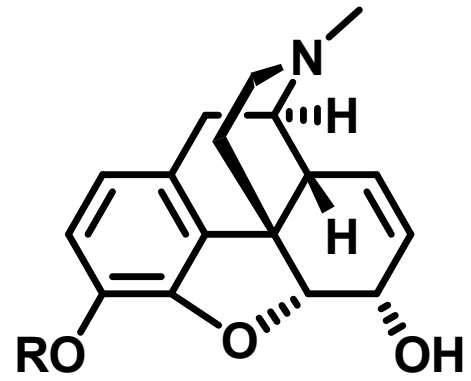
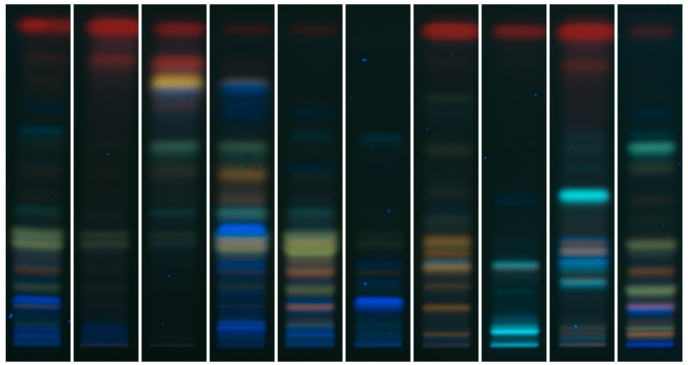
New fluorescent probes for the detection of enzymes inhibitors in complex matrixes

Maël Gainche, Clément Michelin, Elodie Jagu, Norberta Delporte
Clermont Auvergne INP, Sigma Clermont





CESMA group

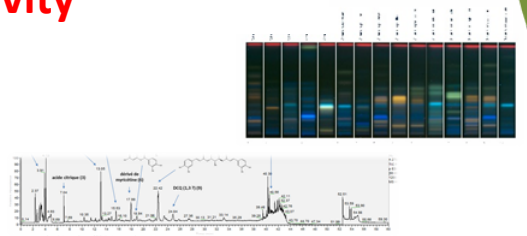
« Conception Extraction Synthesis of bioActive Molecules »

Natural substances

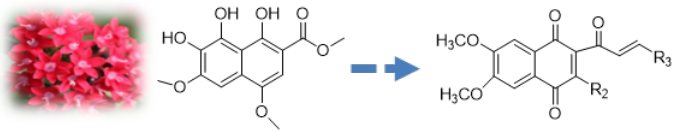
Medicinal chemistry

Extraction of natural matrixes

Phytochemical profile, investigation of complex matrixes, developement of new HPTLC method for screening of bioactivity



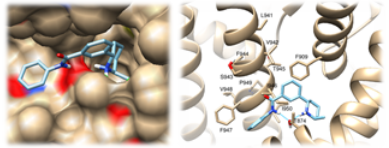
Hemisynthesis
Analog synthesis



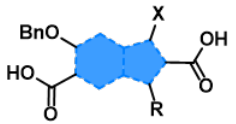
Development of new active principle

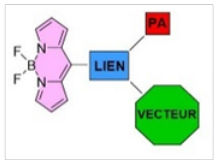
BIOACTIVITY

Inflammation, chronical pain, neuropathic pain, Cancer, antibacterial, etc.



Conception & molecular docking study





SAR
Vectorisation

Study of complex matrixes

Drying

Grinding

Extraction

Active crude extract

Molecule A

Molecule B

Molecule C

Chemical analysis

Chemo or Bioguided fractionnation

Liq/liq partition / Steric exclusion / Prep HPLC / Etc.

