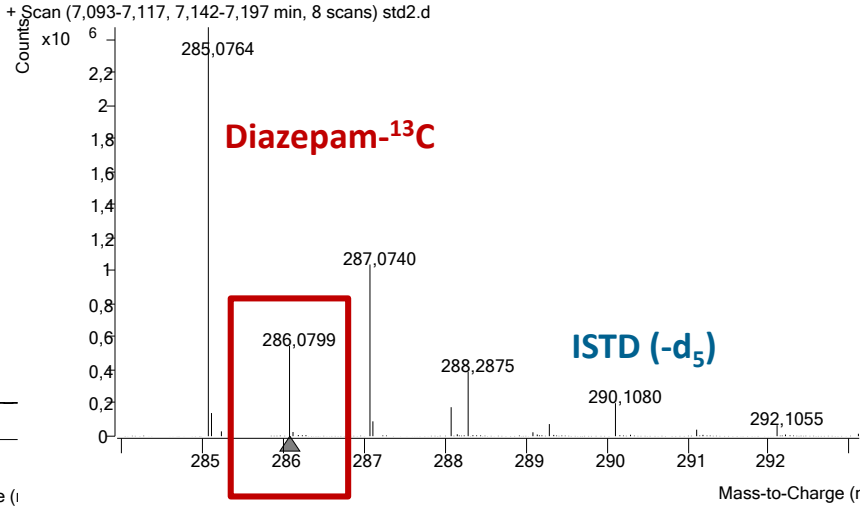


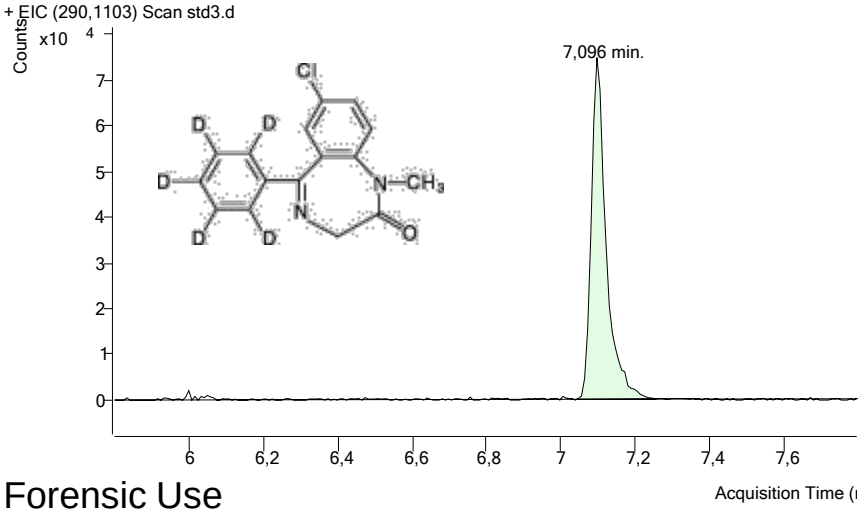


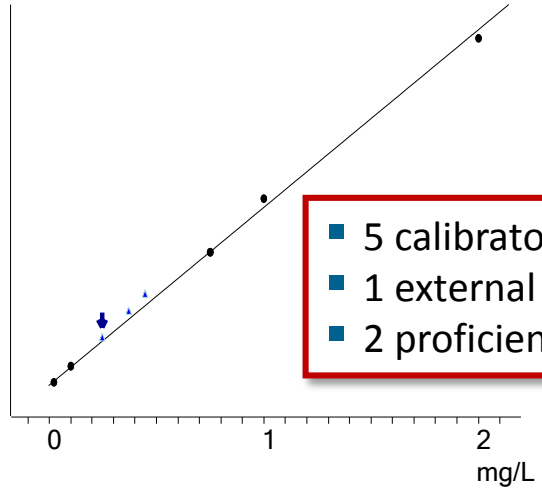
- Signal overloaded
- Quadratic calibration

LC-TOF quantification: diazepam-^{13}C









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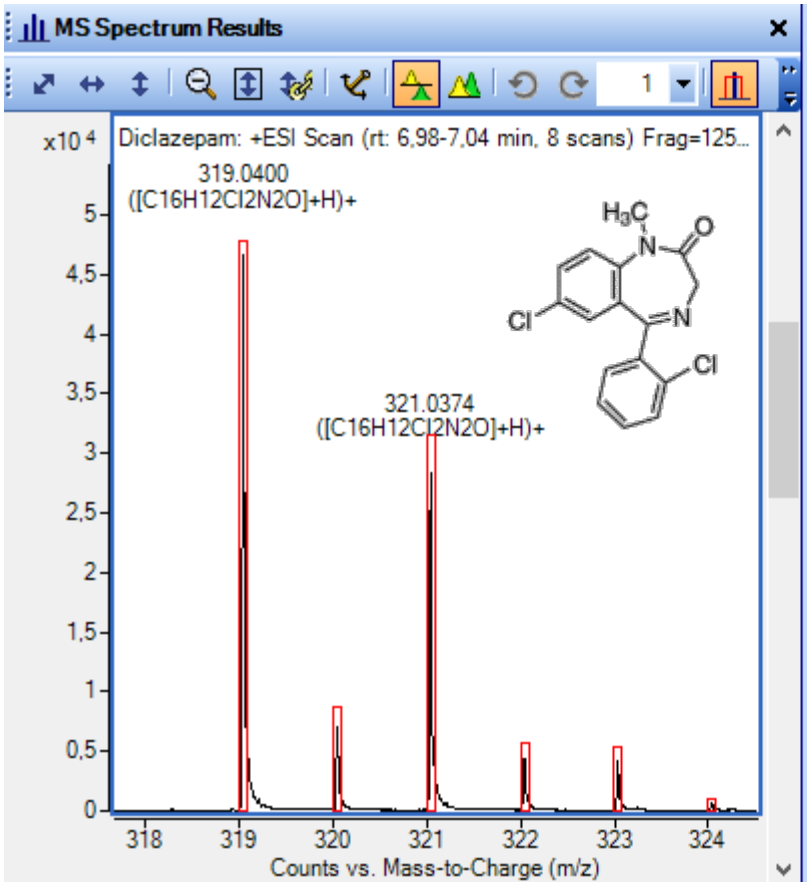
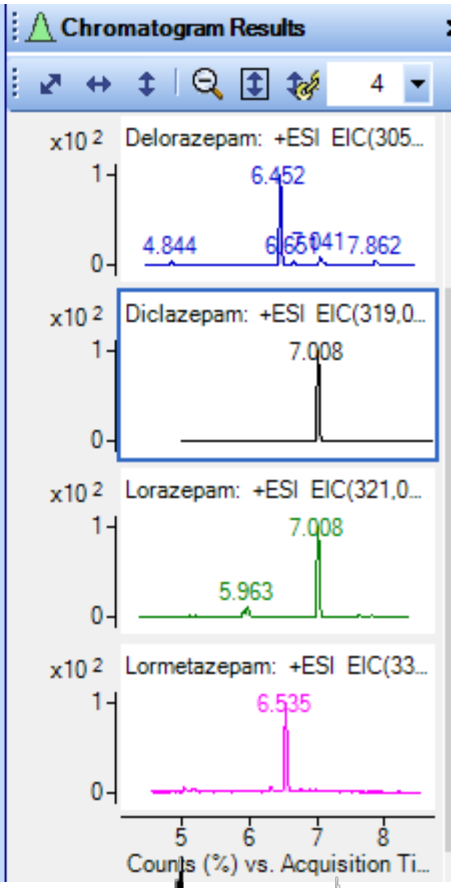
Drug assisted assault case

Label	Formula	RT	Flag	Score	Diff (Tgt, ppm)	m/z	Mass
Delorazepam	C15 H10 Cl2 N2 O	6,45		98,95	-2,53	305,0245	304,0163
Diclazepam	C16 H12 Cl2 N2 O	7,01		98,6	0,36	319,04	318,0328
Lormetazepam	C16 H12 Cl2 N2 O2	6,54		97,24	-1,02	357,0168	334,0272
Lorazepam	C15 H10 Cl2 N2 O2	5,96	low score	60,54	13,87	321,0202	320,0164





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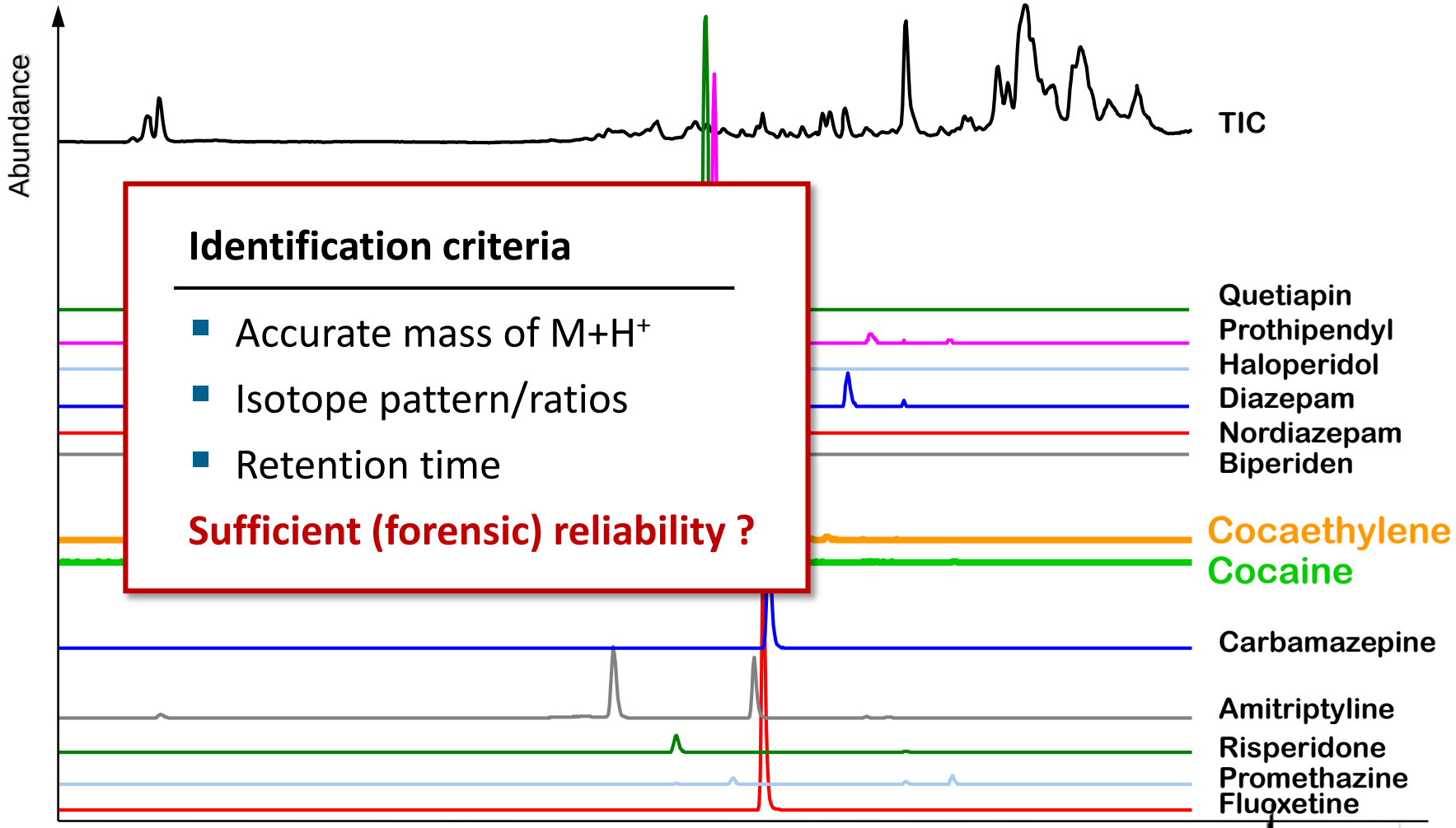
LC-TOF screening: psychiatric hair sample



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LC-TOF: amphetamine in hair with All-Ions

MassHunter PCDL Manager for Forensics and Toxicology - D:\MassHunter\PCDL\Master CAG_10_08_15.cdb

File Edit View PCDL Links Help

Find Spectra

Single Search | Batch Search | Batch Summary | Edit Compounds | Spectral Search | Browse Spectra | Edit Spectra

Mass
Precursor ion:
Tolerance: 200 ppm mDa

Ion polarity:
Ionization mode

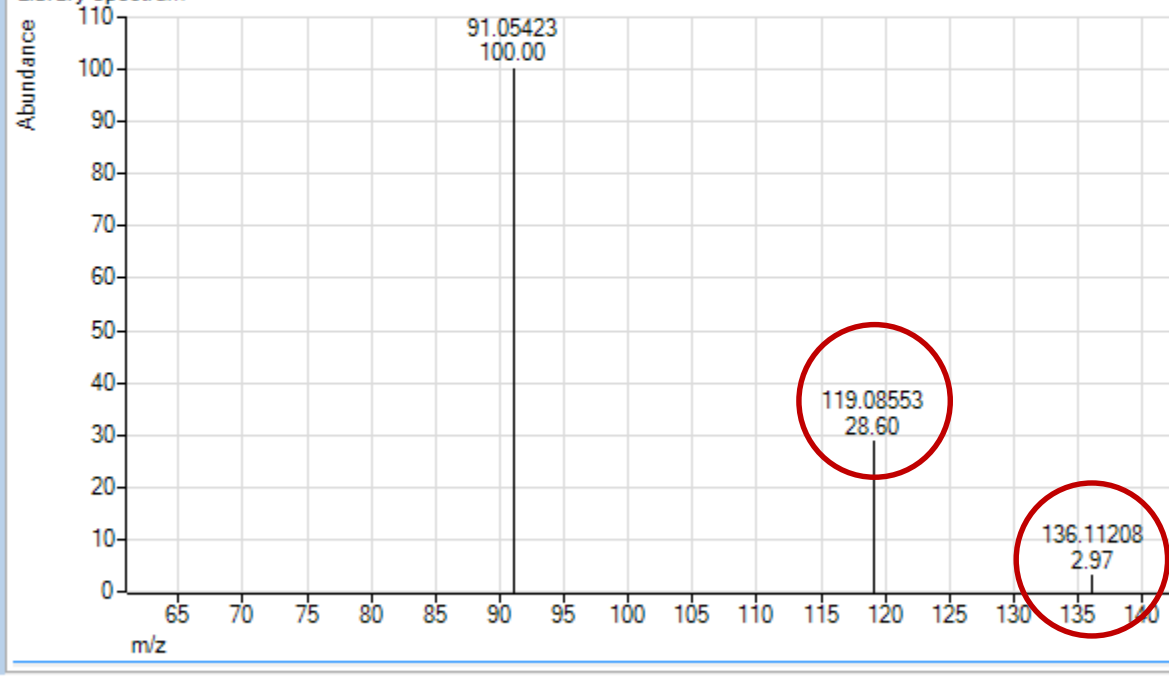
Collision energy
Tolerance: 2.0 eV

Spectra for compound: Amphetamine (Amfetamine)

