Bioassays and other in vitro models and tools

BDS Conference, November 7th, Istanbul

Toine Bovee

RIKILT
WAGENINGEN UR

THIS PRESENTATION

- **A Bioassays**: effect based screening assays for the detection of hormones and EDCs, e.g. in veterinary control

- **B In vitro models for digestion, metabolism and bioavailability**

Why effect based assays for hormones? EU Regulations I

- First: The success with the DR-CALUX®
- Directive 96/23/EC: banns the use of Group A substances
  - Stilbenes, derivatives, salts and esters
  - Antithyreogene compounds
  - Steroids
  - Resorcyclic Acid Lactones (including zeranol)
  - ß-agonists
  - Others, as mentioned in the Annex of Regulation EC 37/2010

However,....
EU regulations II

- Directive 96/22/EC: Prohibits **all substances having hormonal action**

- Regulations EC 178/2002 and EC 882/2004: oblige the member states to identify emerging risks and use validated and accredited methods for control analysis

How to obey to all these laws ?

- The only way is bioactivity screening combined with chemical analytical confirmation and identification using validated and accredited methods for both
- Or...to get rid of the laws. But would that be safe?
- ?

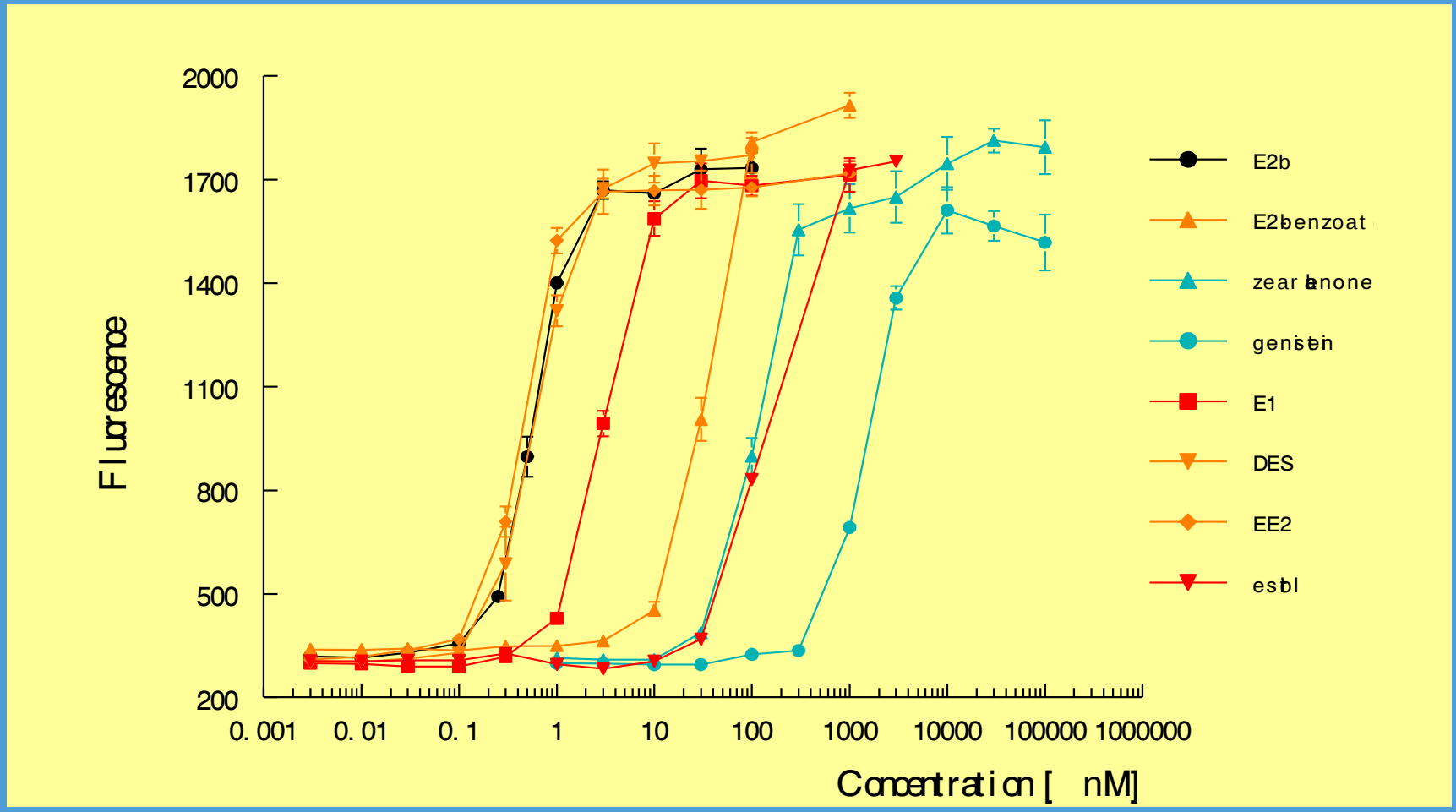
Bioactivity measurements

Transcriptional Activation (TA) bioassays (yeast or mammalian cell based)

- Detect all compounds (structures) that are able to activate the receptor, e.g. the estrogen, androgen, progesterone, glucocorticoid or thyroid receptor. As the main mode of action of all active hormones is by activating their cognate receptor, they fulfil Directive 96/22/EC that prohibits all substances having hormonal action

- Moreover, they are:
  - Sensitive and specific
  - Quick, simple and robust
  - Applicable to urine, feed and preparations

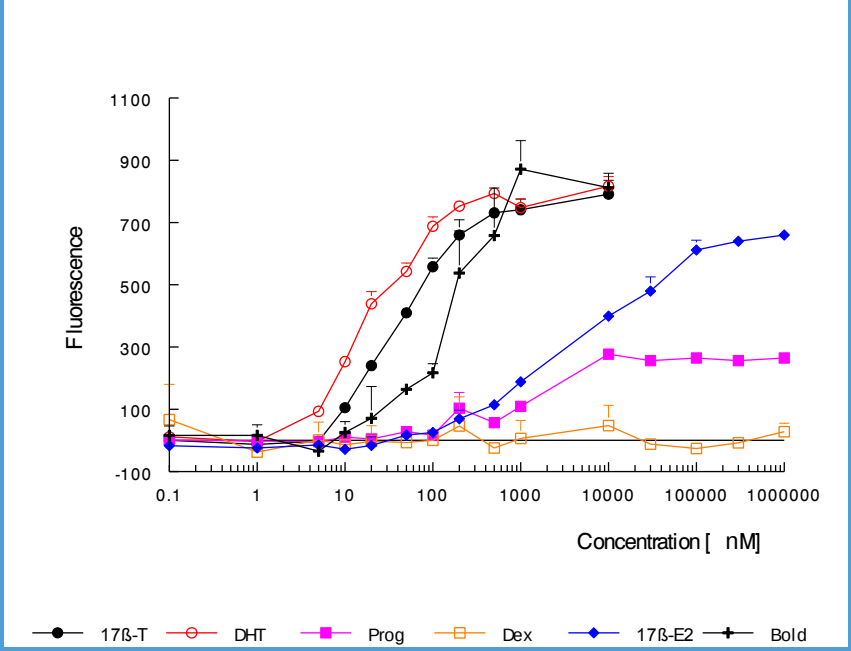
The yeast estrogen bioassay

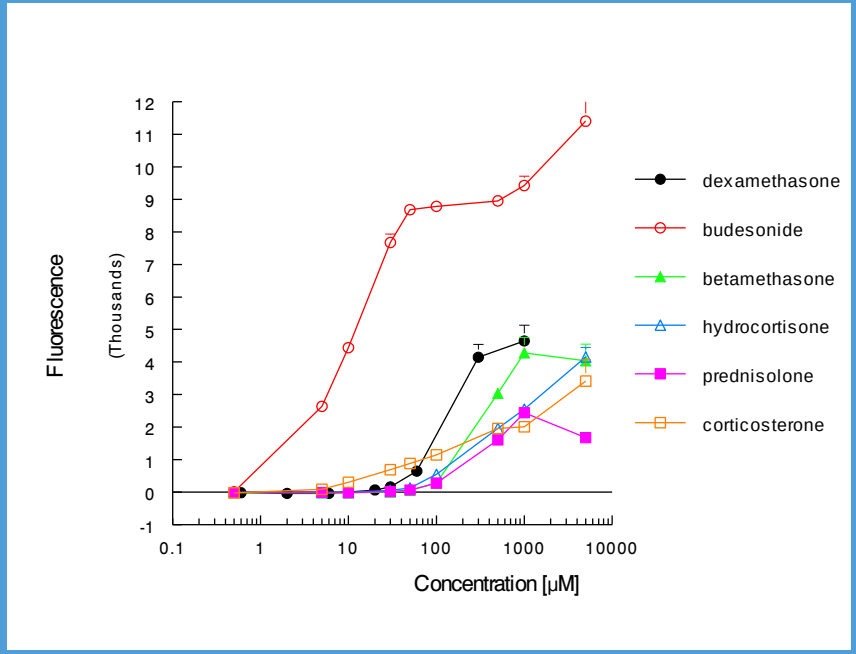


RIKILT
WAGENINGEN UR

Bovee et al., *Gene* **325** (2004) 187-200
Bovee et al., *JSBMB* **91** (2004) 99-109

Similarly we developed a yeast androgen bioassay and yeast corticoid bioassay





RIKILT
WAGENINGEN UR

Bovee et al., *ABC* **389** (2007) 1549-1558
Bovee et al., *ABC* **401** (2011) 873-882

Developed and/or validated bioassays at RIKILT-Institute of Food Safety

- The DR-CALUX® - feed, fat, oil
- The yeast estrogen bioassay - calf urine and feed
- The yeast androgen bioassay - calf urine and feed
- The yeast corticoid bioassay
- The GR-CALUX® - feed
- Receptor-bindingassay β-agonists - feed
- PPARδ bioassay
- The extended steroidogenesis assay - chemicals (OECD)

