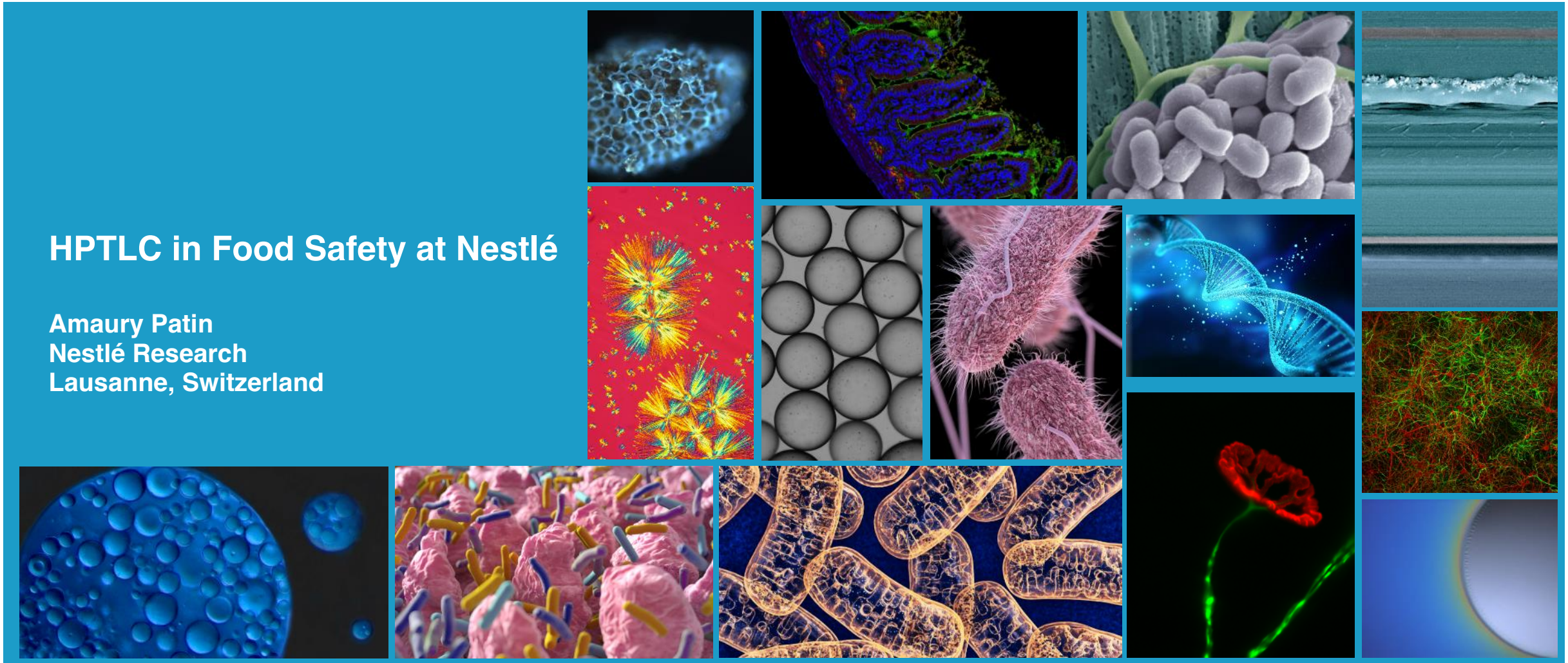
Nestlé Good food, Good life

HPTLC in Food Safety at Nestlé

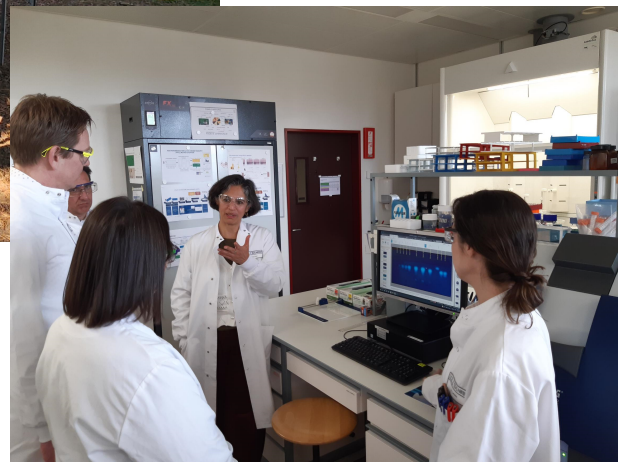
Amaury Patin
Nestlé Research
Lausanne, Switzerland



Nestlé Research

- Nestlé Institute of Agricultural Sciences
- Nestlé Institute of Health Sciences
- Nestlé Institute of Material Sciences
- **Nestlé Institute of Food Safety & Analytical Sciences**
- Nestlé Institute of Packaging Sciences

Biodetection group



sigma CLERMONT

JUSTUS-LIEBIG-UNIVERSITÄT GIESSEN

XENOMETRIX
Swiss Commitment for Bioassays

BDS
BioDetection Systems

CAMAG®

Research and Development

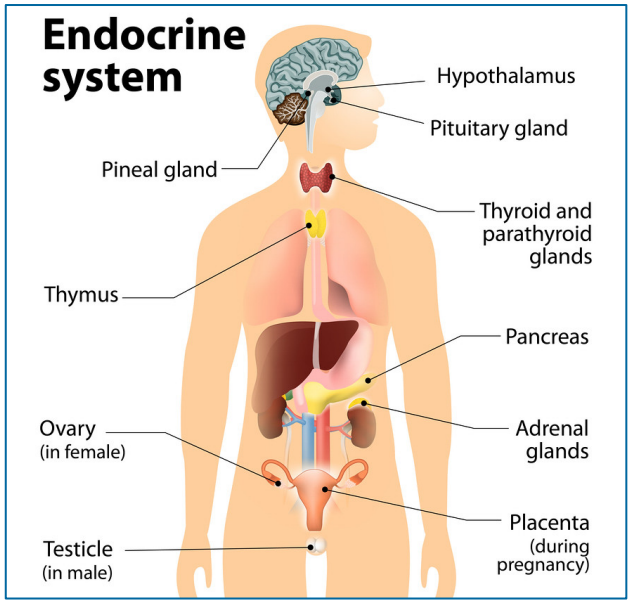
We are developing *in vitro* bioassays to screen our food, ingredient, packaging for substances of toxicological concerns

DNA-Damage

- **Genotoxicity**
- **Mutagenicity**

Benzene, mycotoxins, pyrrolizidine alkaloids…

Endocrine activity



- **Estrogenicity**
- **Androgenicity**
- **Steroidogenesis**
- **Thyroid**

Bisphenol A, phthalates, soy isoflavones, vitamin D

Adulteration

Act of making food worse in quality by adding something to them

Vegetable oils, plant proteins, herbs & spices

Research and Development

Workflow to assess estrogenicity in complex matrices

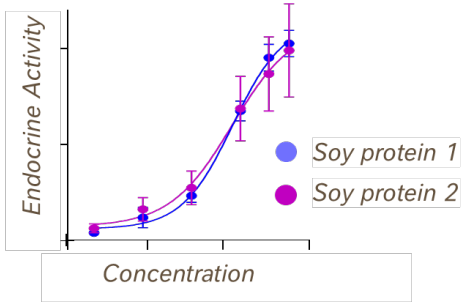
Extraction Sample Prep → **Screen for bioactivity** → **Detect & isolate bioactive(s)** → **Identify & quantify bioactive(s)** → **Concordance & safety assessment**

