The measurement of urinary 5-HIAA using XLC-MS/MS

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Delft

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  - Pathophysiology
  - Biochemistry
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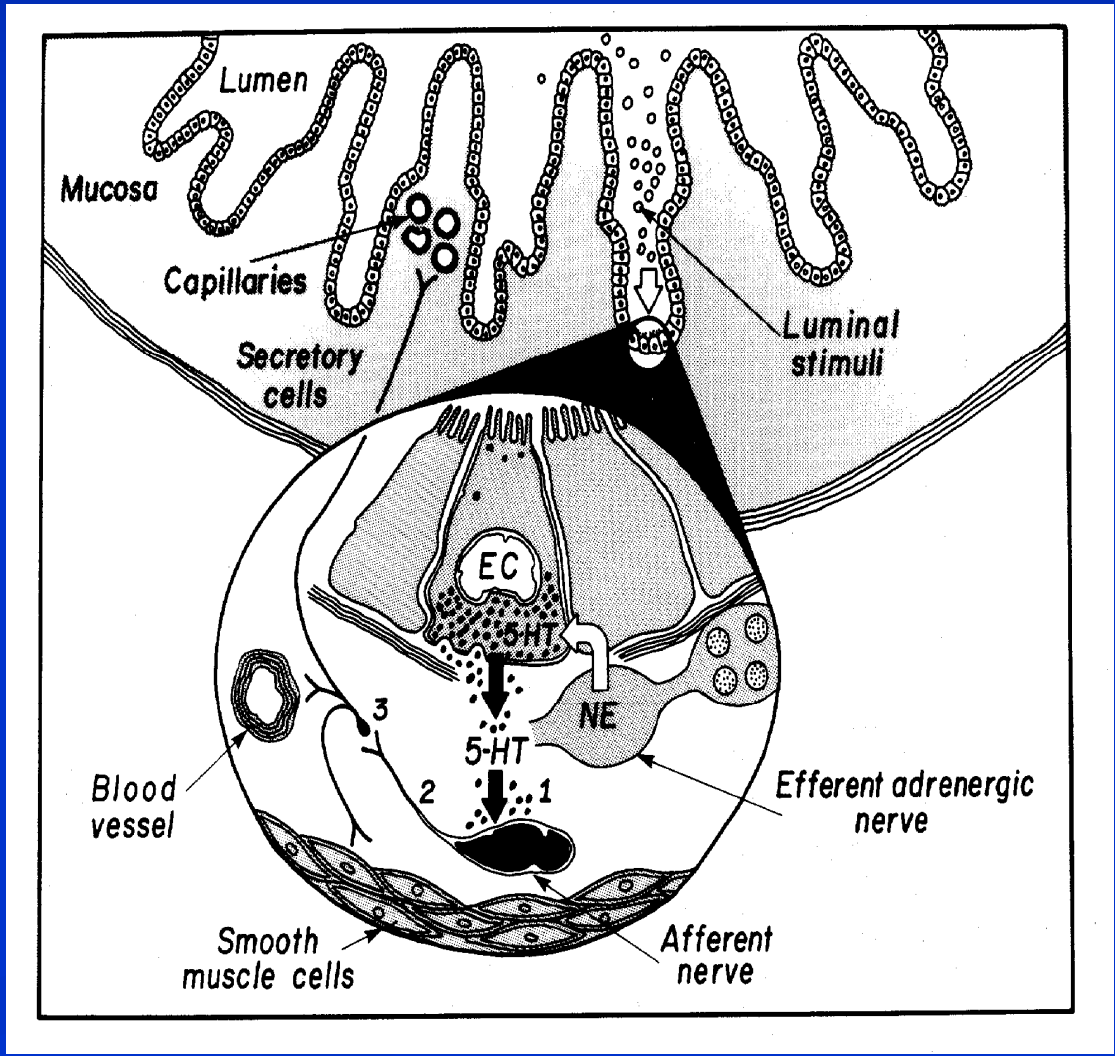
- Future

5-HT/5-HIAA
Physiology

Serotonin

- Widespread in nature
  - Plants, foods, nuts, animals
- Serotonergic neurons (CNS)
  - Synthesis and storage
- Enterochromaffine cells (gut)
  - 80% of total production
  - Storage
  - Release upon different stimuli
- Thrombocytes
  - Storage