+

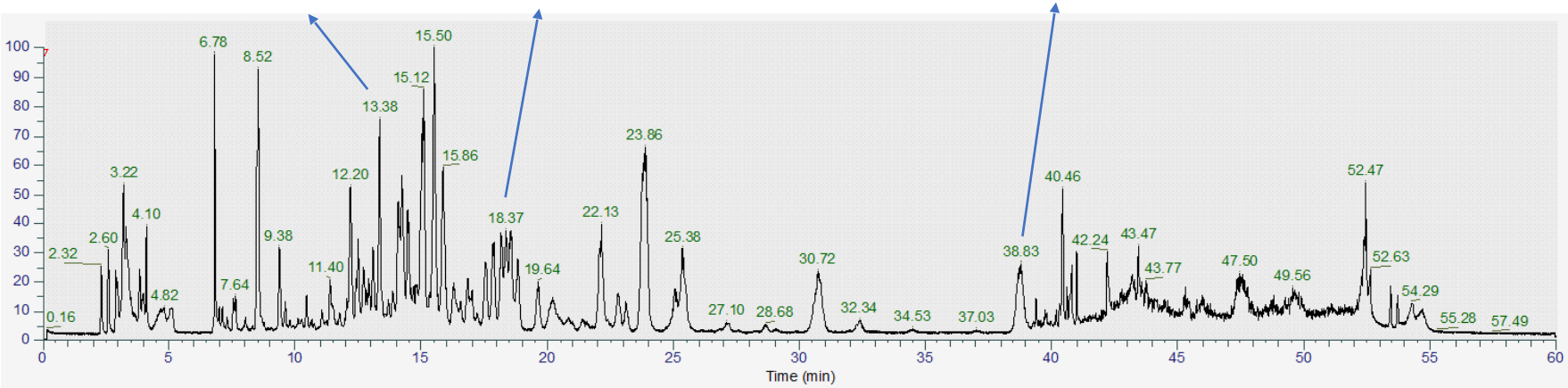
Structural determination

NMR 1D + 2D, LC-MS, LC-MS-MS

Rugosin D

Ellagic acid

Quercetin



Time-consumming approach

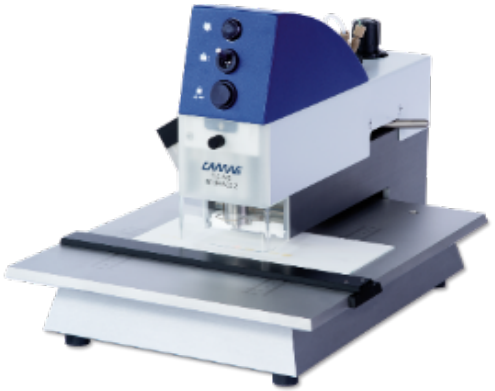
The HPTLC common flowchart

Sampling

Migration

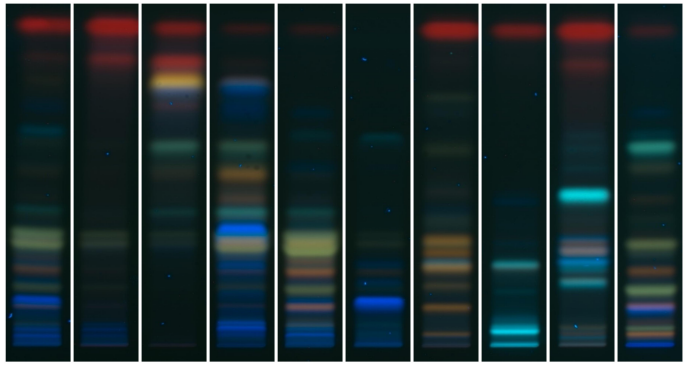
Visualisation

Quantification



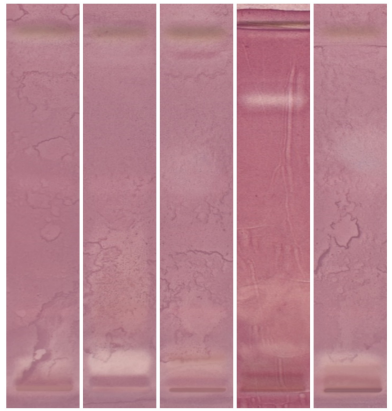
Mass identification interface

Derivatisation



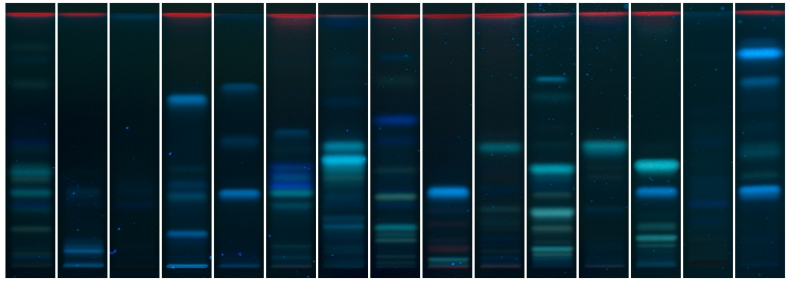
Derivatisation in HPTLC analysis

Derivatisation



Chemical

Chemical characterisation
Family of metabolites
Radical scavenging activity (DPPH)

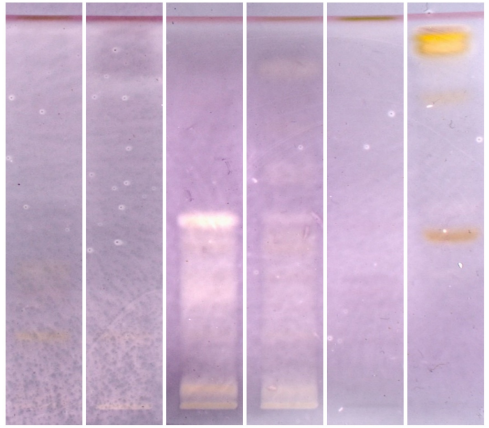


Enzymatic

Inhibition/activation of enzymes
Metabolization

Cellular

Antibacterial compounds
Endocrine disruptor
Cytotoxicity



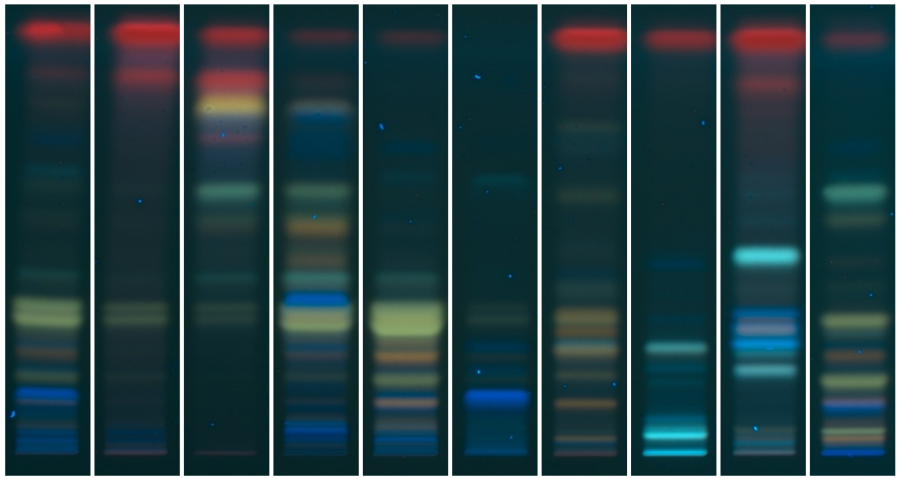
Enzymatic derivatisation in HPTLC analysis

Existing HPTLC enzymatic EDA

- **Tyrosinase**
- **Acetylcholinesterase**
- **Butyrylcholinesterase**
- **α-glucosidase**
- **β-glucosidase**
- **β-glucuronidase**
- **Xanthine oxydase**
- **Cyclooxygenase 1/2**
- **Peroxidase**
- **Aromatase**
- **Neuraminidase**
- **Lipase**
- **α-amylase**
- **Invertase**
- **Dipeptidyl peptidase-4**
- **Monoamine oxidase**
- **Glucose-6-phosphate dehydrogenase**
- **Etc.**