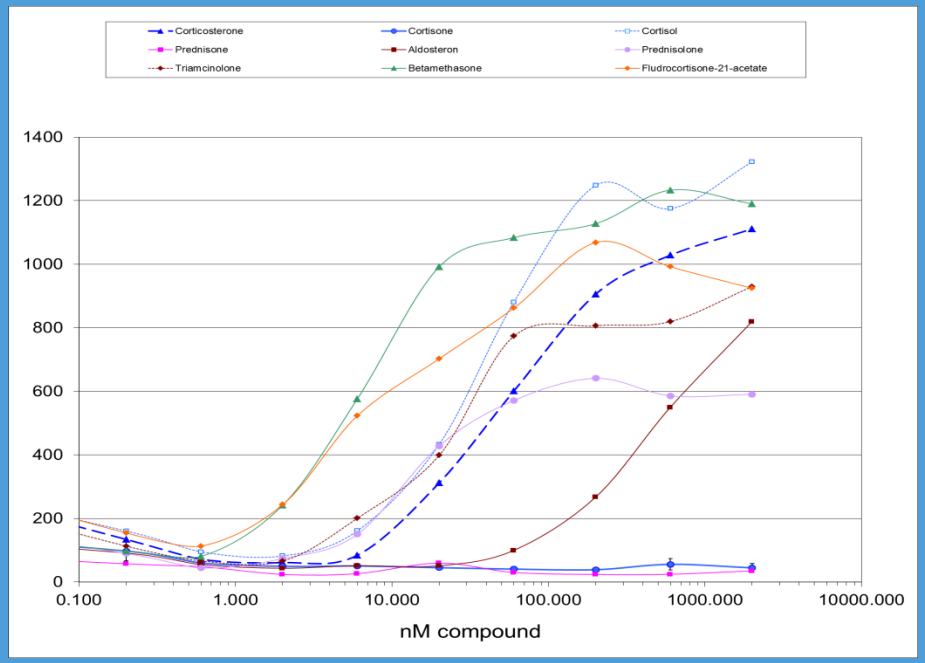
- PR-CALUX® - validation feed on-going

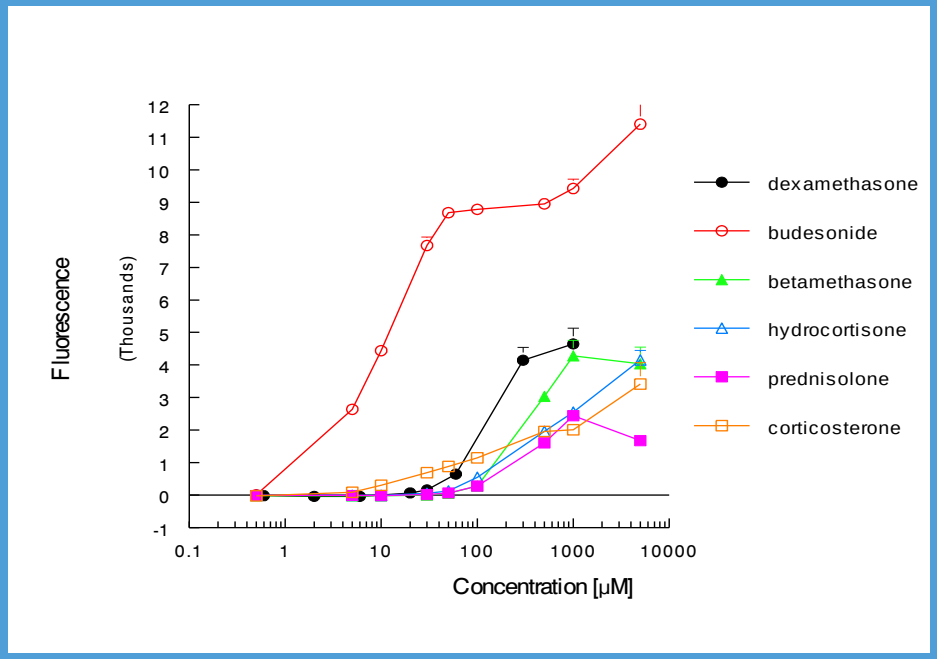
RIKILT
WAGENINGEN UR

- RIKILT glucocorticoid yeast assay:
  - Sufficient sensitivity for screening supplements and preparations (concentrations of 0.5 µg DEX/g)
  - Not sensitive enough for the routine screening of feed samples (<0.1 µg DEX/g)

- Some initial experiments showed promising results for GR-CALUX® bioassay (BDS; U2OS cell line)

Sensitivity GR-CALUX® vs yeast glucocorticosteroid bioassay

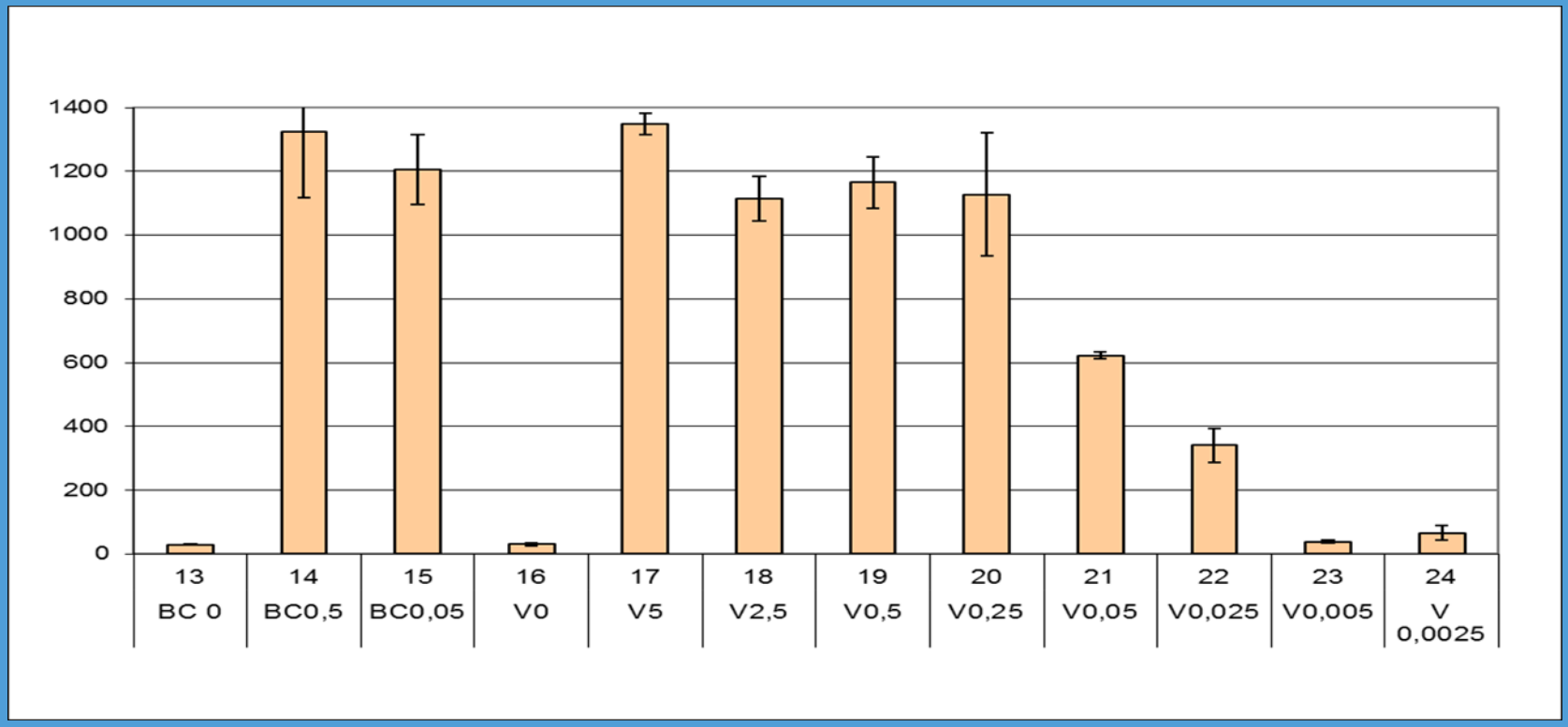




Sensitivity nM vs µM; thus very promising

RIKILT
WAGENINGEN UR

Results for GR-CALUX®
Dexamethasone spiked cattle feed



Sensitivity < 0.1 µg DEX/g; thus promising

RIKILT
WAGENINGEN UR

GR-CALUX® bioassay procedure for screening feed
1) Sample extraction

- Extraction from matrix (1 gram feed) using MeOH/NaAc buffer
- 2 step SPE cleanup of the extract
    - STRATA SDB-L :
        - Apply extract
        - Washing step: MeOH/MilliQ 70/30
        - Elution: Acetone
    - NH2
        - Apply extract, collect runthrough
    - Evaporation, reconstitution in 20 µL DMSO

