

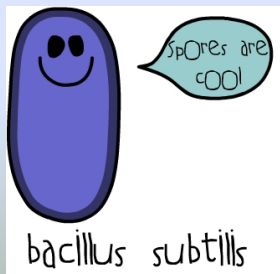


**UMCS**  
MARIA CURIE-SKŁODOWSKA UNIVERSITY

# The Novel TLC-Direct Bioautography Tests for Analysis of Antimicrobials

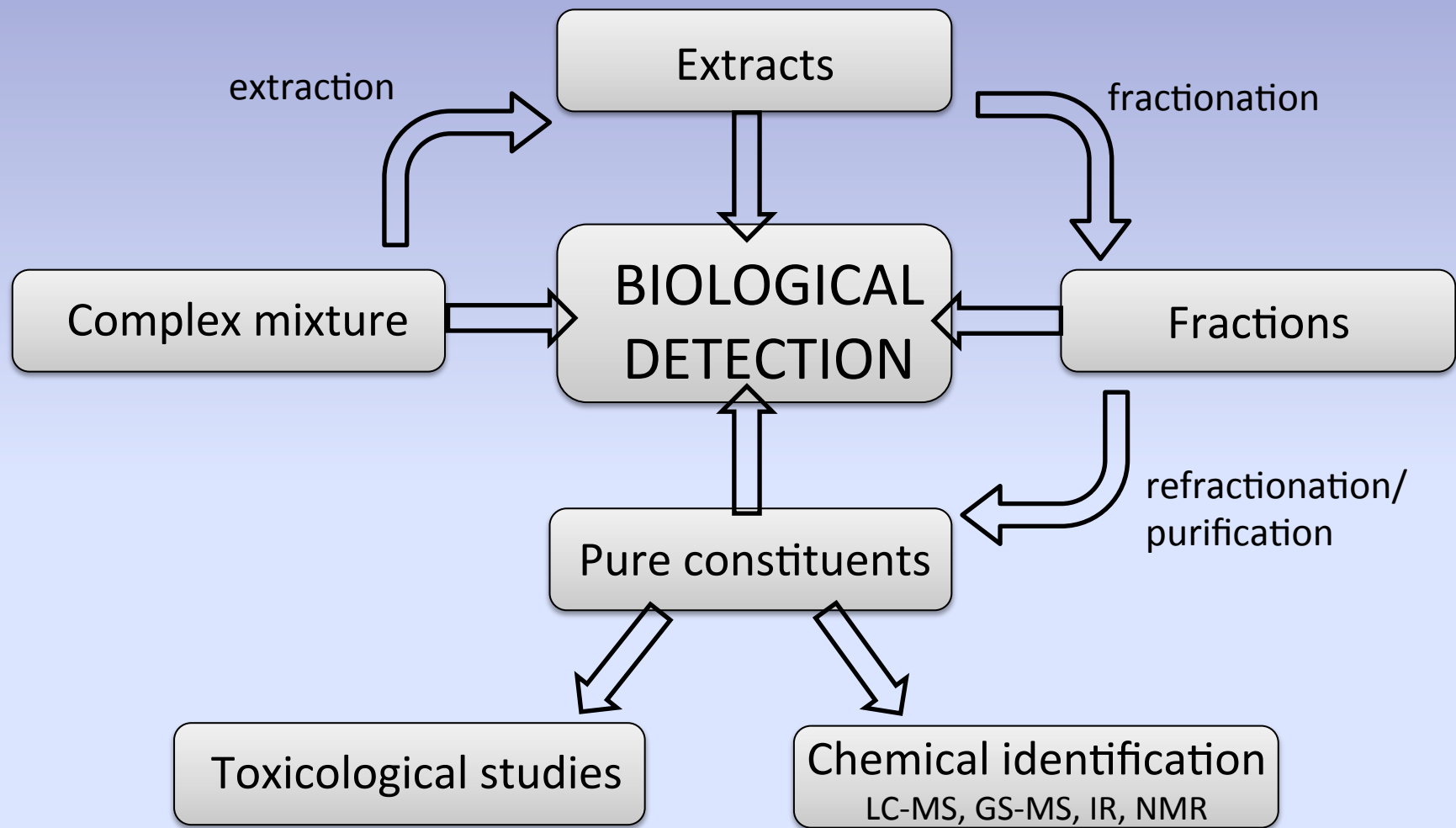
**Irena M. Choma, Edyta M. Grzelak, Wioleta Jesionek**  
M. Curie-Skłodowska University, Lublin, Poland

**Barbara Majer Dziedzic**  
University of Life Sciences, Lublin, Poland



**HPTLC Lyon 2014**







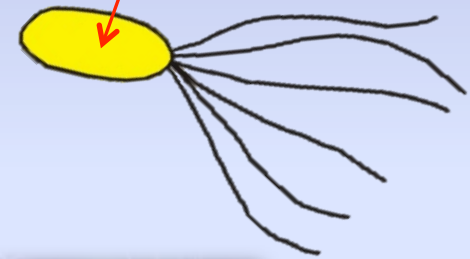
*Cladosporium cucumerinum*



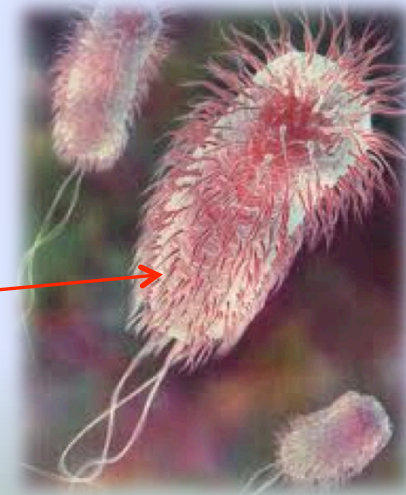
*Candida albicans*



*Aliivibrio fischeri*

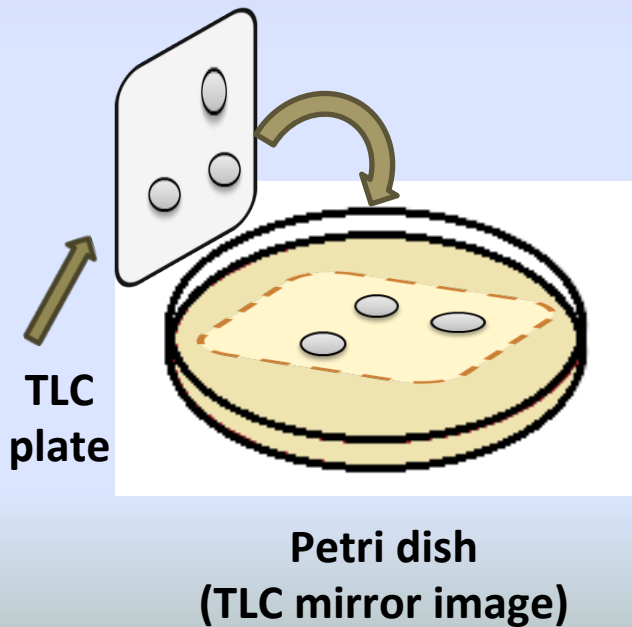


*Escherichia coli*



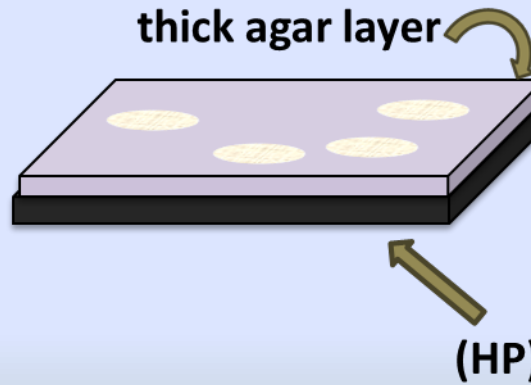
# Bioautography

## contact bioautography



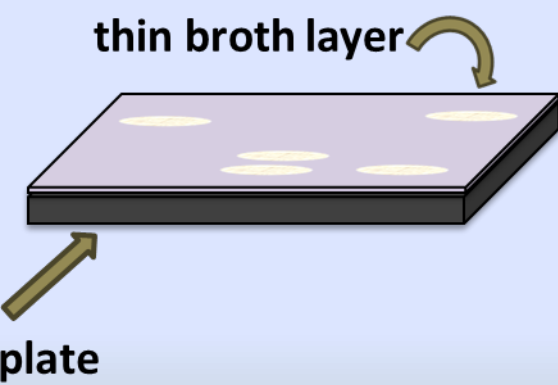
## immersion/overlay bioautography

- immersion in agar
- overlay with agar



## direct bioautography

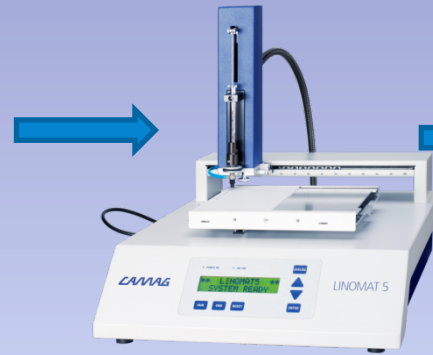
- dipping in broth
- spraying with broth