Optimized QuEChERS

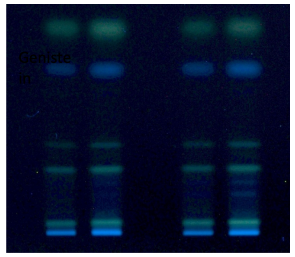
Image from www.palsystem.com

In vitro bioassay
Multiwell-based

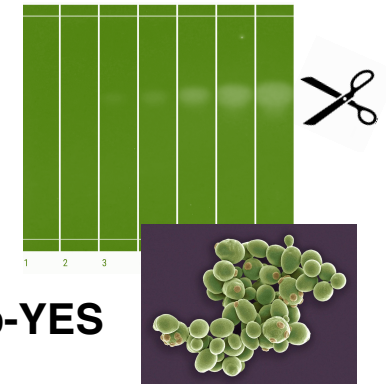
ER-Calux®



Chemical profile

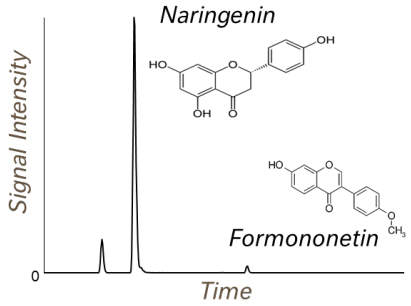


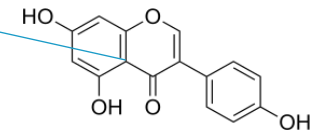
HPTLC-based *in vitro* bioassay

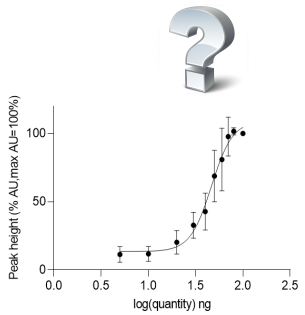


p-YES

Analytical tools:
LC-HRMS, NMR…







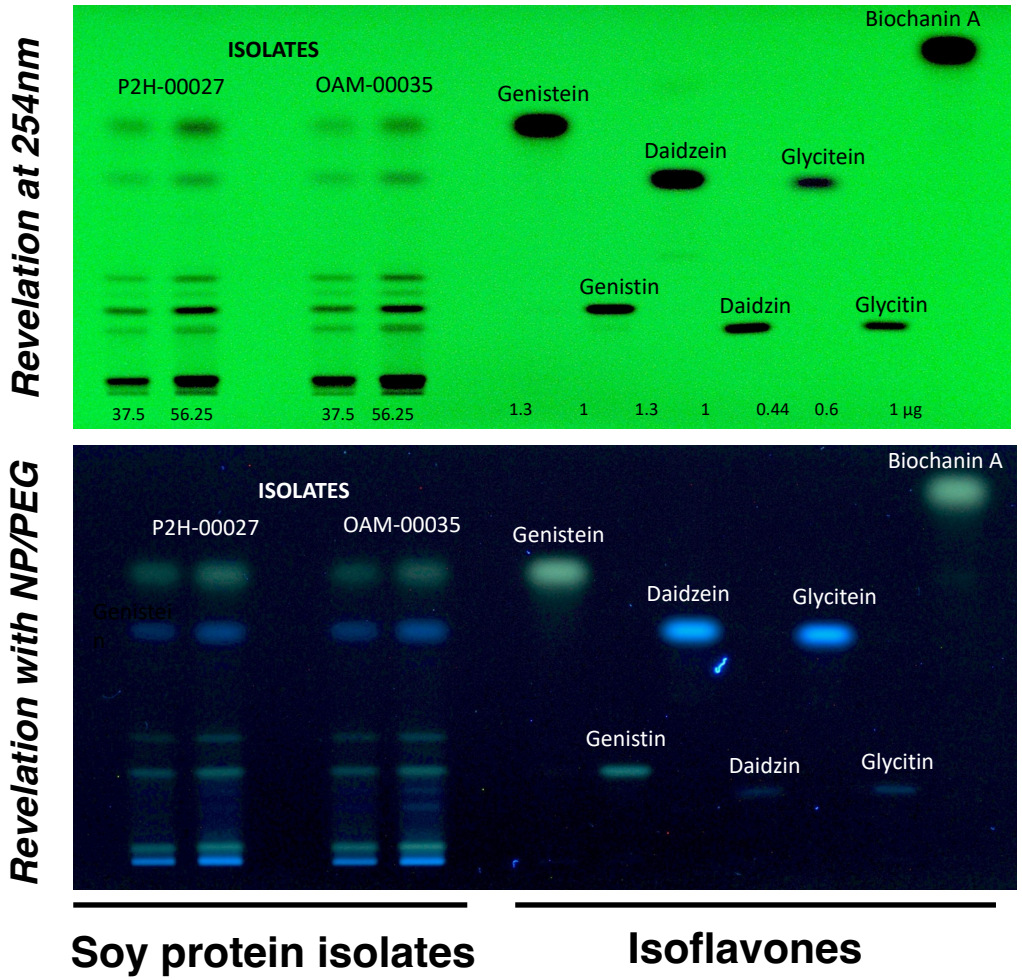
Exposure

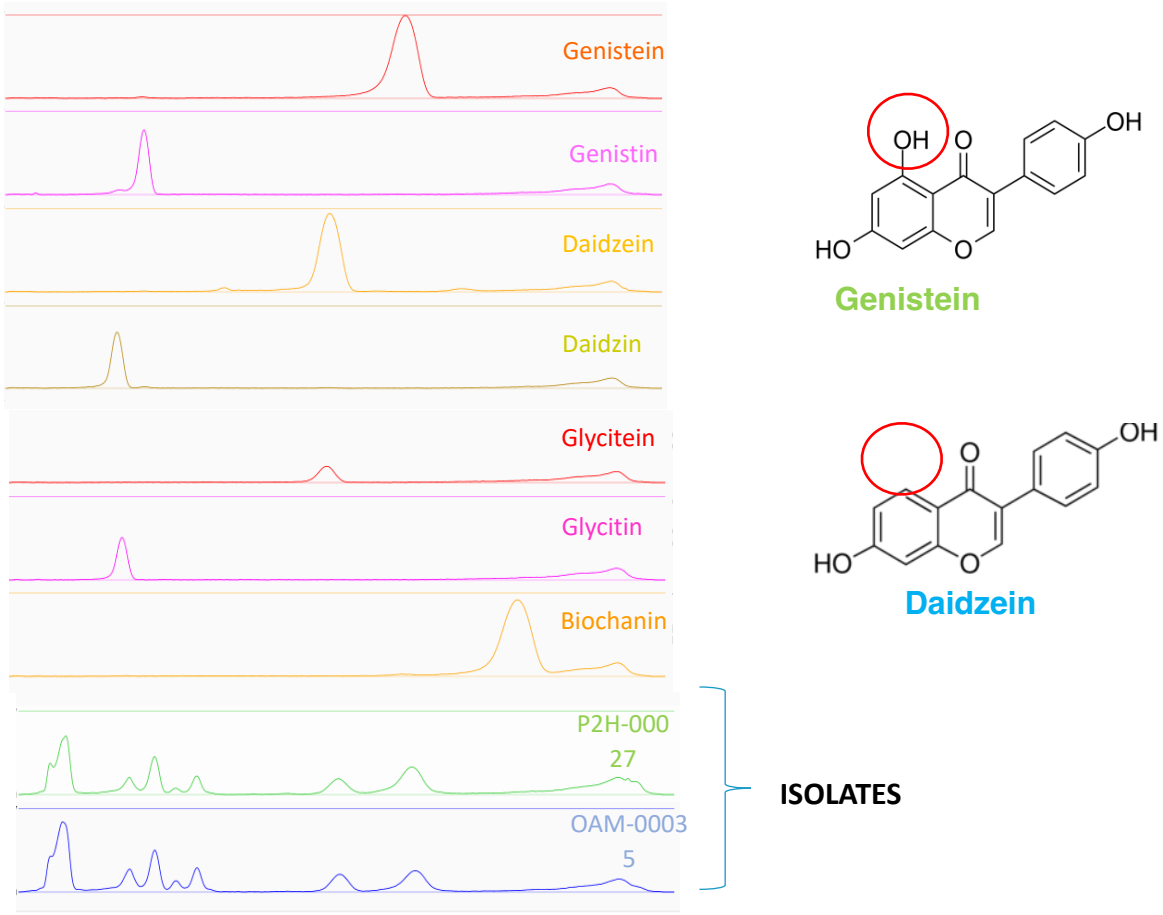
Safe level

Research and Development

p-YES: D. Spira, G. Reifferscheid, S. Buchinger, Journal of Planar Chromatography 26 (2013) 5, 395–401

HPTLC profiling of soy protein isolates vs known isoflavones



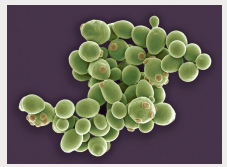


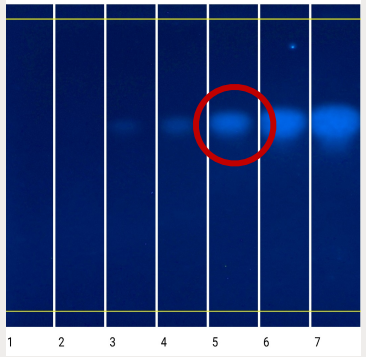
HPTLC chemical profiling confirmed the presence of isoflavones

Research and Development

p-YES & isolation of bioactive band for chemical analysis

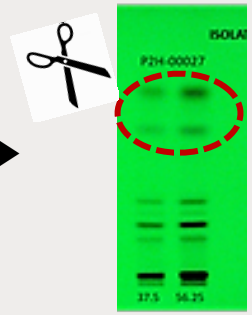
p-YES for estrogenicity





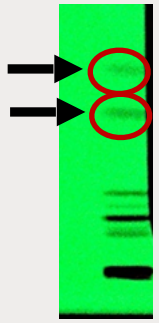
Soy protein isolate

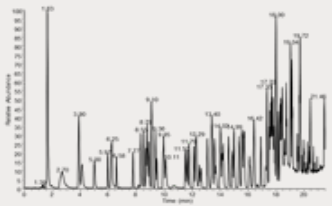
HPTLC profiling & bioactive band recovery



Rf = 0.6
Rf = 0.4

Revelation at 254nm

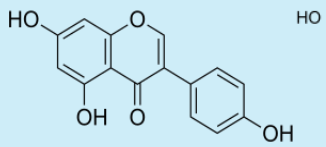




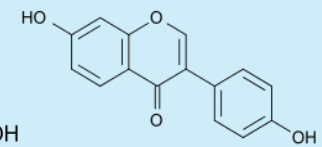
UHPLC-HRMS

Expected isoflavones identified in the bioactive bands

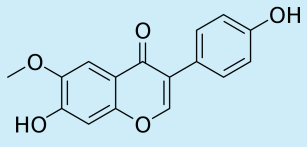
Genistein

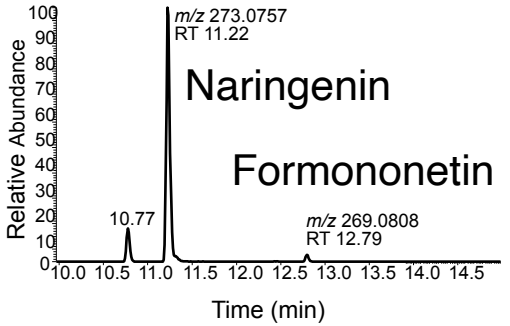


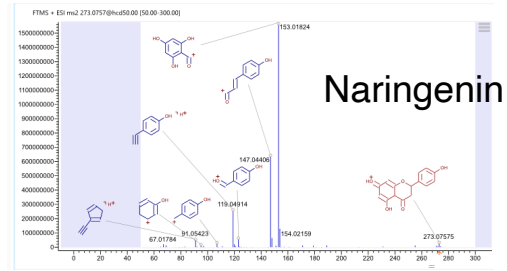
Daidzein

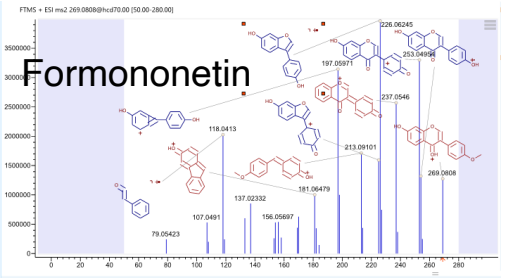


Glycitein









Two additional molecules identified on top of the known isoflavones: Naringenin & Formononetin

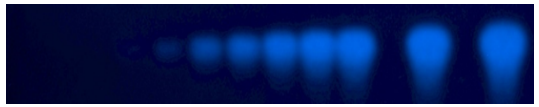
Research and Development

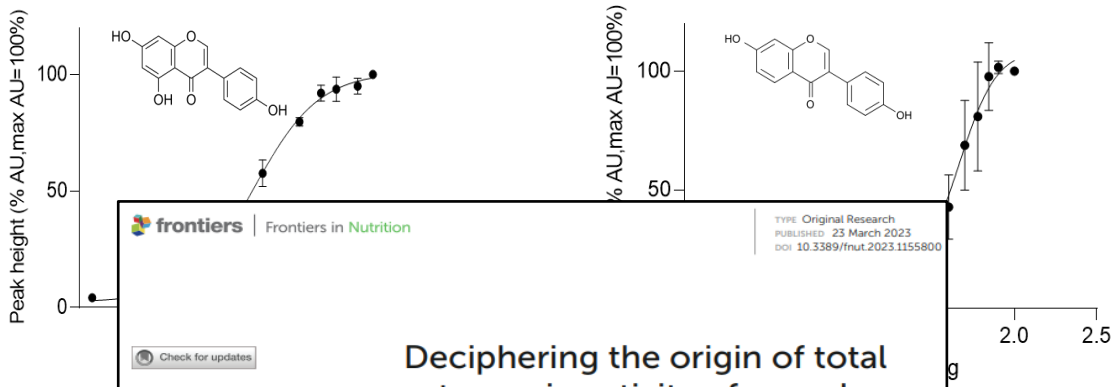
Concordance analysis: Concentration and Activity (p-YES)

Quantification by LC-HR/MS

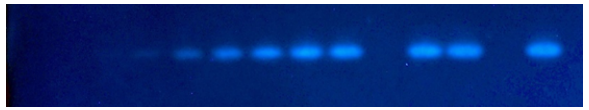
Isoflavones	µg/kg
Biochanin_A	13
Daidzein	3’970
Daidzin	3’960
Genistein	8’600
Genistin	15’500
Glycitein	500
Glycitin	620
Naringenin	5’250
Formononetin	54

Genistein

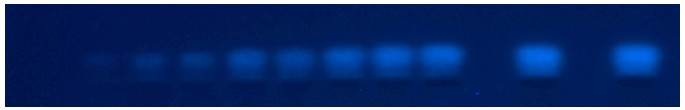


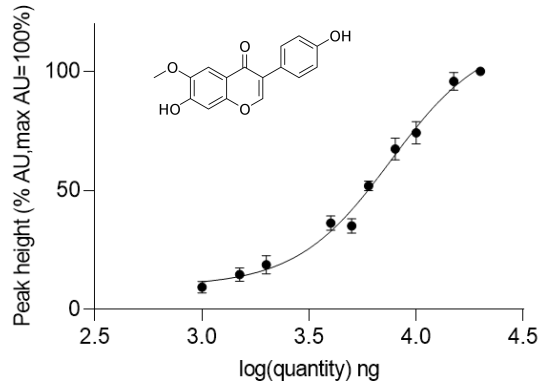


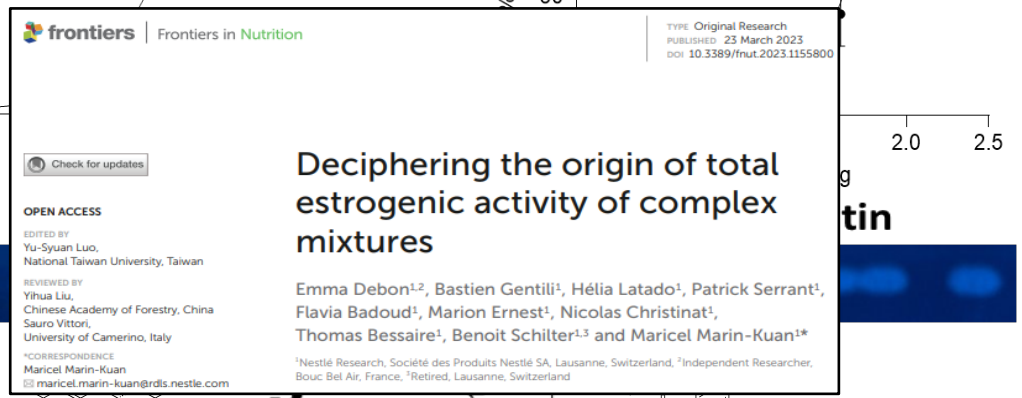
Daidzein



Glycitein







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Deciphering the origin of total estrogenic activity of complex mixtures