RIKILT
WAGENINGEN UR

GR-CALUX® bioassay procedure for screening feed
2) Exposure and measurement

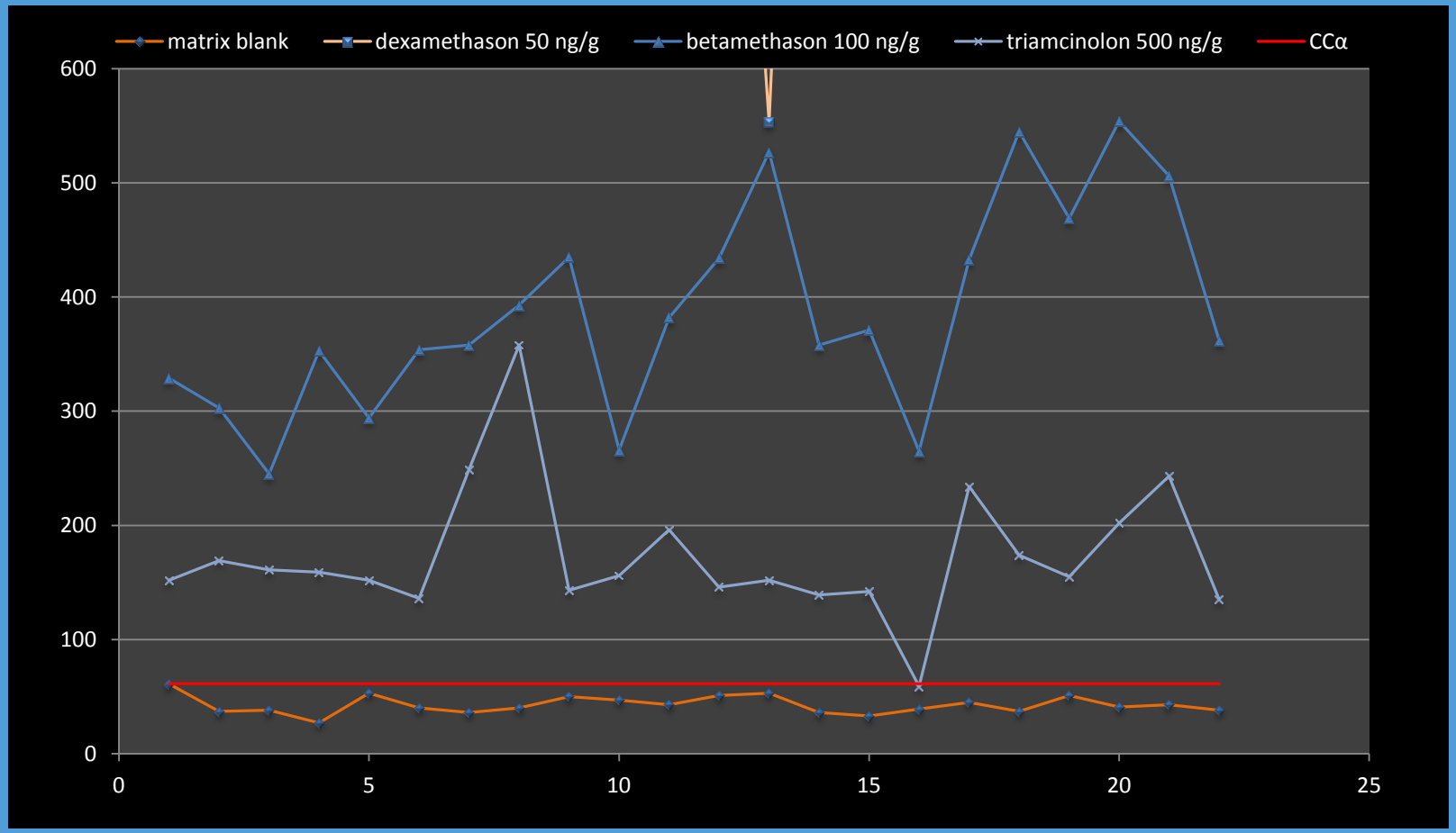
- Dilution of 2 µL extract in 500 µL assay-medium
- Pipette 100 µL of the diluted extract (triplicate) to 96 well plate, containing the GR-CALUX® U2OS cells
- Exposure of 24 h
- Luminescence measurement

The procedure in detail: SOP-A1134

feed – screening of (gluco)corticosteroid activity - bioluminescence

RIKILT
WAGENINGEN UR

Results: CCα and CCβ criterion checks



Bovee TFH, Heskamp HH, Hamers ARM, Brouwer BA, Nielen MWF (2013) Validation of a recombinant cell assay for the detection of glucocorticosteroid activity in animal feed. *FAC* **30**: 264-271.

RIKILT
WAGENINGEN UR

Extend the panel for veterinary control (older animals)

- In collaboration with the Turin University (Sara Divari): up-regulation of the PR-expression in prostate of older animals after administration of estradiol

The added value - Dietary supplements

- Dietary supplements → analysed by LC-MS/MS for 49 steroids.
  - 18 supplements - 11 positive and 7 negative

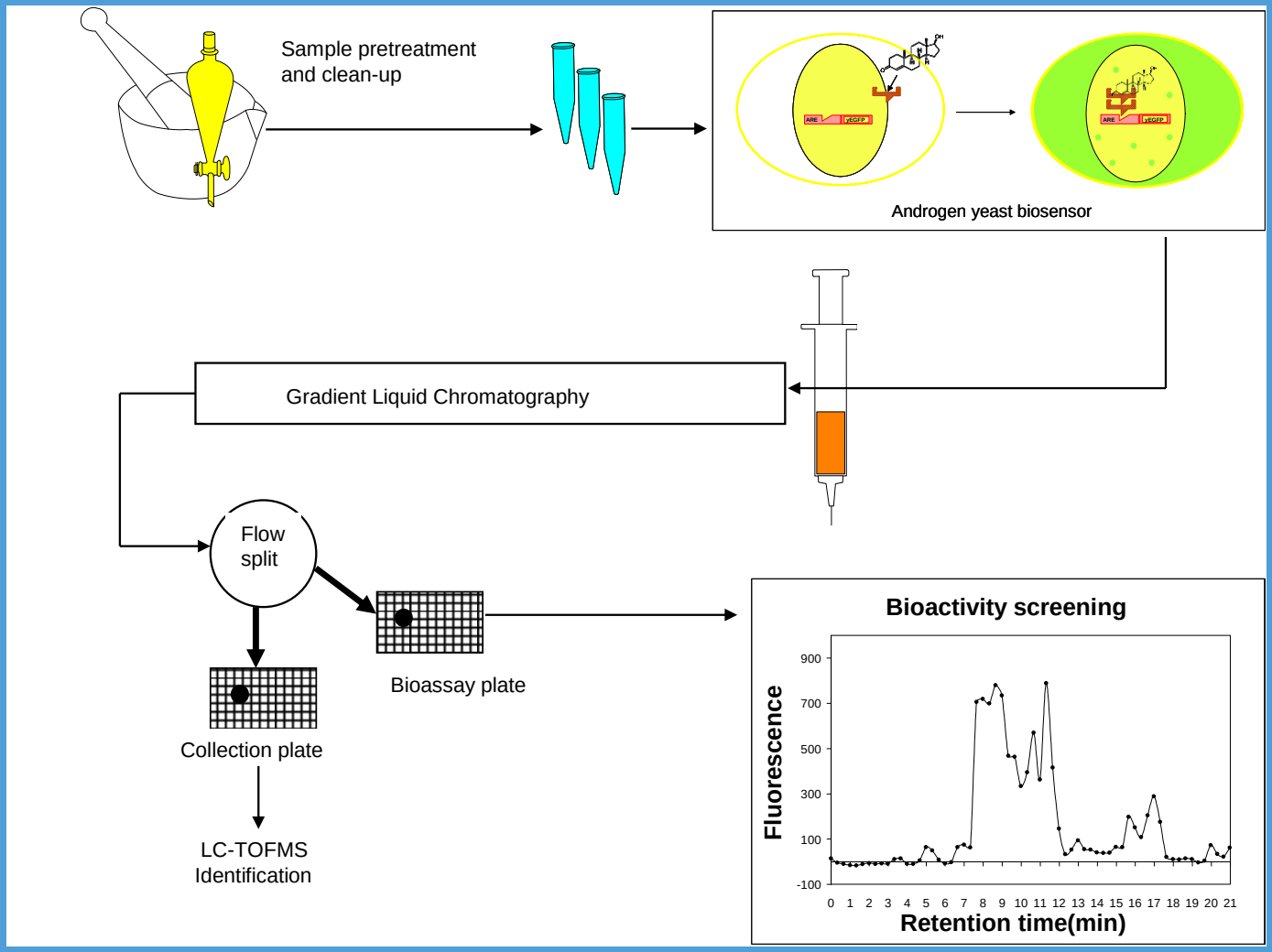
11 positive → also positive in the yeast **androgen** bioassay

7 negative → 2 supplements show androgenic activity in the yeast androgen bioassay