5-HT/5-HIAA
Physiology

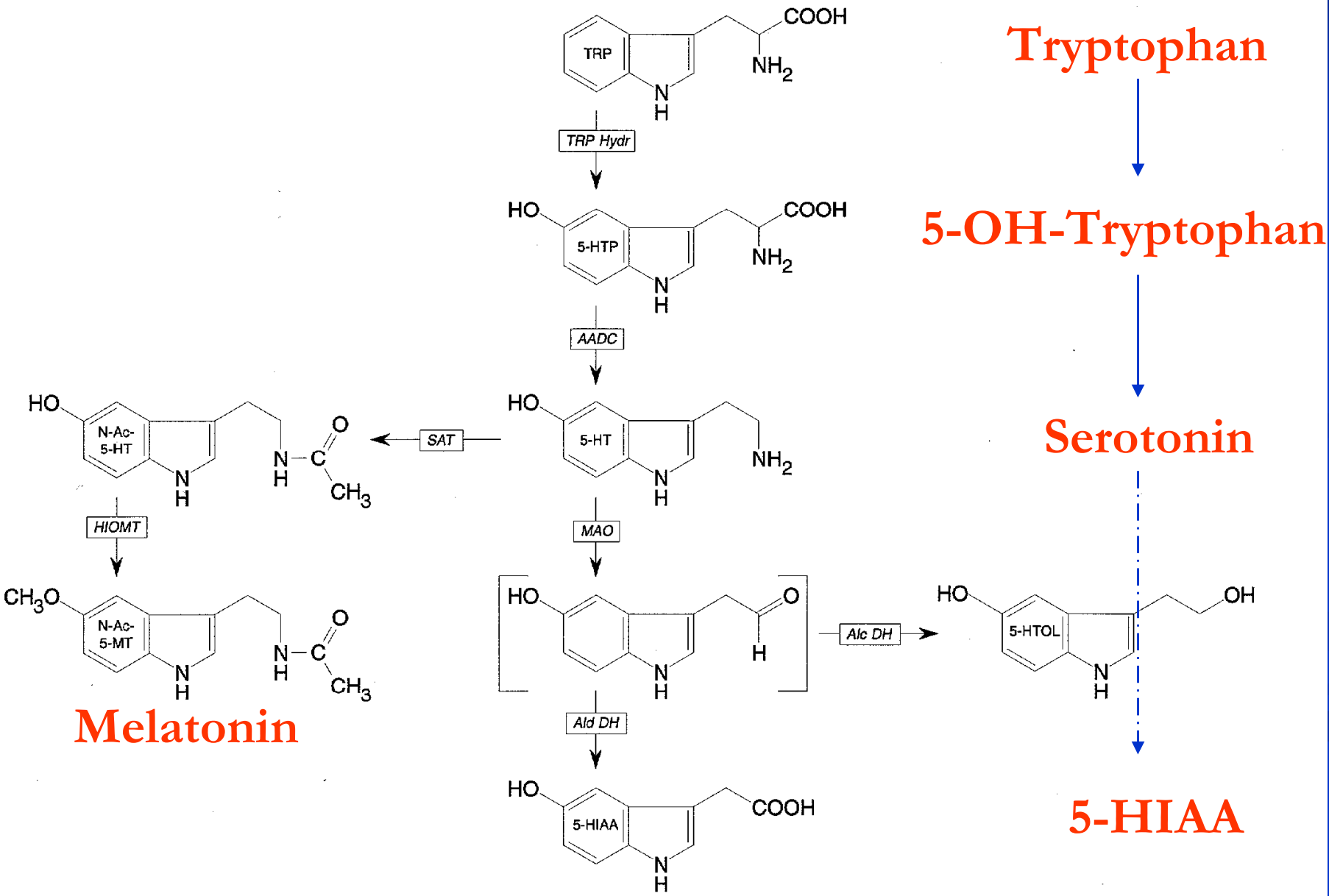
Biosynthesis serotonin

- From essential aminoacid tryptophan
- TRP hydroxylase
- Aromatic amino acid decarboxylase

Metabolism serotonin

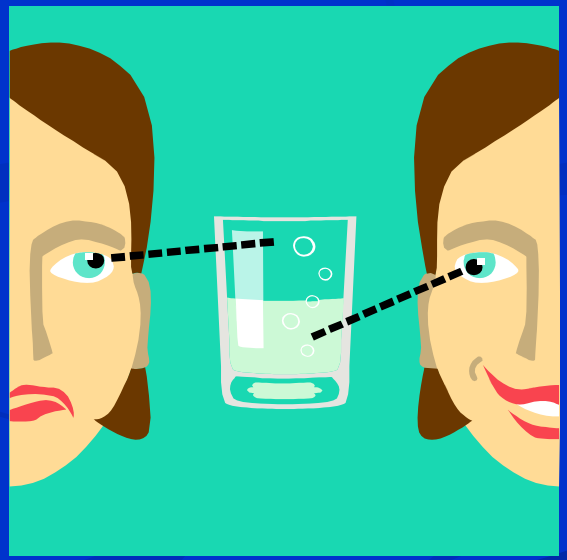
- MAO-A
  - 5-HIAA: most abundant (>98%)
- Aldehyde dehydrogenase
- Conjugation
- In lungs, liver and thrombocytes
- Excretion in urine

5-HIAA
Physiology



5-HT
Physiology

- Neurotransmitter, neuromodulator, hormone
  - Central nervous system
    - Sleep, wake
    - Food intake
    - Sexual activity
    - Mood
  - Peripheral
    - Vasoconstrictor
    - Neurotransmitter GI tract



5-HT
Pathophysiology

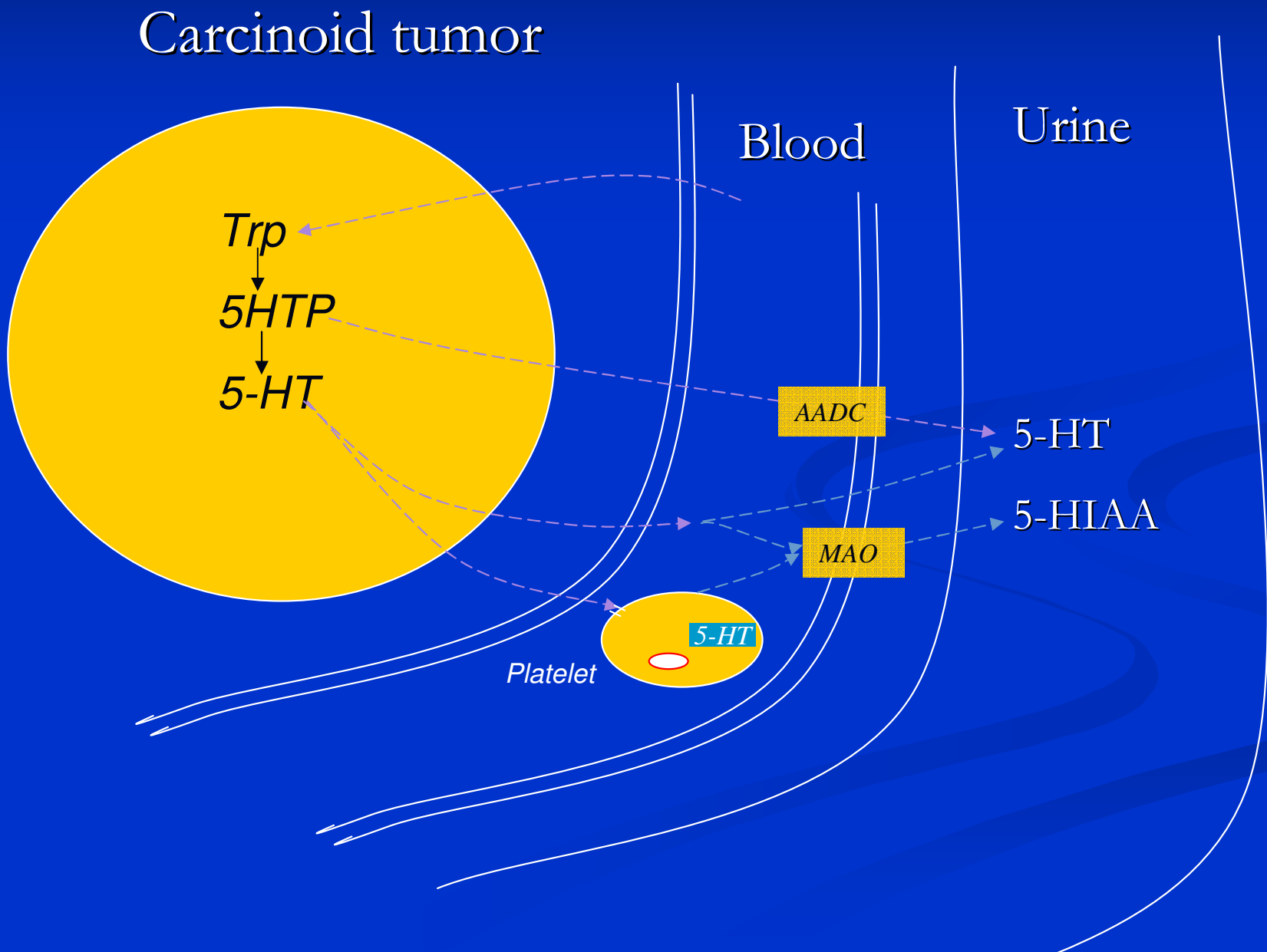
- Central nervous system
  - Eating disorders
  - Anxiety disorders
  - Depression
  - Schizophrenia
  - Autism
- Peripheral
  - Hypertension
  - Migraine
  - Raynaud's syndrome
  - Carcinoid syndrome