Compound Name	Ion Species	Precursor Ion	CE (V)	Po
Amphetamine (Am...	(M+H)+	136.11208	10	Pos
Amphetamine (Am...	(M+H)+	136.11208	20	Pos
Amphetamine (Am...	(M+H)+	136.11208	40	Pos

Graphic | Mass List

Library spectrum

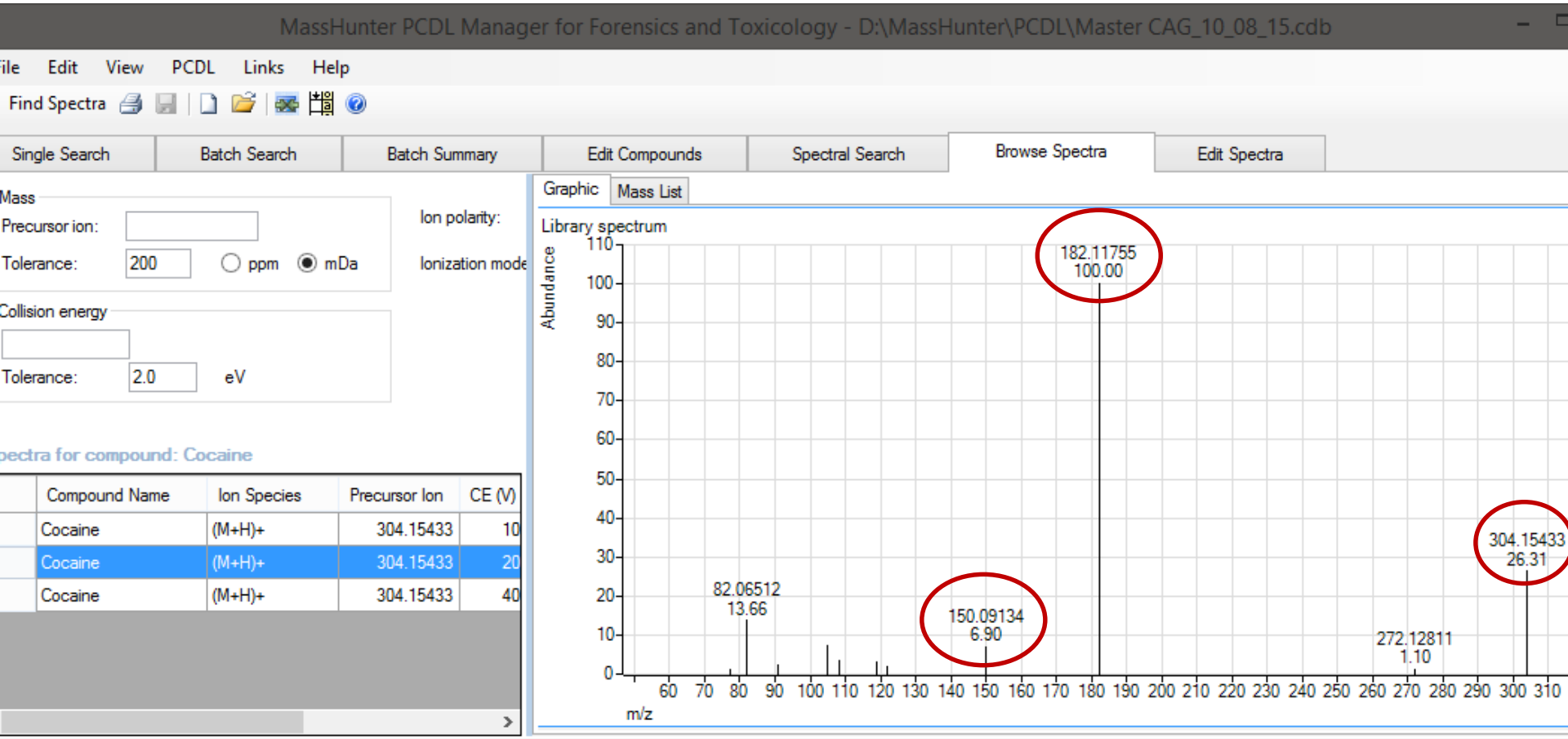


For Forensic Use

Single Search Results: 1 hit for

Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name
Amphetamine (Amfetamine)	C9H13N	135.10480	☐	☐		300-62-9	13852819	1-Phenyl-2-propanamine

LC-TOF All-Ions: cocaine in hair



Single Search Results: 8 hits

Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Spectra
Tropacocaine	C15H19NO2	245.14158	☐	☐		537-26-8	10377	8-Methyl-8-azabicyclo[3.2.1]oct-3-yl benzoate	3
4-Fluorotropacocaine	C15H18FNO2	263.13216	☐	☐		498558-69-3	4321292	8-Methyl-8-azabicyclo[3.2.1]oct-3-yl 4-fluorobenzo...	0
Norcocaine	C16H19NO4	289.13141	☐	☐		18717-72-1	559083	methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-azabicycl...	3
Cocaine	C17H21NO4	303.14706	☐	☐		50-36-2	10194104	Methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-a...	3

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Drugs of abuse in hair (LC-MS/MS)

- Hair decontamination
  shredding with scissors
  ½ h ball mill pulverization
  25 mg typical sample weight
  2 h ultra sonication (1 ml MeOH)
  filtration and evaporation
  100 µl LC solvent
  4 µl injection volume
  ESI+ LC-MS/MS
- Sample2Sample: 12 min

53 Analytes, 28 deuterated internal standards

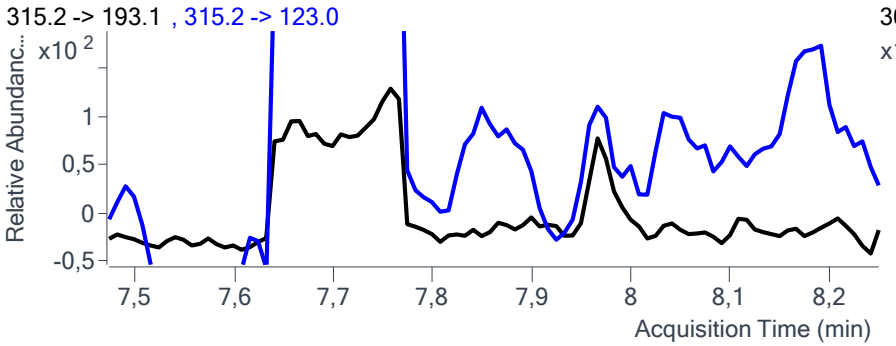
- Cannabis
  Tetrahydrocannabinol (THC), Cannabinol, Cannabidiol
- Amphetamine and derivates
  Amphetamine, methamphetamine, MDMA, MDA, MDE, phenylpropanolamine (norephedrine), methylphenidate
- Cocaine
  cocaine, benzoylecgonine, norcocaine, cocaethylene
- Opiates/opioids
  heroin, 6-acetylmorphine, morphine, 6-acetylcodeine, codeine, dihydocodeine, oxycodone, hydromorphone, methadone+EDDP, buprenorphine+norbuprenorphine, fentanyl+norfentanyl, tilidine+nortilidine, tramadol+O-desmethyltramadol
- Sedatives
  diazepam, nordiazepam, temazepam, oxazepam, flunitrazepam+7-aminoflunitrazepam, bromazepam, alprazolam, clonazepam+7-aminoclonazepam, lorazepam, lormetazepam
  zolpidem, zopiclon, zaleplon,
  diphenhydramine, doxylamine, chlorphenamine
- Others
  ketamine, pregabaline

For Forensic Use

Drugs of abuse in hair (LC-MS/MS)