RIKILT WAGENINGEN UR

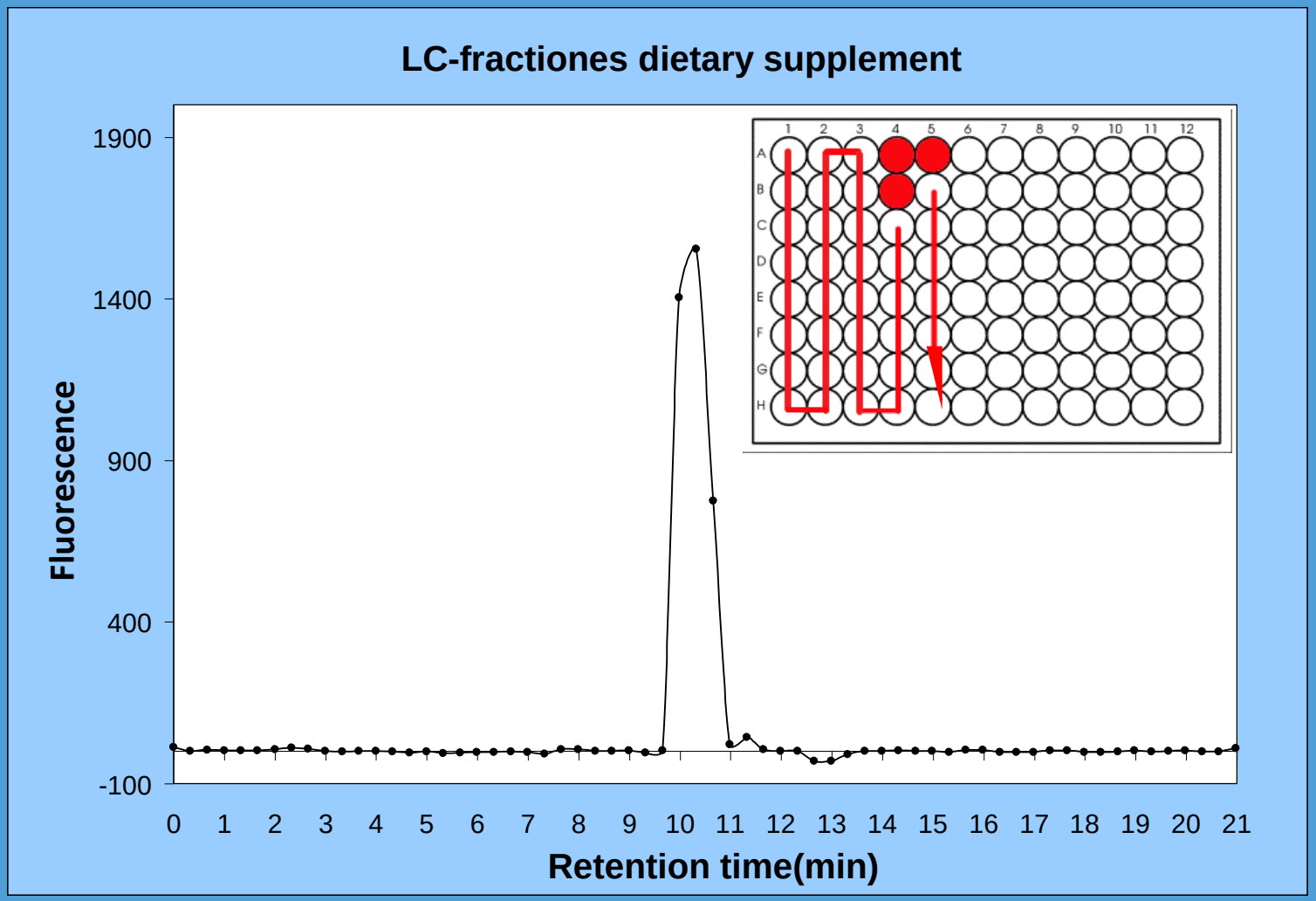
Van Poucke et al., *ACA* **586** (2007) 35-42

Bioassay directed identification of unknowns



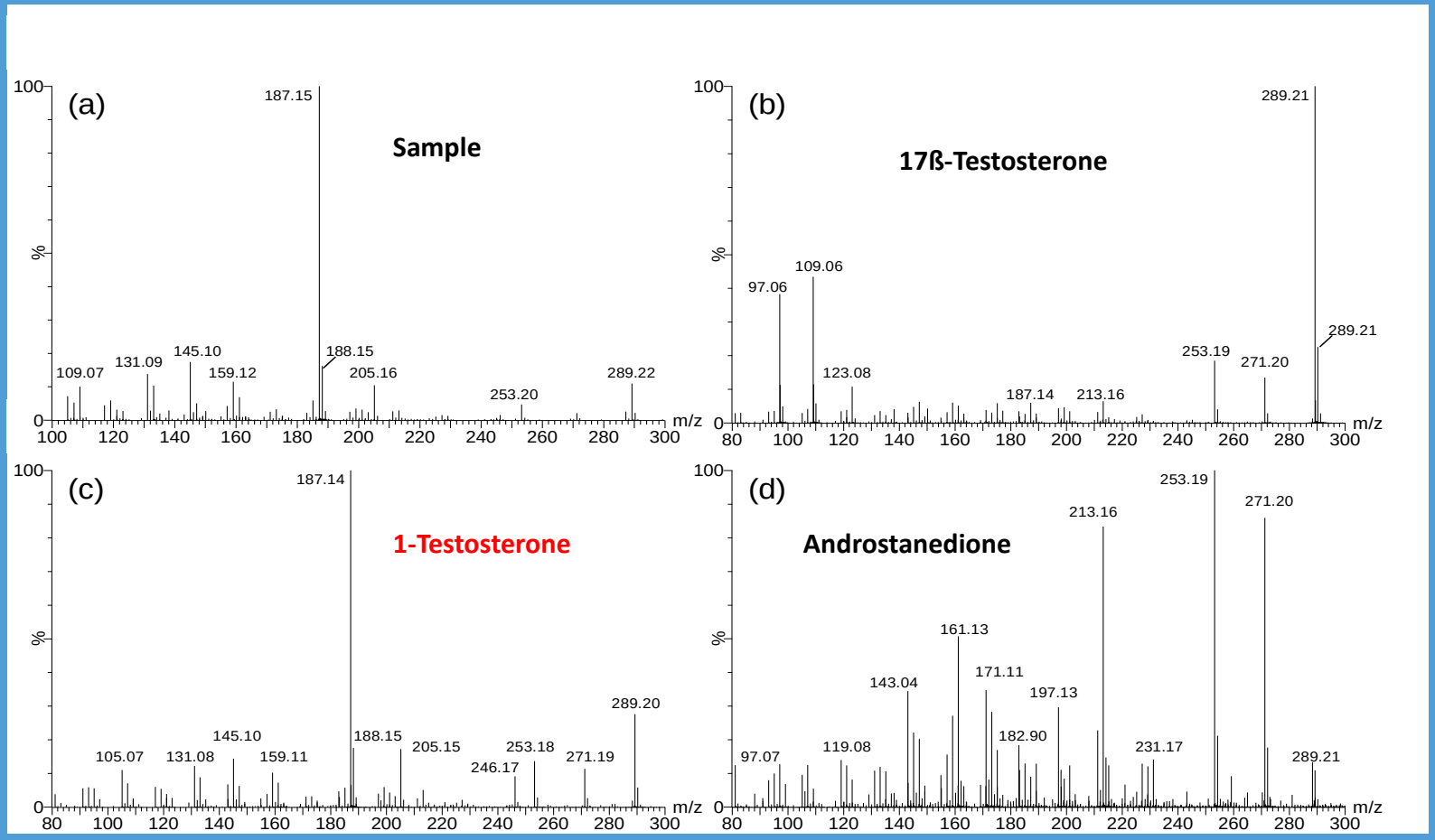
RIKILT
WAGENINGEN UR

Bioassay directed identification of unknowns



RIKILT
WAGENINGEN UR

Dietary supplements in yeast androgen bioassay & LC-MS/MS



The other one contained 4-androstene-3β,17β-diol and 5-androstane-3β,17β-diol

RIKILT
WAGENINGEN UR

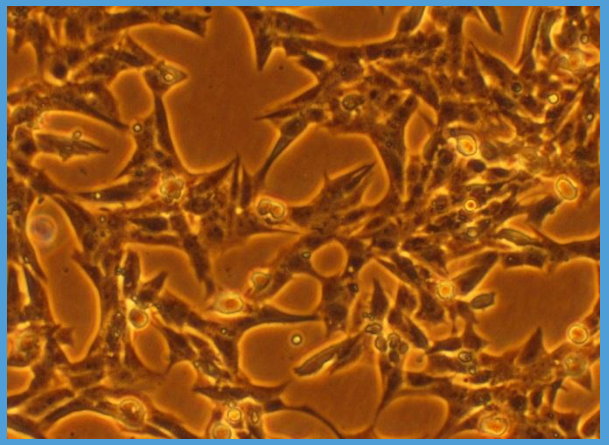
Rijk et al., *ACA* **637** (2009) 305-314

Going a little away from veterinary control

- Going to:
  - Hazard identification – characterization of chemicals (REACH)
  - Alternatives for animal testing (REACH)

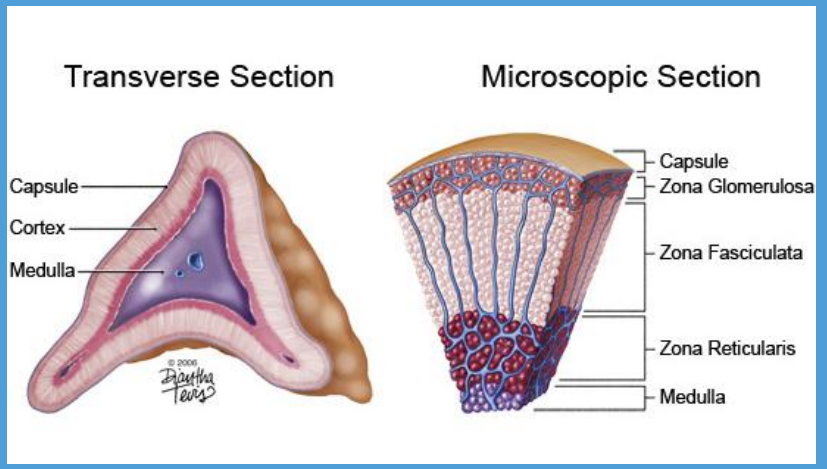
The extended H295R steroidogenesis assay

- Zonally undifferentiated fetal adrenal cells originating from a human adrenocarcinoma