# TLC-DB PROCEDURE



sample



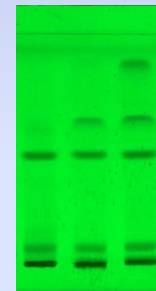
aplication



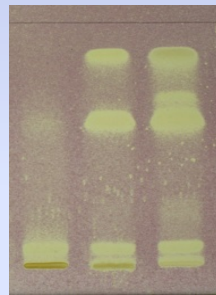
separation



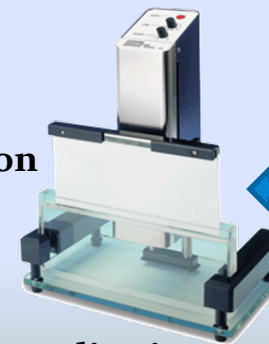
documentation



visualization



Detection of antioxidants

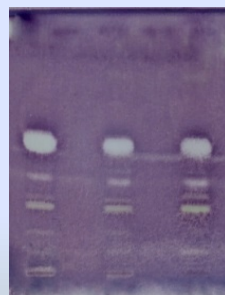


dipping

incubation

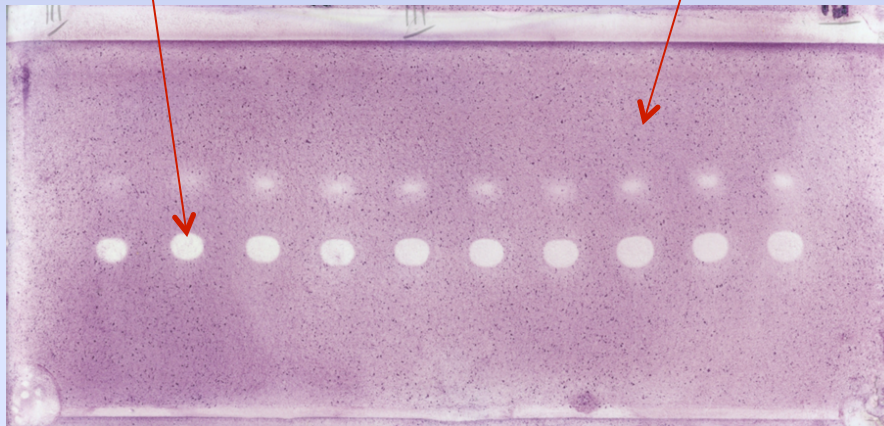
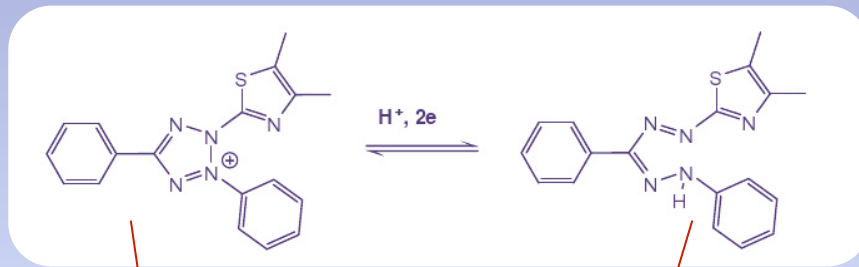


visualization



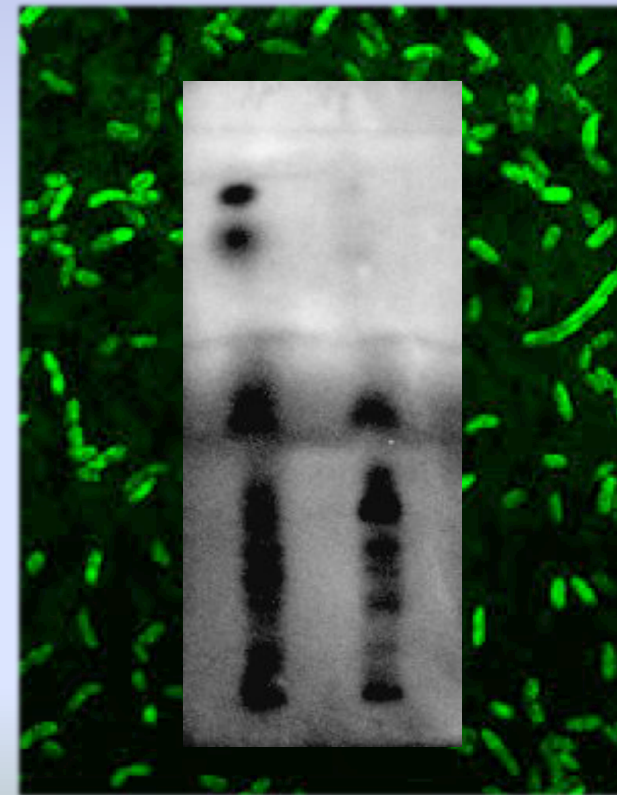
Detection of antimicrobials

# Reduction of tetrazolium salt (MTT) by dehydrogenases of living bacteria

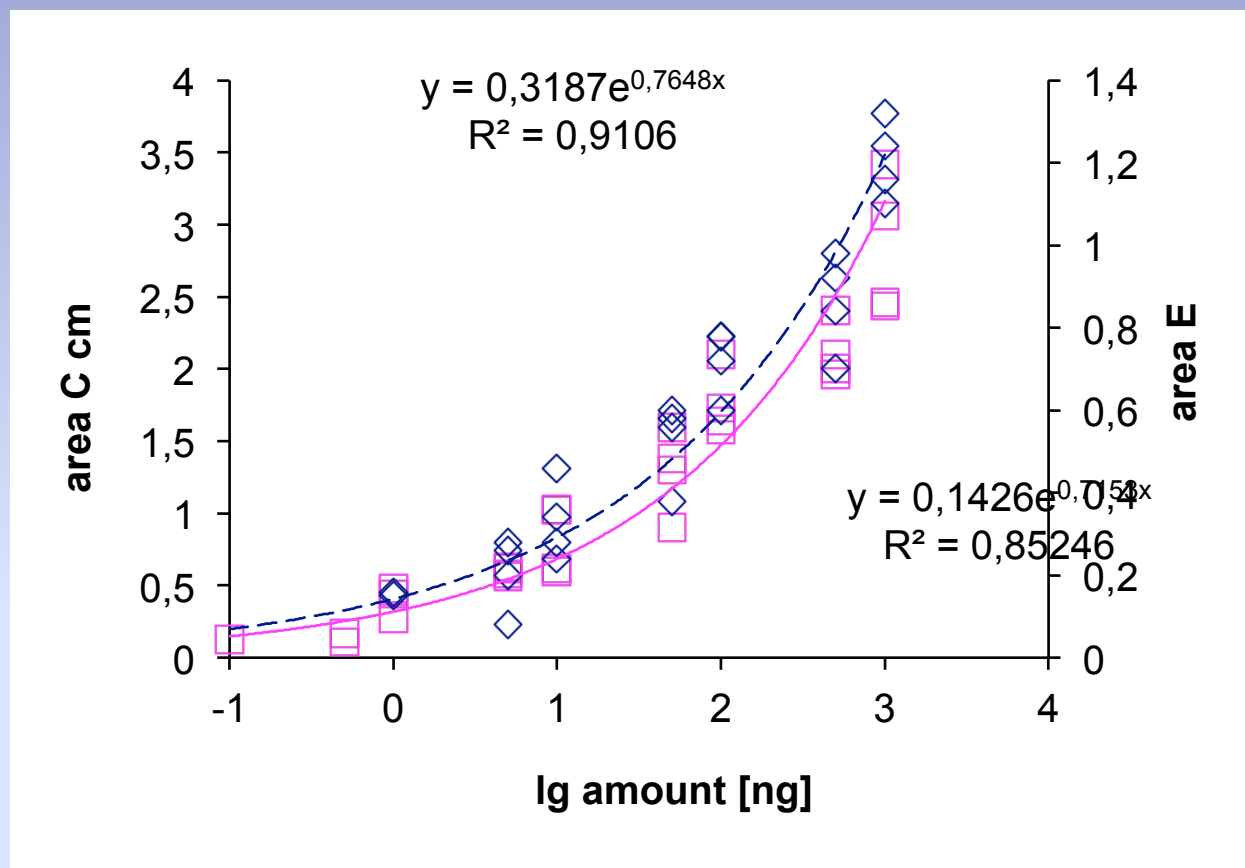
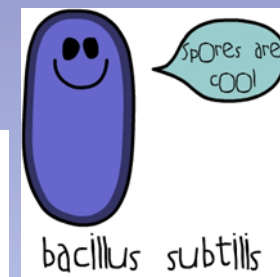


*Bacillus subtilis*

# Extinction of luminescence

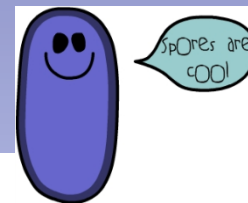


*Aliivibrio fischeri*

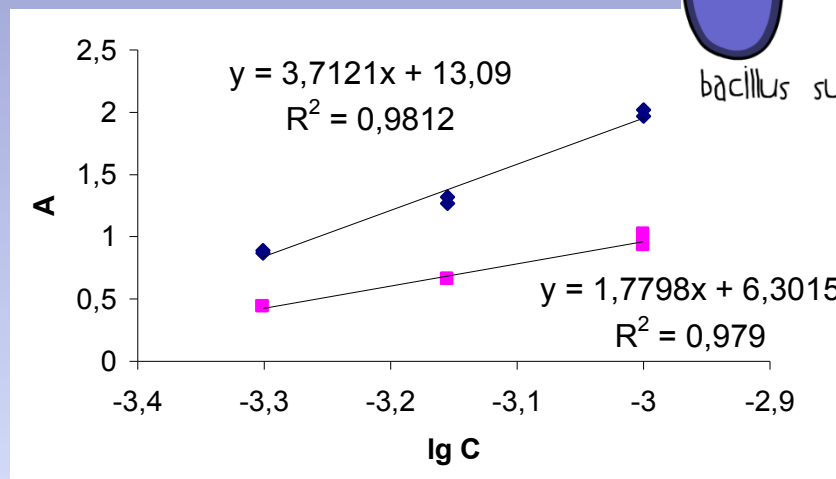
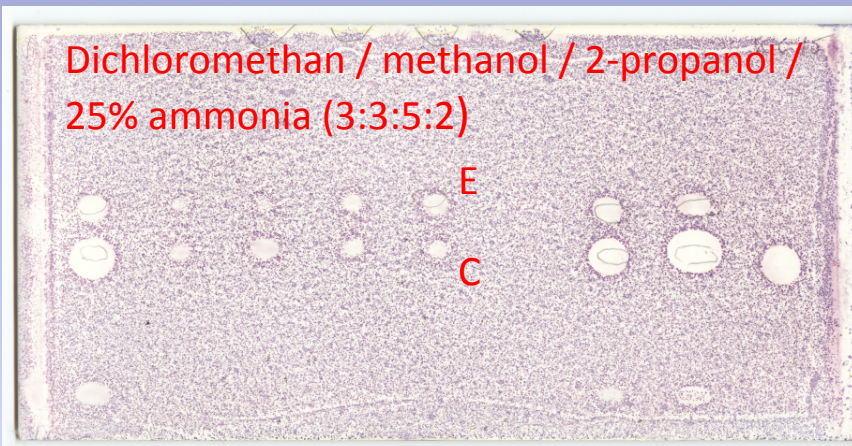


Exponential calibration curves: rhombus – enrofloxacin, square – ciprofloxacin. The data from four bioautograms.