5-HT
Pathophysiology

Carcinoid

- Slowly growing neuro-endocrine tumour
- Incidence ± 2:100.000 population/year
- Classification
  - Foregut
  - Midgut (classic carcinoid)
  - Hindgut
- Carcinoid syndrom
  - Flushing
  - Heart dusease
  - Diarrhea
  - Bronchoconstriction, asthma, wheezing

5-HIAA
Biochemistry



Tumormarkers

- Platelet serotonin
  - Most discriminating indole marker for the detection of carcinoid tumors
  - Especially useful in carcinoids with low serotonin secretion rate
  - Saturation occurs: not suitable for follow-up at high secretion rates
- Urinary 5-HIAA
  - Tumor mass & follow-up

Analytical technique

Fluorometrical detection

Off-line HPLC

- Numerous reagents required
  - MeOH, AcN, ether, acetic acid, NaCl, etc
- Extensive manual sample work-up
  - Acidify sample
  - Pipet sample and IS
  - Add conc. acetic acid
  - Add NaCl
  - Shake for 2 min (extraction)
  - Centrifuge
  - Transfer to new tube
  - Evaporate to dryness under nitrogen
  - Add 2 mL eluens to residual
  - Transfer to vial
- Chromatographic run time: 10 min

On-line HPLC

- Less reagents required
  - AcN, water, formic acid
- No sample work-up
  - Increased reproducibility and repeatability
  - Filling of sample vials
    - Acidify sample
    - Pipet sample and IS
    - Dilute with water
- Run time including sample work-up: 20 min

Analytical technique

XLC

XLC-fluorescence

- Automated combination of techniques
- On-line and in-line
  - SPE: Solid Phase Extraction (Prospect)
  - HPLC: Chromatographic separation
  - Fluorometrical detection
  - Atlas data processing

XLC-MS/MS

- Automated combination of techniques
- On-line and in-line
  - SPE: Solid Phase Extraction (Symbiosis)
  - HPLC: Chromatographic separation
  - MS/MS: Tandem mass spectrometric detection
  - MassLynx data processing

Why method transfer from fluorescence to mass spectrometry?

- To obtain more accurate results
  - MS/MS detection
    - Deuterated IS
    - Increased selectivity and specificity
    - Increased linearity in high concentration range
    - Less interferences
- To reduce total analysis time per sample
- To relieve analists operating old technique

Analytical technique

XLC-MS/MS

Sample vial

XLC-MS-MS

(completely automated)

Data file

Vial to file concept

Analytical technique

XLC-MS/MS

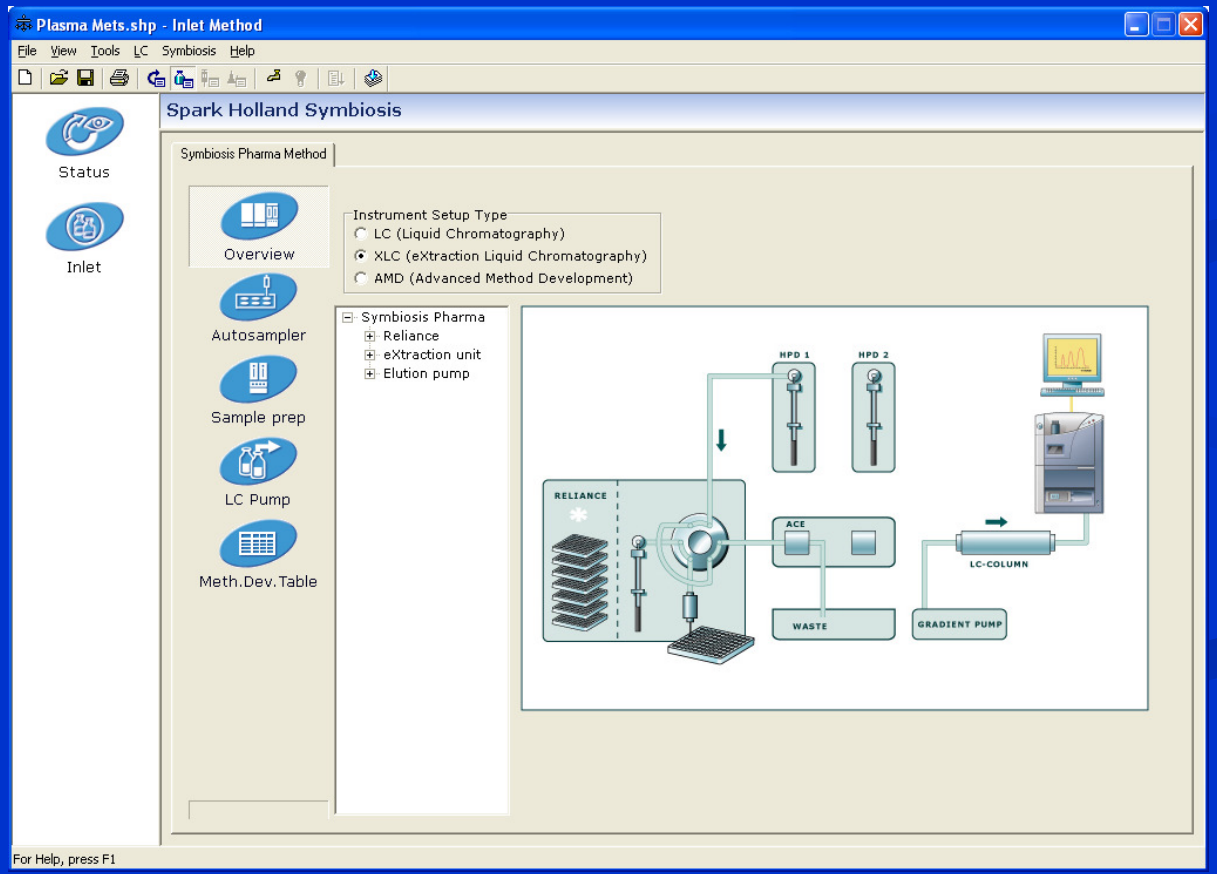
Spark Holland Symbiosis Pharma system
(sample storage, autosampling and SPE)

Quattro Premier
(LC-MS-MS system)