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Genistein

- **Isoflavones are responsible for the estrogenic activity of soy protein isolate**
- **Genistein & Daidzein are the main contributors**

Research and Development

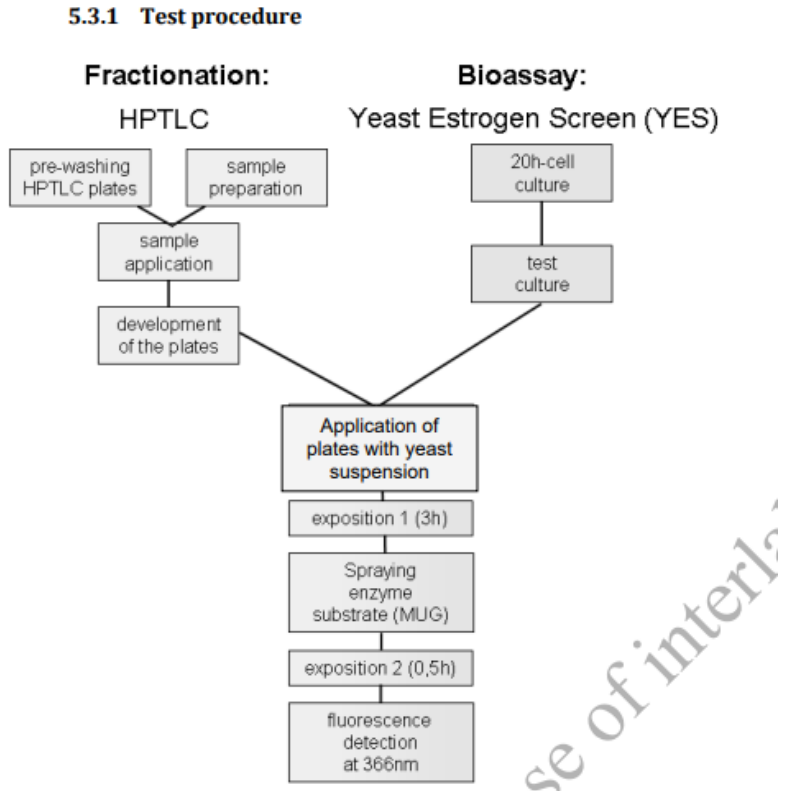
Participation in the Interlab study on p-YES organized by DIN

Deutsches Institut für Normung

Titel en: German standard methods for the examination of water, waste water and sludge - Sub-animal testing (group T) - Part 10: Determination of the estrogenic potential of water and waste water - Planar chromatographic Yeast Estrogen Screen (p-YES) (T 10)

Titel de: Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung - Suborganismische Testverfahren (Gruppe T) - Teil 10: Bestimmung des estrogenen Potentials von Wasser und Abwasser - Planarchromatographischer Hefe-EstrogenTest (p-YES) (T 10)

Titel fr: Méthodes normalisées allemandes pour l'analyse des eaux, des eaux résiduaires et des boues - Testes sous-animaux (groupe T) Partie 10: Détermination du potentiel oestrogénique de l'eau et des eaux résiduaires - Essai d'oestrogénicité



Research and Development

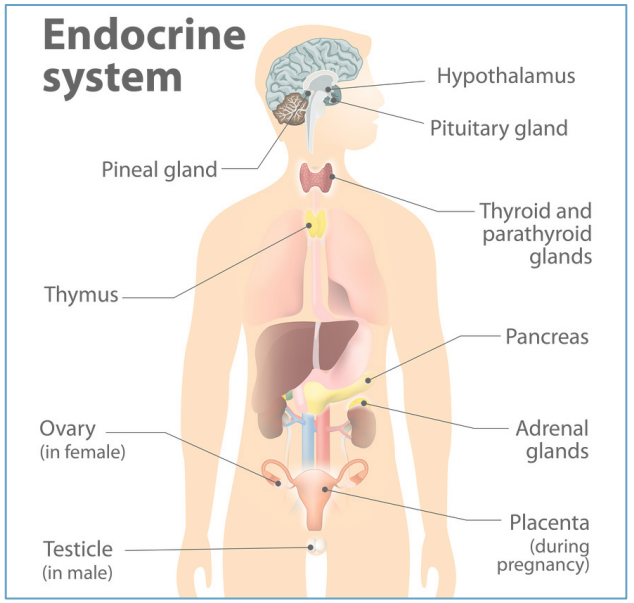
We are developing *in vitro* bioassays to screen our food/ingredient/ packaging for substances of toxicological concerns

DNA-Damage

- **Genotoxicity**
- **Mutagenicity**

Benzene, mycotoxins, pyrrolizidine alkaloids…

Endocrine activity



- **Estrogenicity**
- **Androgenicity**
- **Steroidogenesis**
- **Thyroid**

Bisphenol A, phthalates, soy isoflavones, vitamin D

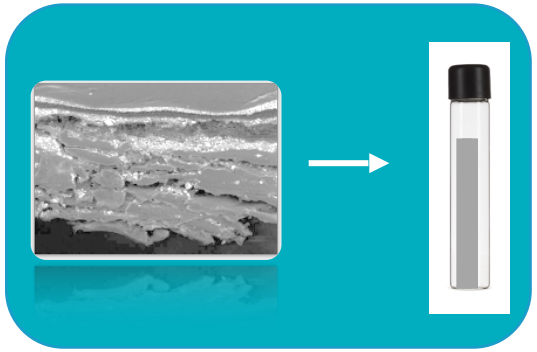
Adulteration

Act of making food worse in quality by adding something to them

Research and Development

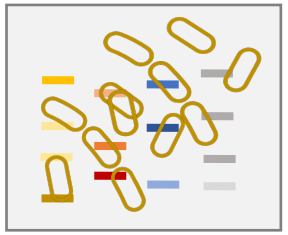
Case study 1: Identification of genotoxicants/mutagens in paper packaging

EXTRACTION
in Hexane/Acetone



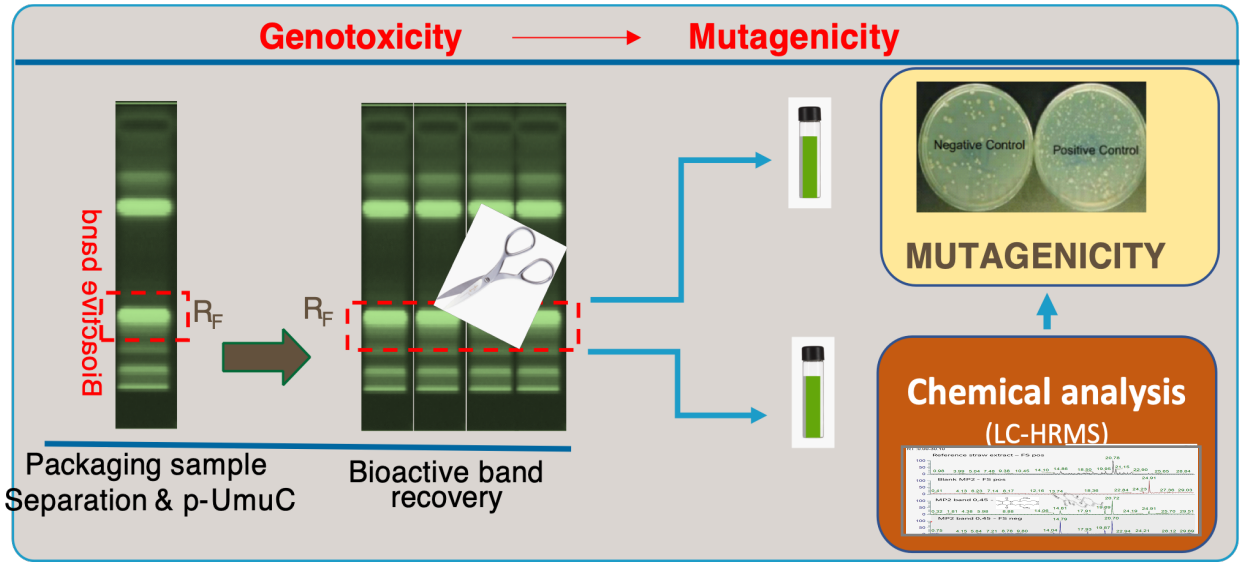
Commercial colored paper material (non-Nestlé product)

Salmonella SOS Umu-C assay



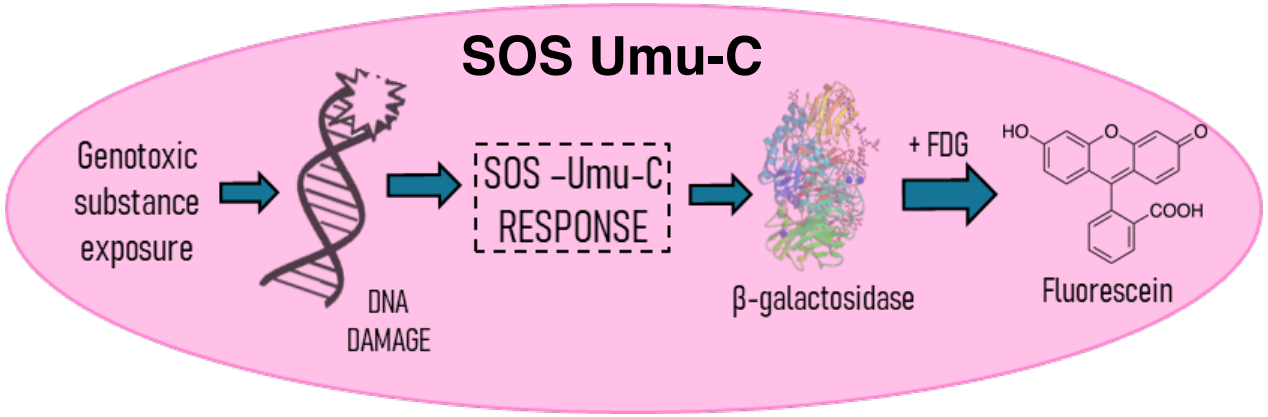
+/- S9 fraction (metabolic activation)