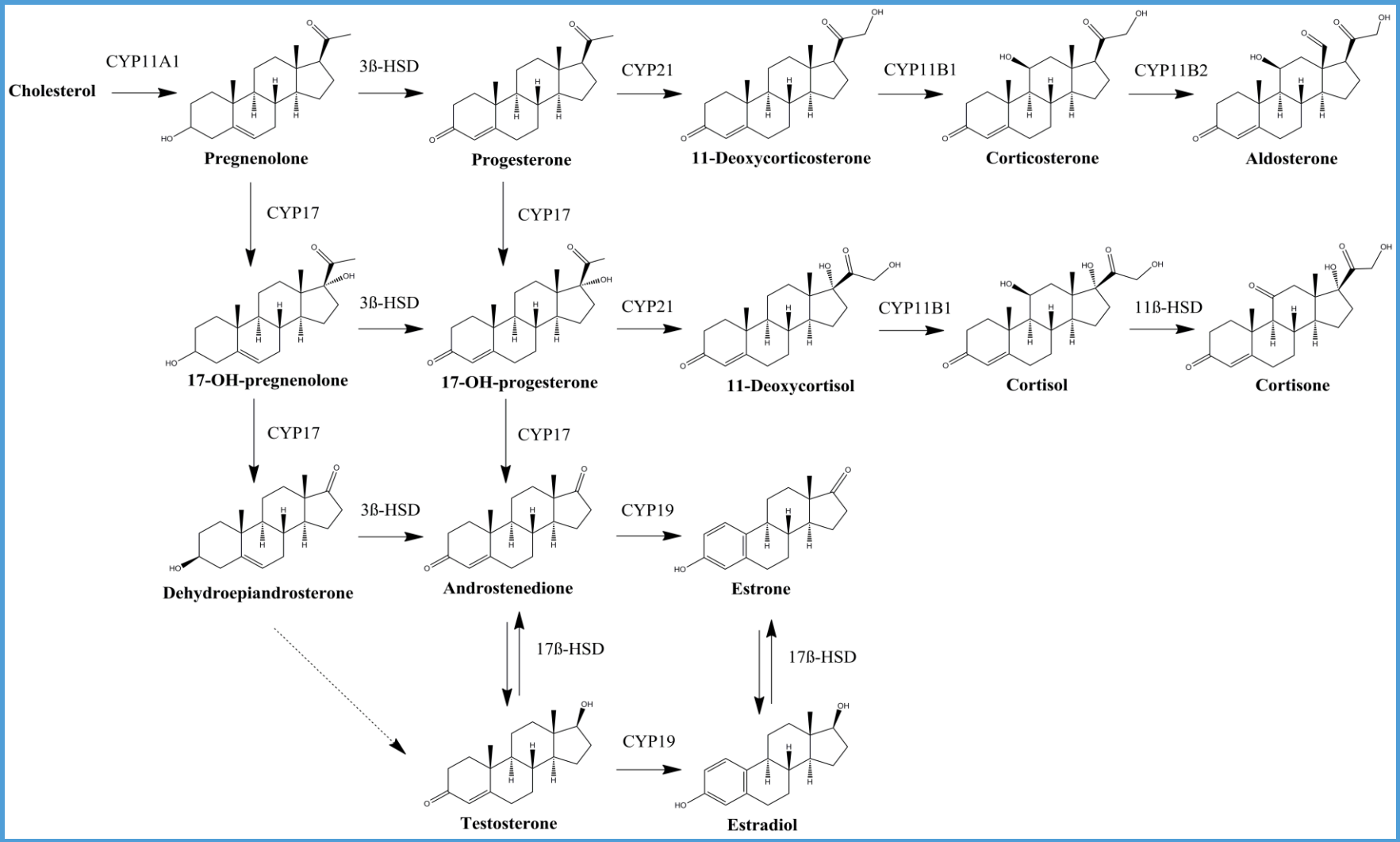
- Express all genes and enzymes involved in steroidogenesis

- OECD validated (TG 456)