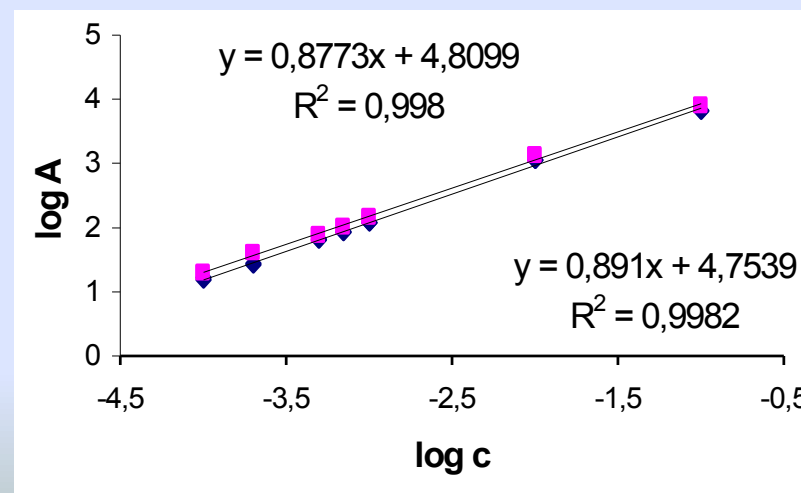
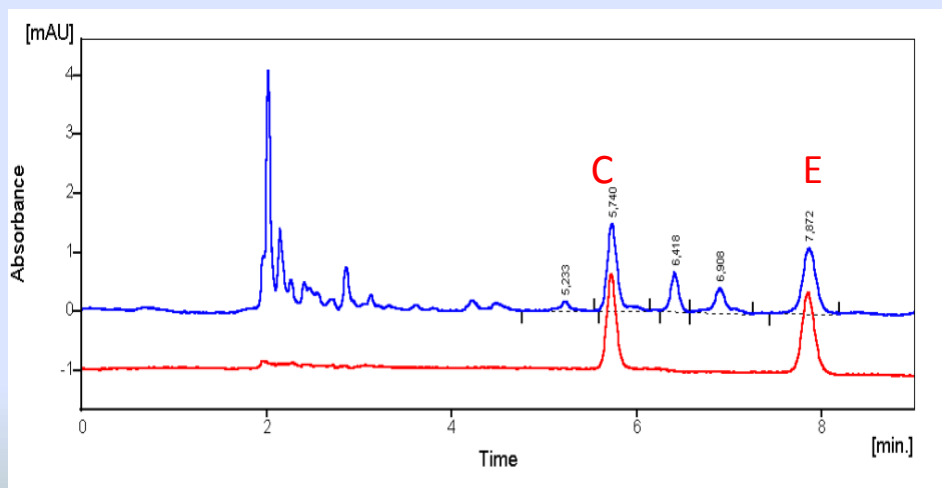
# NP TLC DB



*Bacillus subtilis*



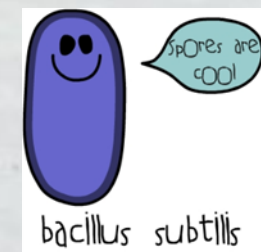
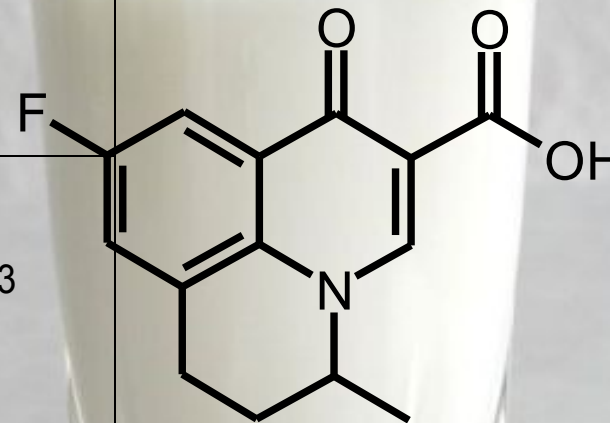
# RP HPLC





Concentration of flumequine in milk	Mean recovery % MSPD-TLC-DB	Mean of the means% (RSD)
10 ppm	56.02 (1 µl) 82.91 (2 µl) 66.88 (3 µl) 77.19 (4 µl) 64.75 (5 µl)  74.07 (1 µl) 79.44 (2 µl) 91.38 (3 µl) 83.74 (5 µl)	75.15±10.97  (14.6)
1 ppm	43.68 (5 µl) 83.77 (10 µl) 81.75 (15 µl)  49.29 (5 µl) 84.34 (10 µl) 89.0 (15 µl) 91.94 (20 µl)	74.82±19.73  (26.36)
0.1 ppm	113.0 (50 µl) 39.77 (50 µl)	76.0
0.05 ppm	70.0 (50 µl) 88.0 (50 µl)	79.0

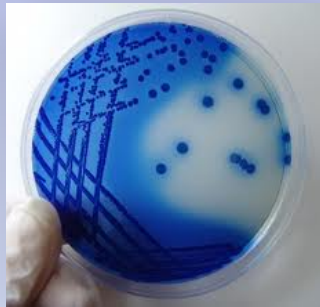
## Flumequine in milk



# Novel tests for TLC-DB



*Escherichia coli*  
(ATCC 25922)  
Gram – bacteria



*Bacillus subtilis*  
(ATCC 6633)  
Gram + bacteria

Optimization:

- pre-incubation - incubation time and temperature (bacterial suspension),
- bulion density,
- type of TLC sorbent,
- incubation time (TLC plates),
- temperature and humidity of the incubation chamber
- preconditioning of TLC plates,

Grzelak E. M., Majer – Dzedzic B., Choma I. M. *JAOAC* 94 (2011):1567-1572 (*E.coli*).

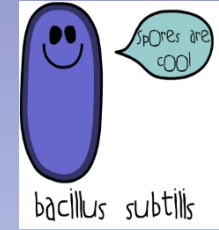
Grzelak E. M., Majer – Dzedzic B., Choma I. M. *JAOAC* 96 (2013): 386-391 (*B.subtilis*).

Grzelak E. M., Jesionek W., Majer – Dzedzic B., Choma I. M. *J. AOAC Int.*, 96 (2013) 1167-1174