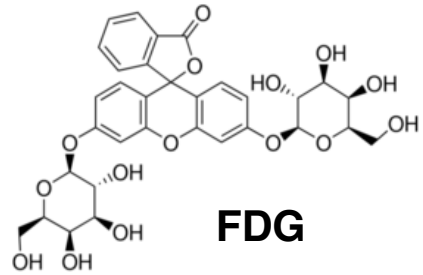
HPTLC coupled to SOS Umu-C assay



Genotoxic bioactive bands

SOS Umu-C

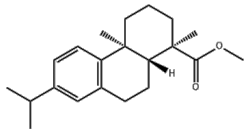




FDG

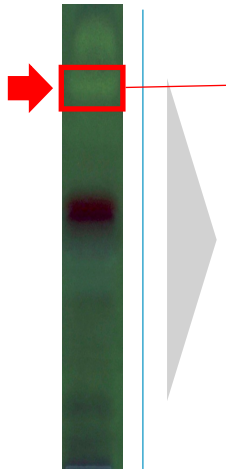
Research and Development

Evaluation of a 1st candidate identified (LC-HRMS) – concordance & mutagenicity



Dehydroabietic acid (DHA), methyl ester

Bioactive band 1

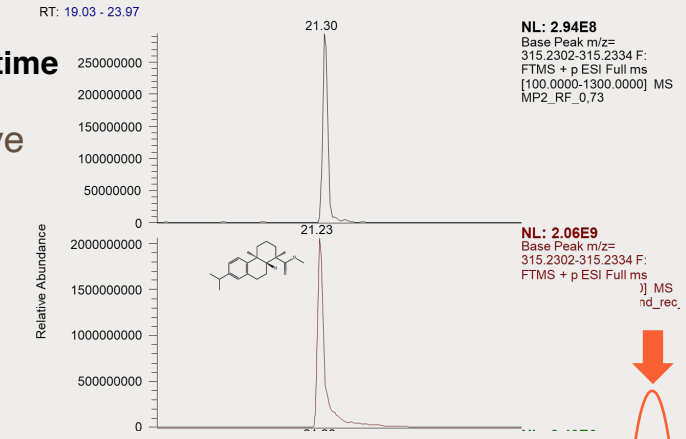


LC-HRMS identification

Retention time

Bioactive band 2

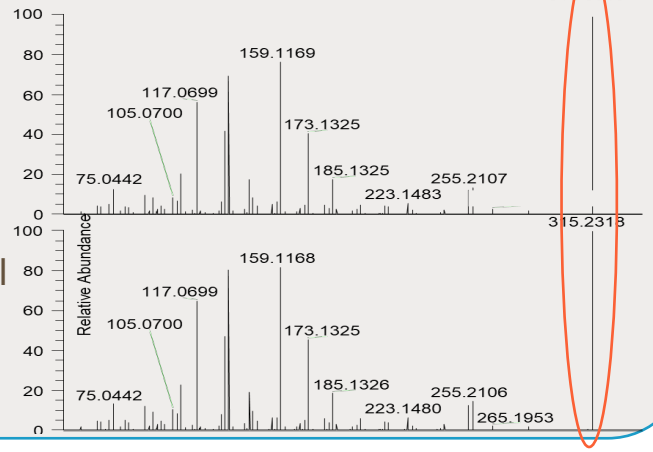
DHA methyl ester



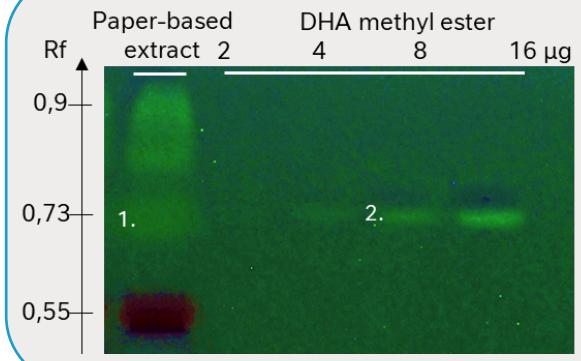
Fragmentation

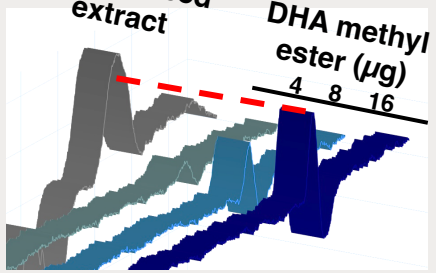
Bioactive band 2

DHA methyl ester

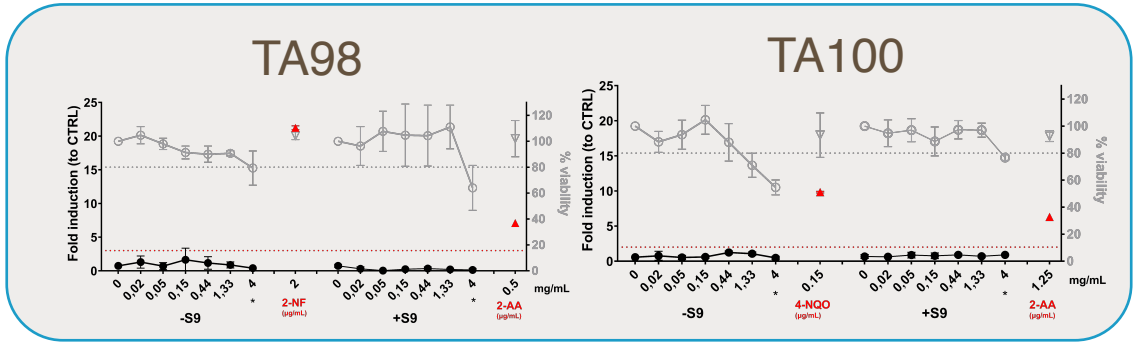


HPTLC-Umu-C confirmation





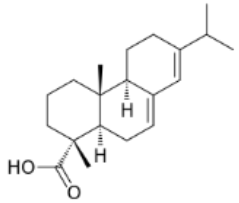
Mutagenicity (AMES_MPF)



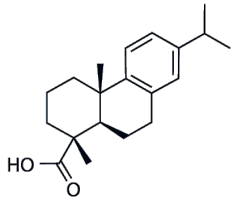
- ✓ Dehydroabietic acid, methyl ester found to be genotoxic and present in paper-extract
- ✓ No **mutagenic** effect was observed for this compound

Research and Development

Evaluation of 2 other candidates – concordance & mutagenicity

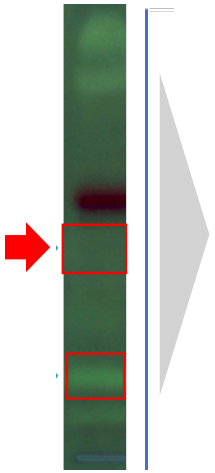


Abietic acid

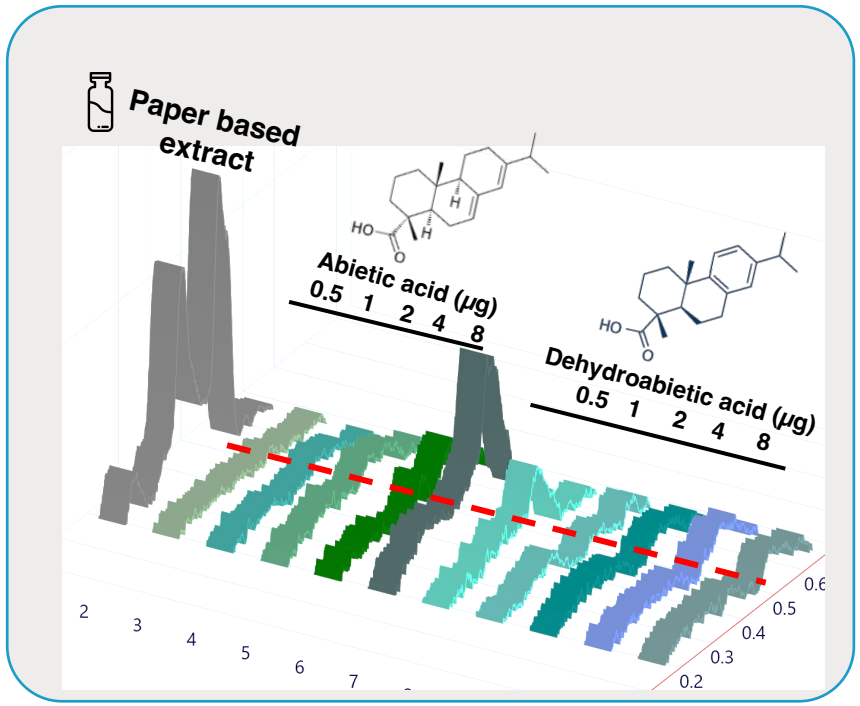


Dehydroabietic acid

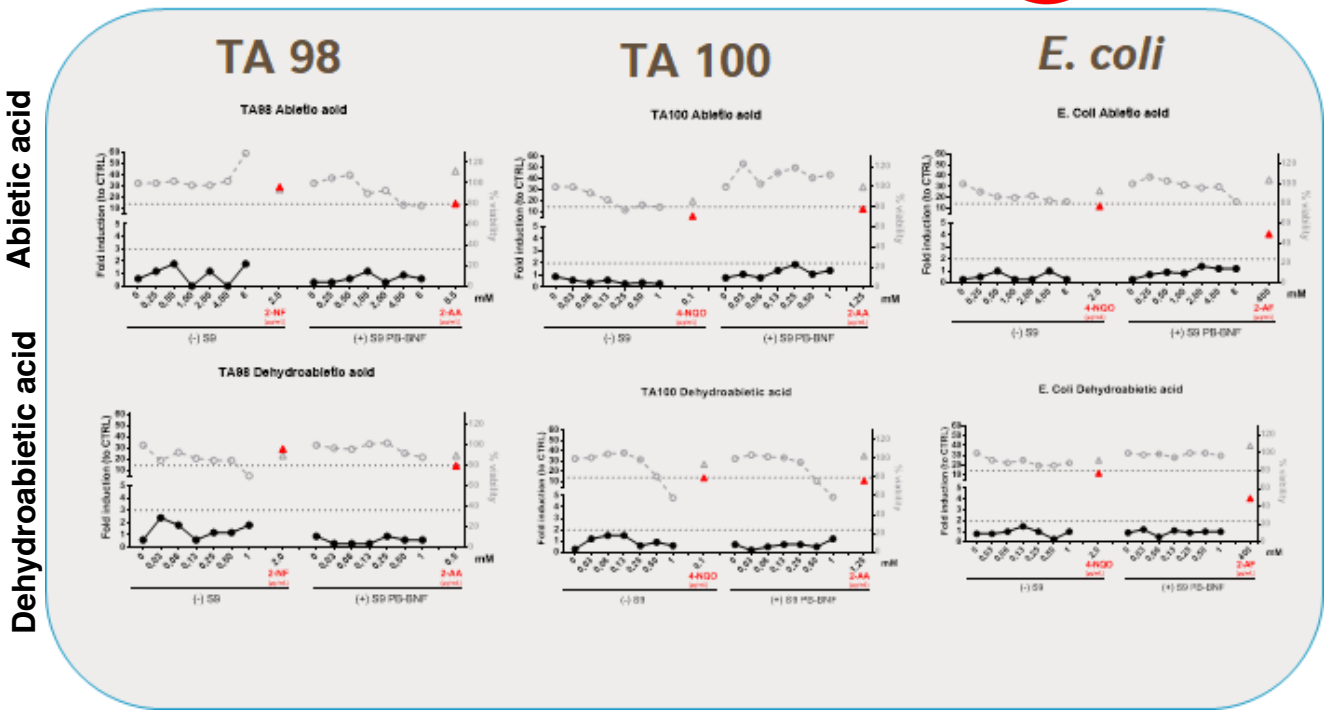
Bioactive band 2



HPTLC-Umu-C genotoxicity



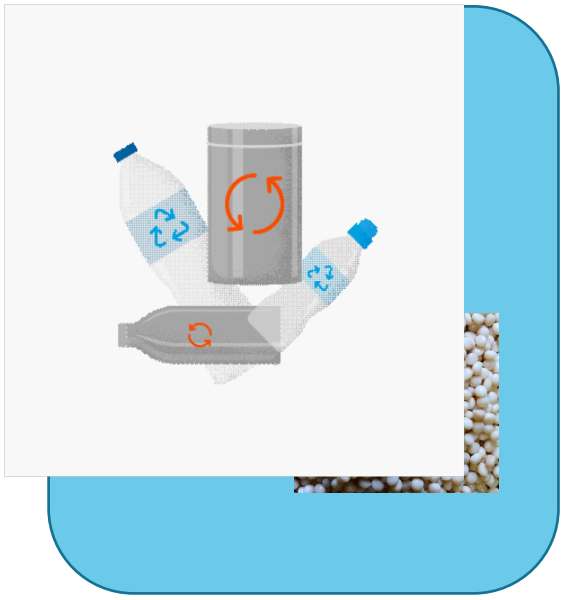
Mutagenicity (AMES-MPF)