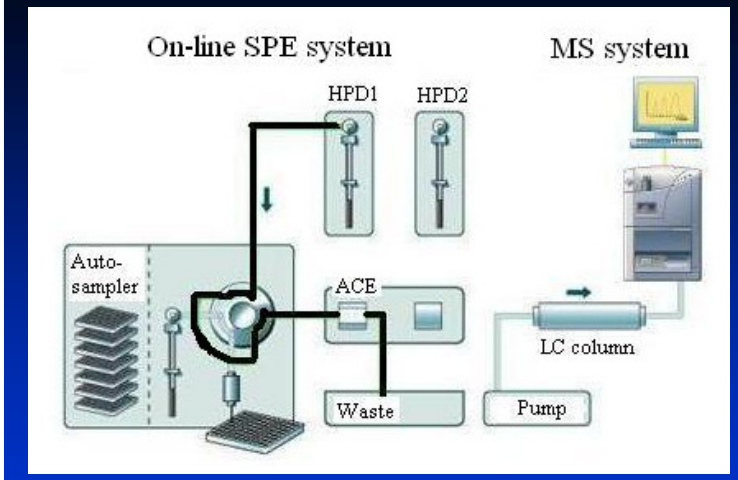
Analytical technique

Automation

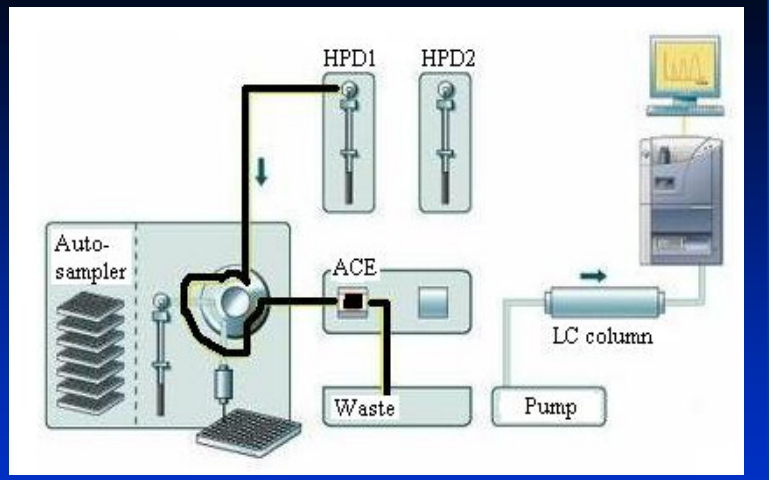
- Symbiosis™ Pharma & Waters® Quattro Premier™
- Both system are coupled by software
- Waters MassLynx software with Symbiosis Pharma driver