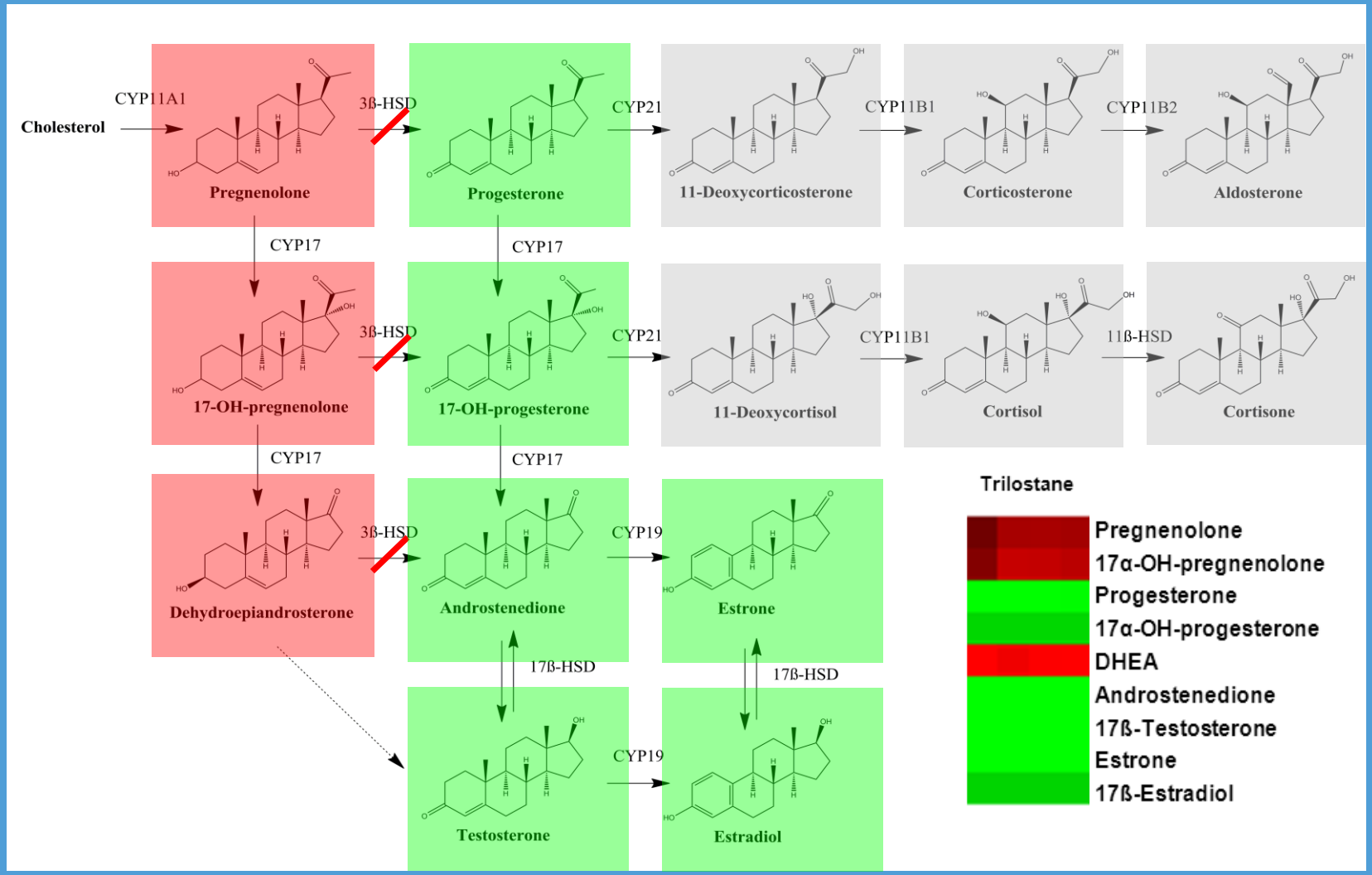
RIKILT
WAGENINGEN UR

The extended H295R steroidogenesis assay



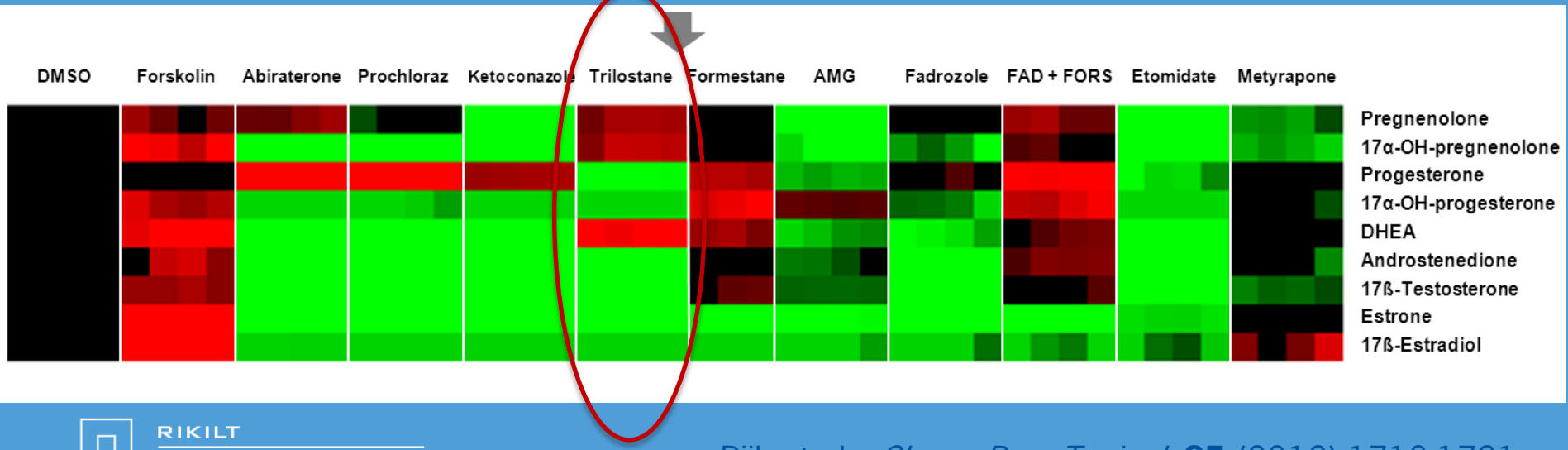
RIKILT
WAGENINGEN UR

Trilostane effects in the H295R assay – GC-MS/MS



Effects in the H295R assay – GC-MS/MS

DMSO	Forskolin	Abiraterone	Prochloraz	Ketoconazole	Trilostane	Formestane	AMG	Fadrozole	FORS + FAD	Etomidate	Metyrapone	Hormone
1.00±0.14	1.79±0.45*	1.97±0.31**	0.87±0.39	0.1±0.00***	1.15±0.16	0.78±0.27	0.17±0.03***	1.00±0.12	2.06±0.37**	0.07±0.02***	0.49±0.11**	Pregnenolone
1.00±0.11	4.53±2.02*	>0.01***	0.04±0.03***	>0.01***	2.65±0.41***	0.85±0.33	0.15±0.05***	0.40±0.17**	1.52±0.13**	0.05±0.00***	0.38±0.05***	17α-OH-pregnenolone
1.00±0.10	1.20±0.19	135±17.2***	6.12±0.34***	2.41±0.09***	0.22±0.04***	2.30±0.71*	0.38±0.03***	1.45±0.10**	4.32±0.31***	0.33±0.10***	0.97±0.06	Progesterone
1.00±0.09	2.70±0.47***	>0.07***	0.34±0.06***	>0.07***	>0.07***	3.21±1.00**	1.62±0.07***	0.49±0.00**	3.20±0.54**	0.07±0.00***	0.77±0.14	17α-OH-progesterone
1.00±0.10	5.15±1.30**	>0.05***	>0.05***	>0.05***	3.97±0.21***	1.98±0.57**	0.40±0.08***	0.30±0.07***	1.68±0.28*	0.10±0.01***	0.95±0.14	DHEA
1.00±0.15	2.40±0.80***	0.01±0.00***	0.01±0.00***	0.00±0.00***	0.01±0.00***	0.82±0.30	0.61±0.12*	0.11±0.03***	1.86±0.25**	0.04±0.01***	0.79±0.23	Androstenedione
1.00±0.06	2.29±0.16***	0.08±0.01***	0.07±0.01***	0.05±0.00***	0.07±0.00***	1.35±0.44	0.57±0.01***	0.19±0.03***	1.40±0.14*	0.05±0.01***	0.58±0.07***	17ß-Testosterone
1.00±0.15	21.9±2.74***	0.09±0.01***	>0.08***	>0.08***	>0.08***	>0.08***	0.22±0.03***	>0.08***	>0.08***	0.30±0.01***	0.09±0.25	Estrone
1.00±0.34	39.8±2.20***	>0.30*	>0.30*	>0.30**	>0.30***	>0.30***	>0.30*	>0.30*	>0.30*	0.35±0.29	2.07±0.74	17ß-Estradiol



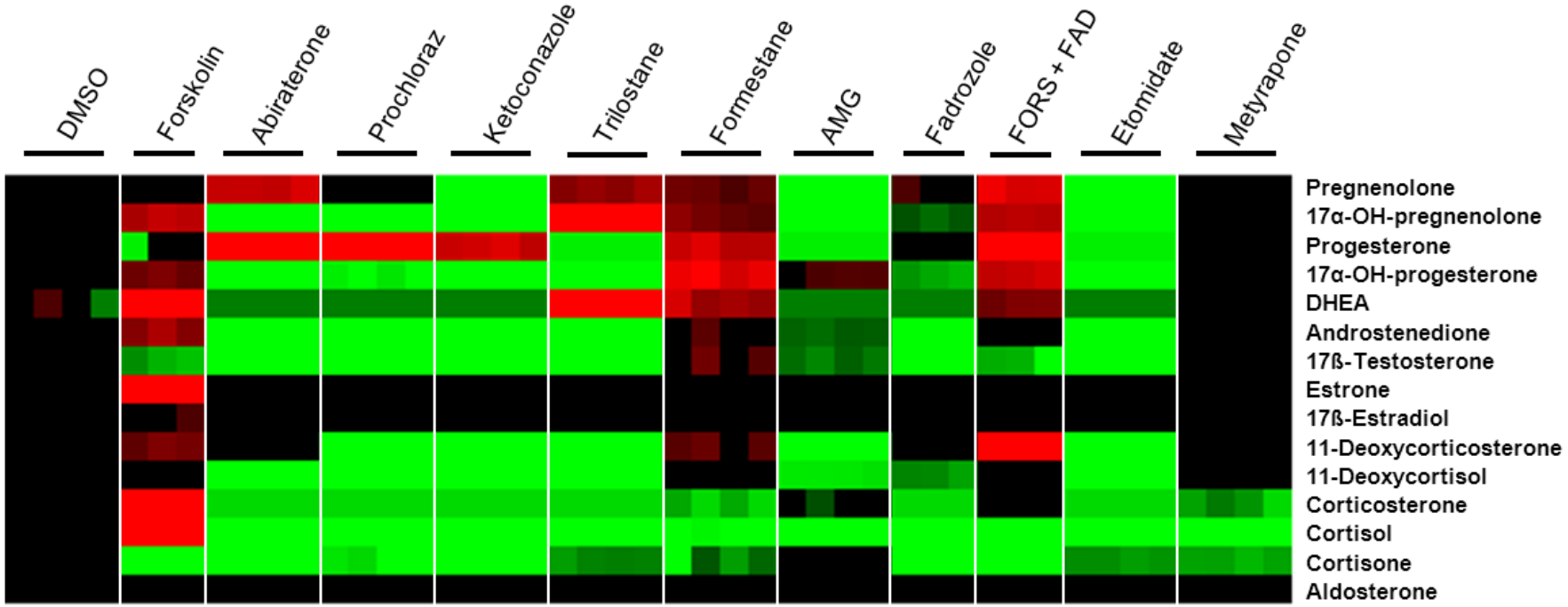
RIKILT WAGENINGEN UR

Rijk et al., *Chem. Res. Toxicol.* **25** (2012) 1710-1731

Effects in the H295R assay

– Hightroughput UPLC-MS/MS

- UPLC-ToF-MS based Metabolomics (targeted search)



Rijk et al., *Chem. Res. Toxicol.* **25** (2012) 1710-1731

The extended H295R steroidogenesis assay

- Monitoring changes in steroid profiles that also unravels the mechanisms of action (predictive value!)

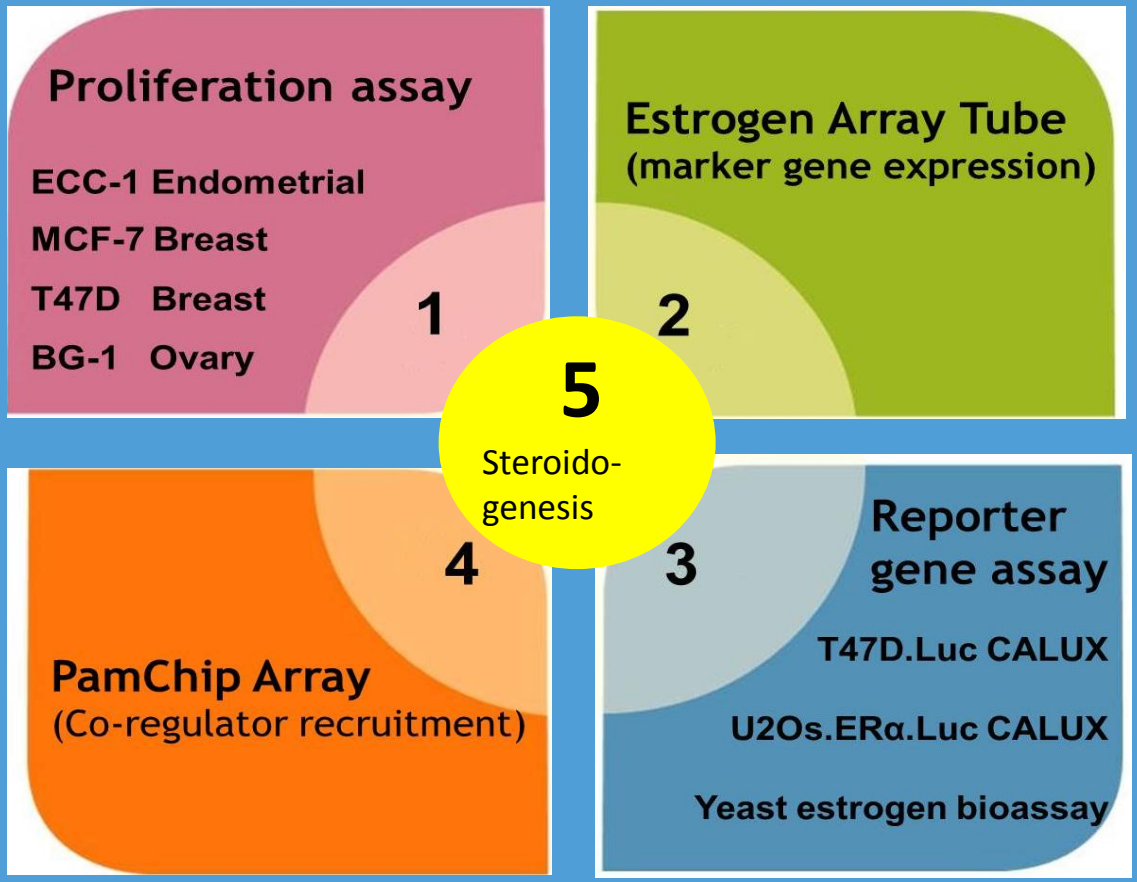
Bioassays for Endocrine Disrupting Chemicals (EDCs)

- The yeast estrogen bioassay (agonists, SERMs and antagonists)
- The yeast androgen bioassay (agonists, SARMs and antagonists)
- The yeast (gluco)corticoid assay (agonists, antagonists)
- The DR CALUX bioassay for dioxins and dl-PCBs
- The U2OS GR CALUX bioassay (for (gluco)corticosteroids)
- The U2OS PR CALUX bioassay (for progestagenes like progesterone)
- The extended H295R steroidogenesis assay
- PPARδ assay
- The receptor-bindingassay for β-agonists

- A LBD-ERalpha bindingassay in combination with MS (BioMS)
- Thyroid transport disruption: TBG and TTR binding assays
- T3 and T4 synthesis: TPO enzyme assay
- Hormone transport disruption: SHBG binding assay
- ELISAs and Luminex methods, e.g. for (gluco)corticosteroids