escherichia coli

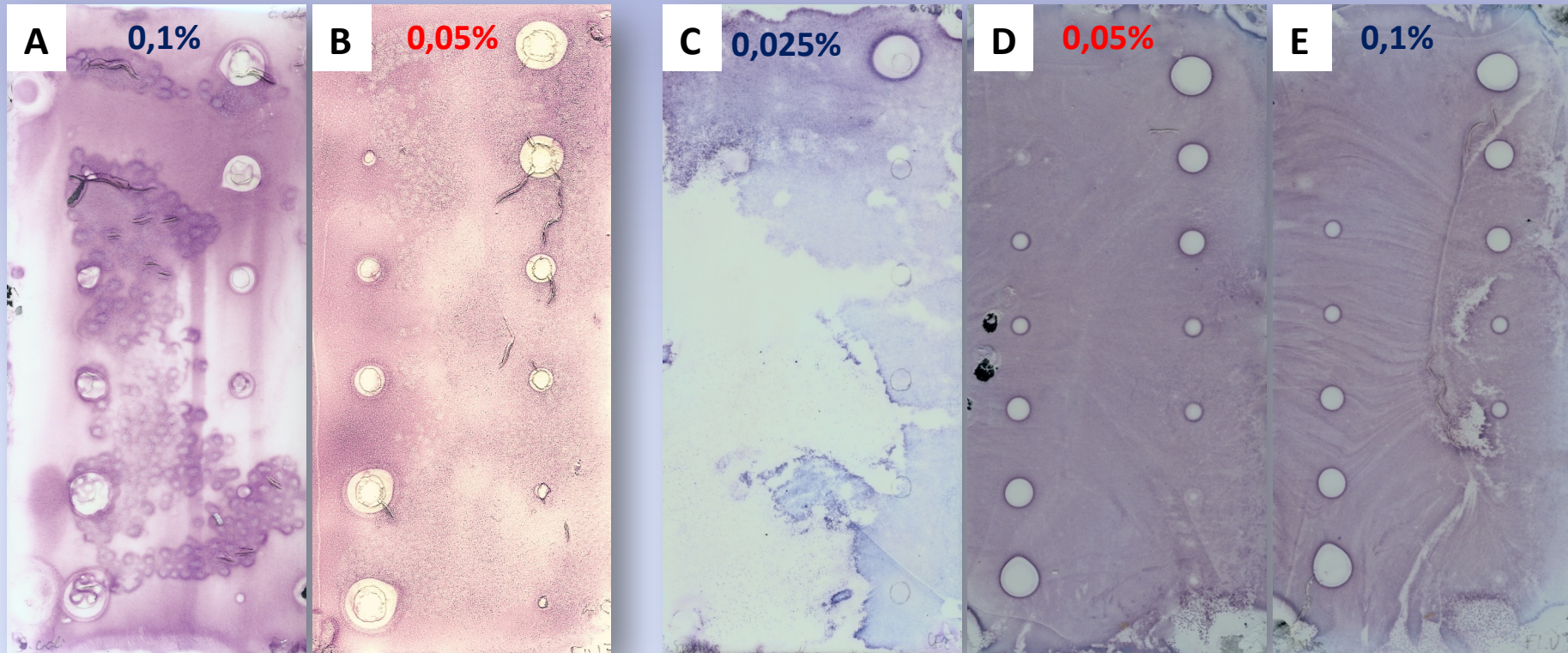
# Density of the bacterial bulion



bacillus subtilis

*Escherichia coli*

*Bacillus subtilis*



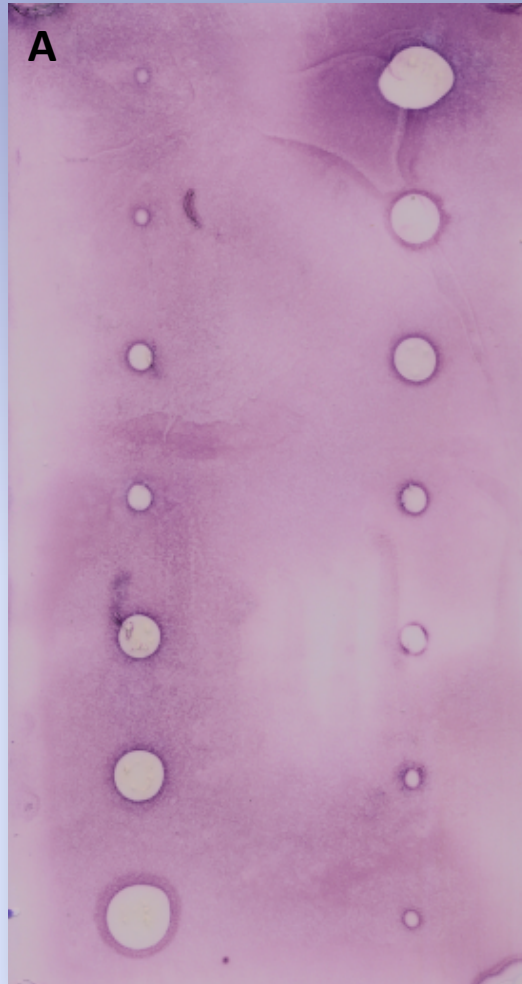
TLC-DB against *E. coli*. M-H bulion (pH  $7,2 \pm 0,2$ ) with the addition of agarose: (A) 0,1%; (B) 0,05%.

TLC-DB against *B. subtilis*. M-H bulion (pH  $7,2 \pm 0,2$ ) with agarose (C) 0,025%; (D) 0,05%; (E) 0,1%.

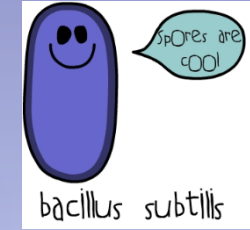
TLC Si60F<sub>254</sub>, 10  $\mu$ L of FLU standards at: 0,5; 0,5; 1,0; 1,0; 5,0; 10,0; 50,0  $\mu$ g/ml  
(up-down at left, down-up at right track)



### TLC-DB against *E. coli*



### TLC-DB against *B. subtilis*



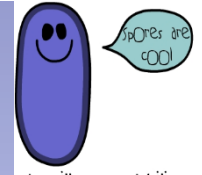
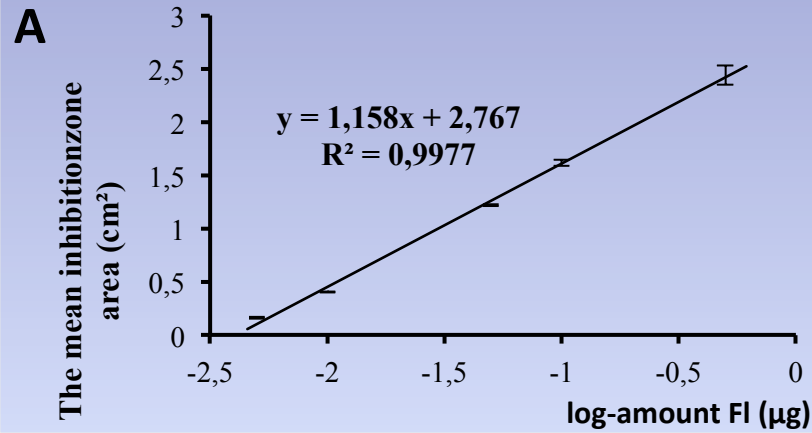
TLC Si60F<sub>254r</sub> 10  $\mu$ L of flumequine standards at: 0,5; 0,5; 1,0; 1,0; 5,0; 10,0; 50,0  $\mu$ g/ml  
(up-down at left, down-up at right track ).



escherichia coli

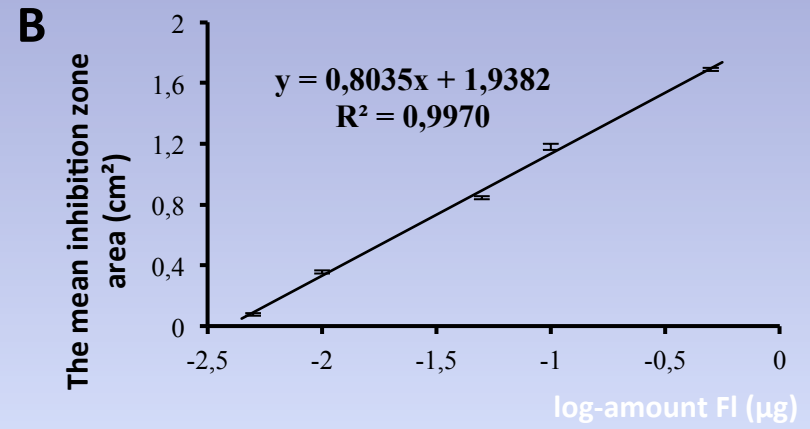
## TLC-DB against *E. coli*

### Intraday assay

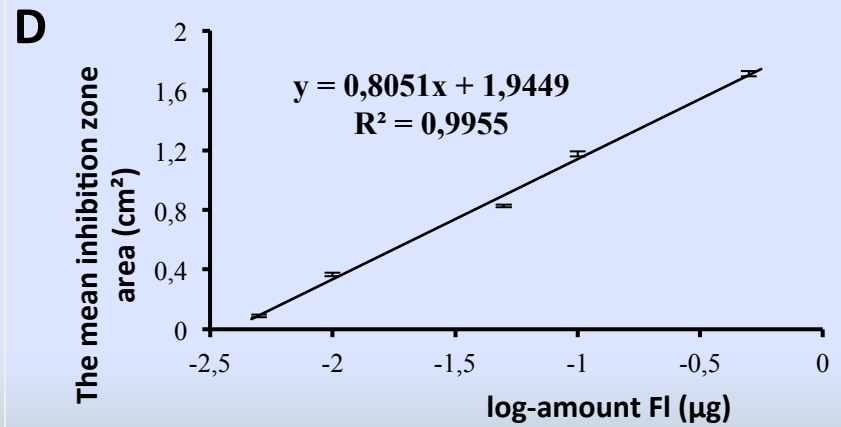
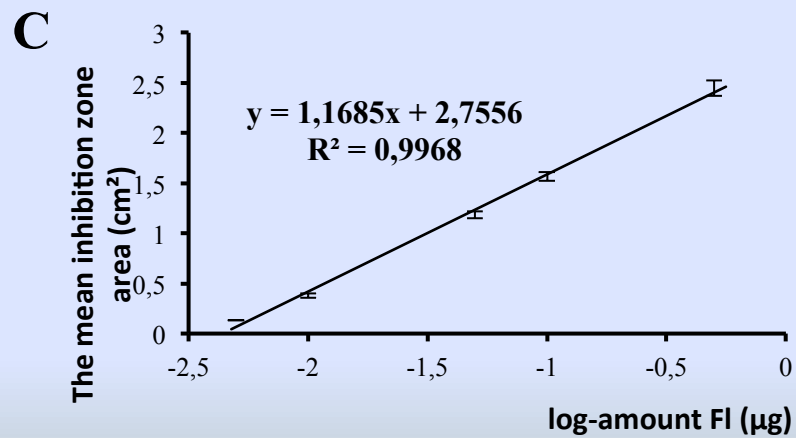


bacillus subtilis

## TLC-DB against *B. subtilis*



### Interday assay

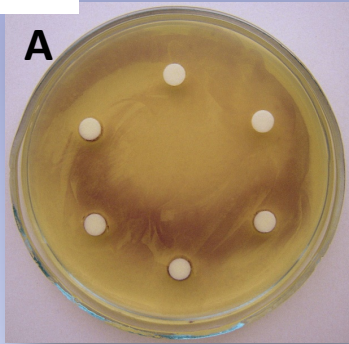




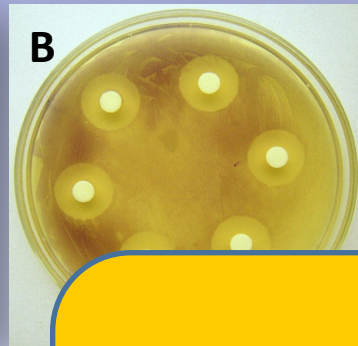
escherichia coli

### Escherichia coli

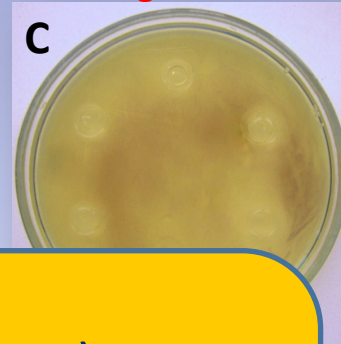
negative



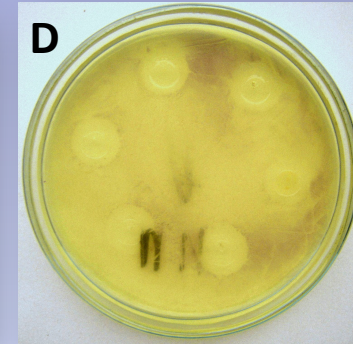
positive



negative



positive

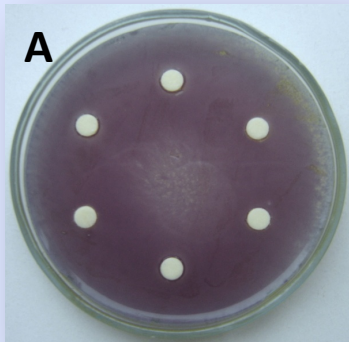


**MIC ( $\mu\text{g}/\text{mL}$ )**  
**TLC-DB 0,5  $\mu\text{g}/\text{mL}$**   
**Disc diffusion assay 50  $\mu\text{g}/\text{mL}$**   
**Cylinder diffusion assay 100  $\mu\text{g}/\text{mL}$**

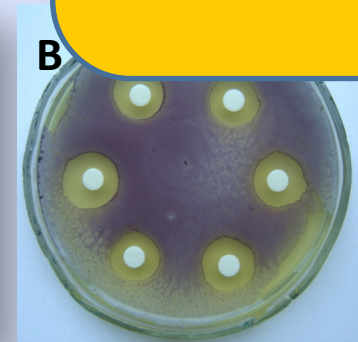
Escherichia coli: Disc diffusion assay (A) negative (0,1  $\mu\text{g}$  FLU per disc); (B) positive (5,0  $\mu\text{g}$  FLU per disc).  
Cylinder diffusion assay (C) negative (0,1  $\mu\text{g}$  FLU per cylinder); (D) positive (5,0  $\mu\text{g}$  FLU per cylinder).