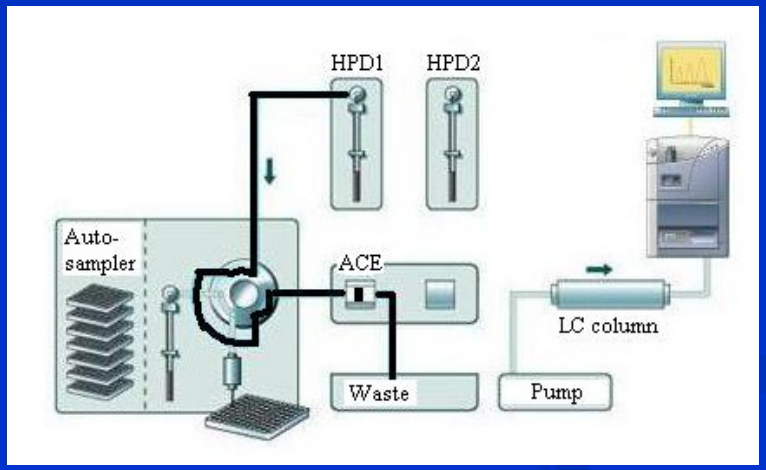
SPE



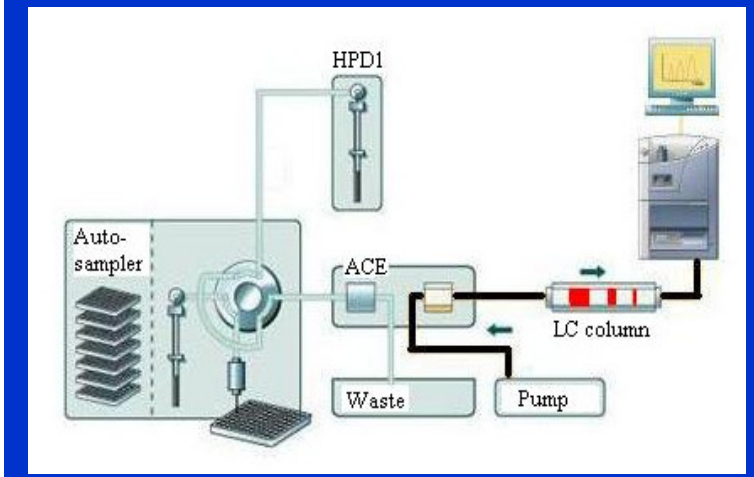
Conditioning/equilibration



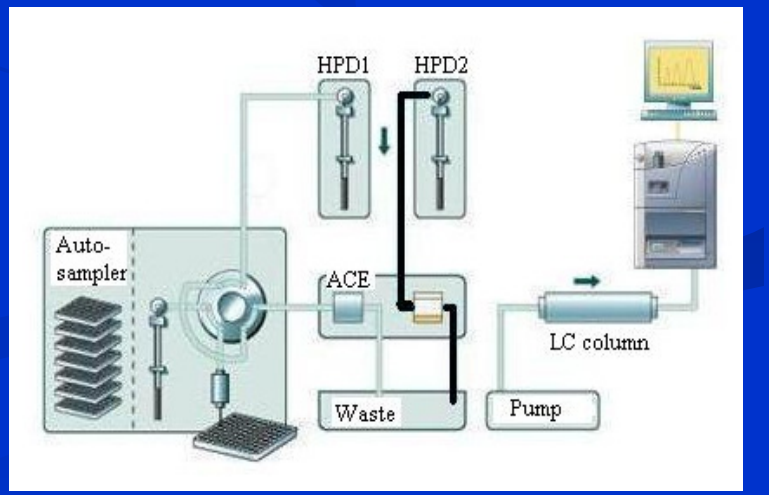
Sample loading



Washing



Elution



Clamp flush

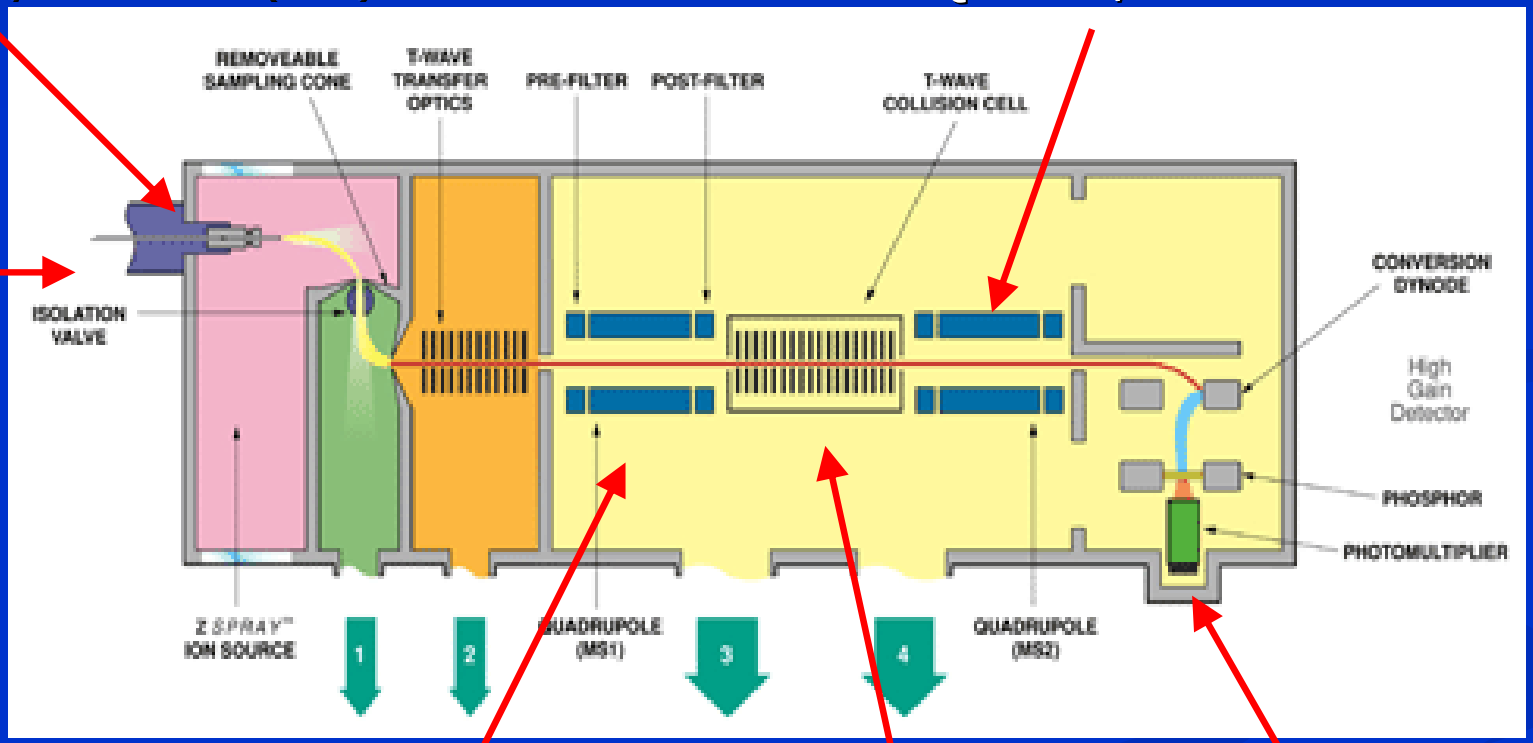
Analytical technique

LC-MS/MS

Inlet probe:
Electrospray ionisatie (ESI)