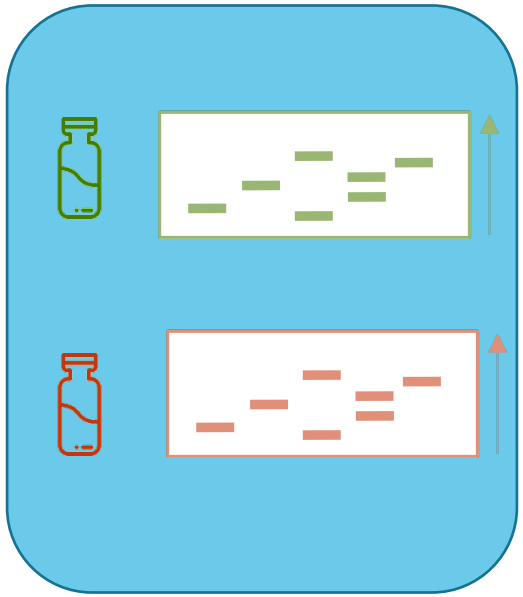


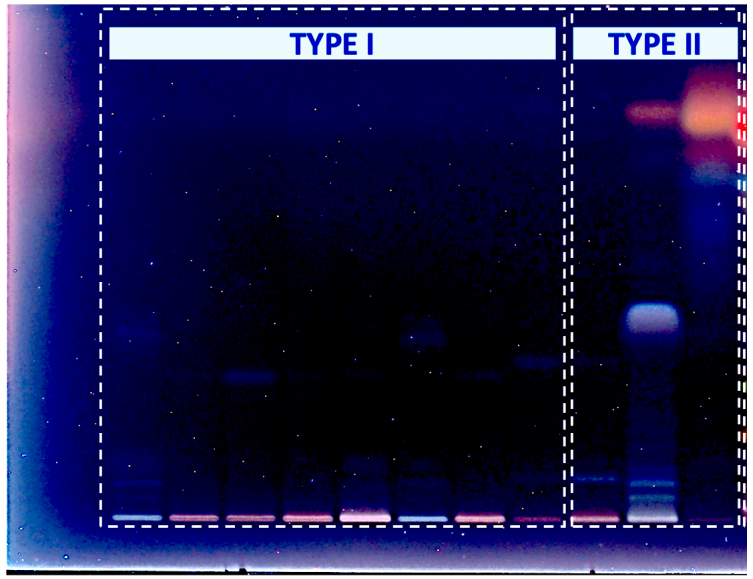
- ✓ Abietic and dehydroabietic acids have been found to be genotoxic and present in the paper-extract
- ✓ No **mutagenic** effect was observed for both of them

Research and Development

Case study 2: Screening of 10 recycled polyolefins* using chemical and effect-based approach for priorization





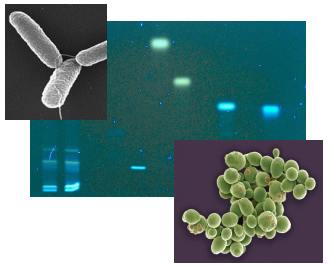


Chemical profiling
(derivatization with p-anisaldehyde)

* Recycled polyolefins not used for food applications at Nestlé

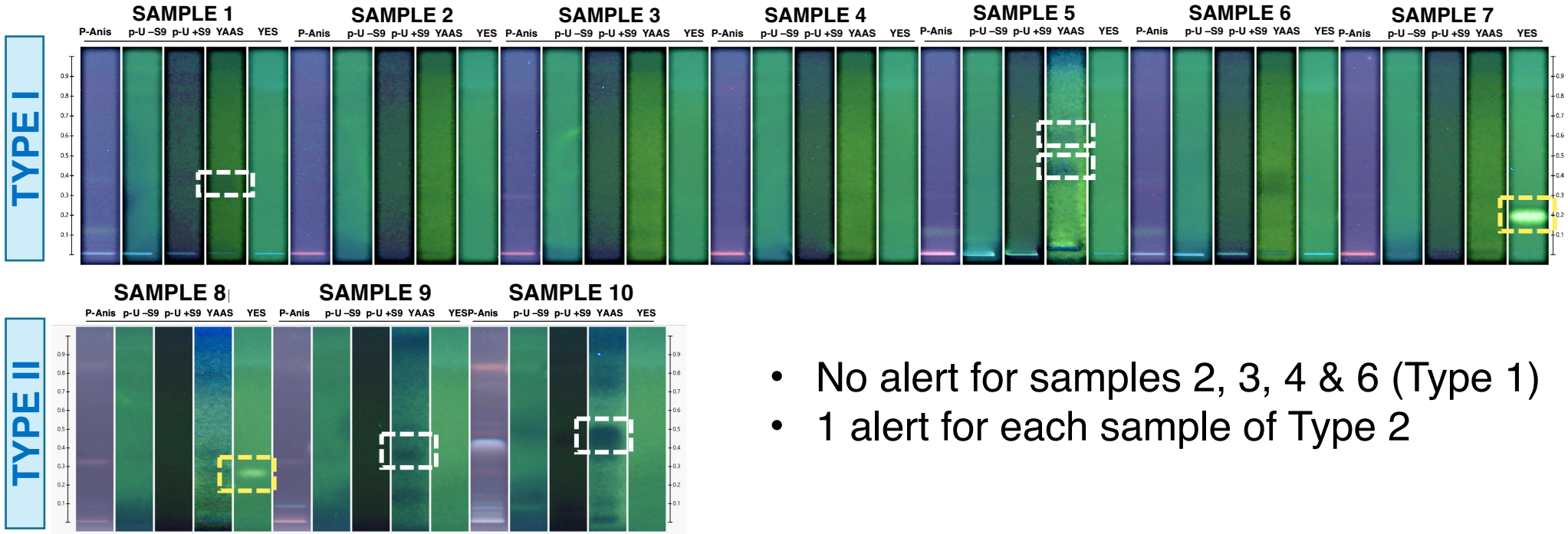
Research and Development

Genotoxicity, estrogenicity and androgenicity evaluation by HPTLC



- Anti-Androgenicity
- Estrogenicity
- Androgenicity
- Genotoxicity (+/- metabolic activation)

Biological effect-based endpoints

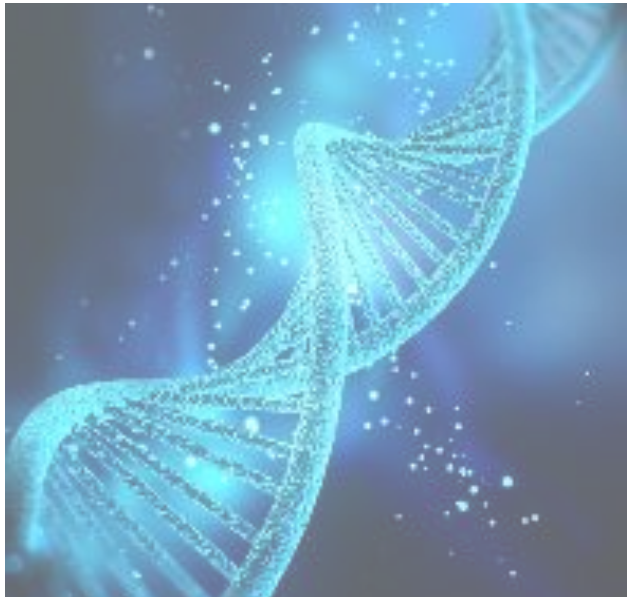


- No alert for samples 2, 3, 4 & 6 (Type 1)
- 1 alert for each sample of Type 2

Research and Development

We are developing *in vitro* bioassays to screen our food/ingredient/ packaging for substances of toxicological concerns

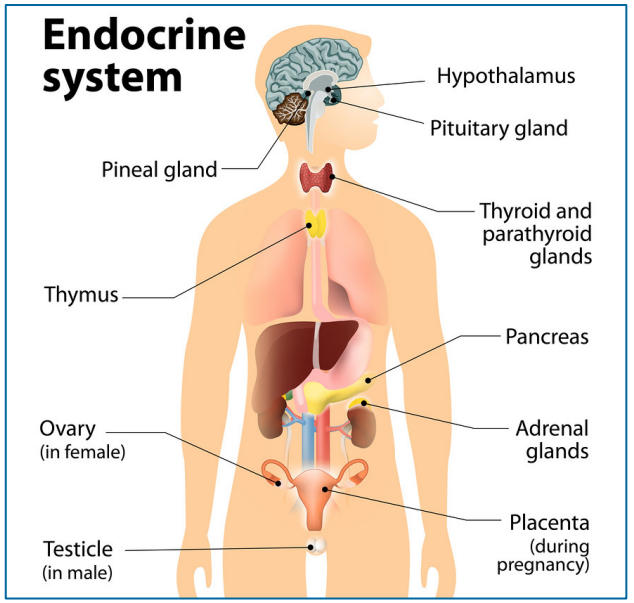
DNA-Damage



- Genotoxicity
- Mutagenicity

Benzene, mycotoxins, pyrrolizidine alkaloids…

Endocrine activity



- Estrogenicity
- Androgenicity
- Steroidogenesis
- Thyroid

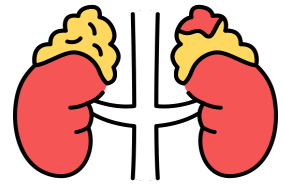
Bisphenol A, phthalates, soy isoflavones, vitamin D

Adulteration

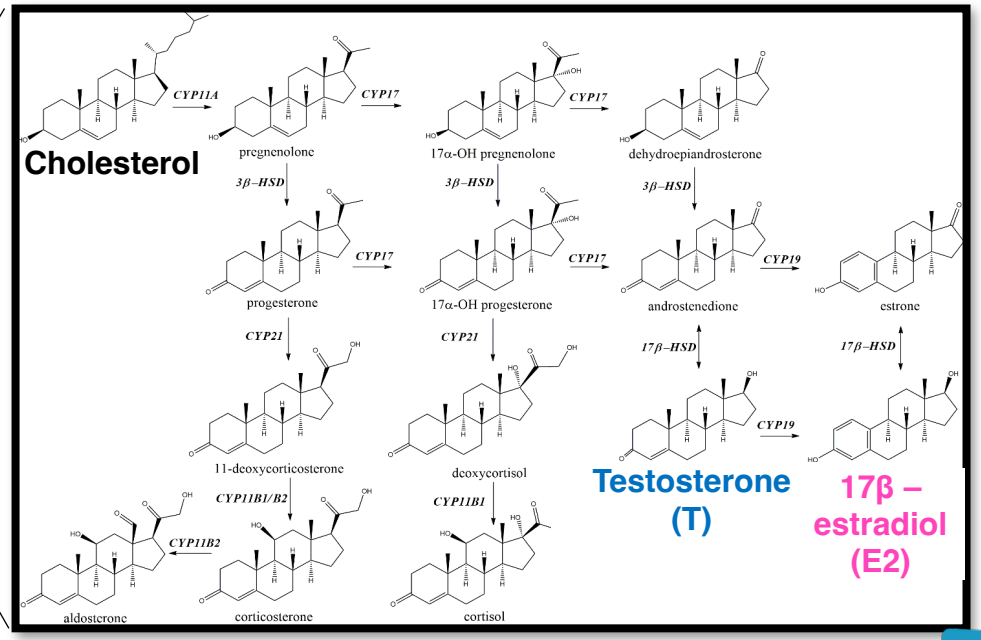
Act of making food worse in quality by adding something to them

Research and Development

Steroidogenesis: definition and main challenges for testing

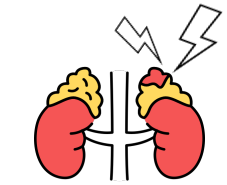


Adrenal glands



Steroidogenesis pathway

Disruption



Adrenal disorders

Hormonal imbalances

Infertility

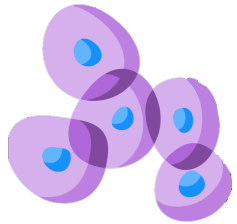
Effects ?

OECD

Test guideline: Use human **model H295R cells**, expressing genes that encode for all key enzymes and producing all steroids hormones

Challenges:

- No recommendation on the **detection method** (i.e ELISA, RIA, LCMS)
- **Required limit of quantification** of **E2** (10 pg/mL) and **T** (100 pg/mL)
- Suitability of the method for testing complex **food matrices** ?