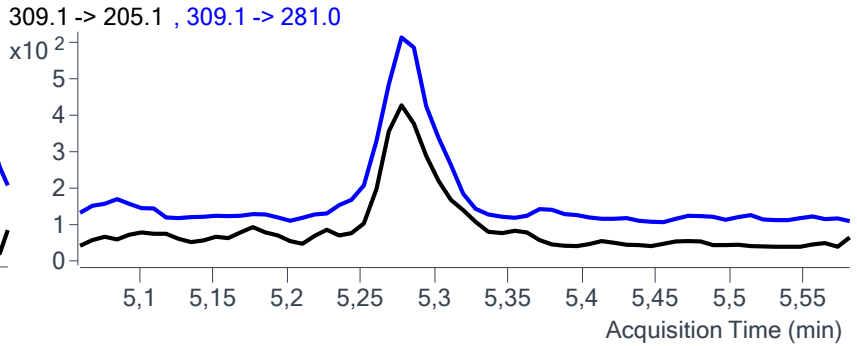
0.01 ng/mg THC

0.02 ng/mg required sensitivity



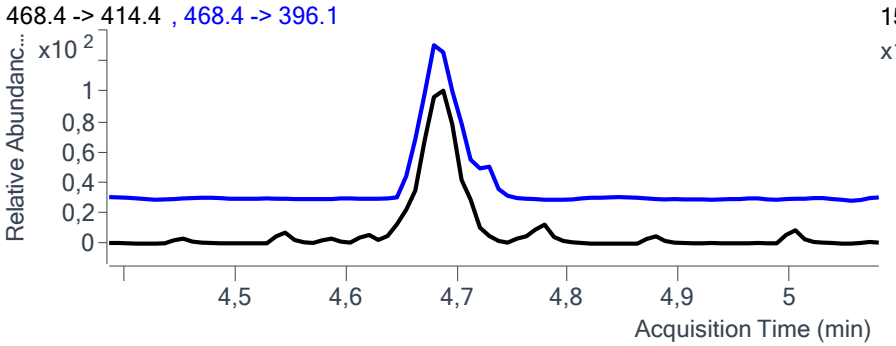
0.025 ng/mg Alprazolam

0.05 ng/mg required sensitivity



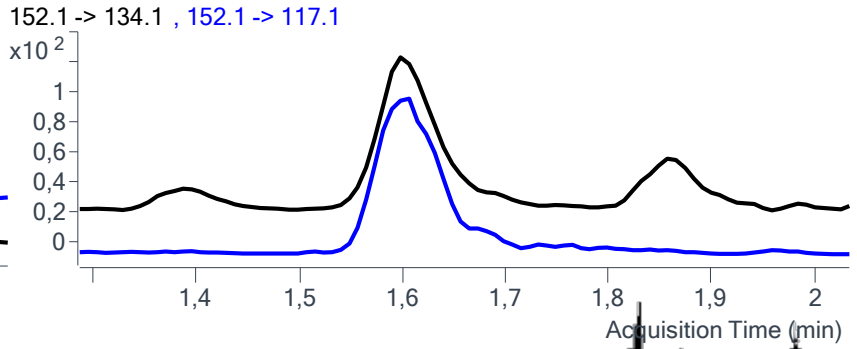
0.025 ng/mg Buprenorphine

0.1 ng/mg required sensitivity



0.025 ng/mg Norephedrine

0.1 ng/mg required sensitivity for amphetamines

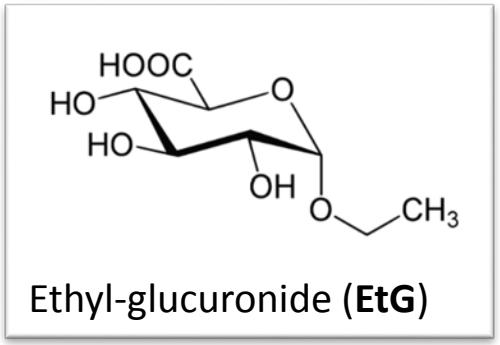


For Forensic Use

Ethyl glucuronide in hair with LC-MS/MS

Applied Biosystems 4000 Q-TRAP, **ESI-**

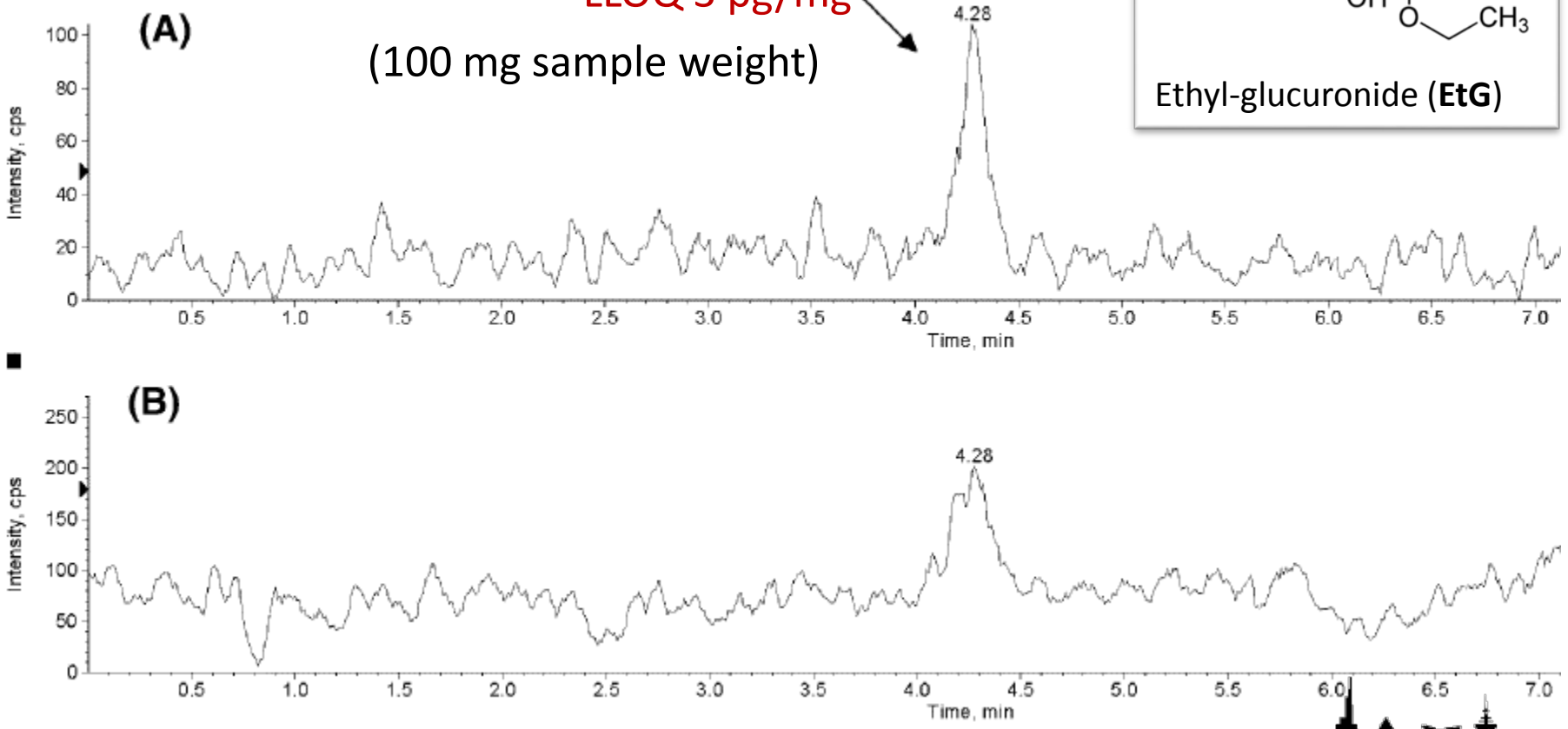
Chrompack Inertsil ODS-3 column, else HILIC columns



Ethyl-glucuronide (**EtG**)

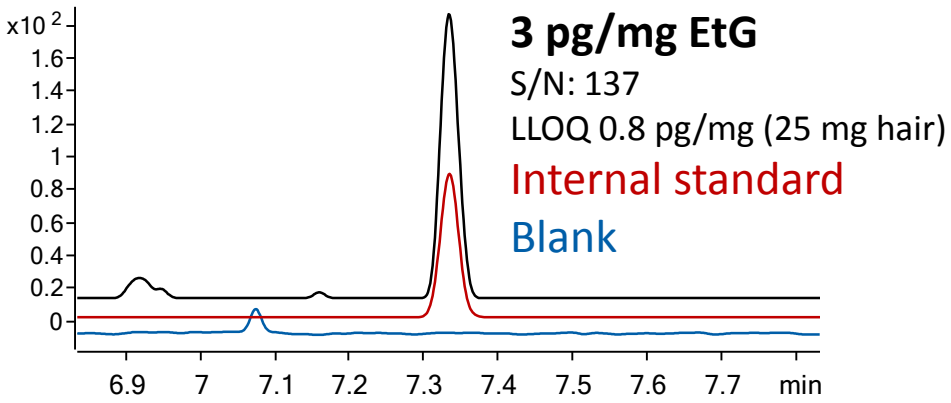
LLOQ 3 pg/mg

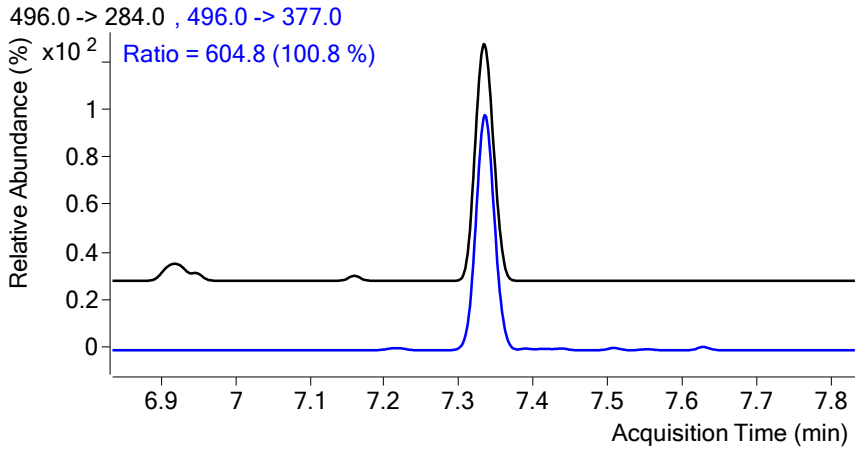
(100 mg sample weight)



J Mass Spectrom 2006, 41:34-42

Ethyl glucuronide in hair with GC-MS/MS





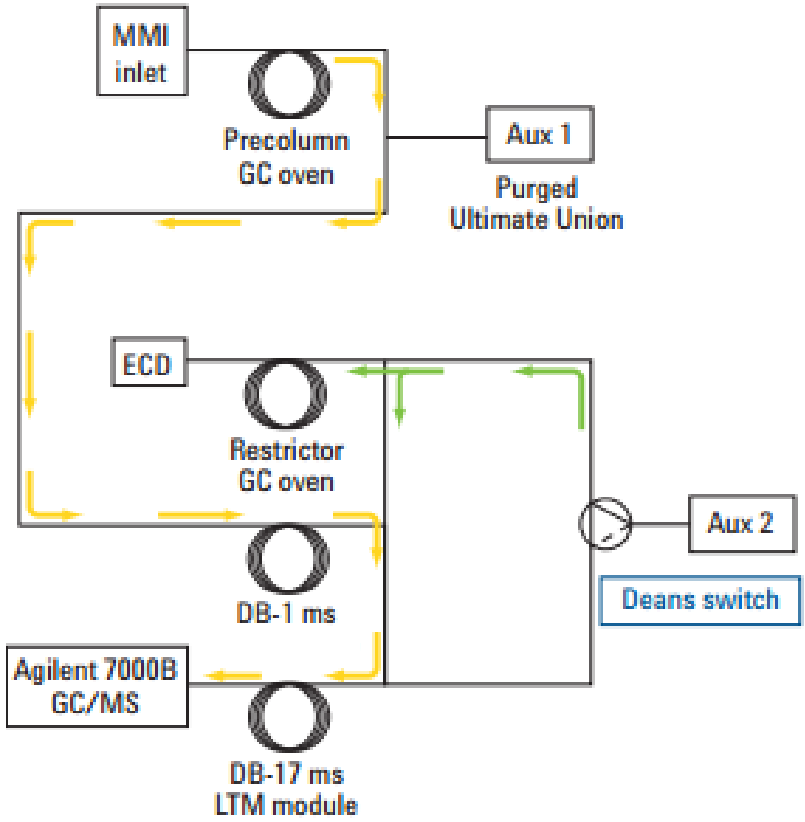
Sensitivity & routine robustness

- Hair decontamination
  shredding with scissors
  25 mg typical sample weight,
  24 h incubation with 0.8 ml water
  filtration and evaporation
  derivatization with 50 µl **PFPA**
  evaporation; 50 µl ethyl acetate
  2 µl injection

- Precolumn: Agilent DB-1ms (1 m)
  Backflush after 1.5 min
  Oven column: Agilent DB-1ms (15 m)
  Deans Switch: 5.5 - 7 min transfer to
  LTM-module: Agilent DB-17ms (15 m)

  NCI$_{(ammonia)}$ GC-MS/MS

- Sample2Sample: 14 min



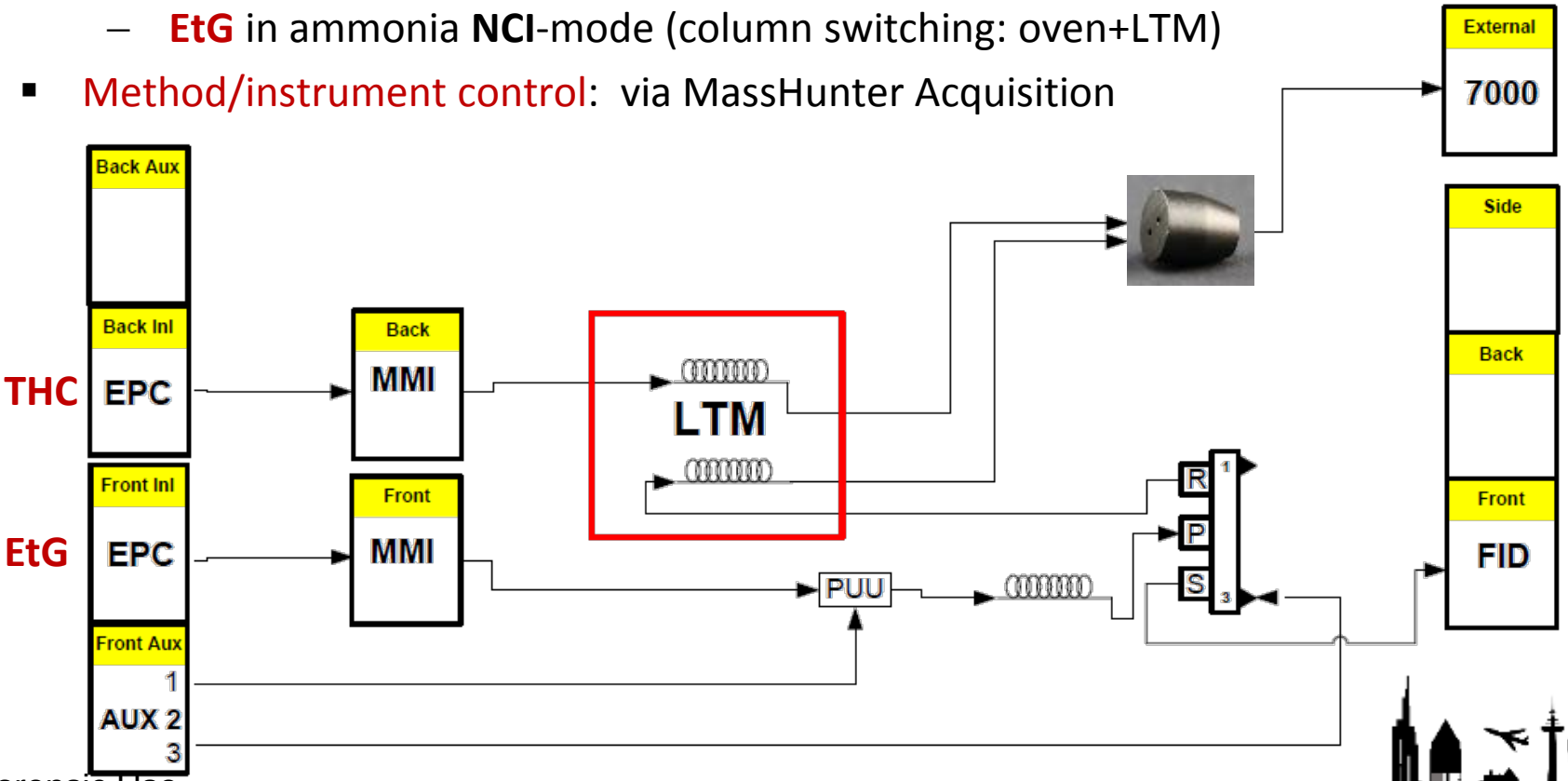
Petzel-Witt et al. (2018) Drug Test Anal. 10:177-183

2 x GC-MS/MS ?