Interest of HPTLC

- ❖ High precision and sensitivity
- ❖ High reproducibility
- ❖ High throughput screening (maximum 23 tracks/plate)



Precaution when performing enzymatic test :

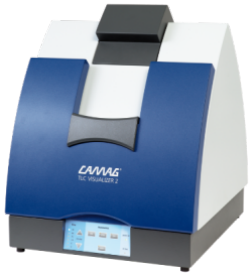
- ❖ Very sensitive method (better with an initial formation)
- ❖ pH sensitive (neutralisation step)
- ❖ Sensitive to organic solvents
- ❖ Price and commercially available enzymes

Focus on acetylcholinesterase EDA

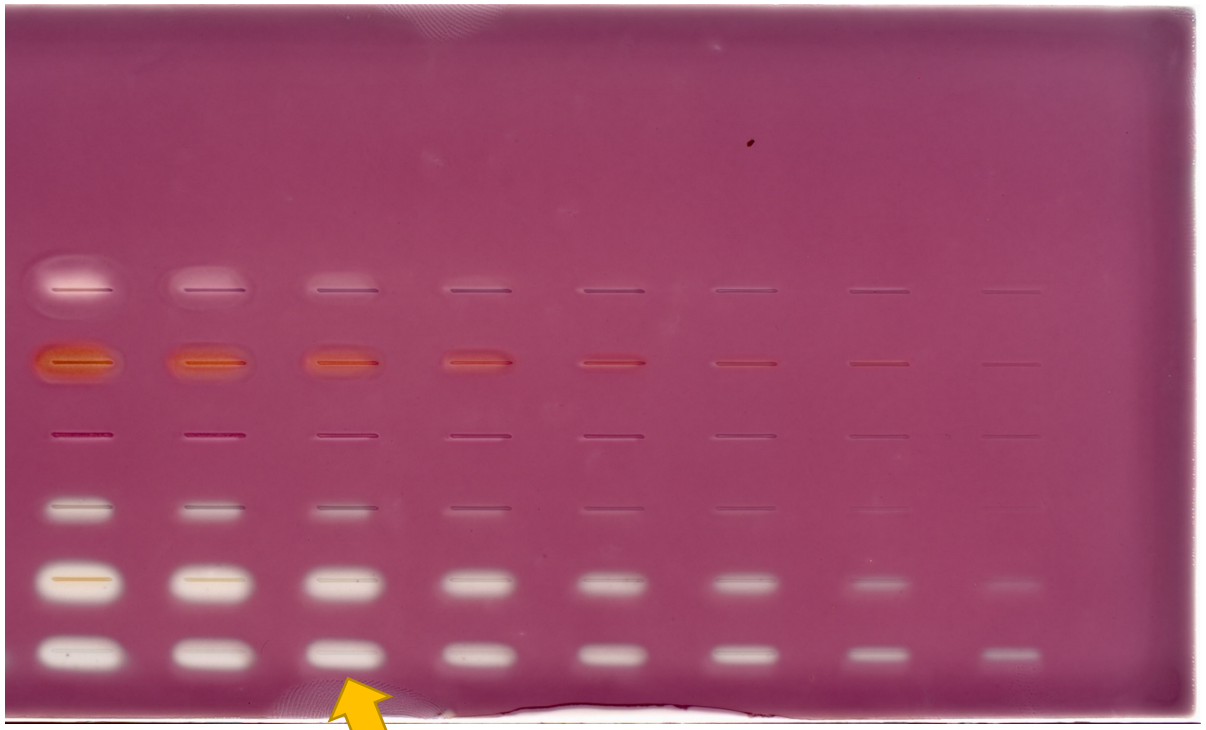
Effect Directed Analysis (EDA)