Quad 2 / MS2

LC kolom



Quad 1 / MS1

Collision cell

Multiplier

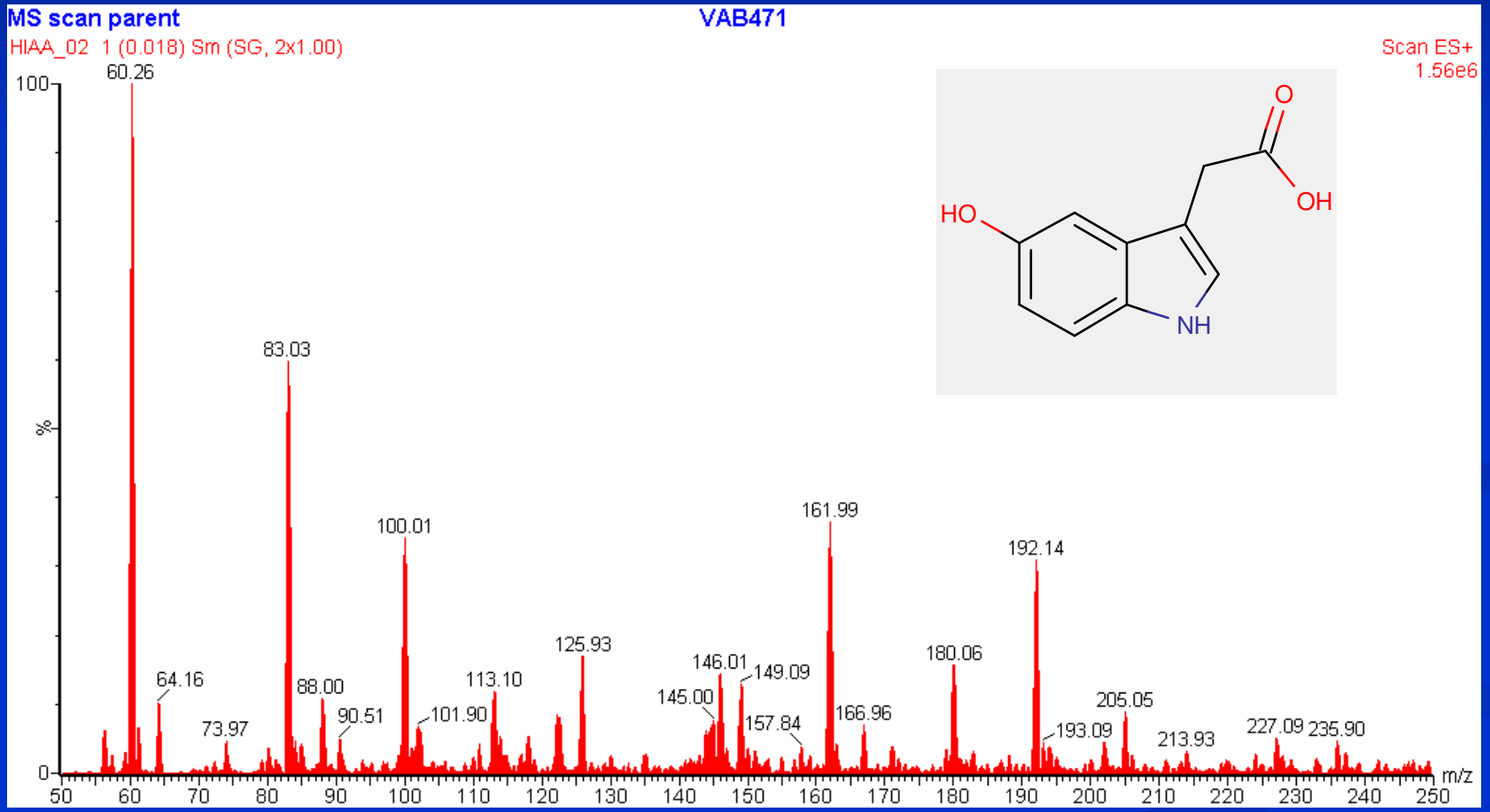
Analytical technique

Method development

- MS/MS
  - Characteristics analyte (acid, alkalic, mixed, neutral)
  - Selection parent ions
  - Selection daughter ions
  - Multiple Reaction Monitoring Mode
- HPLC
  - Selection chromatographic column
  - Optimization chromatographic separation (gradient)
- SPE
  - Selection cartridge sorbent
  - Characteristics analyte (acid, alkalic, mixed, neutral)
  - Matrix
  - Optimization extraction solvents
  - Optimization elution with LC gradient

Method development

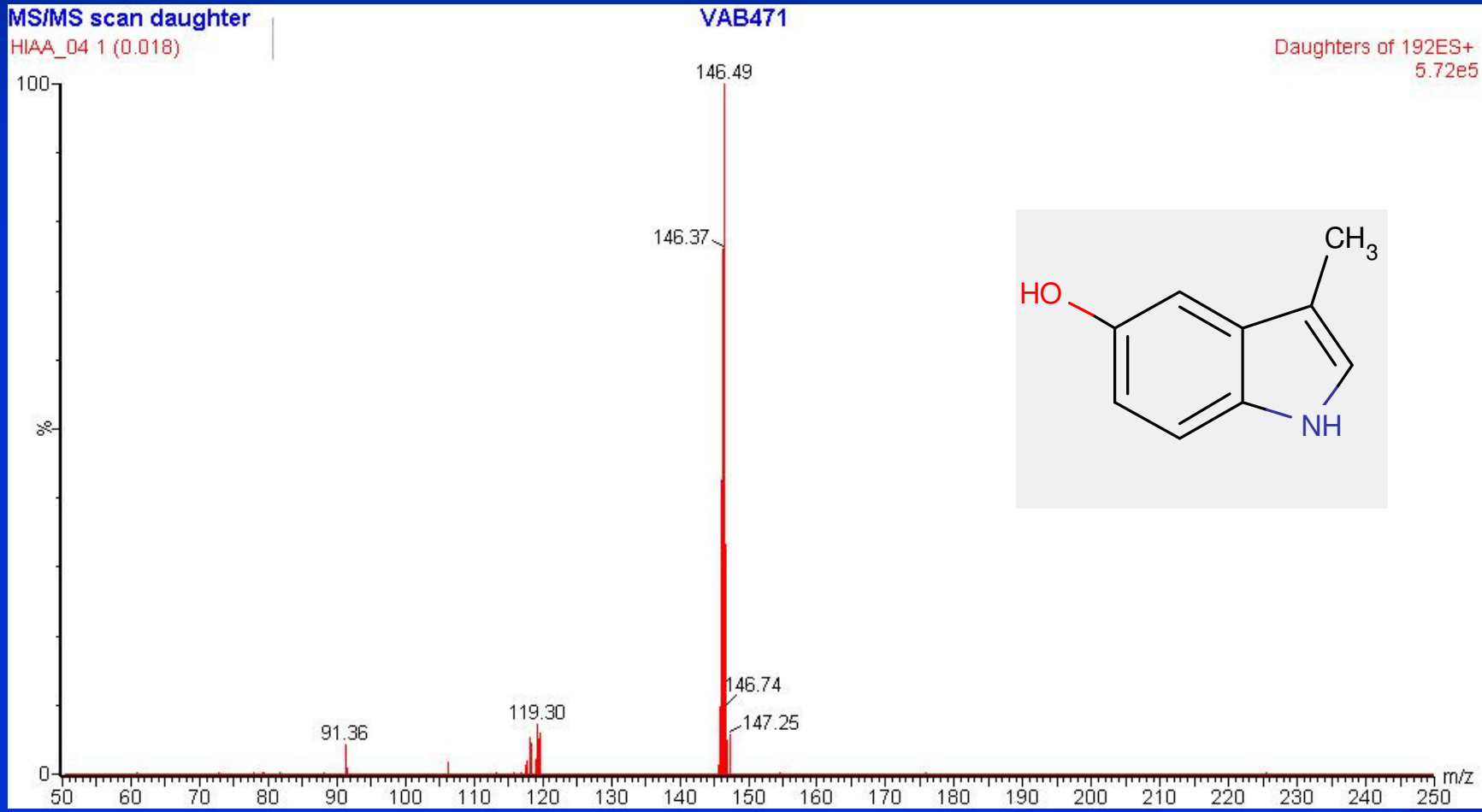
MS



Selected parent ion 5-HIAA

$[M+H]^+$ m/z 192.14

Method development
MS/MS

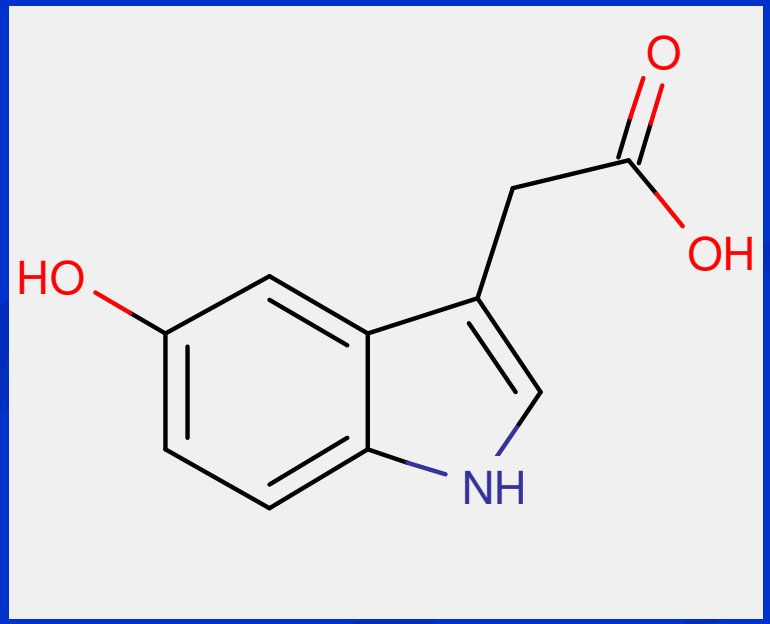


Daughterspectrum of parent 192.14
Selection m/z 146.49 (= M - COOH)