For Forensic Use

Concept and configuration of GC-MS/MS

- 2 Injectors and 2 inlets with double drilled ferrule → MS
- Only target compound analyses, CI-mode, no EI-mass spectra
  - Serum **cannabinoids** in methane **PCI**-mode (1x LTM)
  - **EtG** in ammonia **NCI**-mode (column switching: oven+LTM)
- Method/instrument control: via MassHunter Acquisition



Automation

Solid-phase extraction

- Cannabis
  over night
- Basic drugs
  over day (stability)
- Online sample prep?
  NO ! (too slow, inflexible)
- Future ?
  liquid-liquid extraction

Forensic Toxicology is more than analytics…

- Competence in analytic procedures is necessary
- Complemented by …
  - Pharmacological & toxicological expertise
  - Forensic expertise
  - Legal expertise

- Experience in court hearings
- Teaching
- Scientific research projects

https://www.drugabuse.gov/2016/12/14/pics/trends-statistics/infographics/synthetic-cannabinoids-k2spice-unpredictable-danger

https://www.dytigabuse.com/2016/12/14/pics/trends-statistics/infographics/synthetic-cannabinoids-k2spice-unpredictable-danger

For Forensic Use

Study design

6 subjects

0 mg JWH-018

2 mg JWH-018

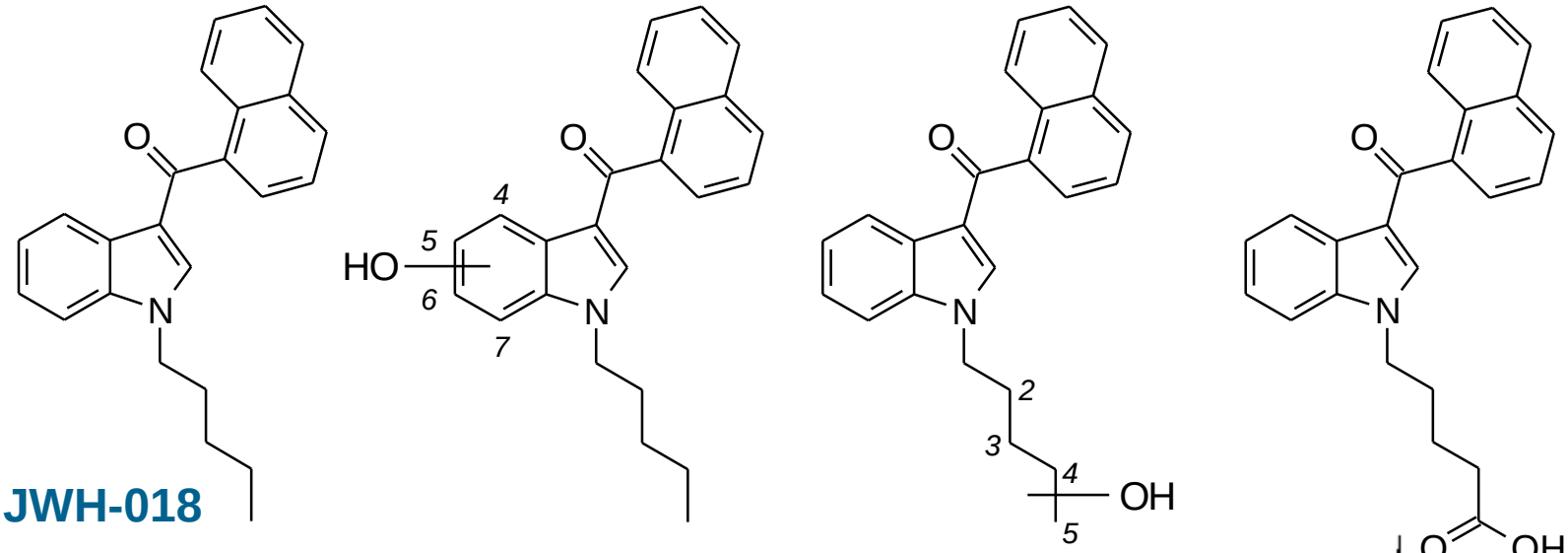
3 mg JWH-018

t = 0 → **12 h**

- JWH-018 as pure substance (no incense)
- Neurocognitive test battery
- 12 x Serum & oral fluid, 5 x urine for pharmacokinetics

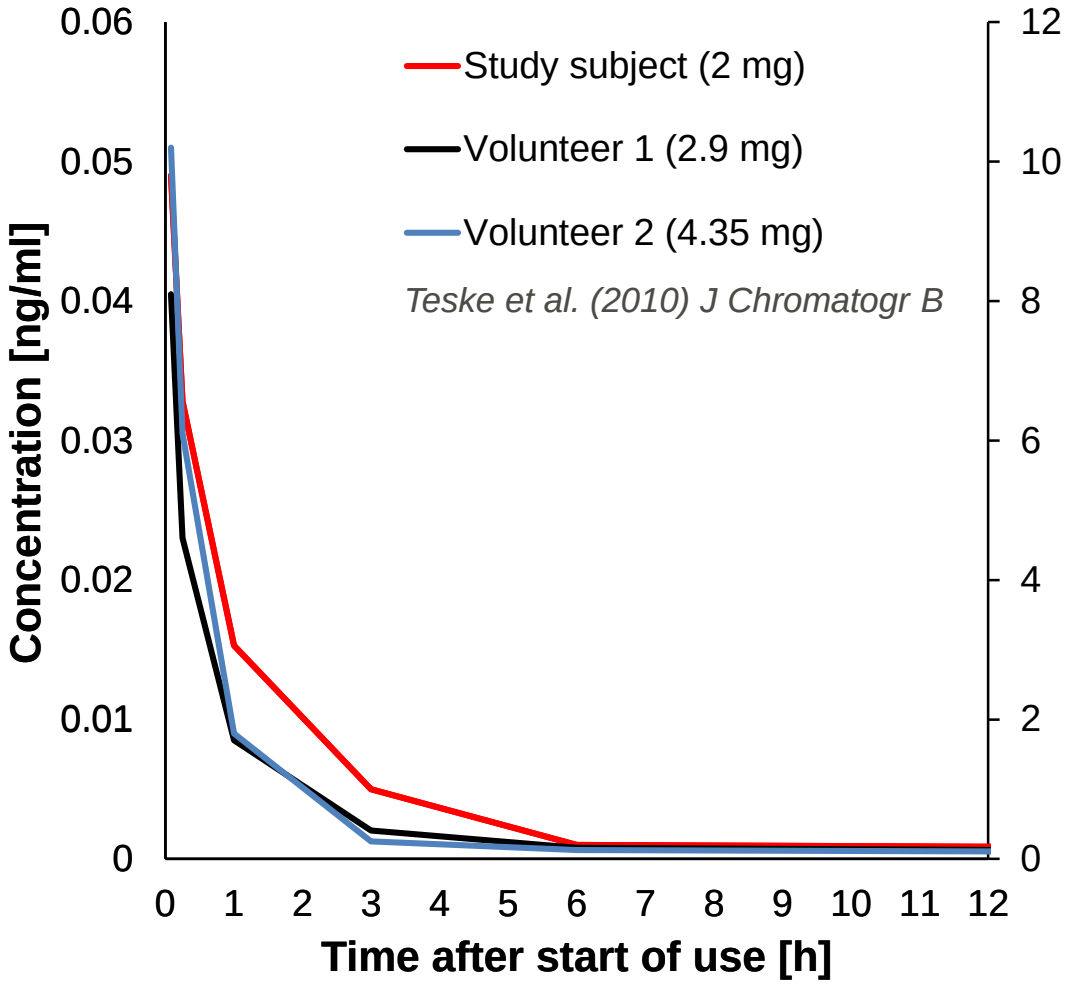
LC-MS/MS targeted method

- Agilent 1290 LC, JetStream ESI, 6460 MS/MS
- Serum liquid-liquid extraction of 0.5 ml
- Oral Fluid (Quantisal) dilution of 0.1 ml with methanol
- Urine liquid-liquid extraction of 0.5 ml, **±GRD**
- **11** Analytes validated (LLOQs Serum <0.01; Oral Fluid <0.1; Urine <0.01 ng/ml)