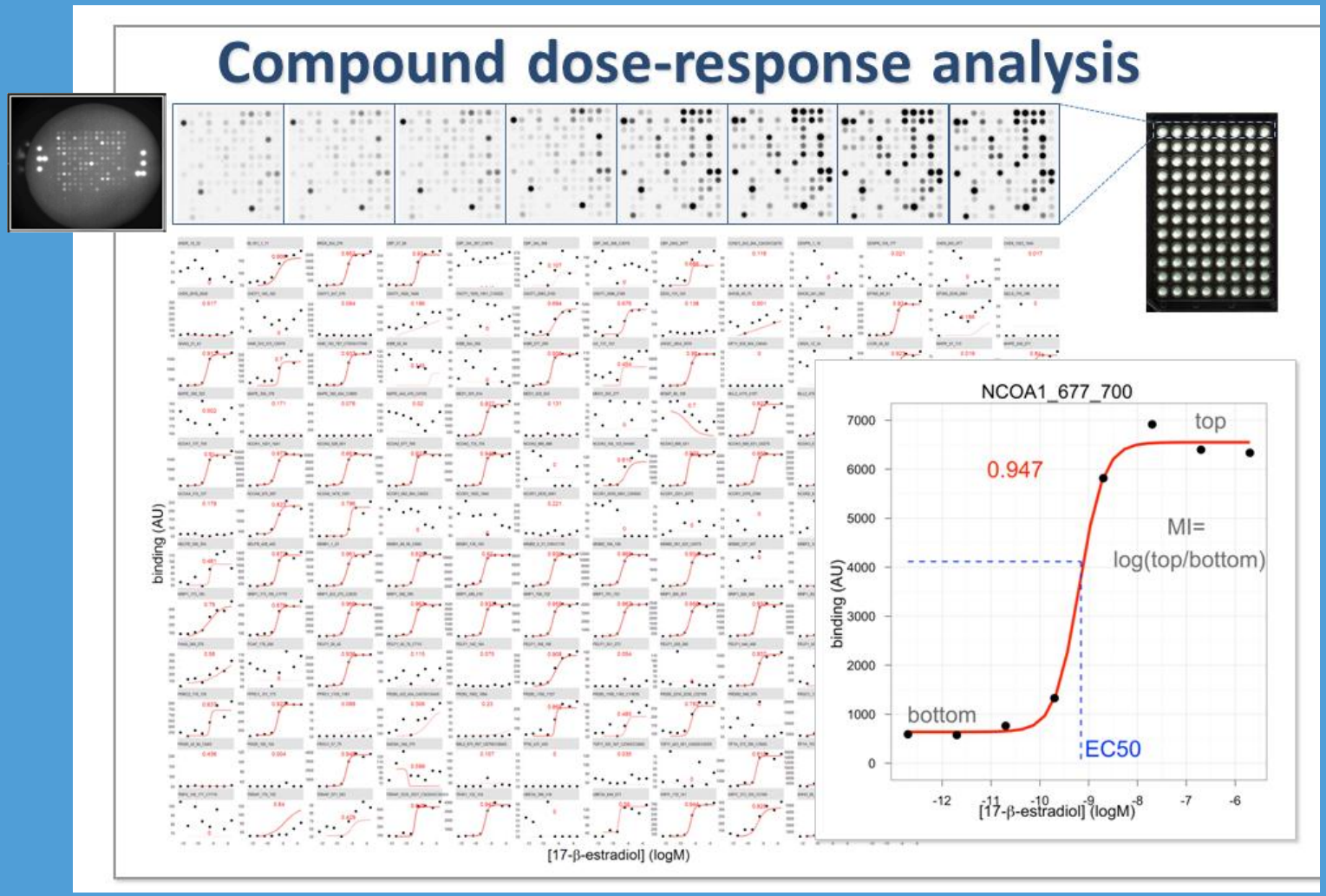
Combining different assays: an *in vitro* testing strategy (ITS) for estrogenicity

1 x
2 x
3 ERα CALUX & yeast assay
4 PamChip Array
5 Extended steroidogenesis



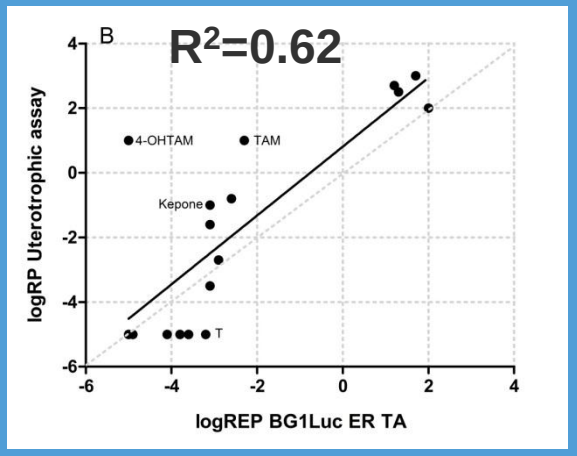
Si Wang, PhD thesis 2013 on alternatives for estrogenicity testing

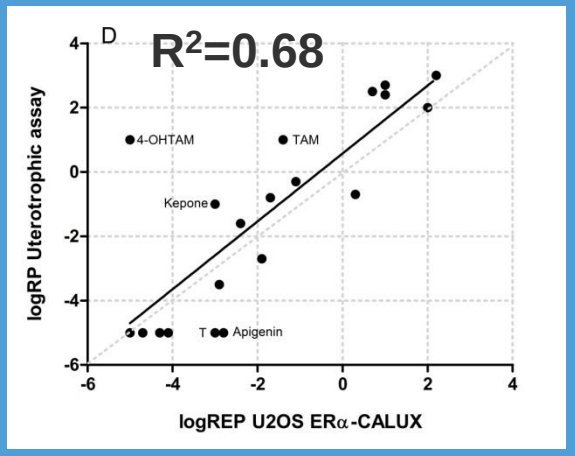
The PamChip® peptide array

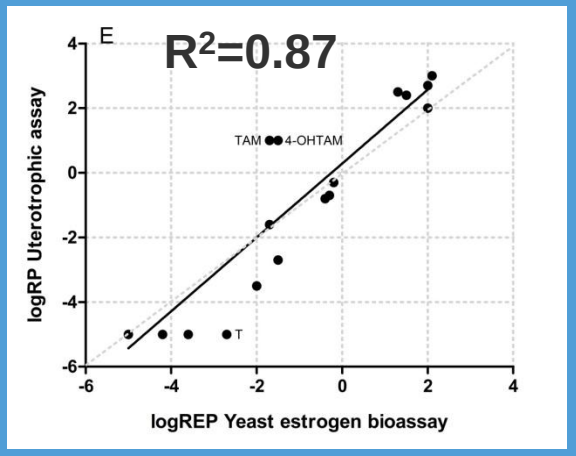


RIKILT
WAGENINGEN UR

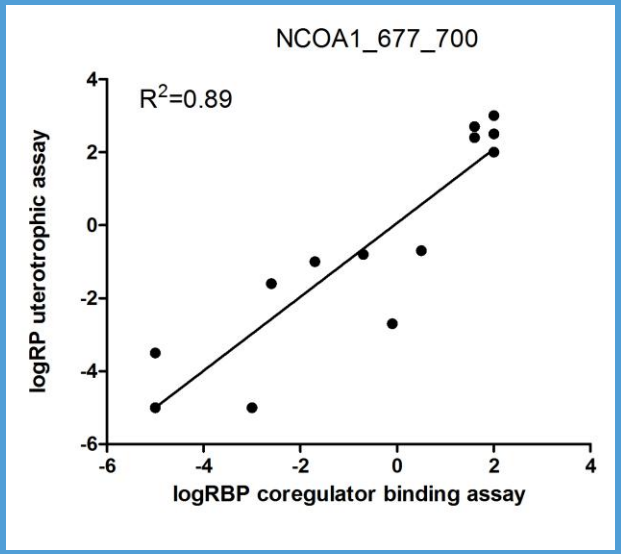
An ITS for estrogenicity







BG-1Luc ER TA
OECD TG457



RIKILT
WAGENINGEN UR

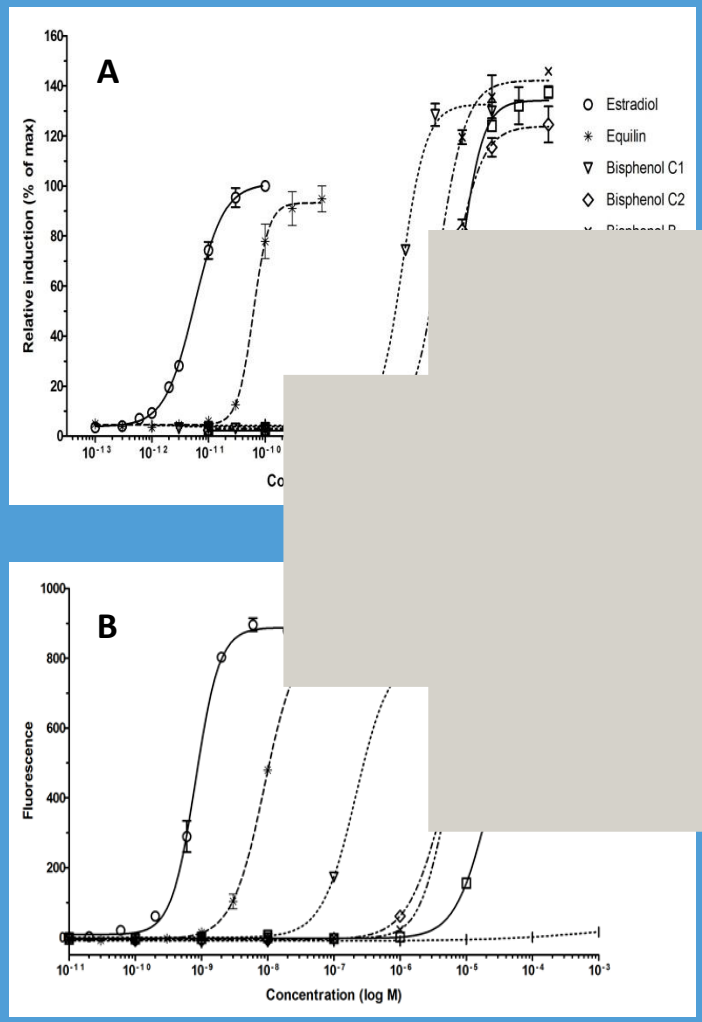
Wang et al., *ALTEX* **30** (2013) 145-157
Wang et al., *J. Appl. Toxicol.* (2013) in press