Sampling

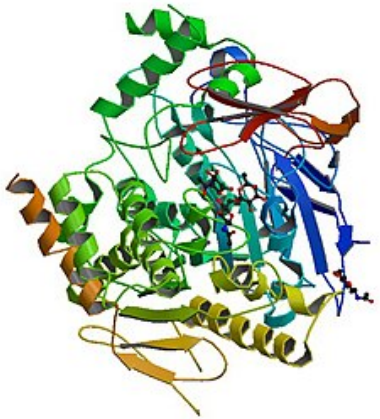
Migration



Visualisation



Spraying



Enzyme

Ex : acetylcholinesterase

1-naphtylacetate

1-naphtol

FBS

Azo dye

Potential inhibitor

Focus on acetylcholinesterase EDA

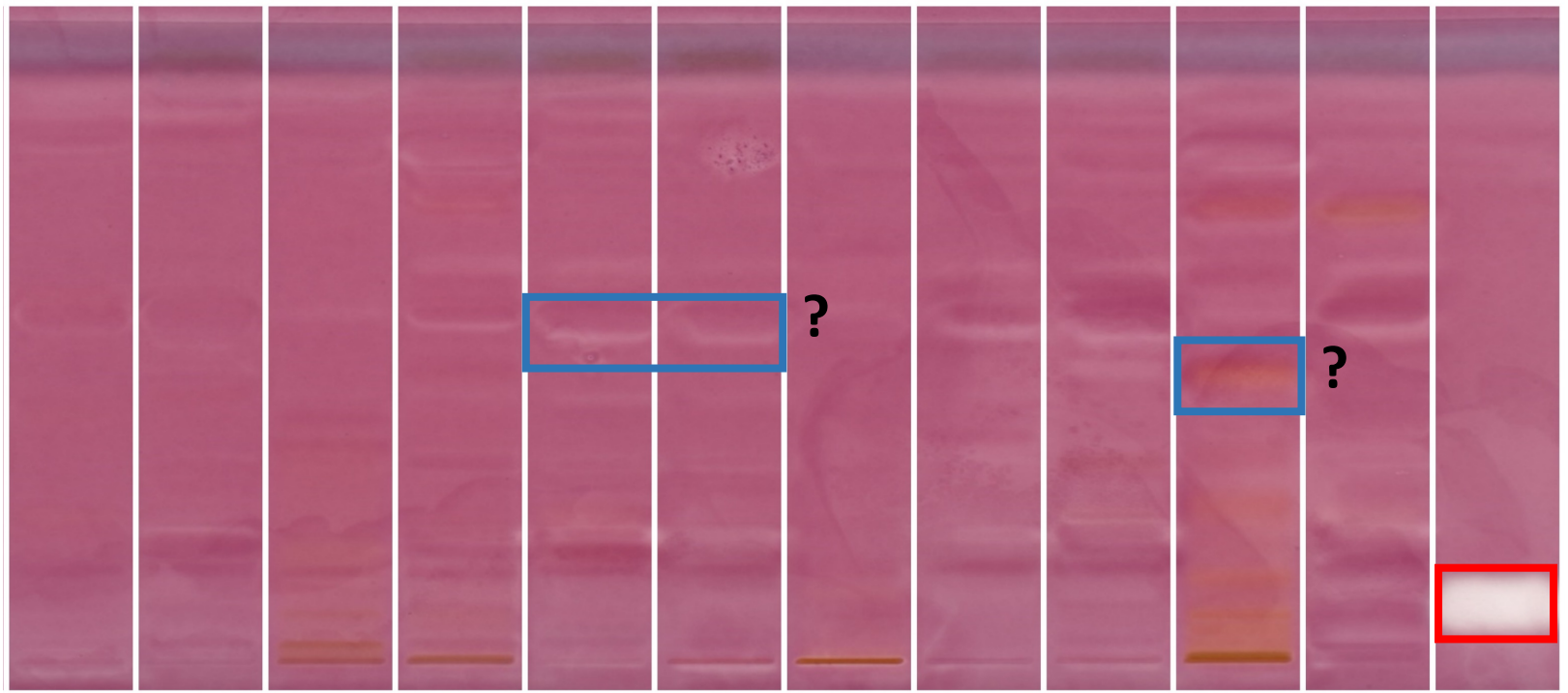
AChE

1-naphtylacetate → 1-naphtol

Azo dye

Fast Blue B Salt (FBS)

Parasitic reaction



Problems :

- Results not clear !
- Interaction/reaction with FBS
- Indirect detection (2 reactions)
- High quantity of extract on plate = poor resolution

Positive control (Galantamine)

Focus on acetylcholinesterase EDA

Galactosidase

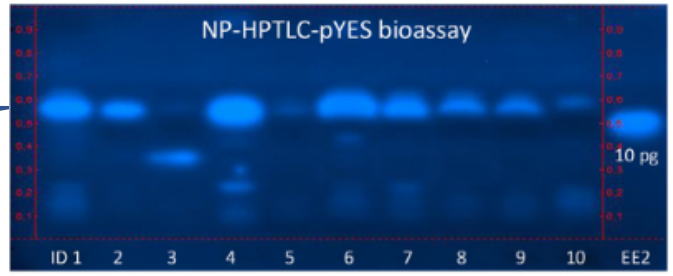
4-methylumbelliferone galactoside (4-MU-Gal)

4-methylumbelliferone (4-MU)

Fluorescent

λ_{ex} = 380 - 390 nm

λ_{em} = 410 nm



P-YES bioassay (endocrine disruptor)

Image taken from : https://doi.org/10.1016/j.chroma.2020.461511

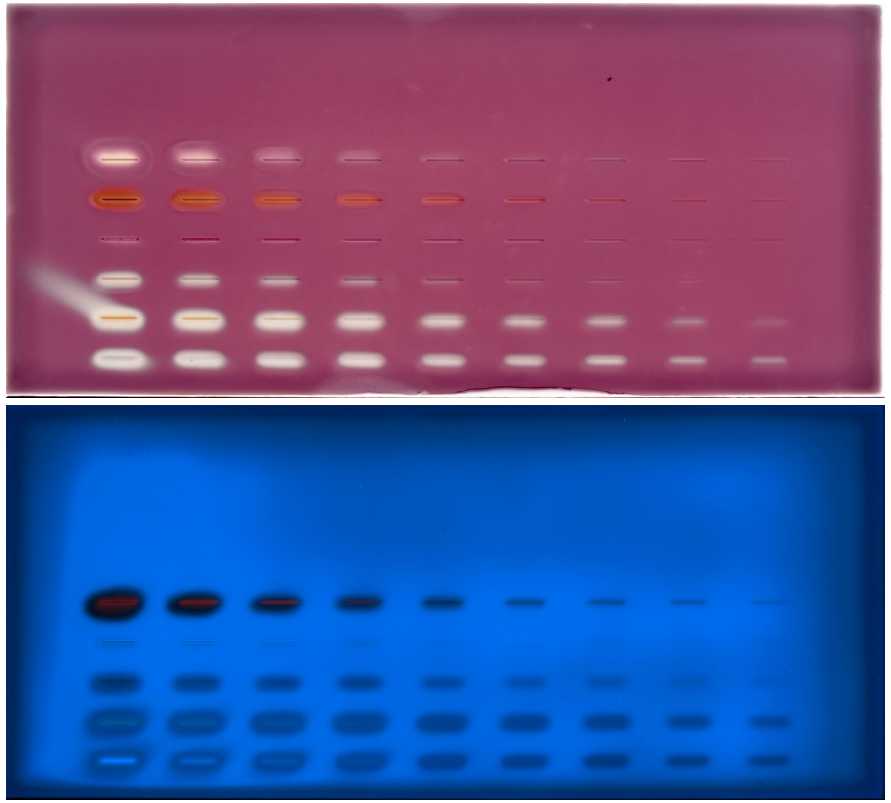
AChE

4-methylumbelliferone acetate (4-MU acetate)