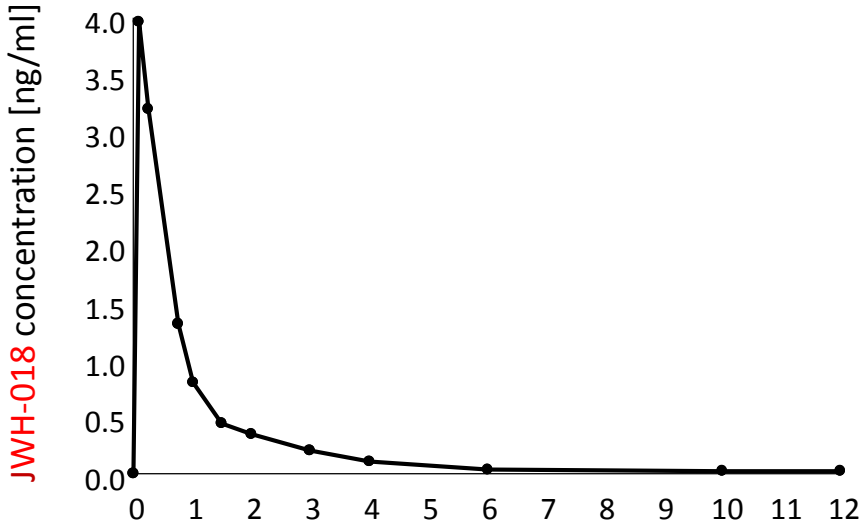


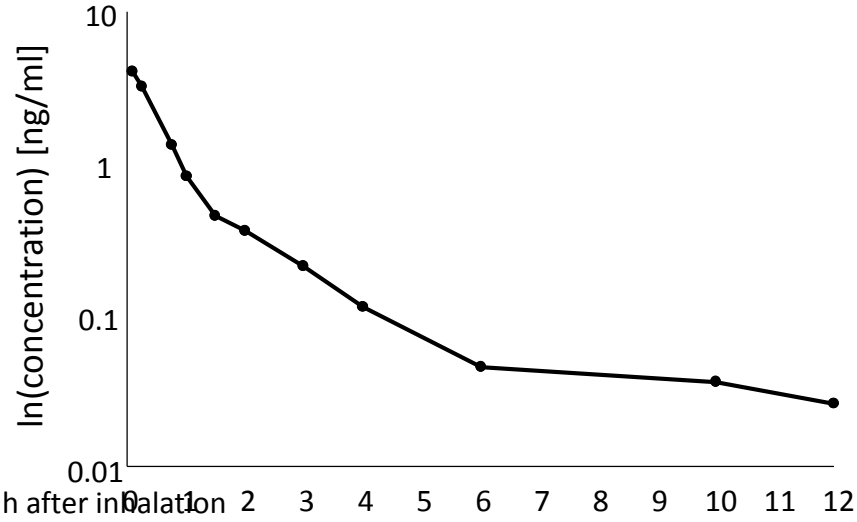
JWH-018

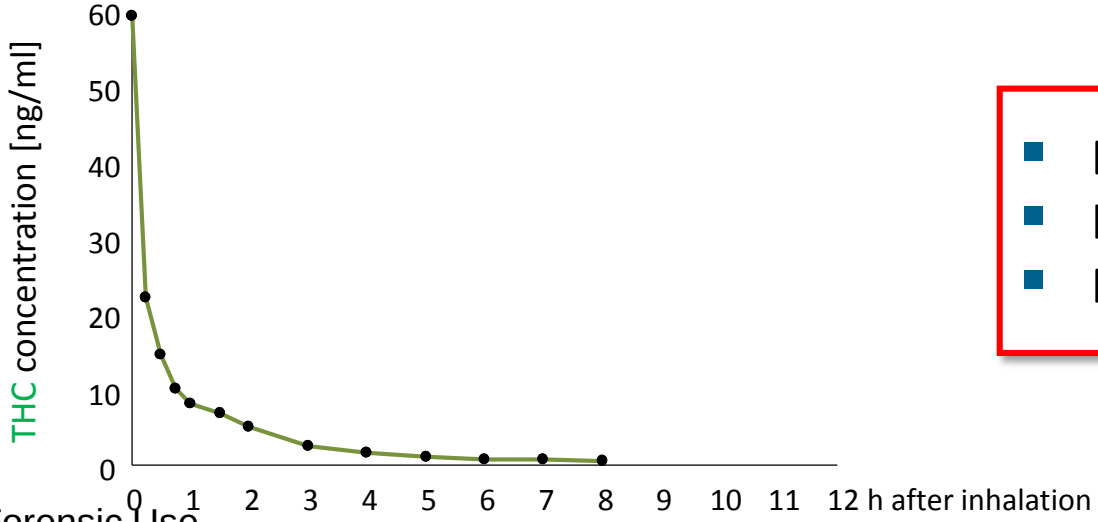
First drug application…



JWH-018 concentration-time curve

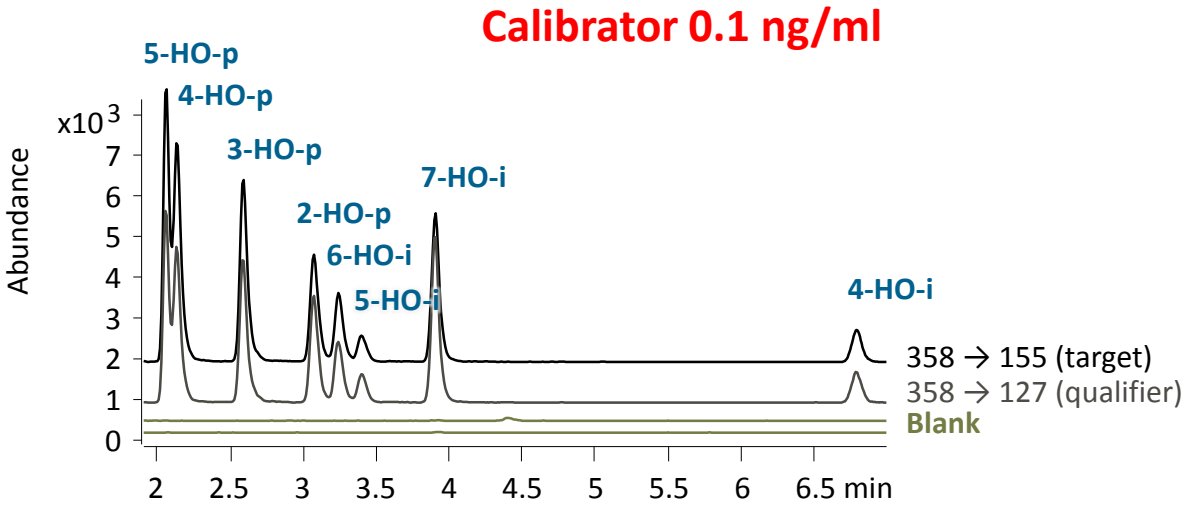


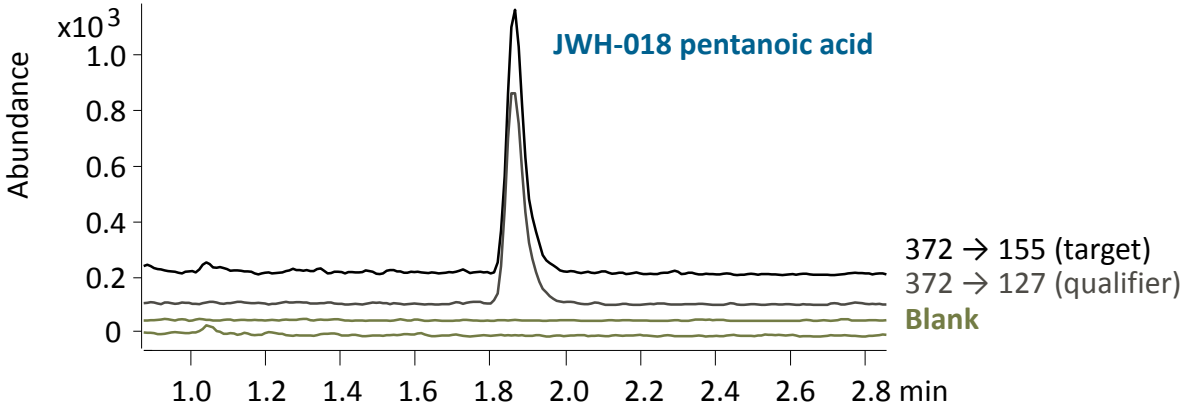




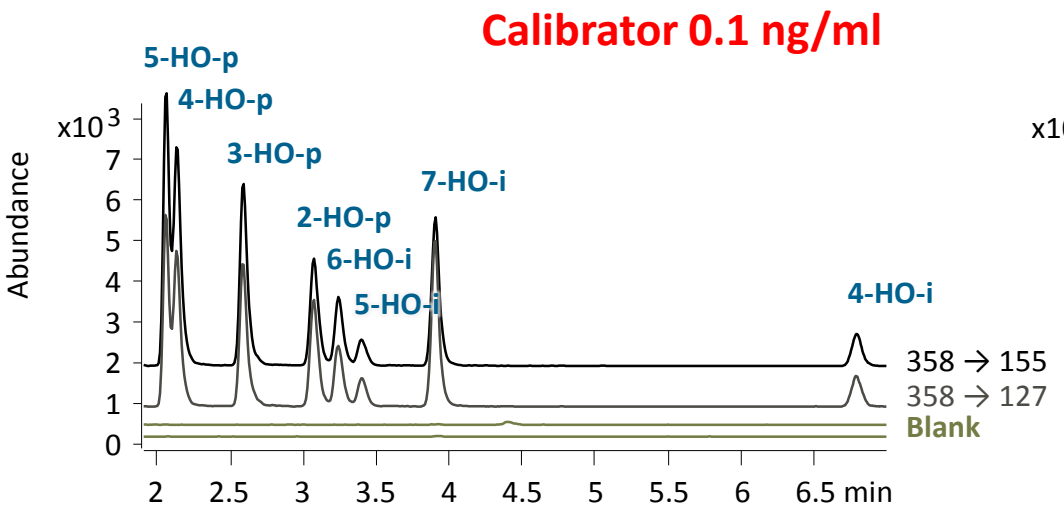
- Distribution phase
- Kinetics similar to THC
- Polyphasic elimination

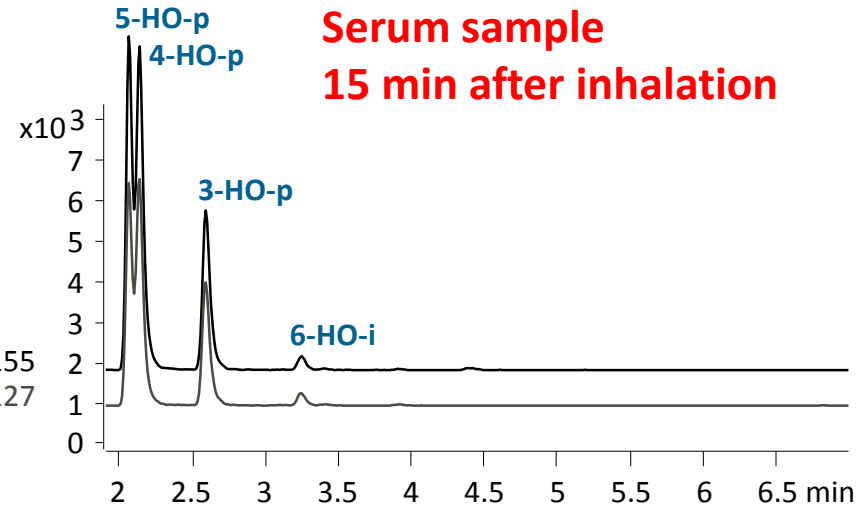
Metabolites in serum

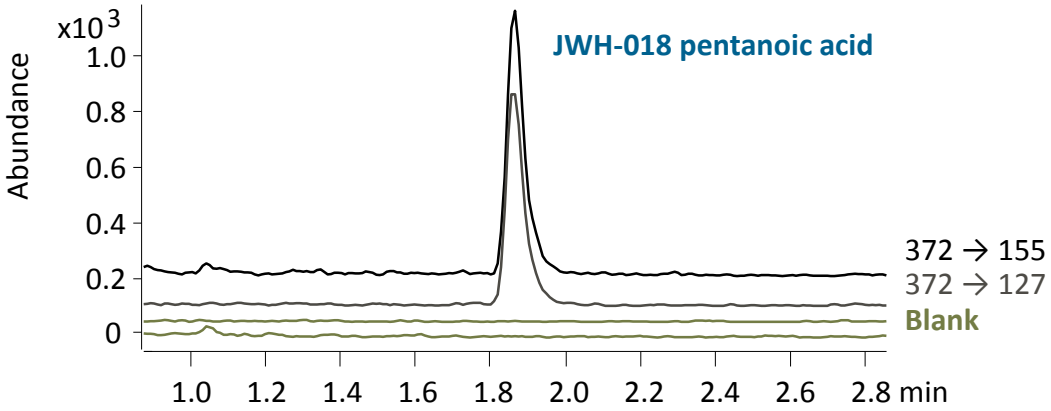


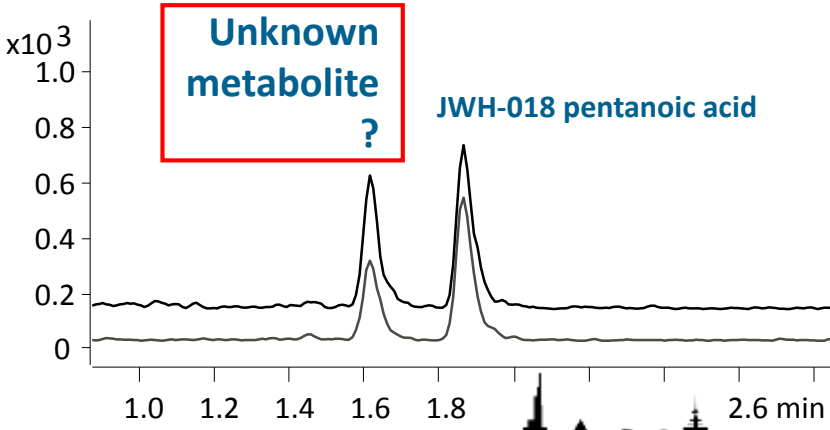


Metabolites in serum





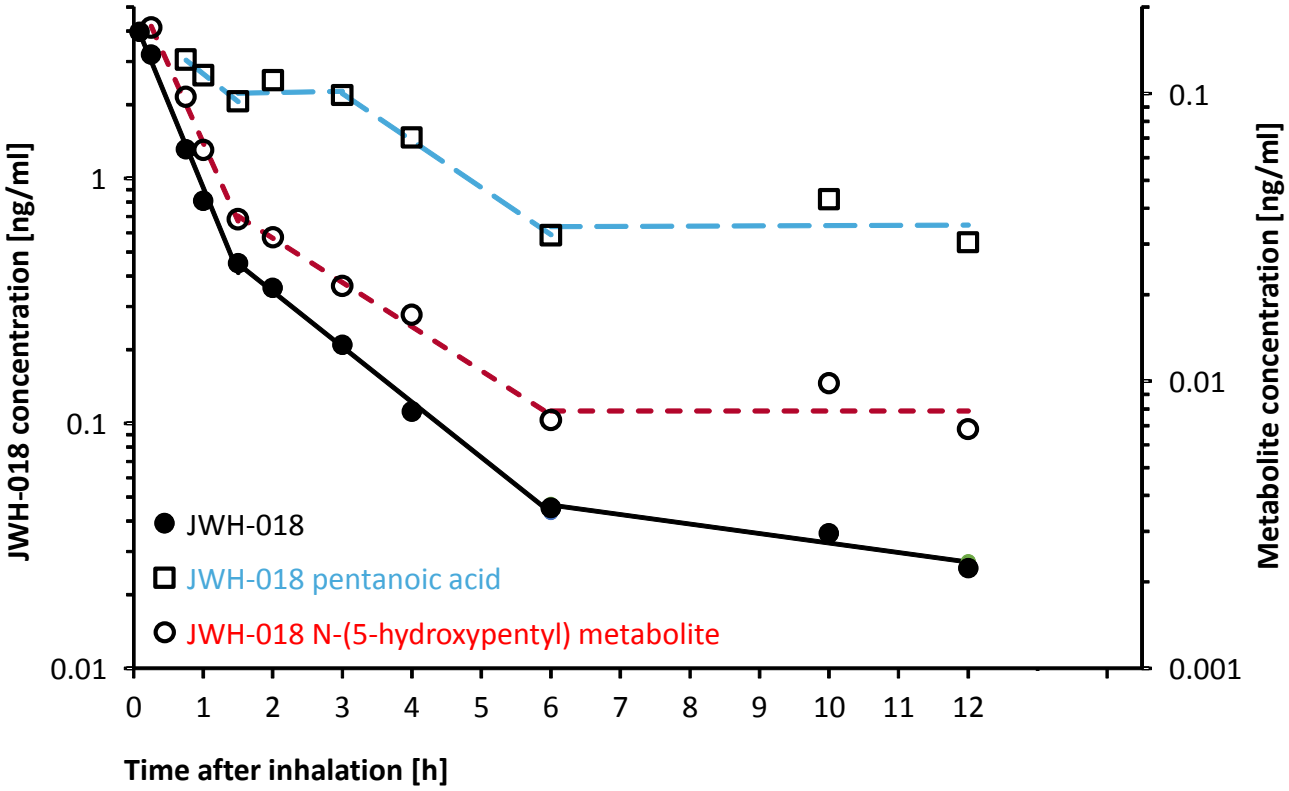




For Forensic Use

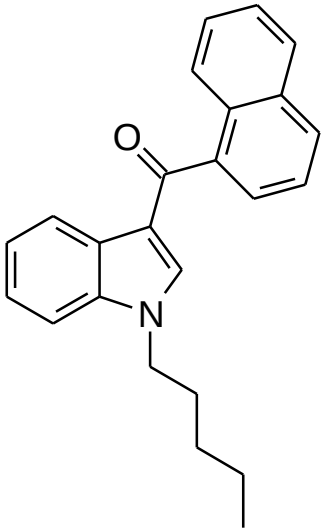
Rechtsmedizin Frankfurt am Main

Concentration-time curves (metabolites)

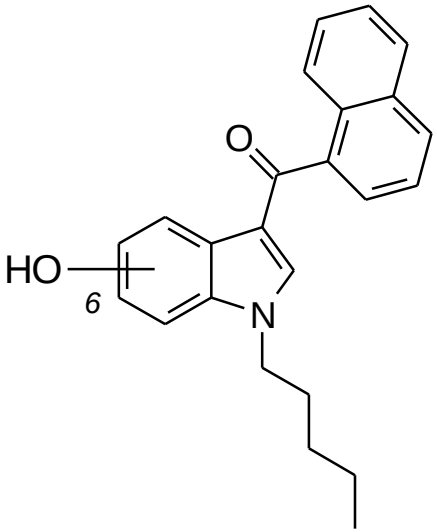


Toennes et al. (2017) J Pharm Biomed Anal 140

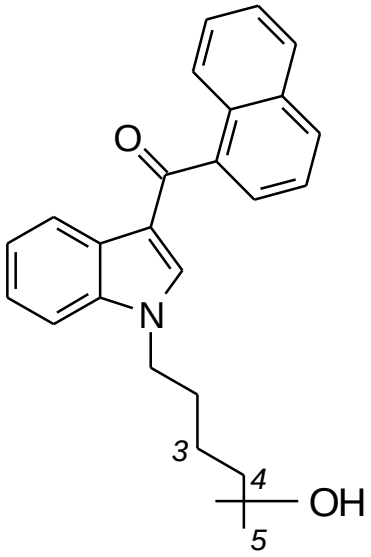
Concentrations [ng/ml]



JWH-018
C_{max} **4.76** (2.90-9.91)
$C_{12\,h}$ 0.03 (0.01-0.15)

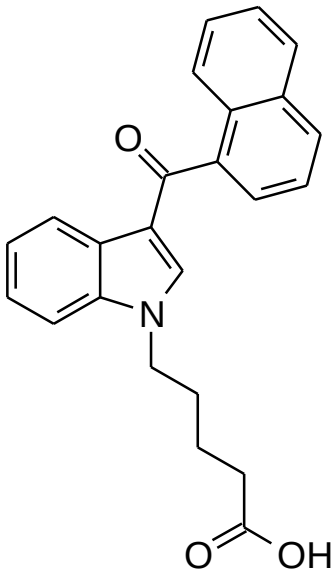


6-HO-indol-JWH-018
C_{max} **0.03** (0.00-0.17)
$C_{12\,h}$ 0.00



5-HO-pentyl-JWH-018
C_{max} 0.16 (0.06-**0.37**)
$C_{12\,h}$ 0.01 (0.00-0.03)

4-HO-pentyl-JWH-018
C_{max} 0.18 (0.07-**0.41**)
$C_{12\,h}$ 0.01 (0.00-0.06)

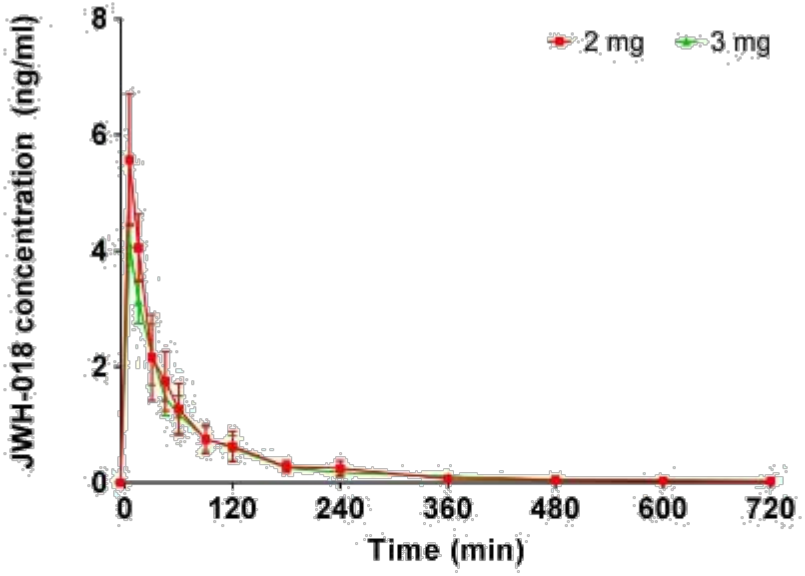


JWH-018 pentanoic acid
C_{max} 0.14 (0.06-**1.02**)
$C_{12\,h}$ 0.03 (0.00-0.13)

Effects: „High“ ?