Bacillus subtilis: Disc diffusion assay (A) negative (0,1  $\mu\text{g}$  FLU per disc); (B) positive (5,0  $\mu\text{g}$  FLU per disc).  
Cylinder diffusion assay (C) negative (0,1  $\mu\text{g}$  FLU per cylinder); (D) positive (5,0  $\mu\text{g}$  FLU per cylinder).

negative



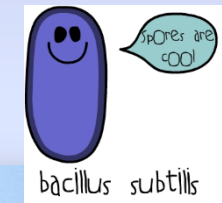
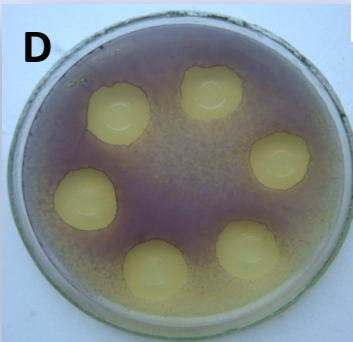
positive



negative



positive



Bacillus subtilis: Disc diffusion assay (A) negative (0,1  $\mu\text{g}$  FLU per disc); (B) positive (5,0  $\mu\text{g}$  FLU per disc).  
Cylinder diffusion assay (C) negative (0,1  $\mu\text{g}$  FLU per cylinder); (D) positive (5,0  $\mu\text{g}$  FLU per cylinder).

# APPLICATIONS



**FOOD**



**PLANTS**

# Essential oils of conifers

TLC-DB: *E. coli*

*B. subtilis*

GC-MS

pine



cypress



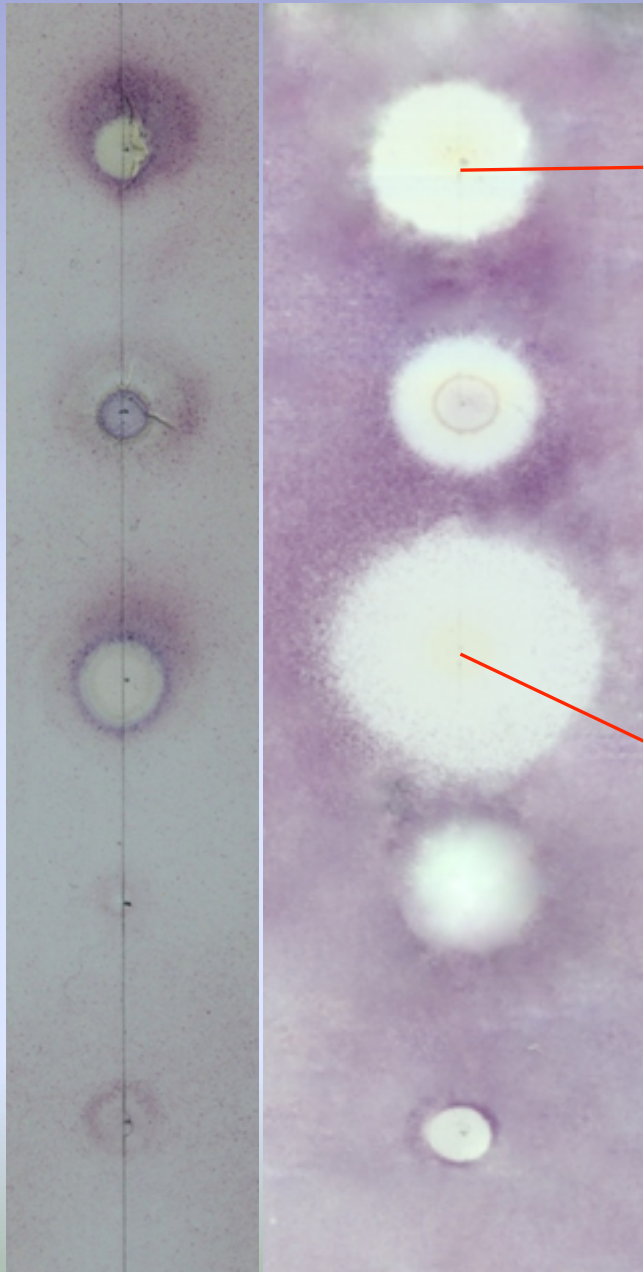
Green Douglas-fir.



juniper

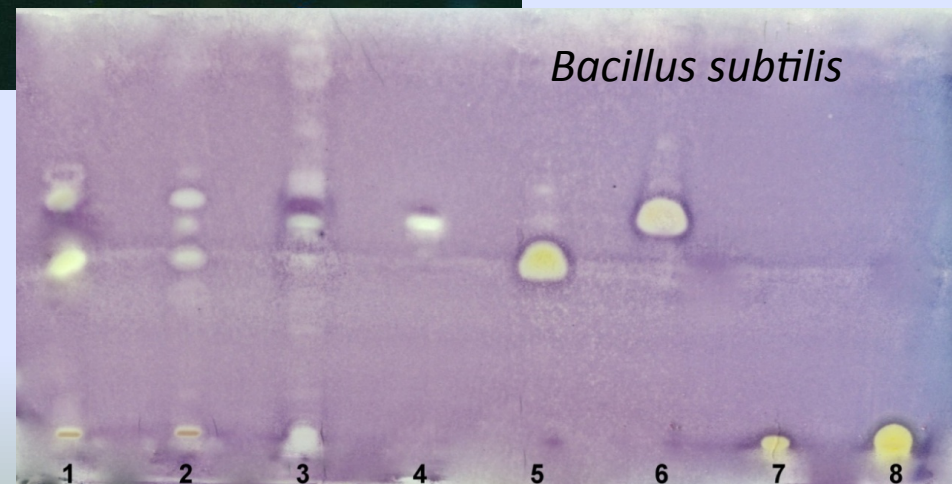
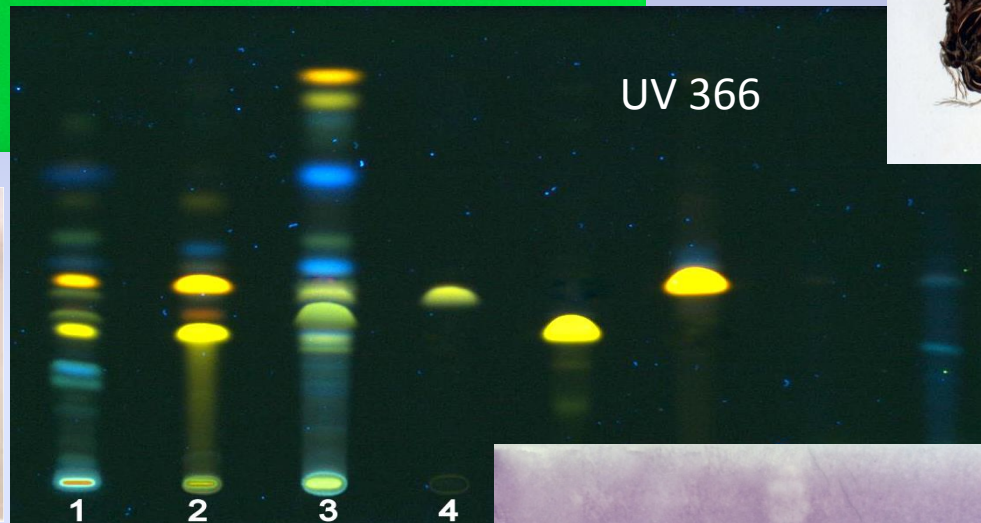
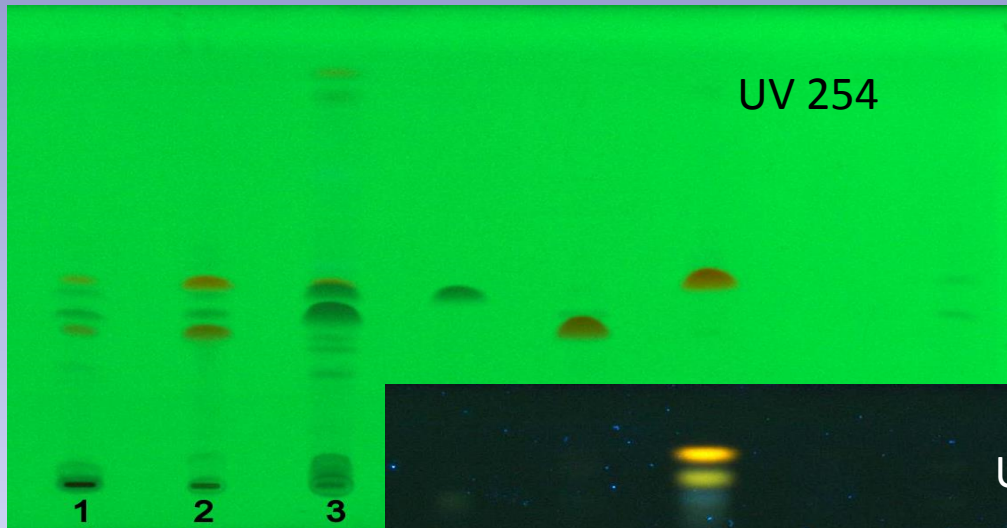