4-methylumbelliferone (4-MU)

False negative : very low probability

False positive: fluorescence quenching



Comparison of EDA AChE using FBS (top) and 4MU (bottom)

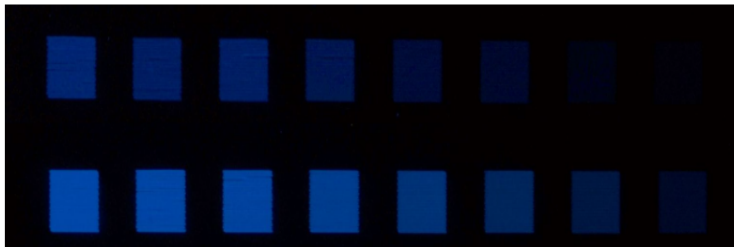
Fluorescent probe methodology

Calibration curve of 4-MU

After sampling

4MU-Ac

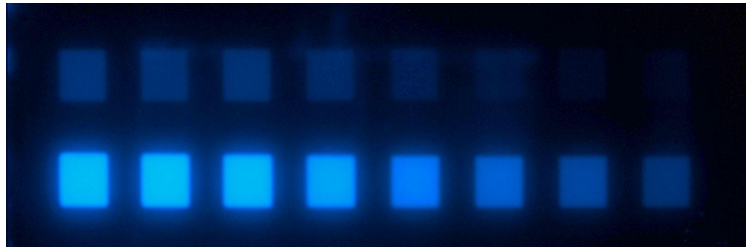
4MU



After plate wetting

4MU-Ac

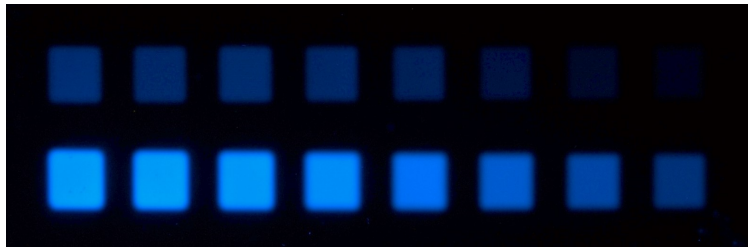
4MU



After drying step

4MU-Ac

4MU



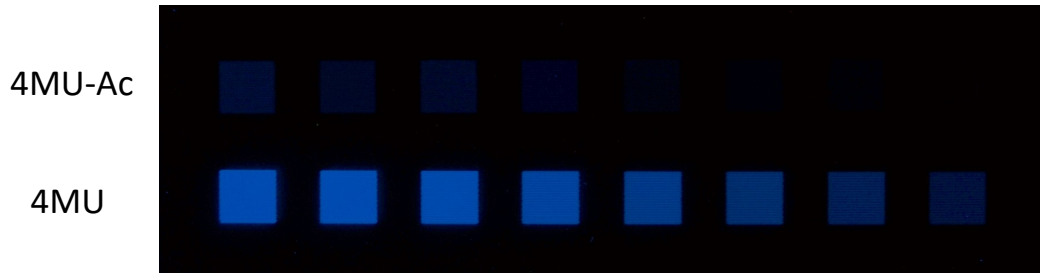
Range from 56.8 to 1.1 nM/cm²
Exposition time : 200 ms

- Small influence of pH on high concentration of 4MU
- High fluorescence delta between Ac-4MU and 4MU
- High contrast between 4MU and the plate

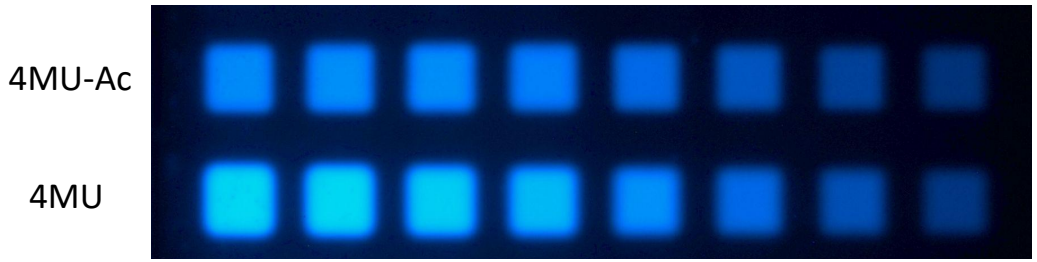
Fluorescent probe methodology

Measurement of AChE activity on plate

Before AChE



After AChE (20 min)