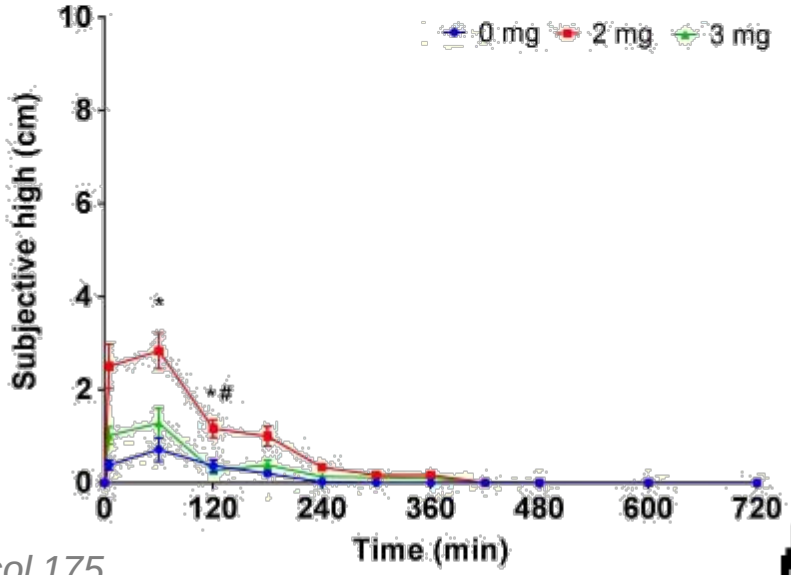
- **C_{Serum}**

  mean±SEM



- **VAS High**

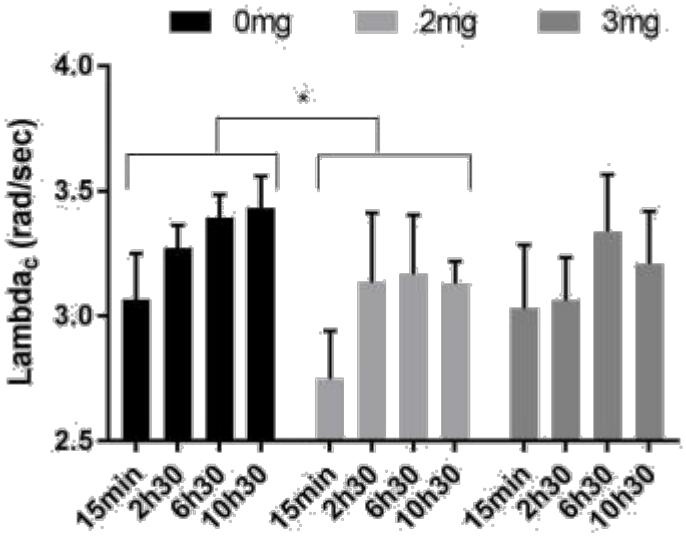
  mean±SEM



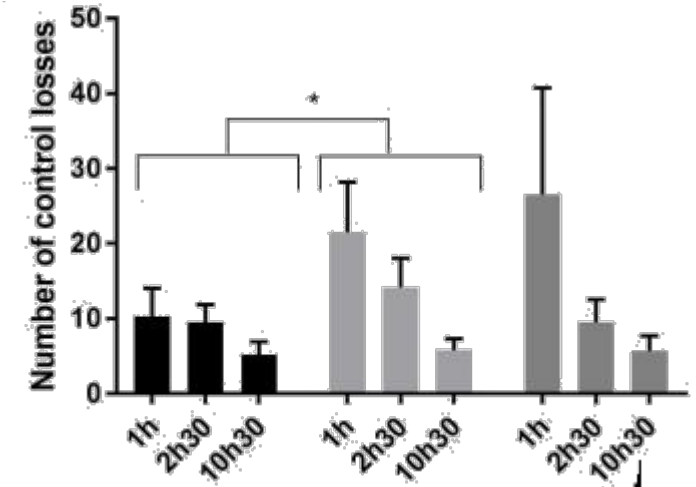
Theunissen et al. (2018) Br J Pharmacol 175

Neurocognitive effects

- **Lambda-c** in the critical tracking task (CTT)



- **Control losses** in the divided attention task (DAT)



Theunissen et al. (2018) Br J Pharmacol 175

Correlation of dose and effects or PK ?

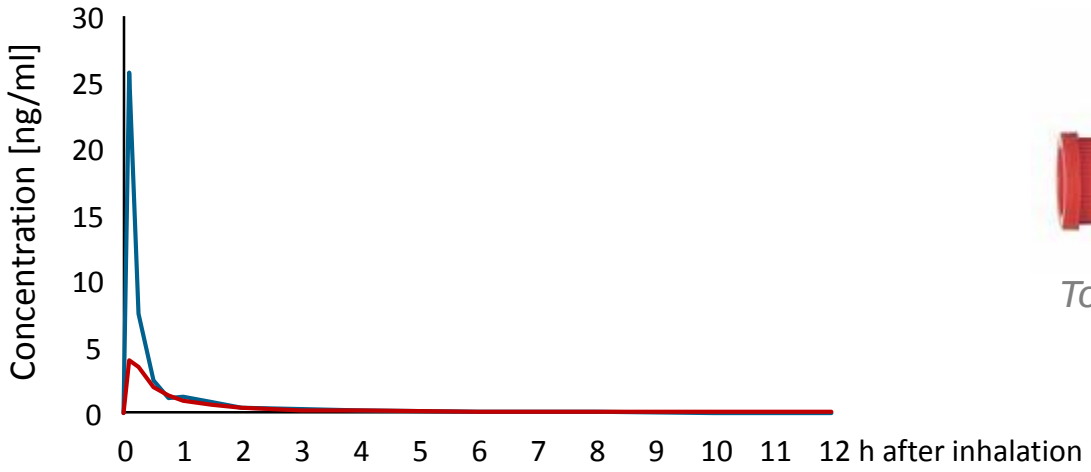
- No significant difference in any PK property between 2 and 3 mg dose

- Dosing efficiency using „Maastricht crack pipe“ ?

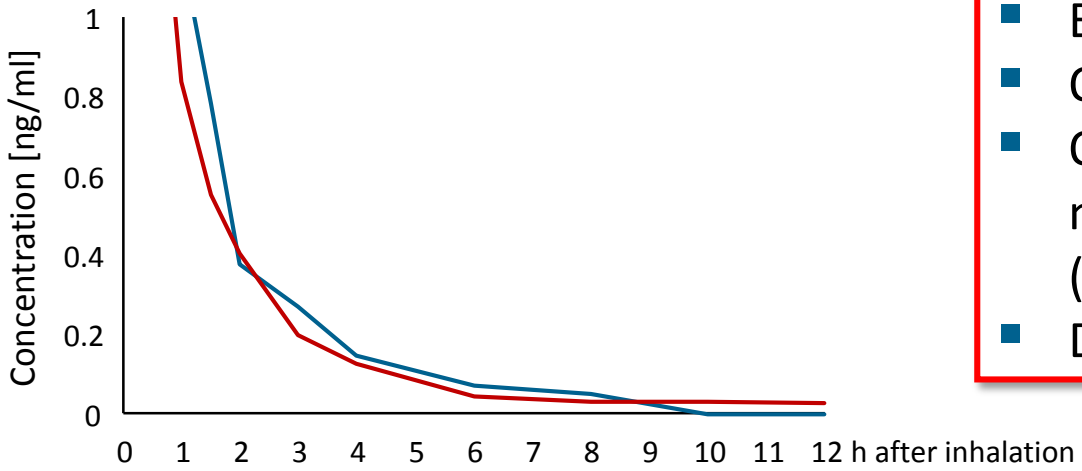
- Analysis of residues:

  0.09 to 2.10 mg
  (**median 1.14 mg**)

JWH-018 in oral fluid vs. serum

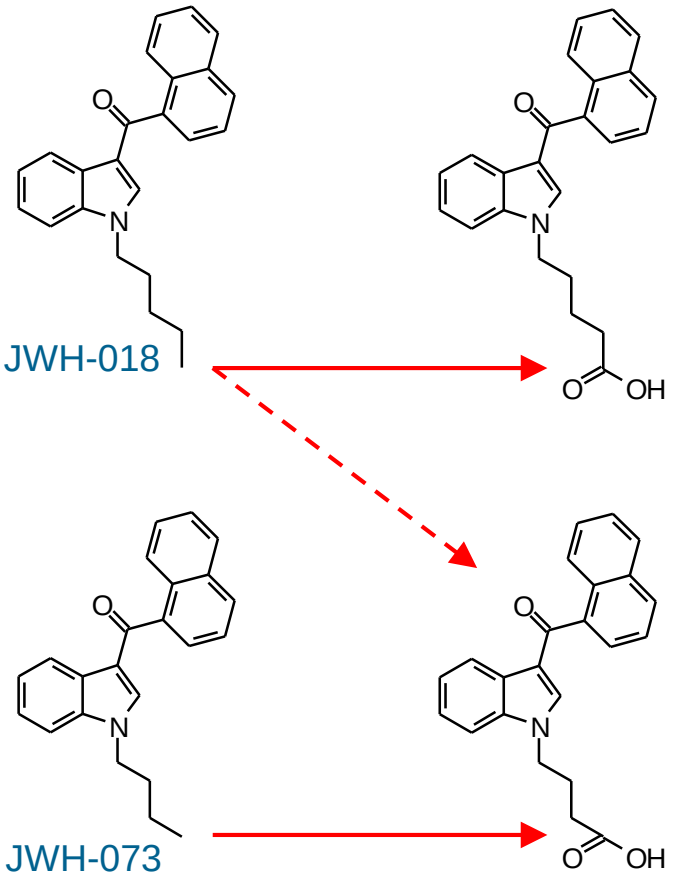


Toennes et al. (2018) Drug Test Anal 10



- Extensive elimination < 1 h
- OF ≈ serum (@ ≥ 1 h)
- OF/S ratio:
  median 1.4, IQR 0.5 – 2.8
  (Min 0.05 – Max 554)
- Detectability: OF < S

Urine: JWH-073 butanoic acid as metabolite?