Research and Development

Steroidogenesis testing (H295R cells): development of detection methods

Available methods | External laboratories

External collaboration

LC-MSMS

UPLC-MSMS detection method: **E2 and T**

- Limitation of cell model: E2 detected but not quantified in some samples
- Despite that, E2 level increase observed

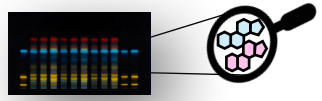
Internal development

CALUX (modified method)

E2 or T changed levels detected through E/A reporter gene assay

- Limitation: no distinction between direct effect on gene regulation & steroid production

HPTLC



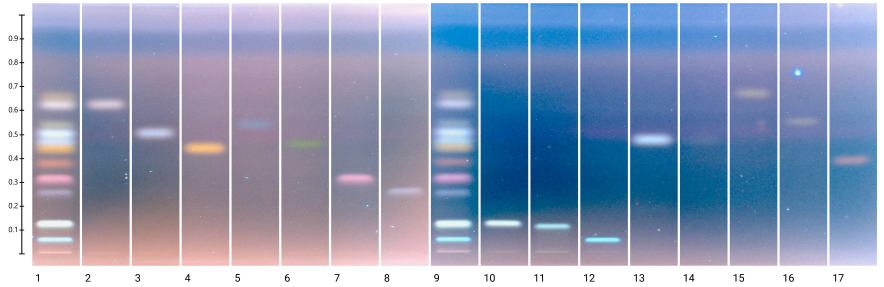
to detect E2 and T

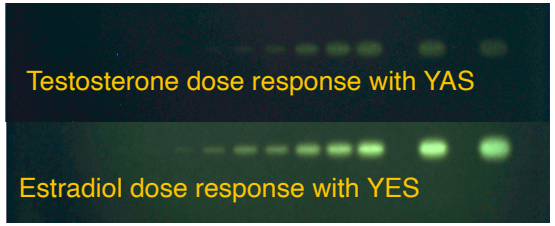
STOP I. Coupled with immunostaining

STOP II. Coupled with steroids revelator

III. Coupled with Yeast estrogen/ androgen reporter gene assay

Steroid separation on HPTLC plate





LOQ (pg/ mL)

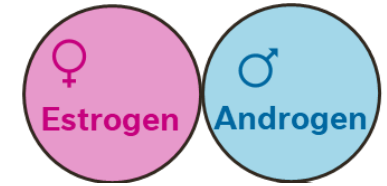
1160

1.7

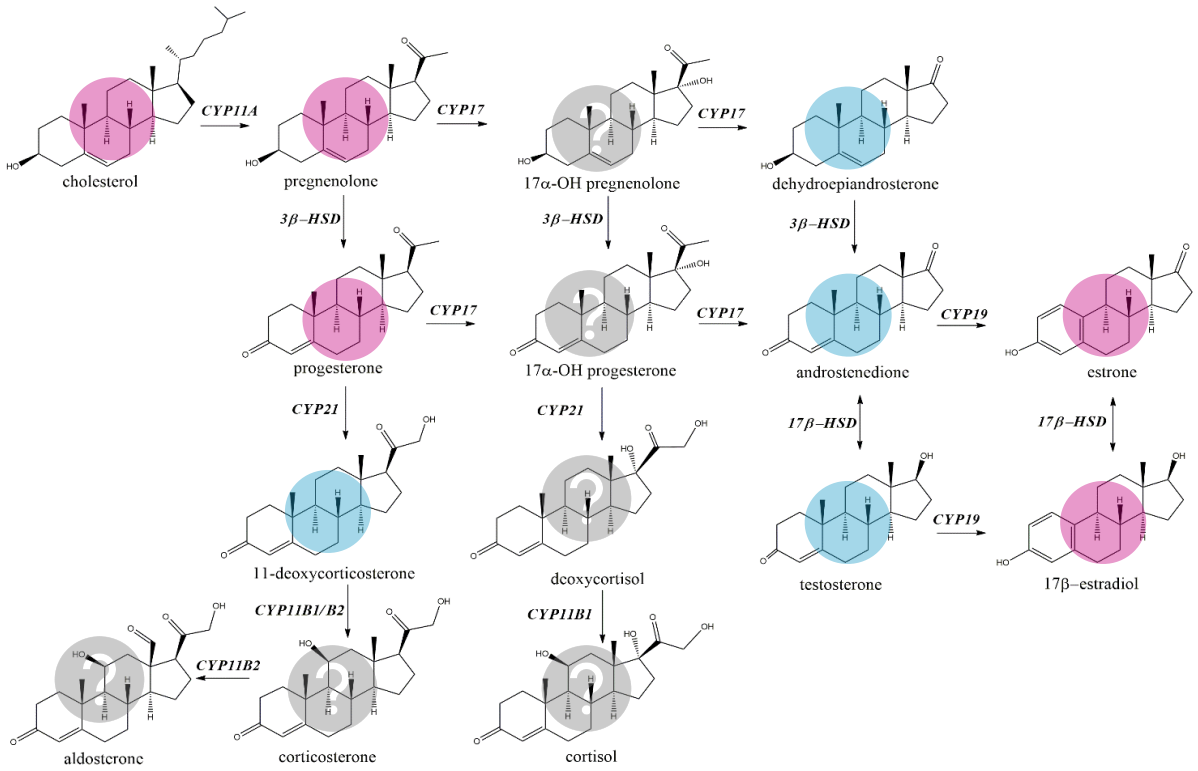
- Promising preliminary results → **need further validation**

Research and Development

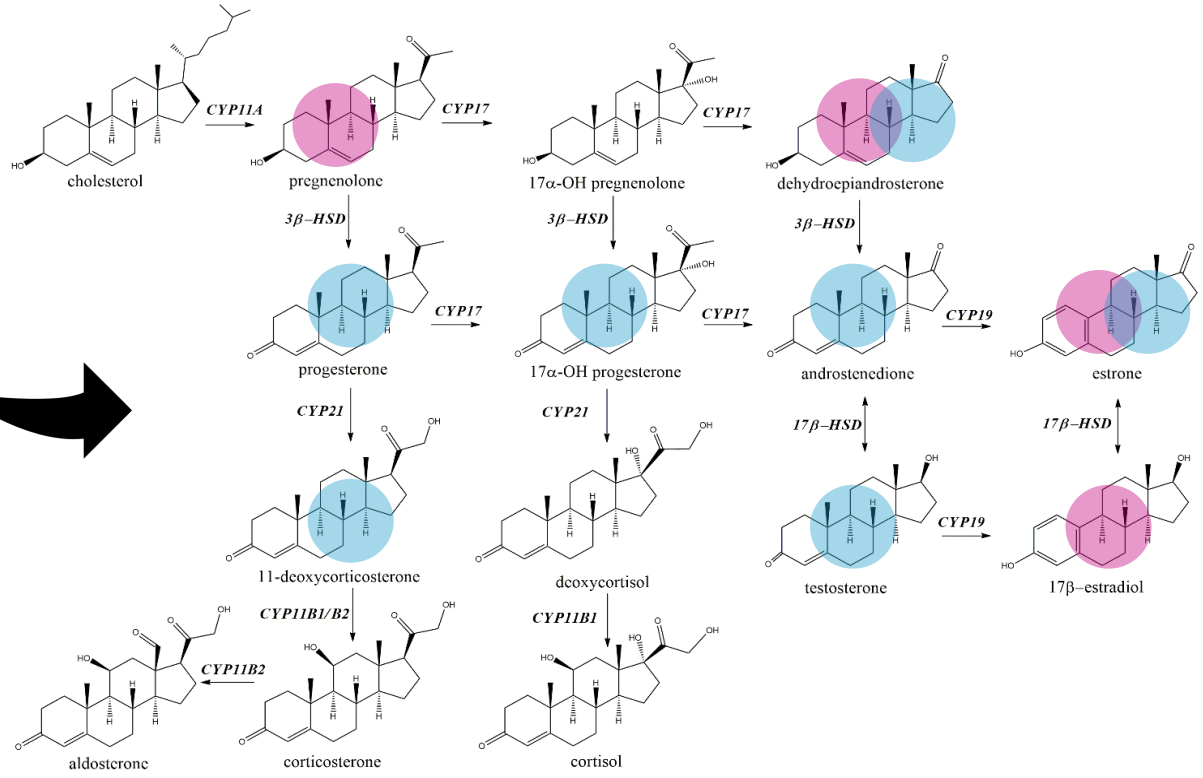
E or A activity of steroids from literature vs pYES/pYAS bioassays



From literature



From our p-YES/p-YAS bioassays

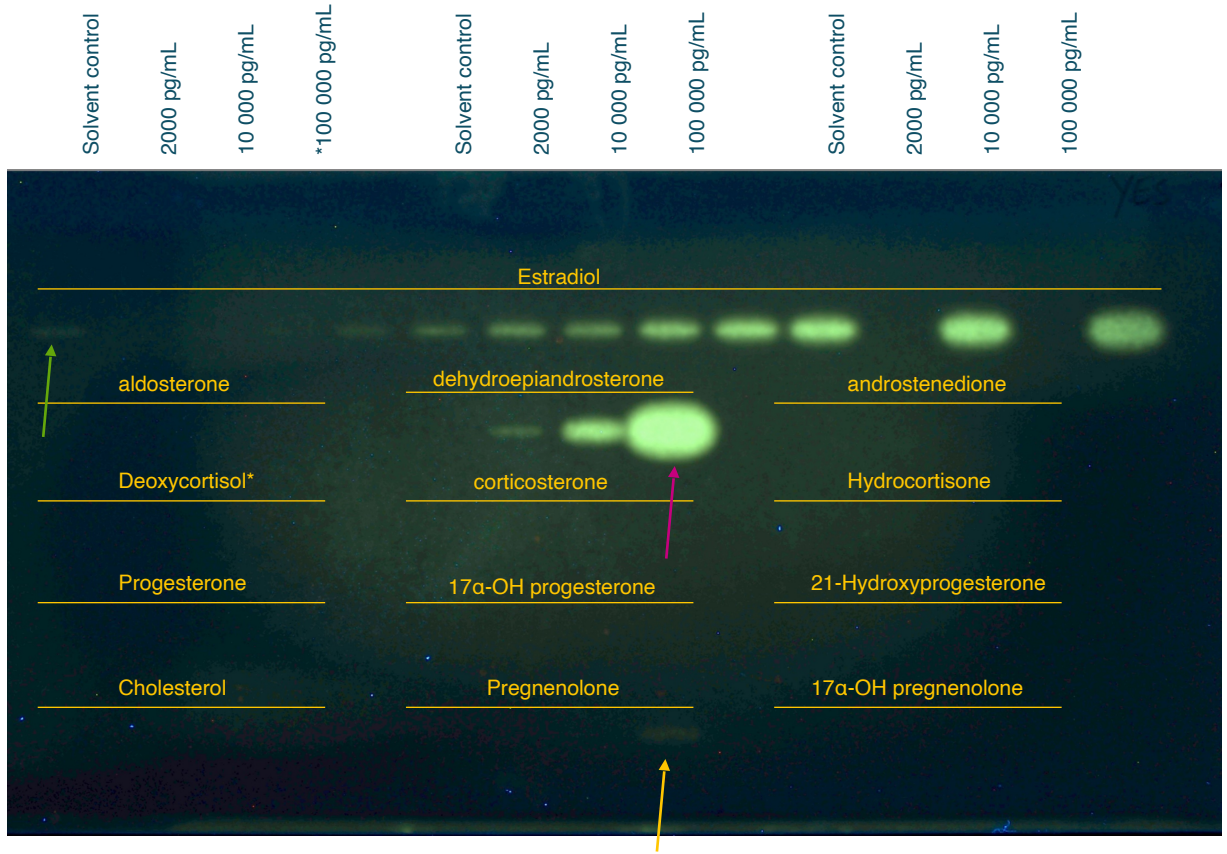


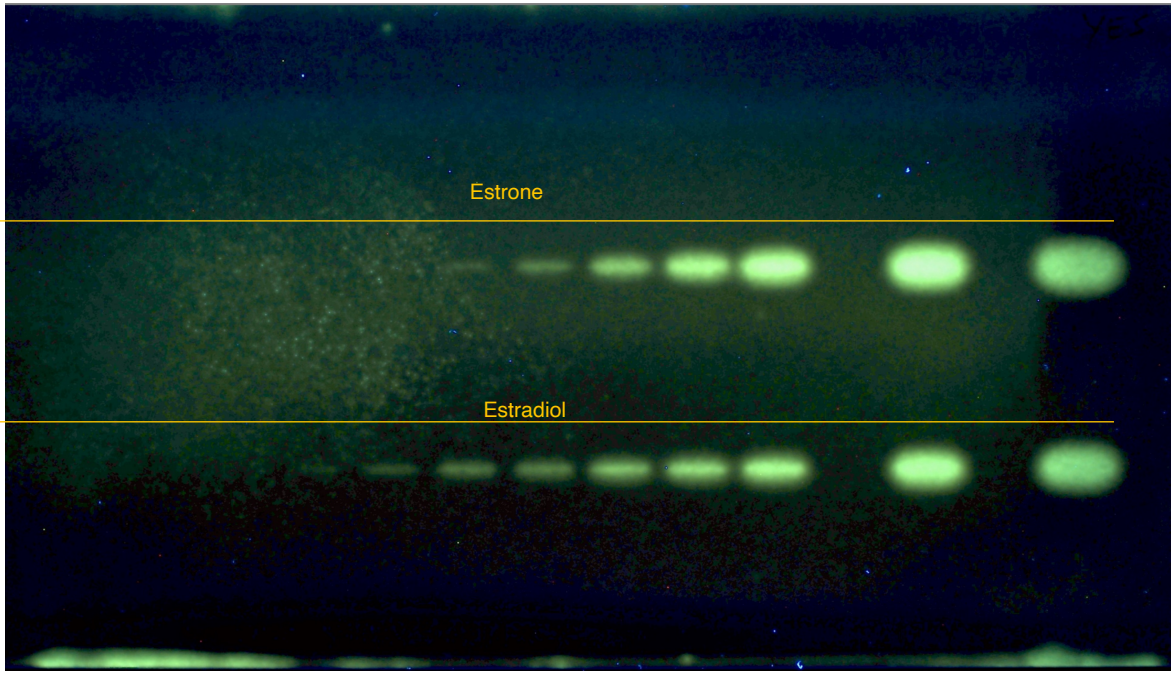
- Would it be possible to quantify E2 and T using our p-YES/p-YAS bioassays?
- Enough sensitivity to reach the low limit of detection?
- Can the different hormones be separated by HPTLC?