Method development
XLC

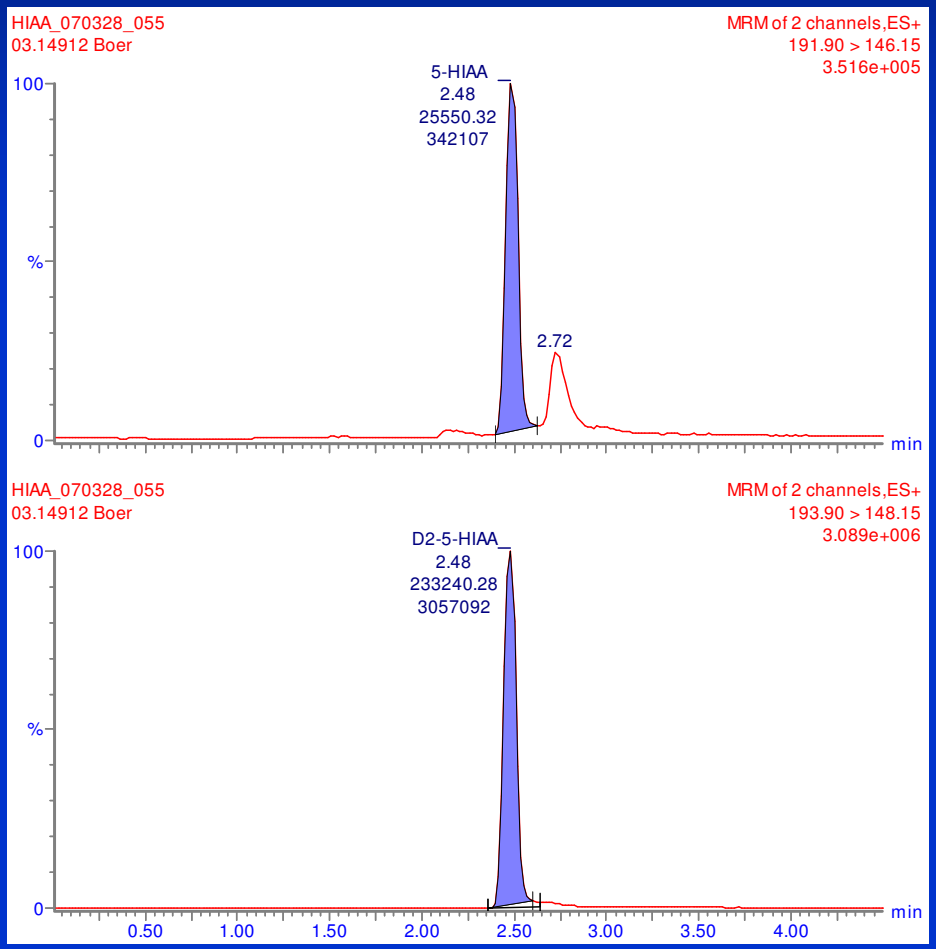
Analytical characteristics 5-HIAA

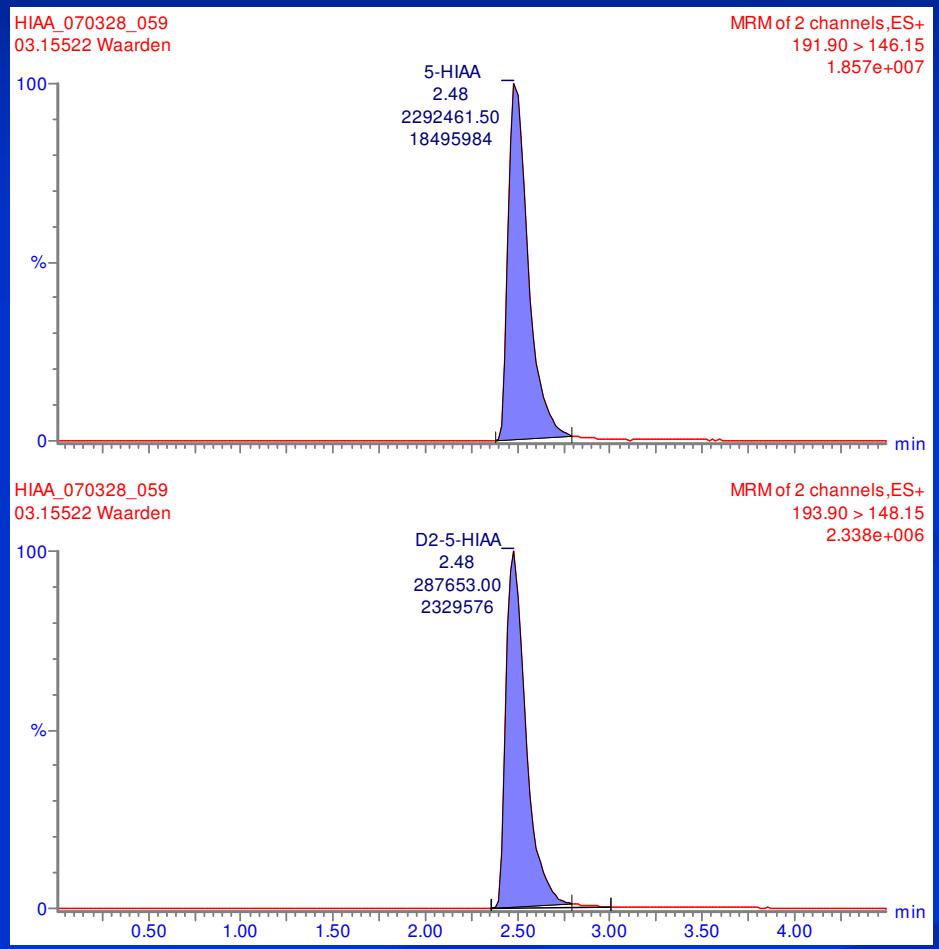
- Weak acid
- Hydrofobic interactions
  - C18 column
  - Hydrofobic SPE
- Acidic/organic gradient
  - Total run time 5 min