Range from 56.8 to 1.1 nmol/cm²
Exposition time : 200 ms

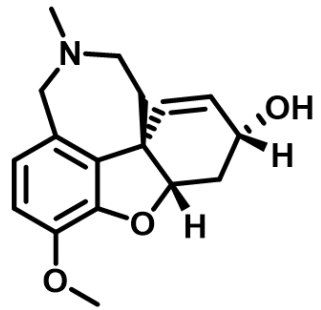
AChE transform Ac-4MU into 4MU

Fluorescent probe methodology

Measurement of Galantamine inhibitory activity

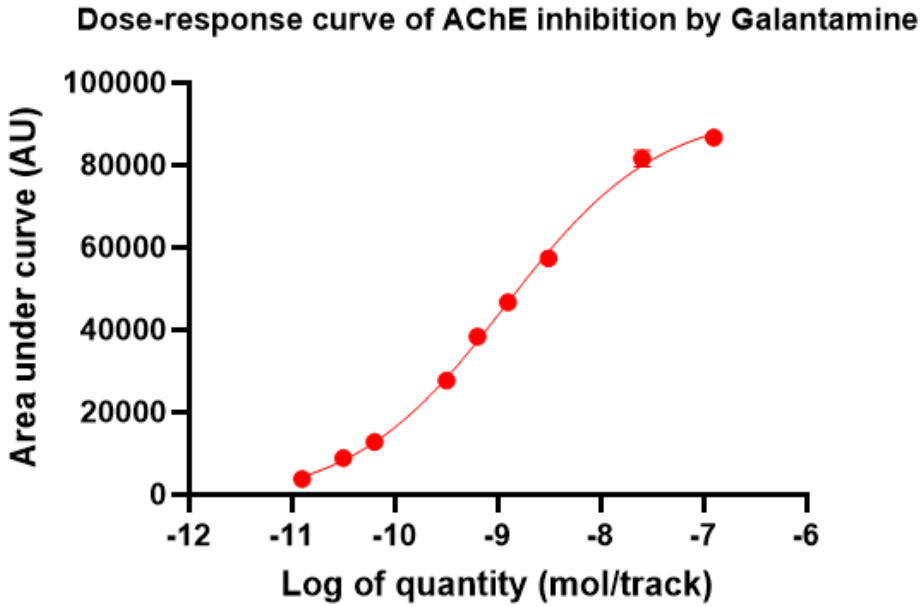
Increasing quantity of Galantamine

Photodensitometry using ImageJ



Galanthamine

Range from 109.7 to 0.0109 nmol
(n = 6) ; Average RSD = 9.7%



Pseudo IC_{50} ≈ 1 nmol / track

Fluorescent probe methodology

Method validation (using galantamine) and comparison with FBS

Chromogenic probe

Specificity : interaction with FBS and compounds possible

	Regression equation	16.046X + 129.91
	Correlation coefficient	0.988
	LOD (in ng/spot)	0.93 ± 0.05
	LOQ (in ng/spot)	3.09 ± 0.18
	Linearity range (ng/spot)	5 - 50
	Intra-day precision (RSD %)	Inter-day precision (RSD%)
50 ng	7.5%	0.9%
20 ng	7.6%	7.3%
10 ng	10.9%	11.3%
5 ng	12.7%	16.0%
2 ng	15.2%	17.7%
Average	10.8%	10.6%

Fluorescent probe

Specificity : necessity of AChE to change fluorescence

	Regression equation	685.17X + 682.19
	Correlation coefficient	0.991
	LOD (in ng/spot)	0.29 ± 0.02
	LOQ (in ng/spot)	0.95 ± 0.06
	Linearity range (ng/spot)	1 - 20
	Intra-day precision (RSD %)	Inter-day precision (RSD%)
50 ng	13.5%	3.4%
20 ng	18.2%	8.0%
10 ng	17.9%	15.6%
5 ng	14.4%	4.8%
2 ng	14.5%	19.5%
Average	15.7%	10.3%

Robustness : 3 different manipulator + preparation of fresh solution every time

Every concentration was analysed with **n = 18**

Lower LOD/LOQ by a factor of 3.2 !