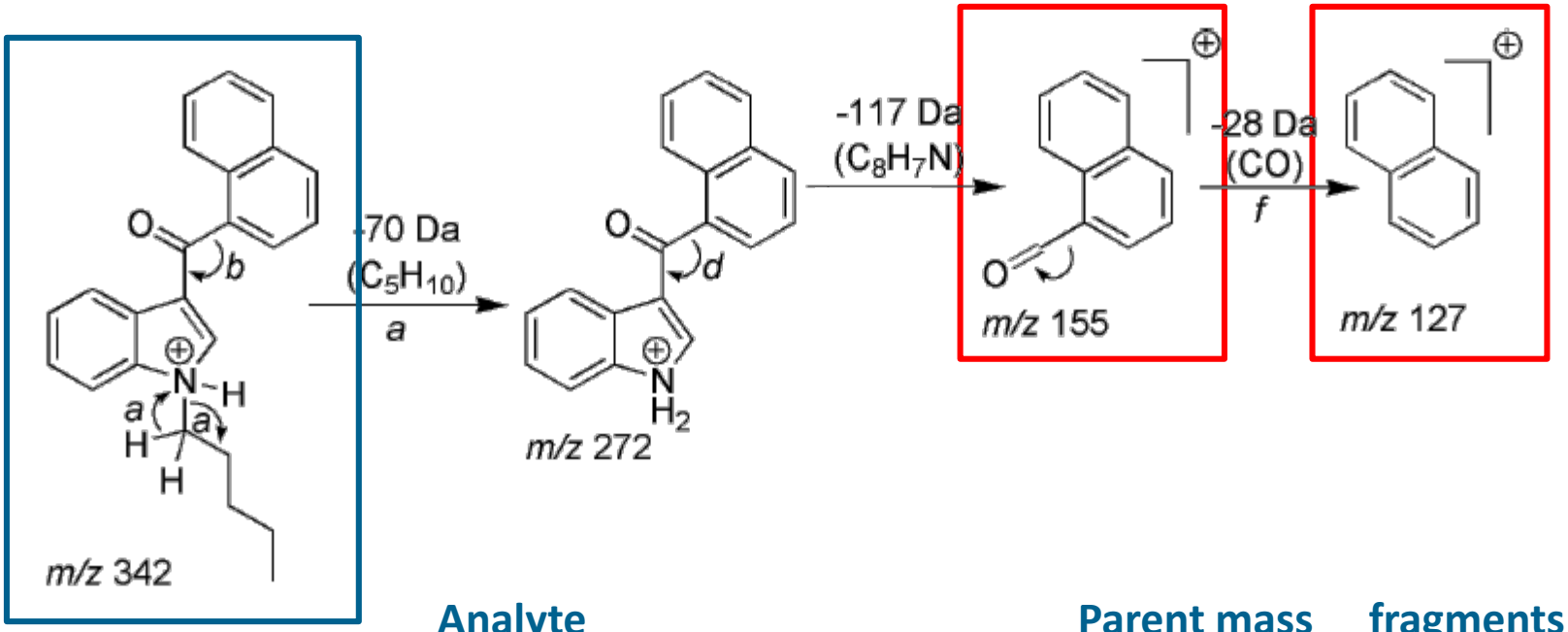
JWH-018 pentanoic acid

Hutter et al. (2012) J Mass Spectrom

JWH-073 butanoic acid

Moran et al. (2011) Anal Chem

Selection of fragments for MRM

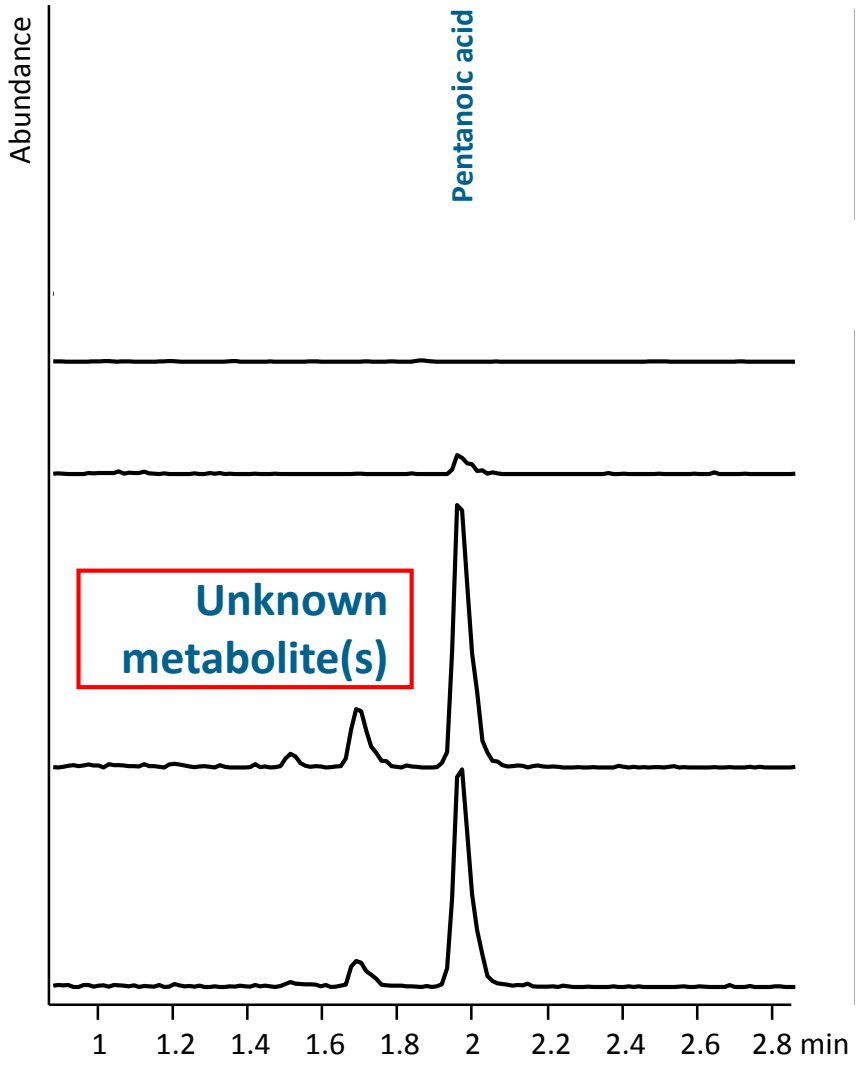


JWH-018

Analyte	Parent mass	fragments
JWH-018	m/z 342	155, 127
Pentanoic acid (+16+16-2)	m/z 372	155, 127
Hydroxylation chain/ring (+16)	m/z 358	155, 127
Butanoic acid (-12-2+16+16-2)	m/z 358	155, 127

Möller et al. (2011) Drug Test Anal

Metabolites in urine

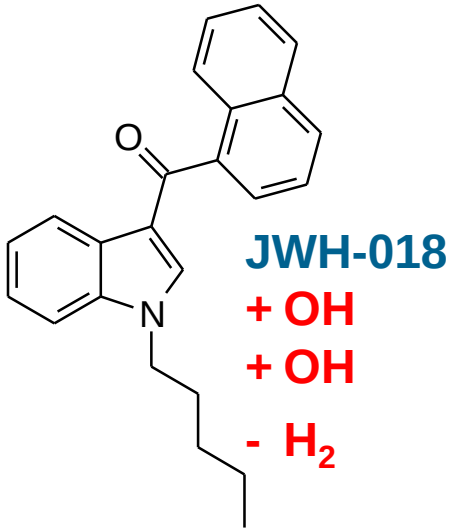


Wintermeyer et al. (2010) Anal Bioanal Chem
Möller et al. (2011) Drug Test Analysis

Several isomeric metabolites:

dihydroxylated and dehydrogenated

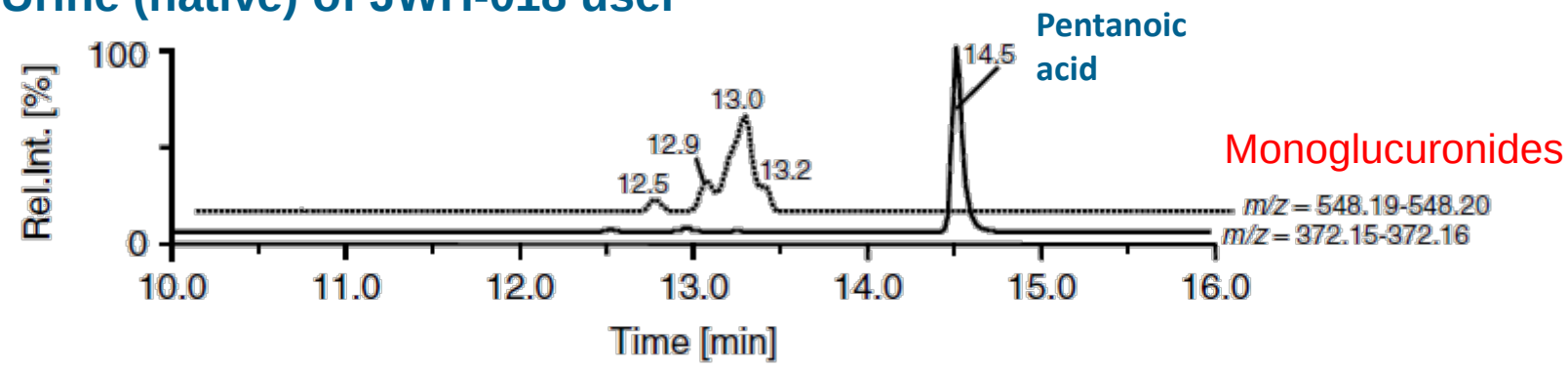
- In-vitro metabolism study
- Detected in one authentic urine sample
- Glucuronidated



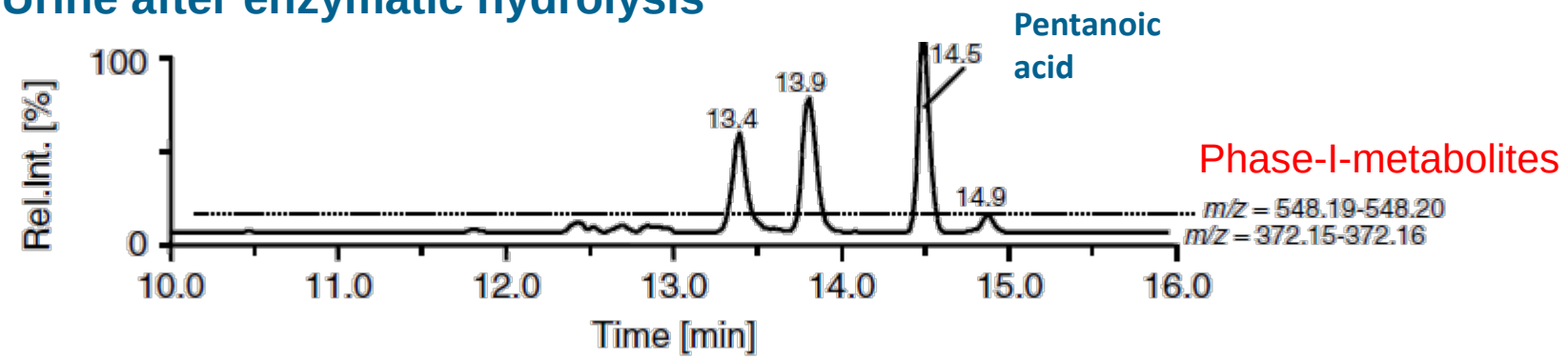
Toennes et al. (2017) J Pharm Biomed Anal 150

Hydroxy-keto metabolites

Urine (native) of JWH-018 user



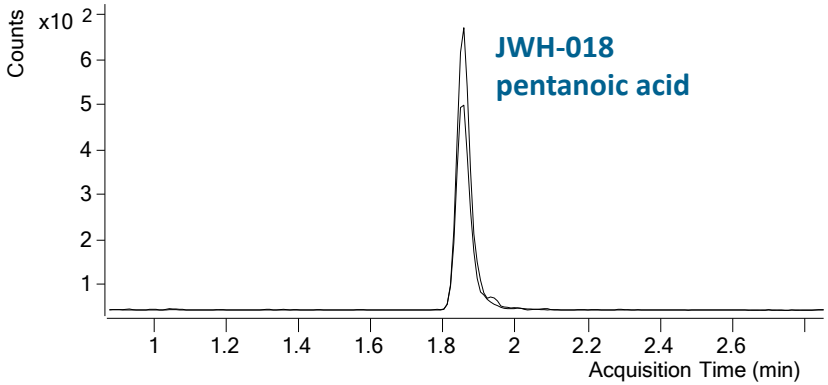
Urine after enzymatic hydrolysis



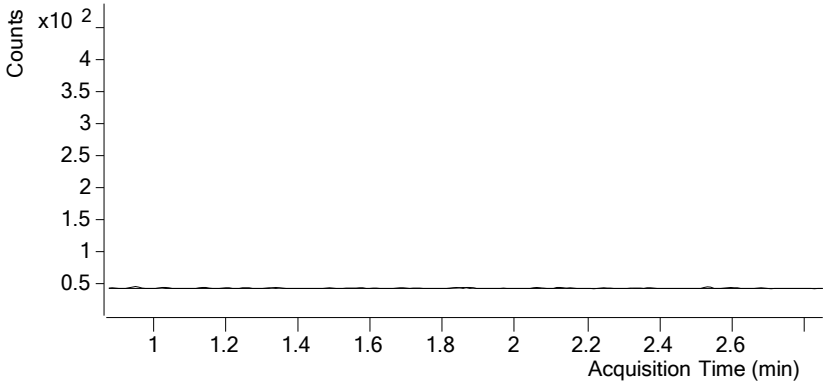
Möller et al. (2011) Drug Test Anal
Watanabe et al. (2016) Forensic Sci Int

Regular metabolite !

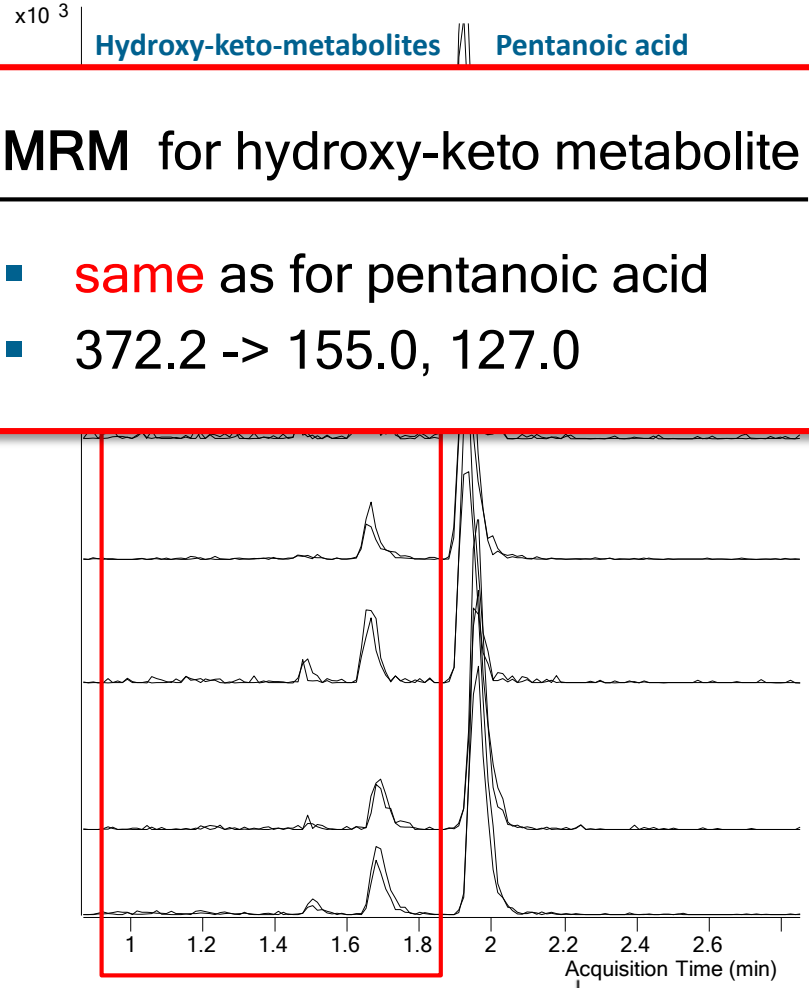
Calibrator



Baseline (representative subject)