Results

Validation





Healthy control

10.3 mmol/L

1.7 mmol/mol kreat

High sample carcinoid patient

797.5 mmol/L

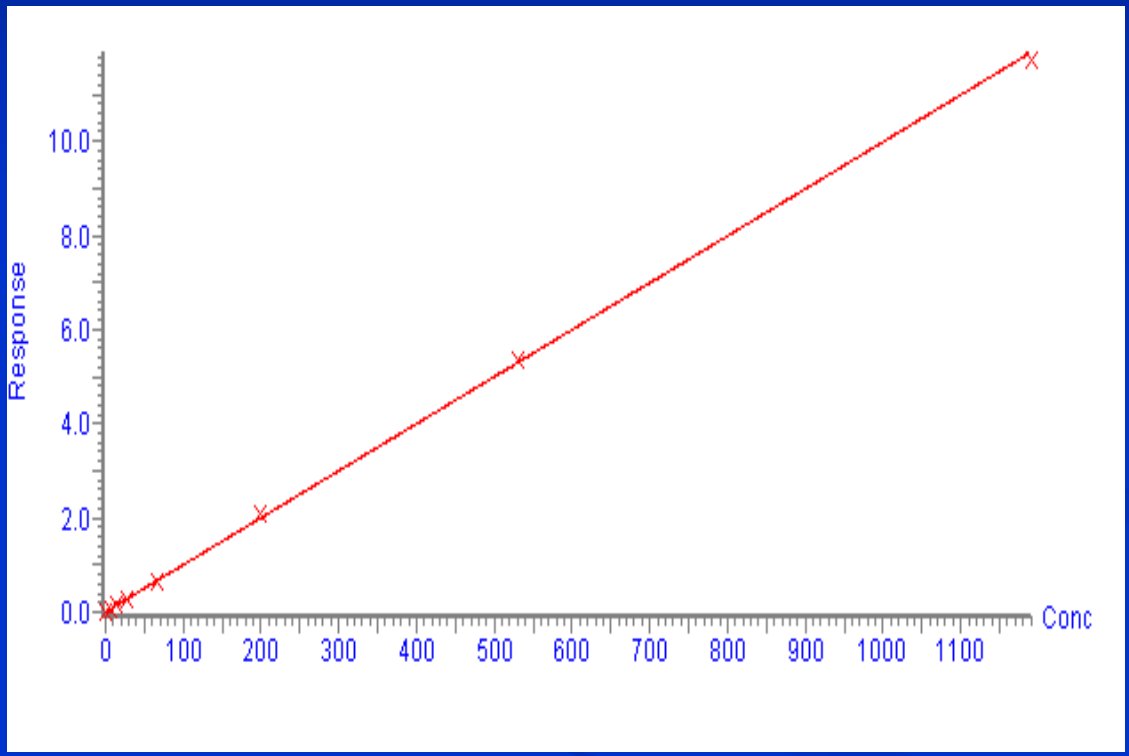
249.2 mmol/mol kreat

Results
Validation

Linearity in calibration-range

0-1200 mmol/L

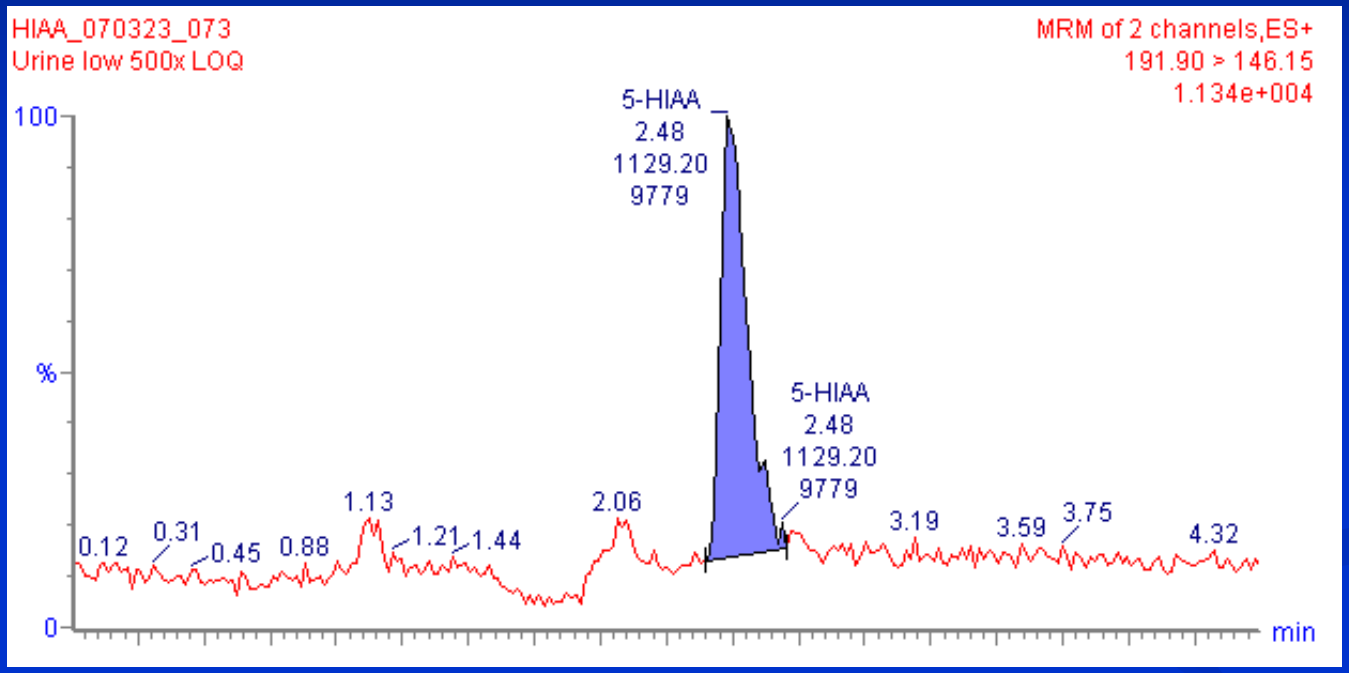
$R^2 > 0.99$



No carry-over (<0.1%)

Reuse cartridges (30 times)

Results
Validation



High sensitivity: LOD < 0.1 nmol/L
LOQ: 0.1 nmol/L

5-HIAA: 0.1 nmol/L
S:N = 25:1

Results
Validation

	Intra-assay		Inter-assay	
	Mean ± SD mmol/L	**CV** %	**Mean ± SD** mmol/L	**CV** %
5-HIAA				
Low	13.69 ± 0.12	0.91	13.57 ± 0.67	4.93
Medium	257.15 ± 3.58	1.39	252.17 ± 9.15	3.63
High	989.12 ± 7.70	0.78	968.55 ± 21.53	2.22

Precision
- Intra-assay variation
- Inter-assay variation

- Absolute recovery standards XLC vs LC
  - > 96 %
- Recovery cartridge
  - > 81%
- Stability in vial
  - 48 hr at 4 °C, 10 °C and room temperature

Conclusion

Promising technique in clinical chemistry

- Automated
- High-throughput
- Easy method switching
- Sensitive, specific and selective
- High quality, low variable costs

Future

Conversion of more clinical biomarkers to XLC-MS/MS mode

Acknowledgements

UMCG

- Department of Pathology and Laboratory Medicine
  - Ido Kema
  - Jan van der Molen
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Questions?