Less possibility of incorrect results

Fluorescent probe methodology

Method validation (using galantamine) and comparison with FBS

ELSEVIER

Journal of Chromatography A

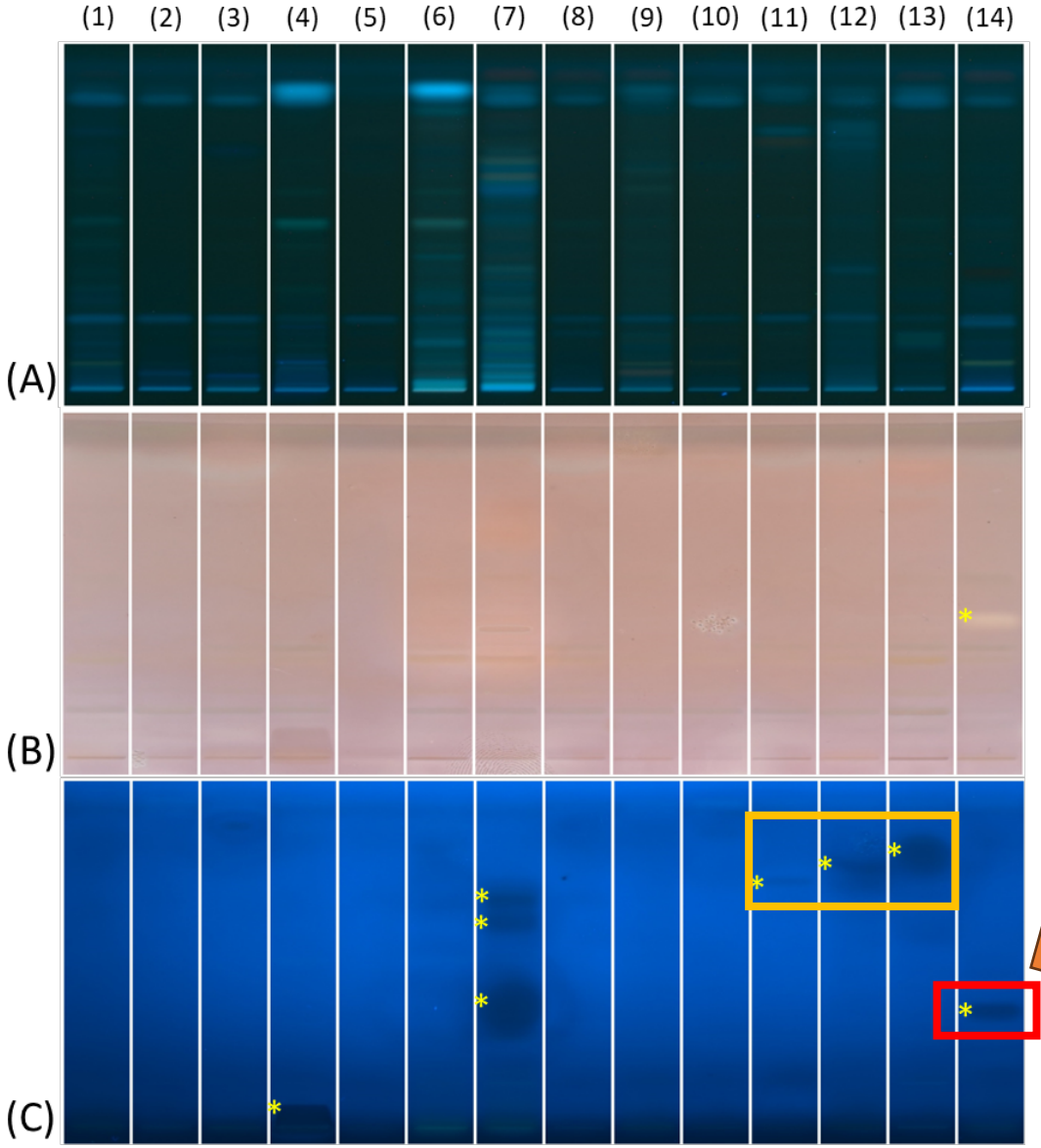
Volume 1708, 11 October 2023, 464330

Fluorescent probe for the detection of acetylcholinesterase inhibitors using high performance thin layer chromatography effect-directed assay in complex matrices

M. Gainche, N. Delporte, C. Michelin, E. Jagu

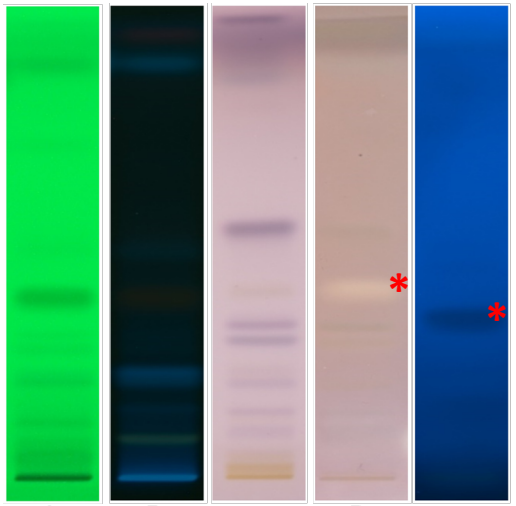
https://doi.org/10.1016/j.chroma.2023.464330

Fluorescent probe for screening



Xerocomus chrysenteron
(Boletaceae)

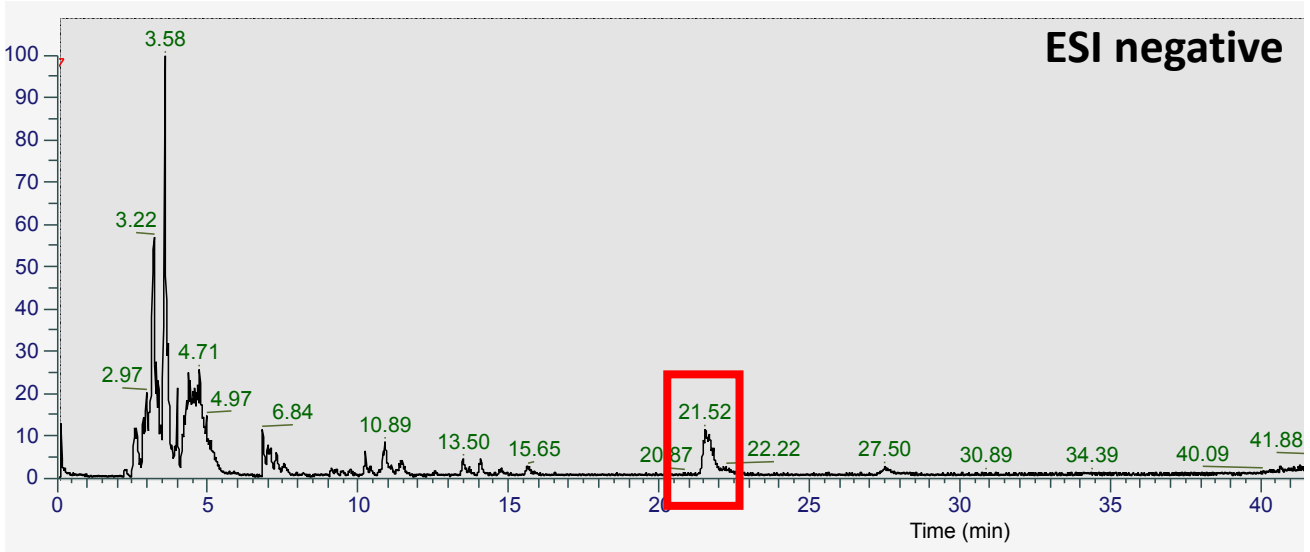
MeOH extract HPTLC fingerprinting



A – 254 nm
B – 366 nm
C – p-anisaldehyde (Vis)
D – AChE EDA using 1-naphtylacetate/FBS
E – AChE EDA using 4-MU acetate