thuja



Compound	RI	Douglas (%)	Juniper (%)	Thuja (%)	Pine (%)	Cypress (%)
$\alpha$ - thujene	930	2,97	3,08	*	1,63	*
$\alpha$ - pinene	940	14,63	27,44	1,66	40,38	*
camphene	960	*	*	1,71	6,38	*
sabinene	979	1,75	40,92	*	*	*
$\beta$ -pinene	987	20,07	1,30	*	2,2	*
$\beta$ - myrcene	991	1,36	4,28	1,65	2,54	2,21
$\alpha$ - terpinene	1016	*	*	2,99	16,08	*
p- cymene	1024	0,65	1,20	*	*	*
D-limonene	1036	*	*	0,67	*	88,51
$\beta$ - felandrene	1037	0,88	5,93	1,66	1,05	*
Z-ocimene	1049	5,10	0,69	*	*	*
$\gamma$ - terpinene	1065	1,20	2,40	2,94	*	*
6- camphenon	1092	3,69	2,14	0,53	1,52	*
(+)-3-thujene	1100	*	*	38,16	*	*
$\alpha$ - thujene	1128	*	*	28,63	*	*
lavandulol	1165	*	*	9,48	*	*
Z-dihydrocarvon	1192	*	2,59	1,08	*	*
verbenone	1205	*	*	2,68	*	*
bornyl acetate	1294	45,34	*	3,05	*	*
butyl butanoate	1344	*	*	*	3,52	*
terpinyl acetate	1354	*	*	*	*	1,86
isolongifolene	1401	*	0,35	*	6,76	*
aromadendrene	1441	*	1,61	*	4,1	0,07
phenethyl isovalerate	1490	*	*	*	2,07	*
$\alpha$ - muurolene	1500	*	*	*	2,94	*
homo- $\gamma$ -bisabolene	1515	*	*	*	7,96	*
$\Sigma$ (%)		100	100	100	100	100

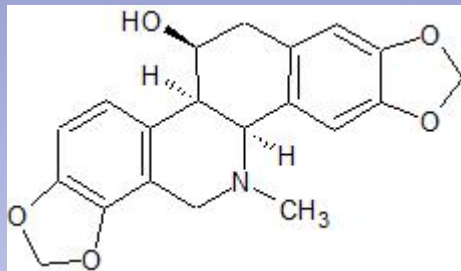




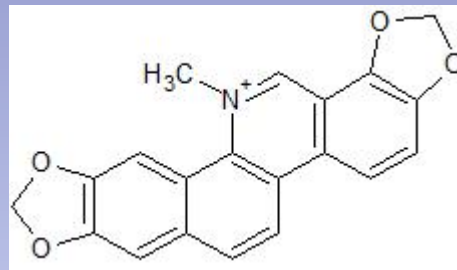
1. Ukrain
2. Quaternary alkaloids
3. Tertiary alkaloids
4. Chelidone
5. Chelerythrine
6. Sanguinarine
7. Coptisine
8. Berberine

**Mobile phase: chloroform : ethyl acetate : methanol (80:15:5)**

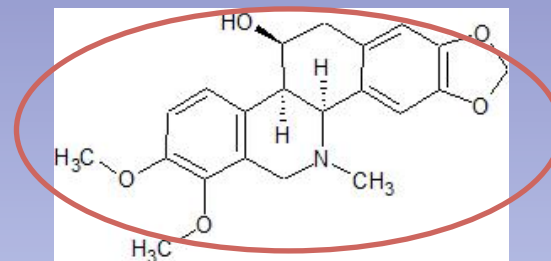
**POSTER  
P36**



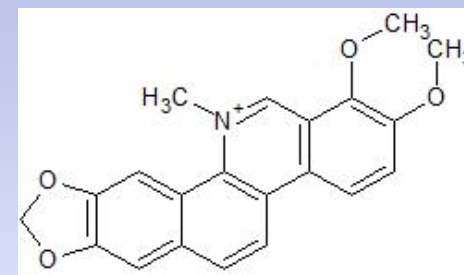
**F7 - chelidone**



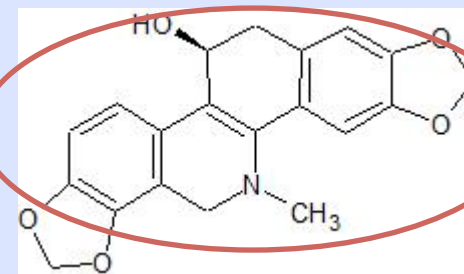
**F7 - sanguinarine**



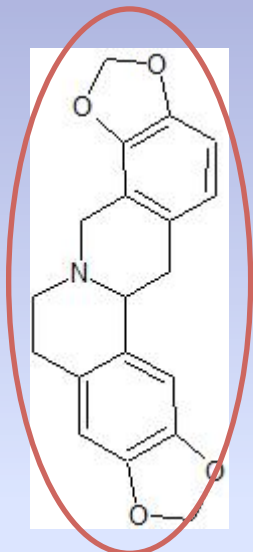
**F8 -  $\alpha$ -homochelidonine**



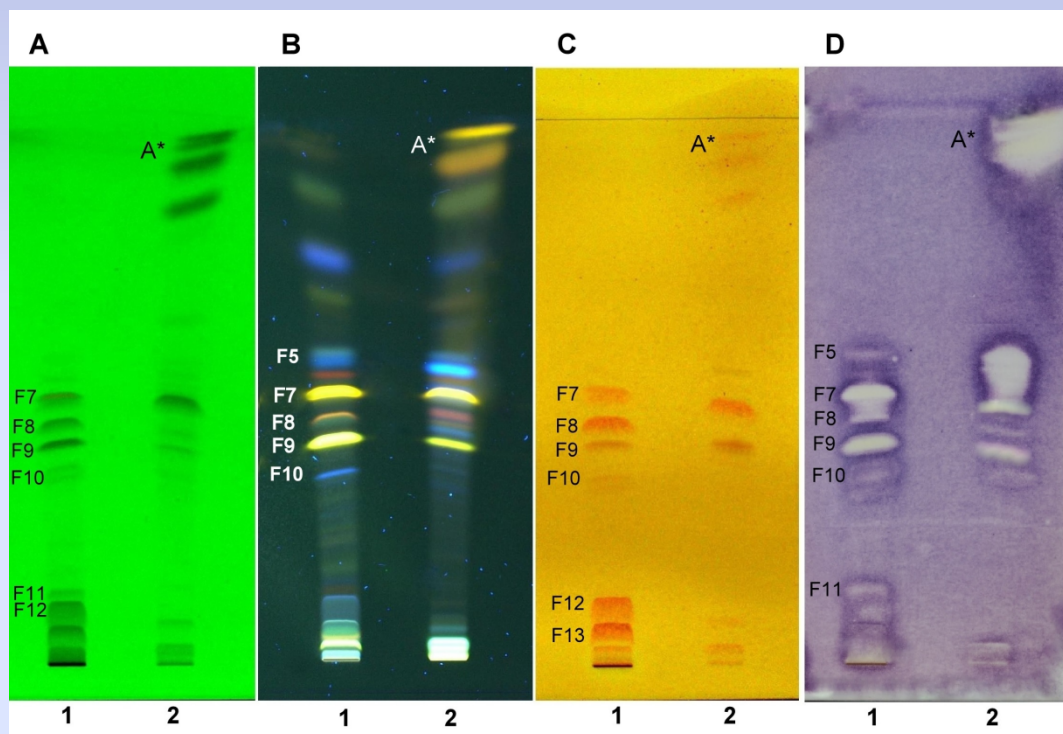
**F9 - chelerythrine**



**F10 - dihydrochelidonine**



**F5 - stylopine**



**Ukrain™ (1) and *C. majus* alkaloid extract (2).**

A - 254 nm, B - 366 nm, C - Vis-Drageendorff reagent, D - TLC-DB *B. subtilis*.

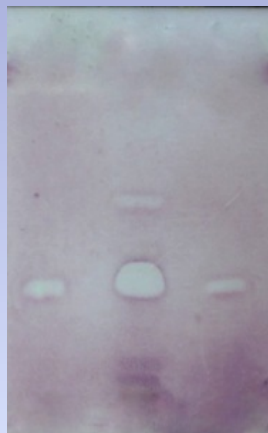
Applied volume: 15  $\mu$ L. Mobile phase: chloroform : ethyl acetate : methanol (77:15:8)

# *Mentha x piperita* L.

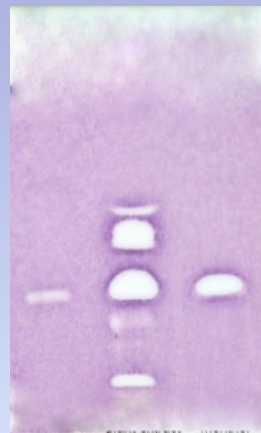
A) AS reagent



B) *E. coli*



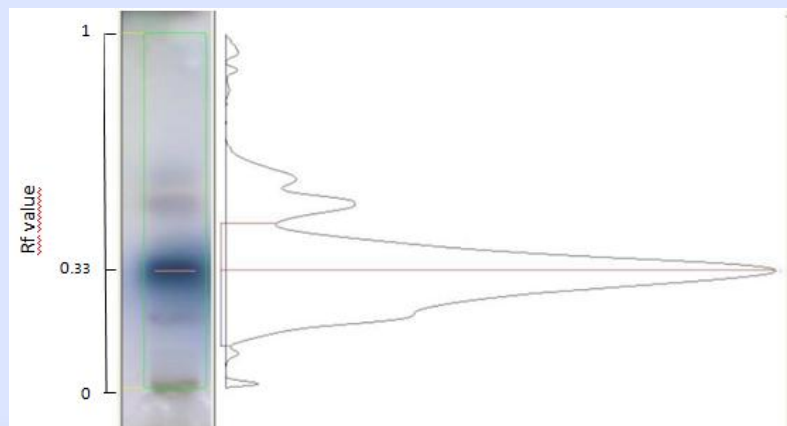
C) *B. subtilis*



1 2 3

1 2 3

1 2 3

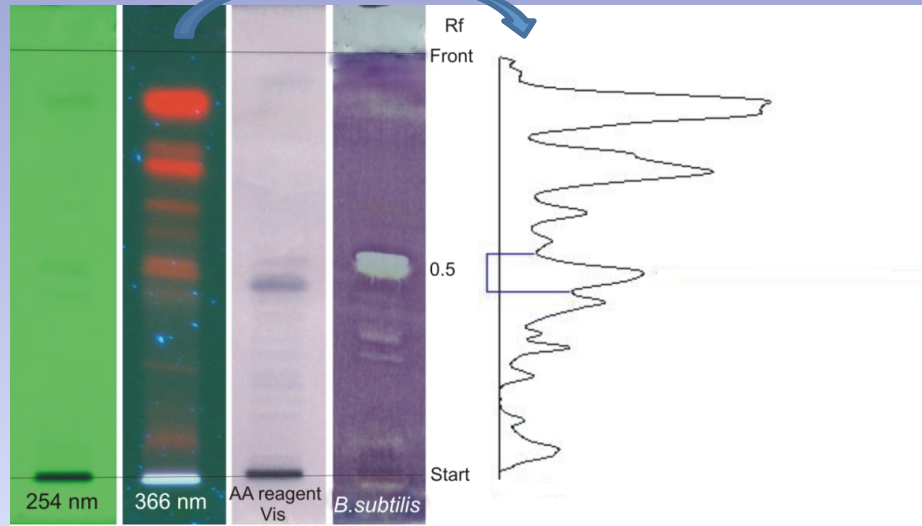


Mobile phase: toluene/ethyl acetate (93/7)