Research and Development

Detection of the estrogenic hormones by pYES

Derivatized - Visualization 366 nm

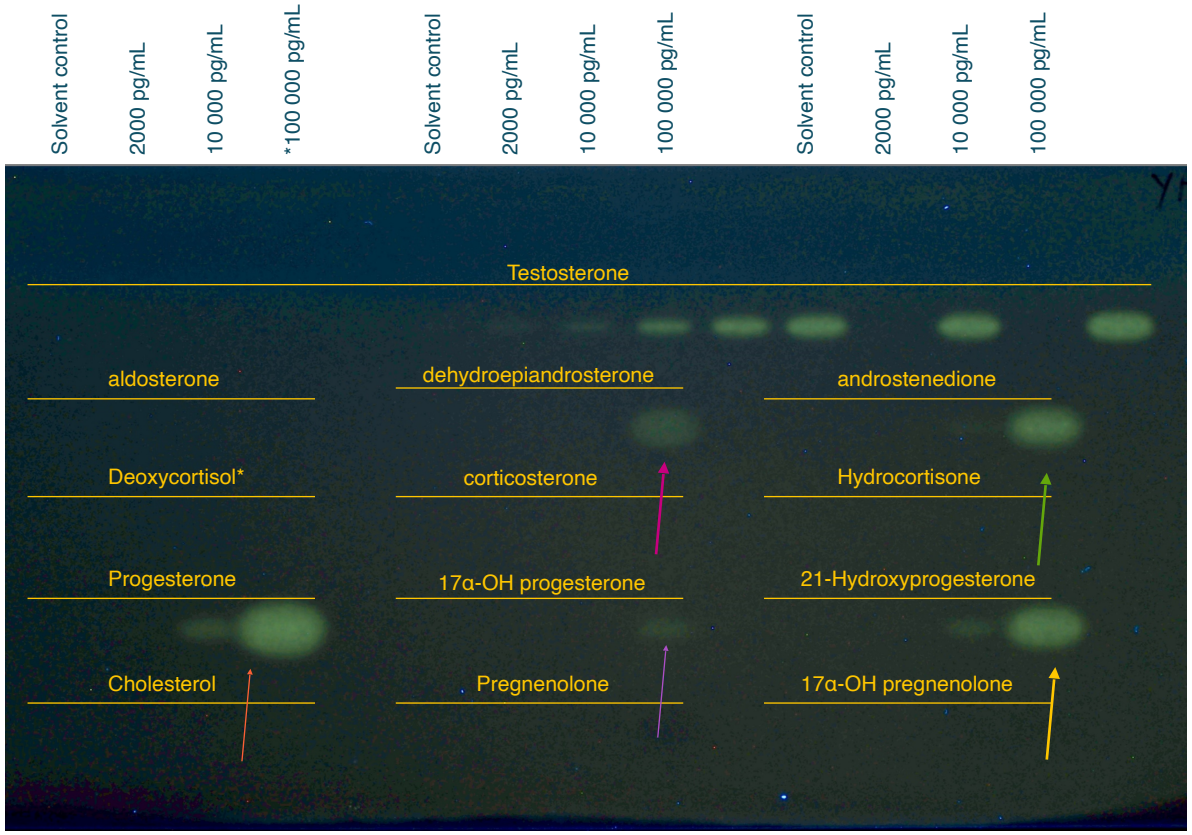


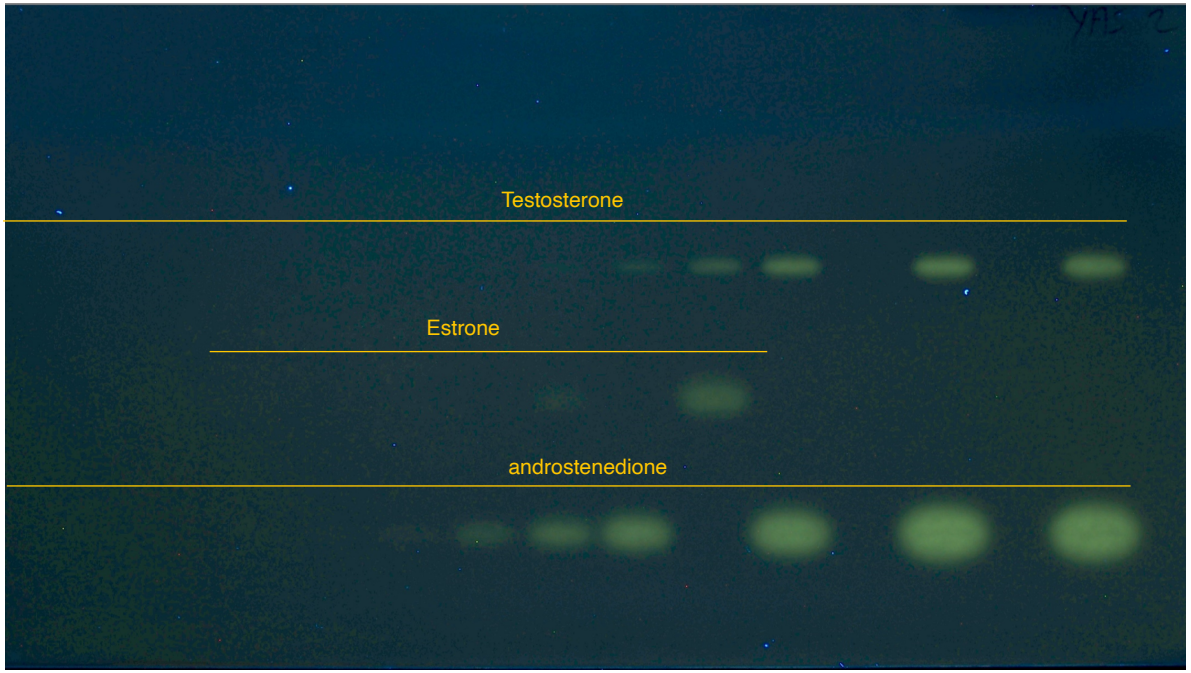


Research and Development

Detection of the androgenic hormones by pYAS

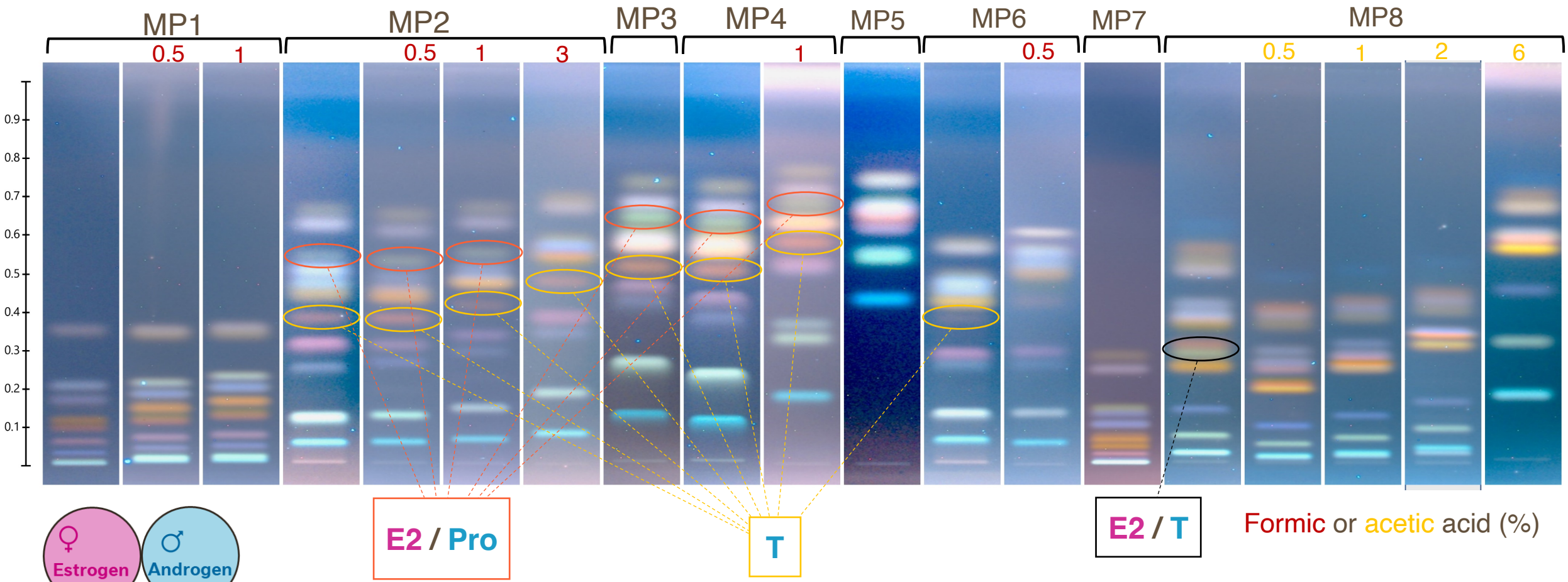
Derivatized - Visualization 366 nm





Research and Development

Mobile Phase optimisation to separate hormones with the same bioactivities



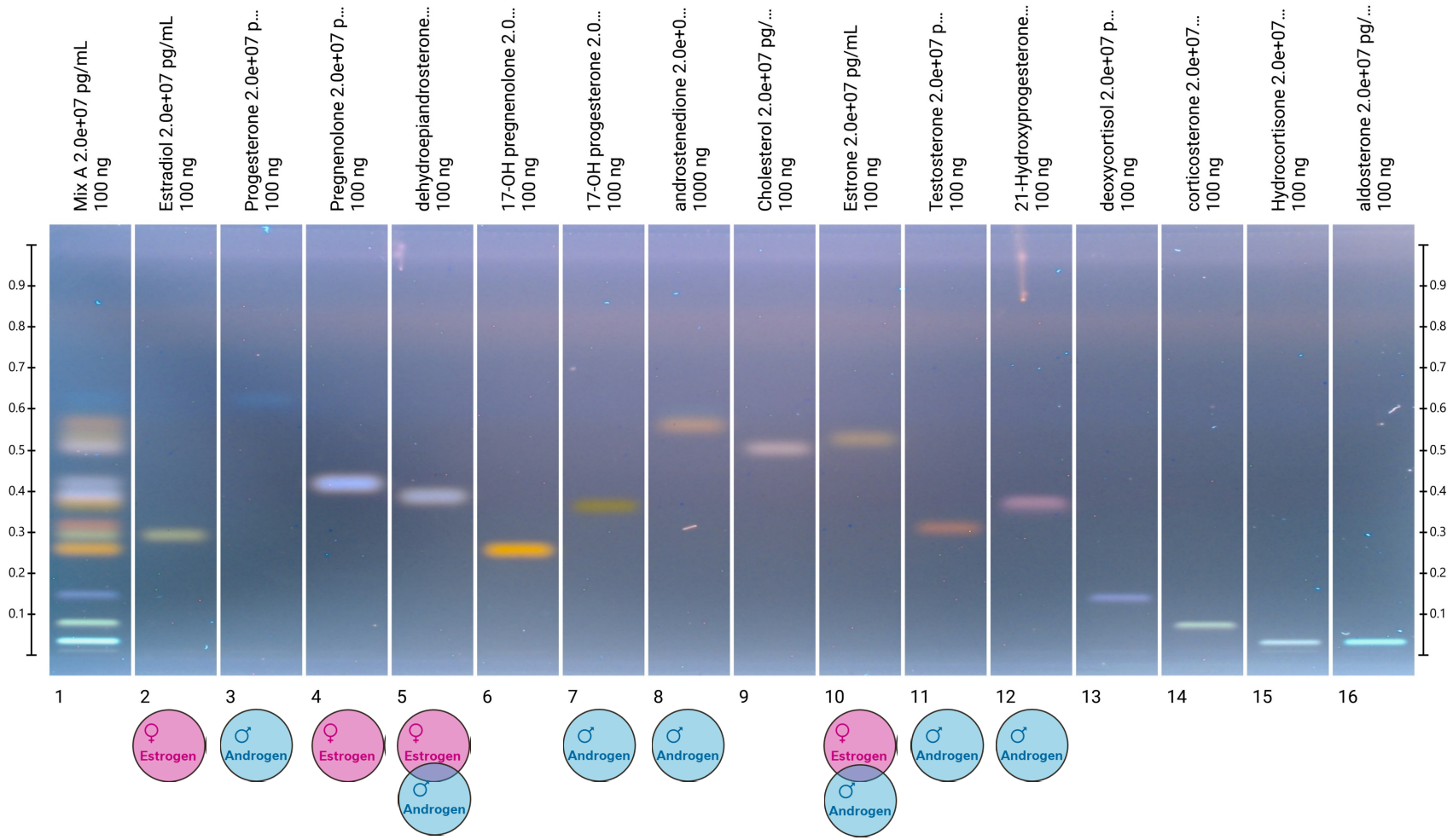
Estradiol (E2)
Pregnenolone (Pre)
DHEA
Estrone (E1)

Testosterone (T)
Androstenedione (AN)
Progesterone (Pro)
17α-OH progesterone
11-deoxycorticosterone

- **MP1** not polar enough
- **MP2 + formic acid** (max 1%) efficient to separate **E2** and **T**, **BUT** low pH not suitable for bioassays
- **MP8** suitable for **E2** and **T**

Research and Development

Optimized mobile phase for the separation of the hormones with the same bioactivity (E and/or A)



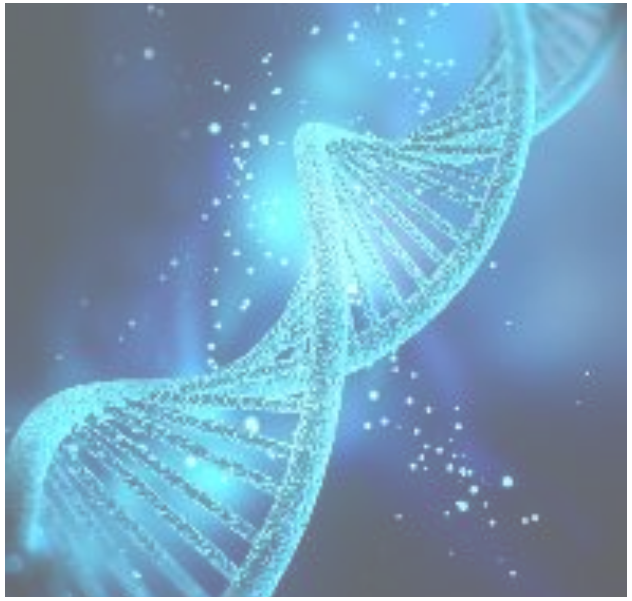
Reference substance	R_F
Progesterone	0.63
Androstenedione	0.56
Estrone	0.53
Cholesterol	0.50
Pregnenolone	0.42
DHEA	0.39
17-OH-progesterone	0.37
21-hydroxyprogesterone	0.37
Testosterone	0.31
Estradiol	0.30
17OH – pregnenolone	0.26
deoxycortisol	0.14
Corticosterone	0.07
hydrocortisone	0.03
aldosterone	0.03

- Limit of detection reached for E2 & T and separation from other hormones with same bioactivity
- Test in the H295R cell line on going

Research and Development

We are developing *in vitro* bioassays to screen our food/ingredient/ packaging for substances of toxicological concerns

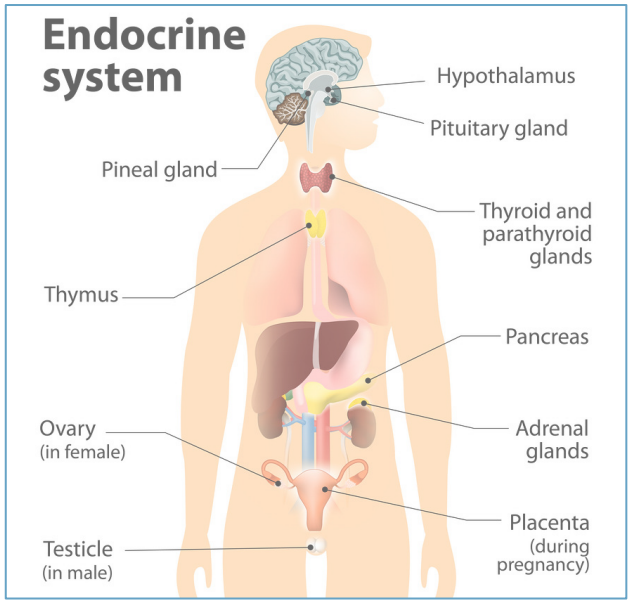