* Active metabolites

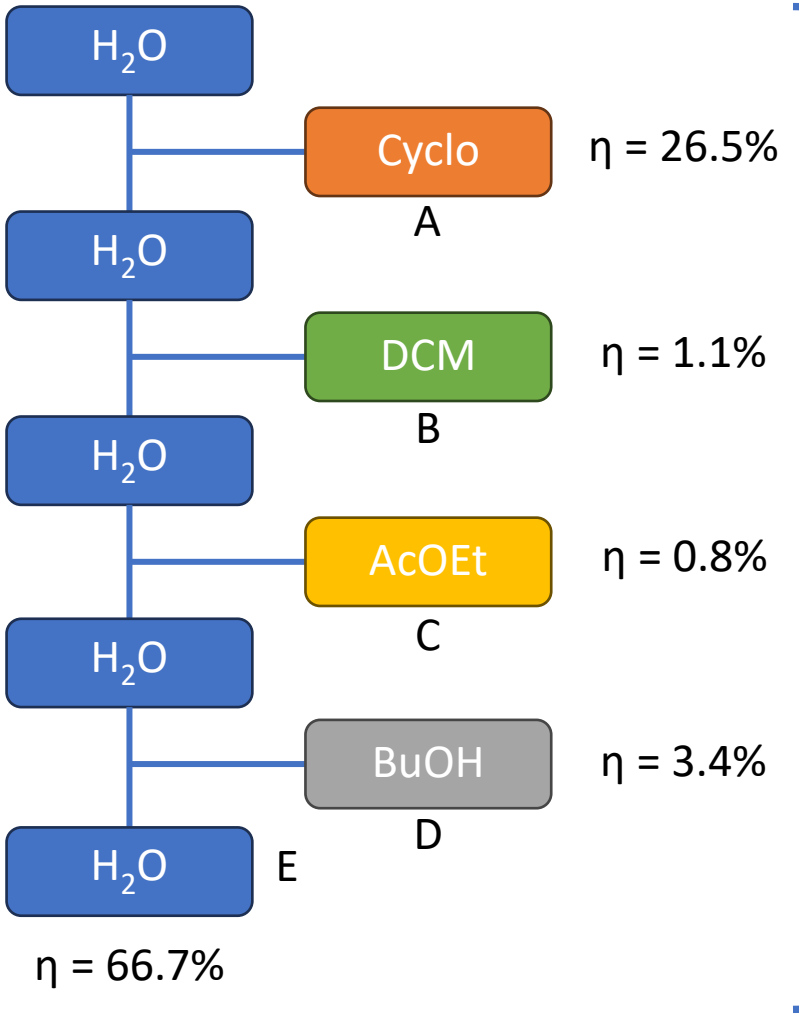
Extract concentration : **50 µg/spot**



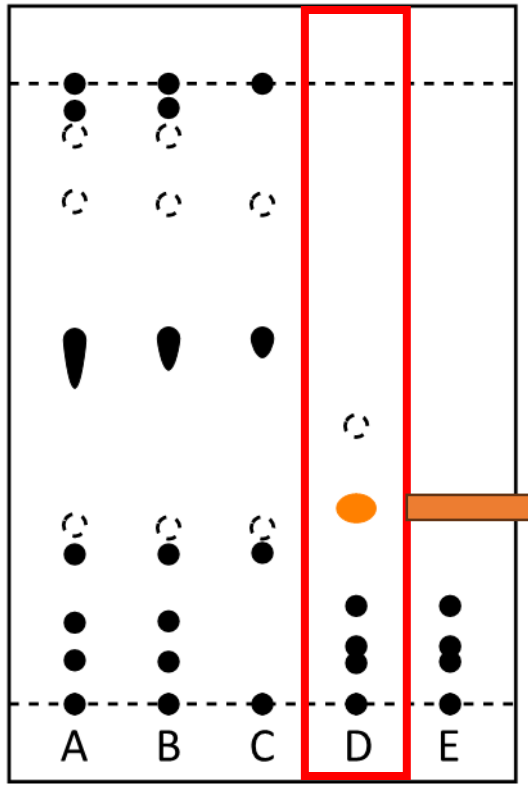
A : FLD 366 nm ; **B :** EDA AChE FBS (Vis) ; **C :** EDA AChE 4MU-Ac (366 nm)

Fluorescent probe for screening

Investigation of *X. chrysenteron*



5 fractions of increasing polarity



Variegatic acid

Variegatic acid HPTLC:

A (top) – 366 nm

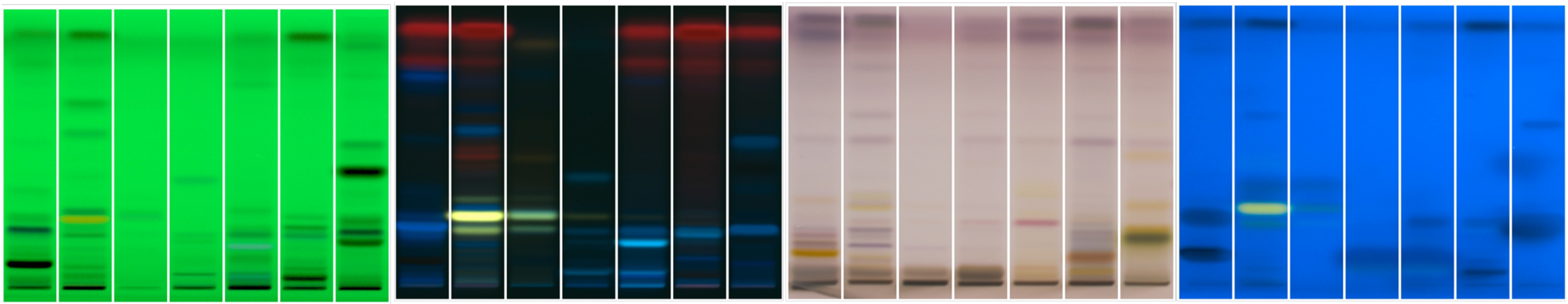
B (middle) – AChE EDA using 1-naphtylacetate/FBS

C (bottom) – AChE EDA using 4-MU acetate

Spectrophotometric test in progress

Work in progress and perspectives

- Measurement of kinetic parameters
- Comparison of IC_{50} on plate
- Modification of probe
- Design and synthesis of new probes for other enzymes
- Validation of all the analytical method
- Screening on natural matrixes and purification of active compounds



Thanks for your attention !