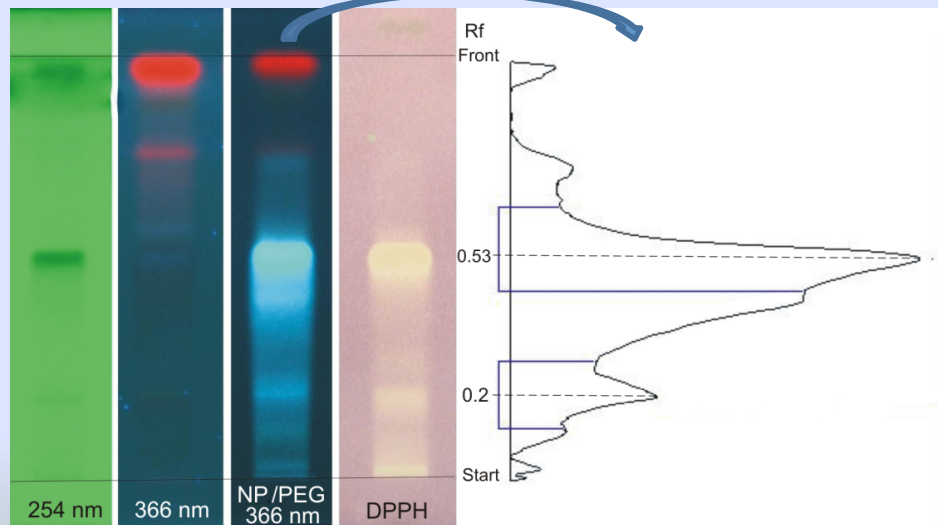
- 1 mentha essential oil
- 2 mentha extract
- 3 menthol



# Melisa officinalis L.



chloroform /ethyl acetate/methanol 1 /30/10

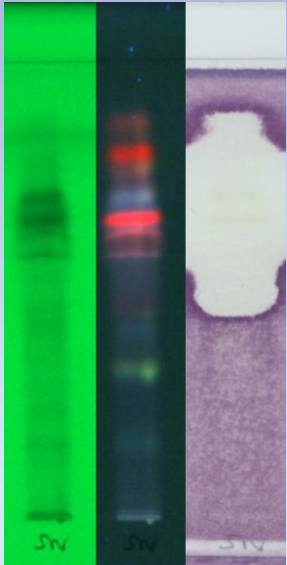


ethyl acetate /methanol / water 75/15/10



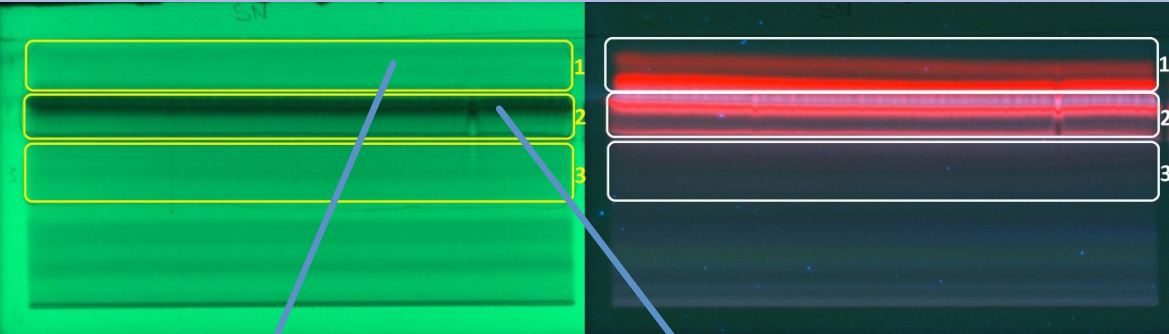
# *Pinus sylvestris* L.

TLC

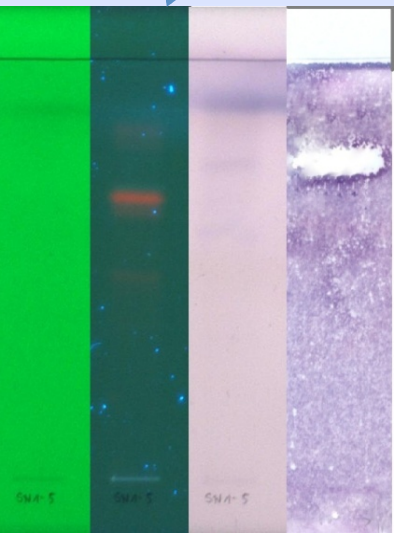


*B. subtilis*

PLC



chloroform/ methanol/10 % acetic acid 40/10/1



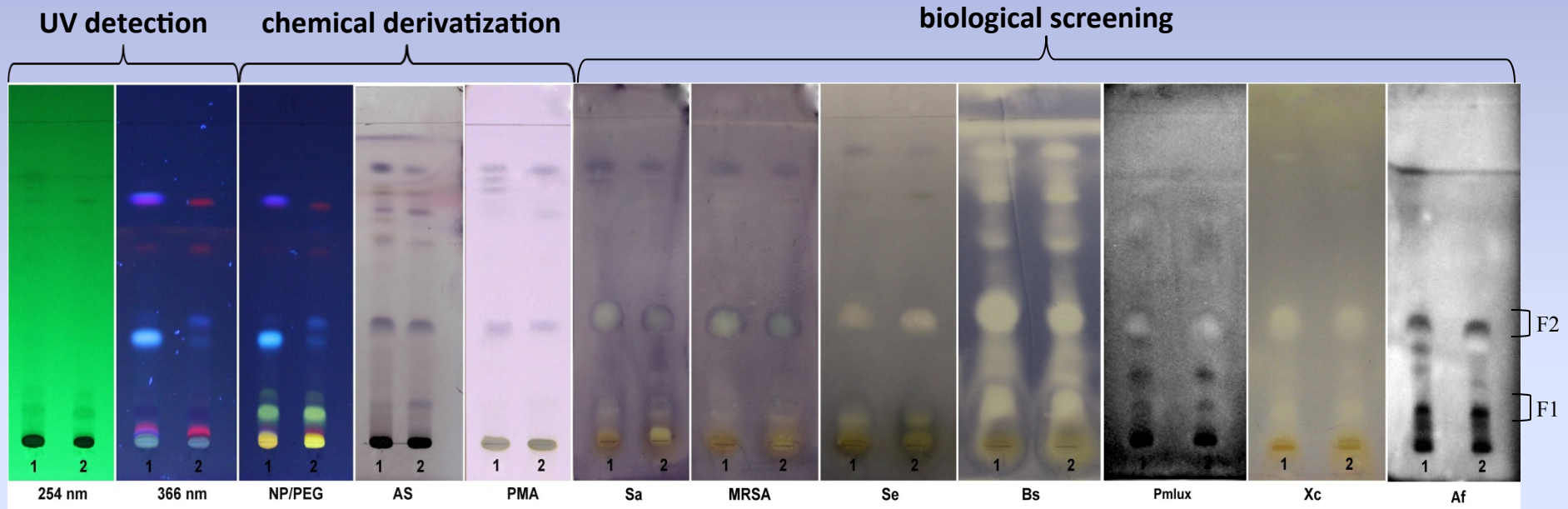
chloroform/ diethyl ether/ methanol 30/10/1

POSTER  
P51

1-Chamomile (*Matricaria chamomilla* L.)

2-Yarrow (*Achillea millefolium* L.)

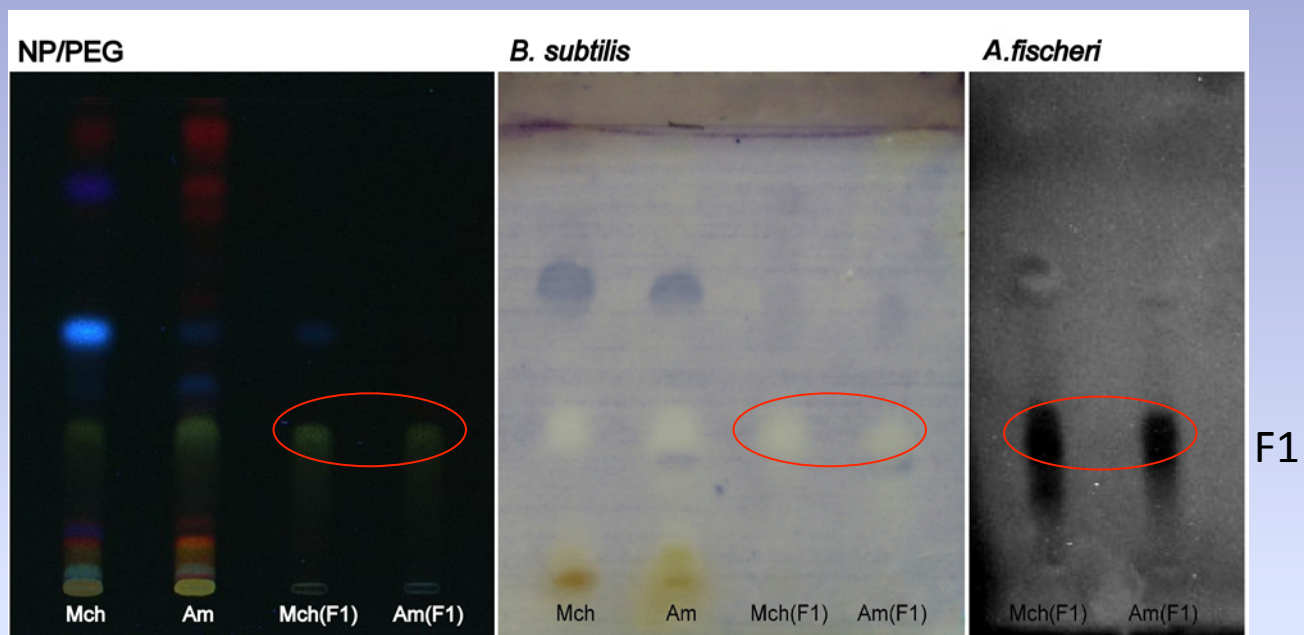
Thin-layer chromatography – direct bioautography