Urine samples of every subject (1 h, GRD)



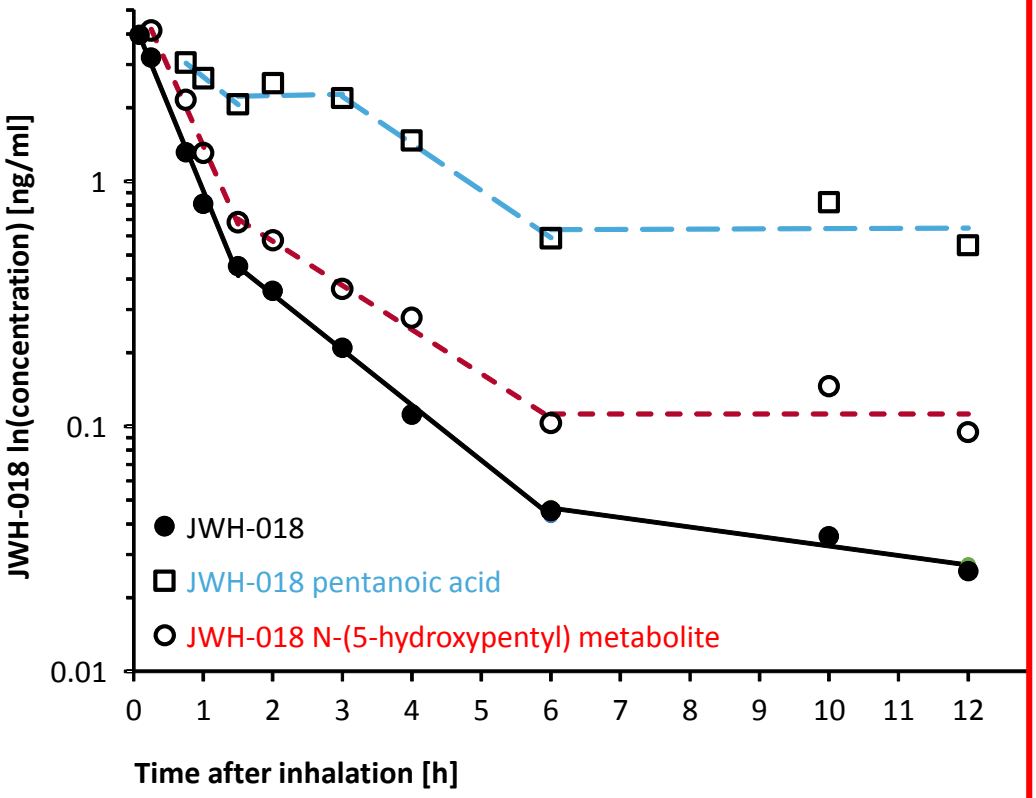
MRM for hydroxy-keto metabolite

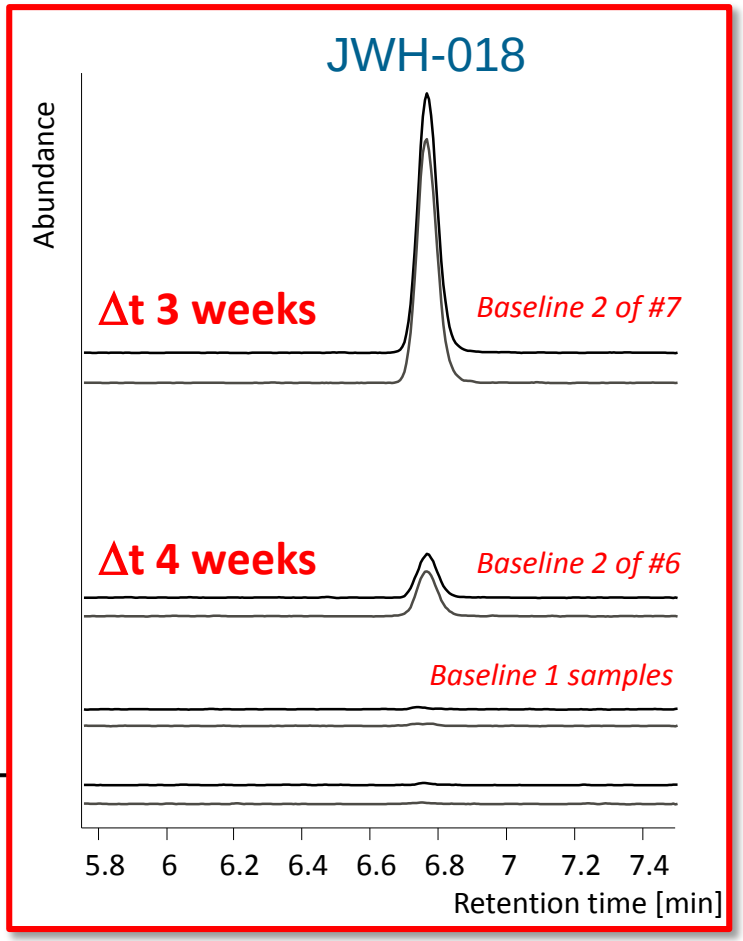
- same as for pentanoic acid
- 372.2 -> 155.0, 127.0

For Forensic Use

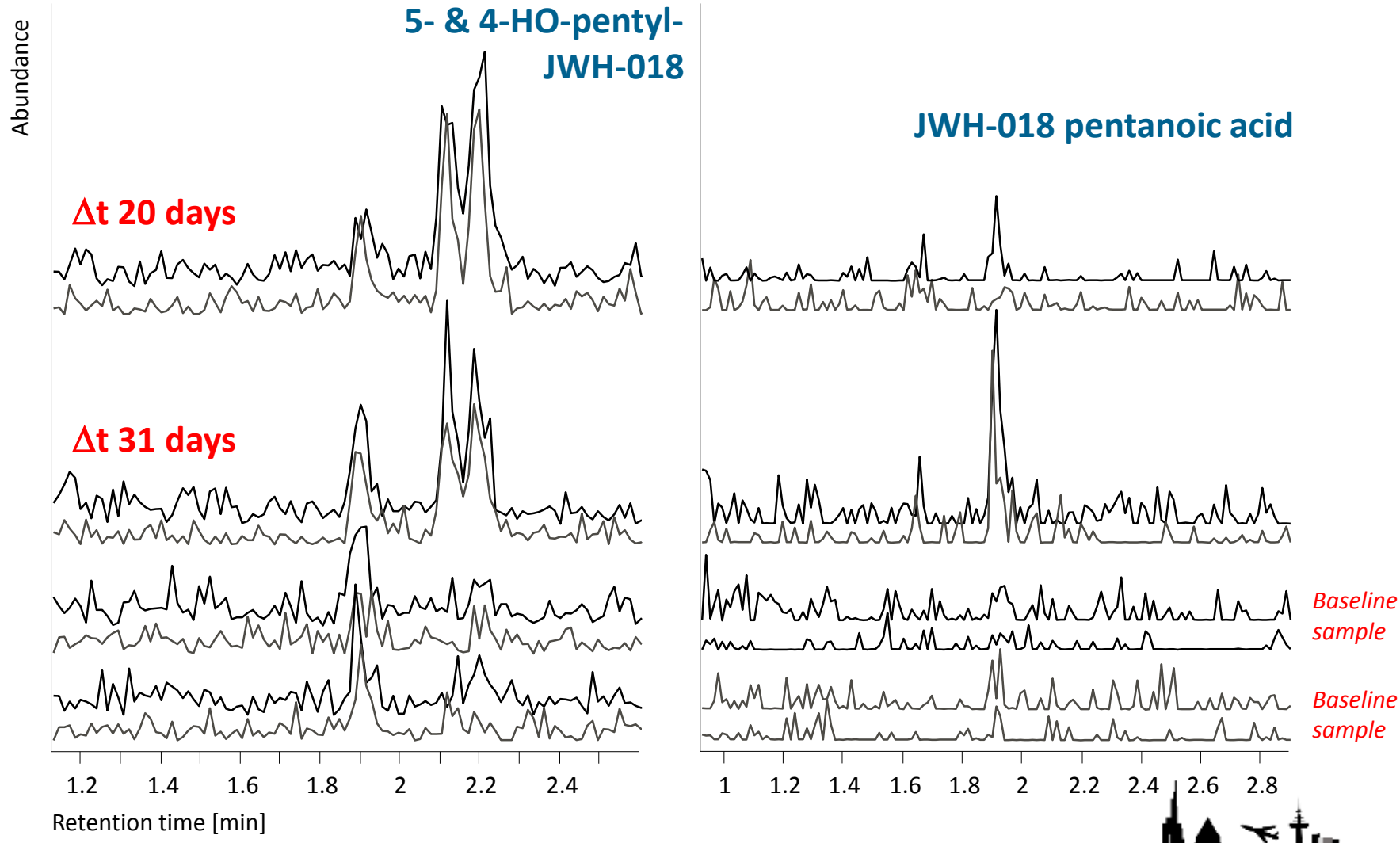
Rechtsmedizin Frankfurt am Main

„Baseline serum“: surprisingly positive





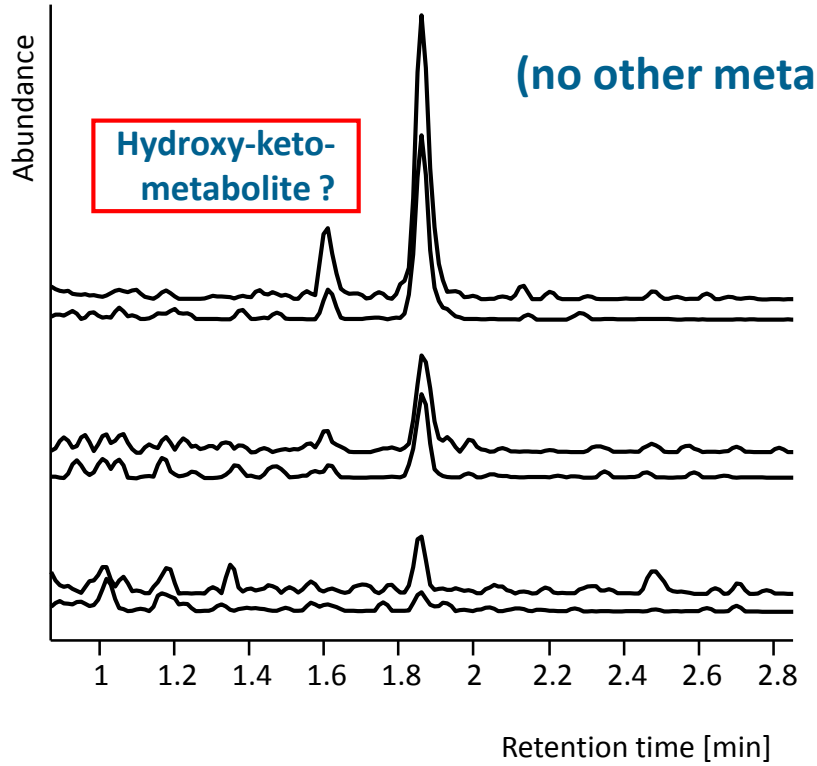
„Baseline serum“: metabolites



„Baseline urine“: samples also positive

JWH-018 pentanoic acid

(no other metabolites)



2 weeks later *(prior to placebo)*

3 weeks later *(prior to 3 mg condition)*

First urine sample *(prior to 2 mg condition)*

c.f. Hegstad et al. (2015) J Anal Toxicol

Main study results

- JWH-018 and 6 metabolites in serum

- No JWH-018, but 13 glucuronidated metabolites excreted in urine, main target is the pentanoic acid (+ hydroxy-keto-metabolite)

- Multi-compartement kinetics in serum and OF
  ⇒ Similar to THC
  ⇒ Shorter detection time in OF

- Prolonged elimination
  ⇒ accumulation probable
  ⇒ lasting effects or tolerance

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European Union

Bund gegen Alkohol und Drogen im Straßenverkehr (B.A.D.S.)

Part of PREDICT (www.predictnps.eu)

Collaborators

- **Team at Maastricht, The Netherlands**
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- **Team at Frankfurt/Main, Germany**
  Anna Geraths, Werner Pogoda, Alexander Paulke, Cora Wunder, Stefan W. Toennes

- **Publications**
  - Theunissen Hutten. Mason, Toennes, Kuypers, Sousa Fernandes Perna, Ramaekers (2018) Neurocognition and subjective experience following acute doses of the synthetic cannabinoid JWH-018: a phase 1, placebo-controlled, pilot study. Br. J. Pharmacol. 175: 18–28
  - Toennes, Geraths, Pogoda, Paulke, Wunder, Theunissen, Ramaekers (2018) Pharmacokinetic Properties of the synthetic cannabinoid JWH-018 in oral fluid after inhalation. Drug. Test. Anal. 10: 644-650.
  - Toennes, Geraths, Pogoda, Paulke, Wunder, Theunissen, Ramaekers (2017) Excretion of metabolites of the synthetic cannabinoid JWH-018 in urine after controlled inhalation. J. Pharm. Biomed. Anal. 150: 162–168
  - Toennes, Geraths, Pogoda, Paulke, Wunder, Theunissen, Ramaekers (2017) Pharmacokinetic properties of the synthetic cannabinoid JWH-018 and of its metabolites in serum after inhalation. J. Pharm. Biomed. Anal. 140: 215–222

- **Further studies are in progress …**

Educational study results

What *else* did we learn from the study ?

Staff & scientists: analytical procedures

- Liquid-liquid extraction of parent together with metabolites
- Impression of sensitivity of LC-MS/MS procedure
- Oral fluid collection device:

  usability, analysis procedure, sensitivity, stability

Forensic toxicologists / scientists

- Pharmacokinetics, especially inter-individual variability

Conclusion

Key elements of forensic expertise (in Germany) are …

… availability of complementary **analytical instruments**,

… intelligent/efficient usage of **limited ressources**,

… competent **staff** and forensic-toxicological **experts**,

… increase in competence by **teaching** and **research**.