DNA-Damage



- **Genotoxicity**
- **Mutagenicity**

Benzene, mycotoxins, pyrrolizidine alkaloids…

Endocrine activity



- **Estrogenicity**
- **Androgenicity**
- **Steroidogenesis**
- **Thyroid**

Bisphenol A, phthalates, soy isoflavones, vitamin D

Adulteration

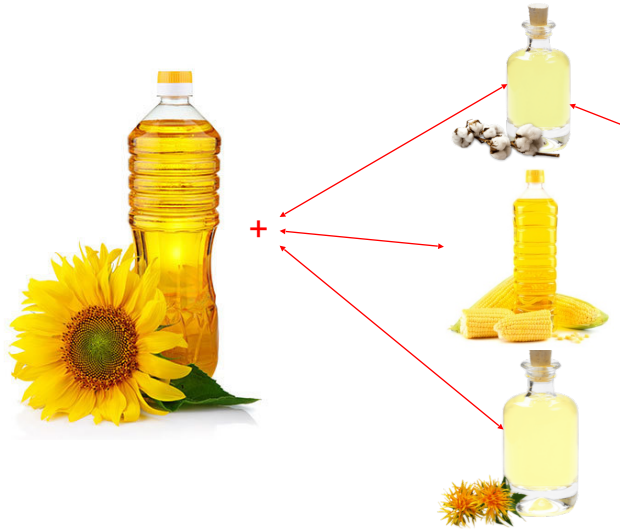
Act of making food worse in quality by adding something to them

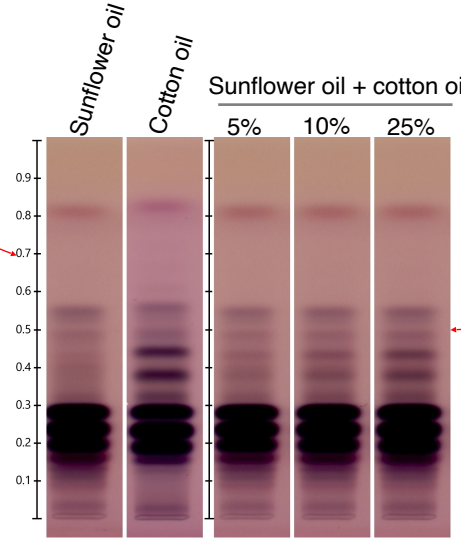
Vegetable oils, plant proteins, herbs & spices

Research and Development

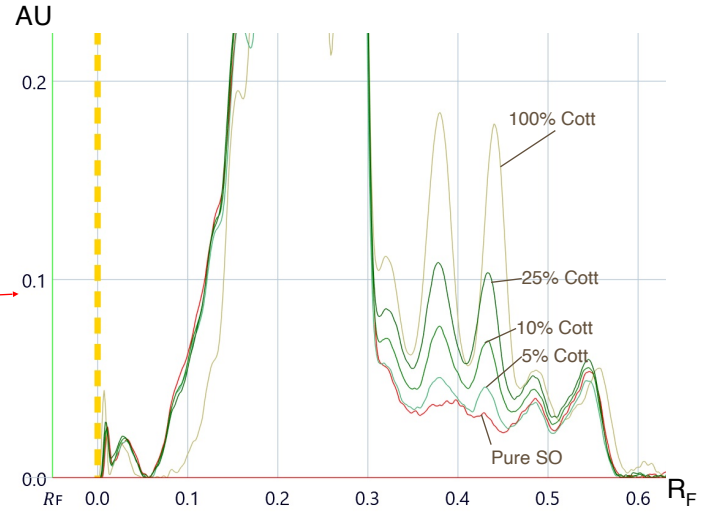
Detection of the adulteration of edible oils by HPTLC

1 Sunflower oil adulterated by edible oils



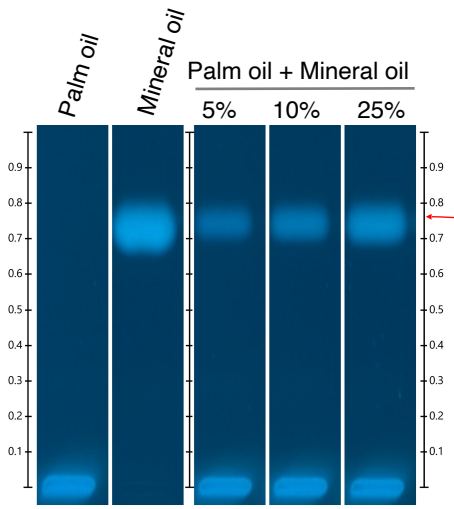


Fingerprint

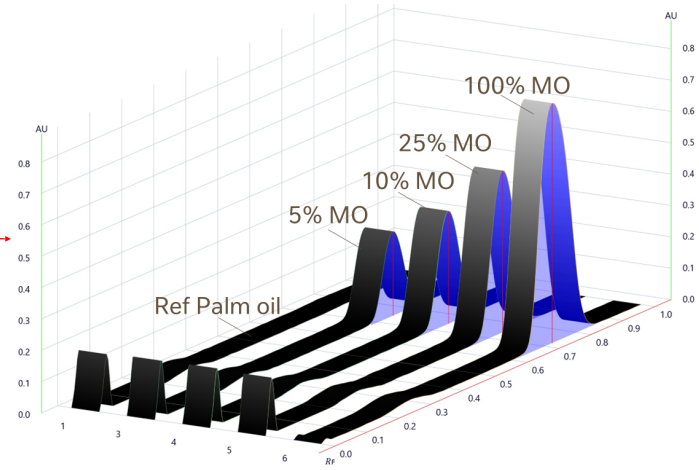


Adulteration levels detected from 5%

2 Palm oil adulterated by mineral oils



Specific peak



HPTLC efficient to detect adulteration of various ingredients like edible oils, plant proteins, herbs & spices… adulterated with different types of adulterants

Research and Development

Conclusions

- Anchoring HPTLC to bioassays (genotoxicity & endocrine activity) is a promising approach in Food Safety Research
- Facilitates the identification of bioactive compounds responsible for genotoxic and endocrine activity
- Other applications of HPTLC can be envisaged like rapid method for raw material analysis against adulteration
- HPTLC is a valuable tool for Reseach & Development and Quality Control

Research and Development

Acknowledgements

Biodetection Group

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CAMAG®

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Research and Development