Mobile phase: chloroform : acetone (90:10 v/v)

- Bacterial strains:
- *Staphylococcus epidermidis* (Se)
  - *Staphylococcus aureus* (Sa)
  - methicillin-resistant *Staphylococcus aureus* (MRSA)
  - *Xanthomonas campestris* pv. *vesicatoria* (Xv)
  - *Pseudomonas syringae* pv. *maculicola* (PsmLux)
  - *Aliivibrio fischeri* (Af)
  - *Bacillus subtilis* (Bs)

# LC-TOF/MS identification of fraction 1 (F1)

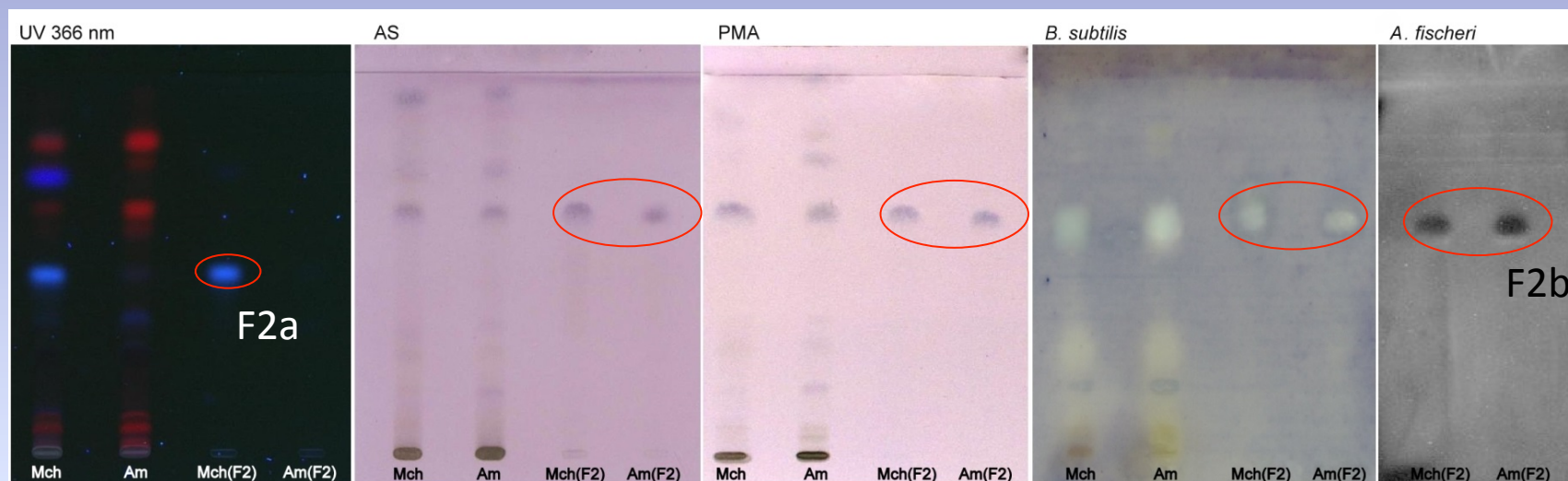


Mobile phase: chloroform : ethyl acetate (60:40 v/v)

LC-TOF/MS results [M-H]<sup>+</sup>

	Fractions	t <sub>R</sub>	Formula	Theoretical monoisotopic mass [M-H] <sup>+</sup>	Measured m/z (ppm Diff)	Compound
(Am) – <i>Achillea millefolium</i> L.	F1	6.6	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	271.0601	271.0601 (0.03 ppm)	Apigenin
(Mch) – <i>Matricaria chamomilla</i> L.	F1				271.0601 (0 ppm)*	

# LC-TOF/MS identification of fraction 2 (F2)



Mobile phase: chloroform : diethyl ether (60:40 v/v)

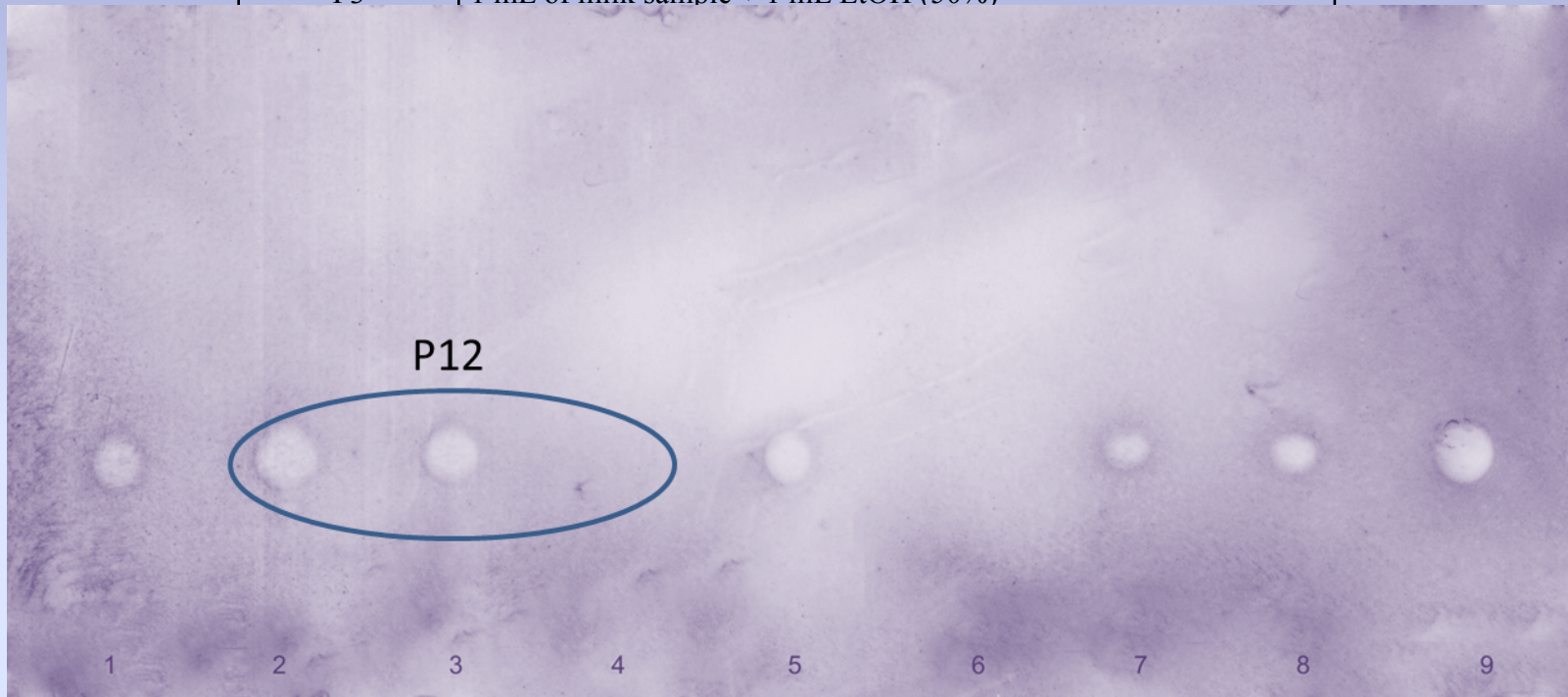
LC-TOF/MS results [M-H]<sup>+</sup>

	Fractions	t <sub>R</sub>	Formula	Theoretical monoisotopic mass [M-H] <sup>+</sup> m/z	Measured m/z (ppm Diff)	Compound
<b>(Mch) – <i>Matricaria chamomilla</i> L.</b>	F2a	5.0	C <sub>9</sub> H <sub>6</sub> O <sub>3</sub>	163.039	163.0387 (1.74 ppm)	Umbelliferone
	F2b	6.9	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	279.2319	279.2314 (1.53 ppm)	α-Linolenic acid
<b>(Am) – <i>Achillea millefolium</i> L.</b>	F2b				279.2317 (0.43 ppm)	



Procedure	
P1	3 mL of milk sample + 1 mL TFA (99%)
P2	1 mL of milk sample + 3 mL TFA (99%) + 200 $\mu$ L HCl (1M)
P3	1 mL of milk sample + 1 mL EtOH (50%)

## Deprotonization procedures



TLC-DB (1, 5) flumequine standard (2.5 ppm), (2, 3) supernatants obtained from procedure P12, (4) blank sample 8S (obtained from procedure P8), (6) blank sample 9S, (7, 8) supernatants obtained from procedure P7, (4, 5) supernatants obtained from procedure P8, (6) flumequine standard (5.0 ppm), (7, 8) supernatants obtained from procedure P9.

P15	1 mL of milk sample + 0.5 mL EtOH (96%)
*P16	1 mL of milk sample + 1 mL EtOH
*P17	1 mL of milk sample + 0.5 mL EtOH + 200 $\mu$ L HCl (1M)



**UMCS**  
MARIA CURIE-SKŁODOWSKA UNIVERSITY



Plant Protection Institute  
**MTA • ATK**



UNIVERSITY OF PÉCS  
MEDICAL SCHOOL

Dr. Ágnes M. Móricz

Dr. habil. Györgyi Horváth

*Thank you for your kind attention*



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