Combining different assays: an *in vitro* testing strategy (ITS)

- The example of Bisphenol A
  - ITS estrogenicity: BDS U2OS-ERα-CALUX®, RIKILT yeast estrogen bioassay, PamChip® peptide array, extended H295 steroidogenesis assy
  - Extended with: the BDS U2OS-AR-CALUX® and RIKILT yeast androgen bioassay

- This goes beyond the ITS for estrogenicity testing (replace the *in vivo* uterotrophic assay: OECD TG440)

An extended ITS for the Bisphenols



A: BDS U2OS ERα-CALUX®

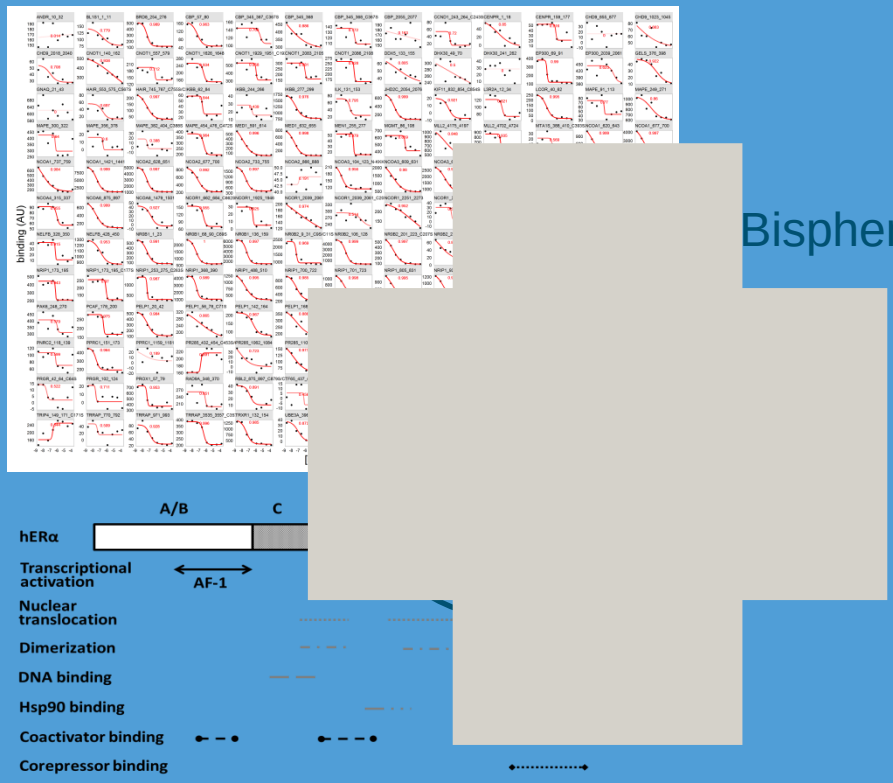
B: RIKILT Yeast Estrogen Bioassay

Wang et al., in preparation

RIKILT
WAGENINGEN UR

An extended ITS for the Bisphenols

Bisphenol B, C1 and C2 are AF2 antagonists

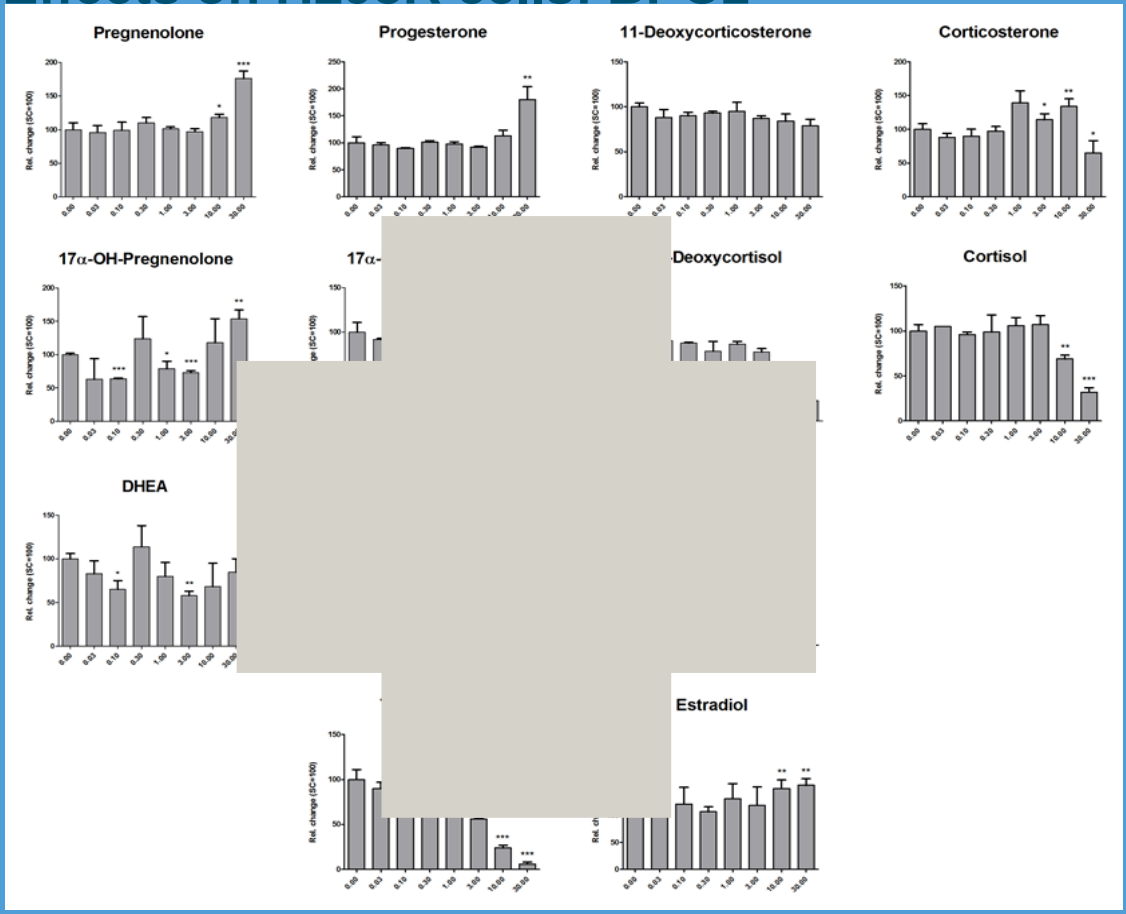


Wang et al., in preparation

RIKILT
WAGENINGEN UR

An extended ITS for the Bisphenols

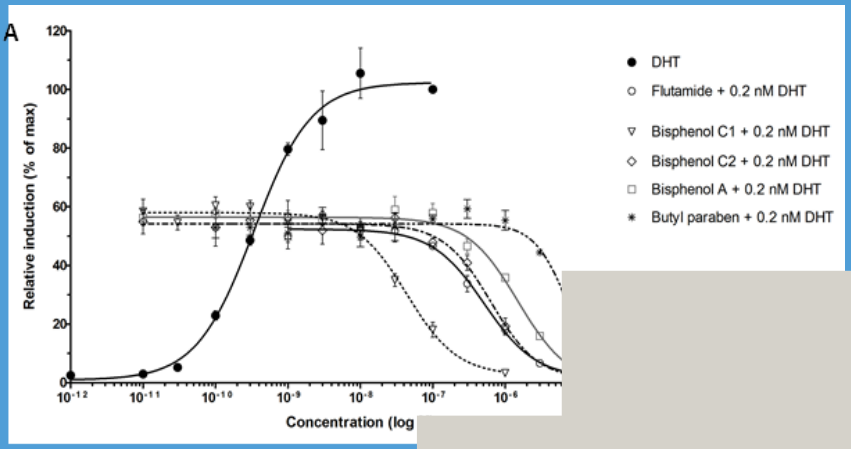
Effects on H295R cells: BPC1



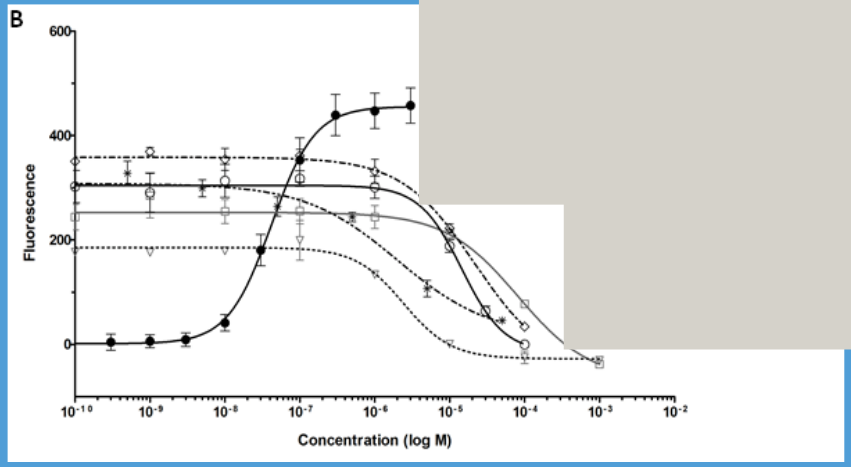
Wang et al., in preparation

RIKILT
WAGENINGEN UR

An extended ITS for the Bisphenols



A: BDS U2OS AR-CALUX®



B: RIKILT Yeast Androgen Bioassay

RIKILT
WAGENINGEN UR

Wang et al., in preparation

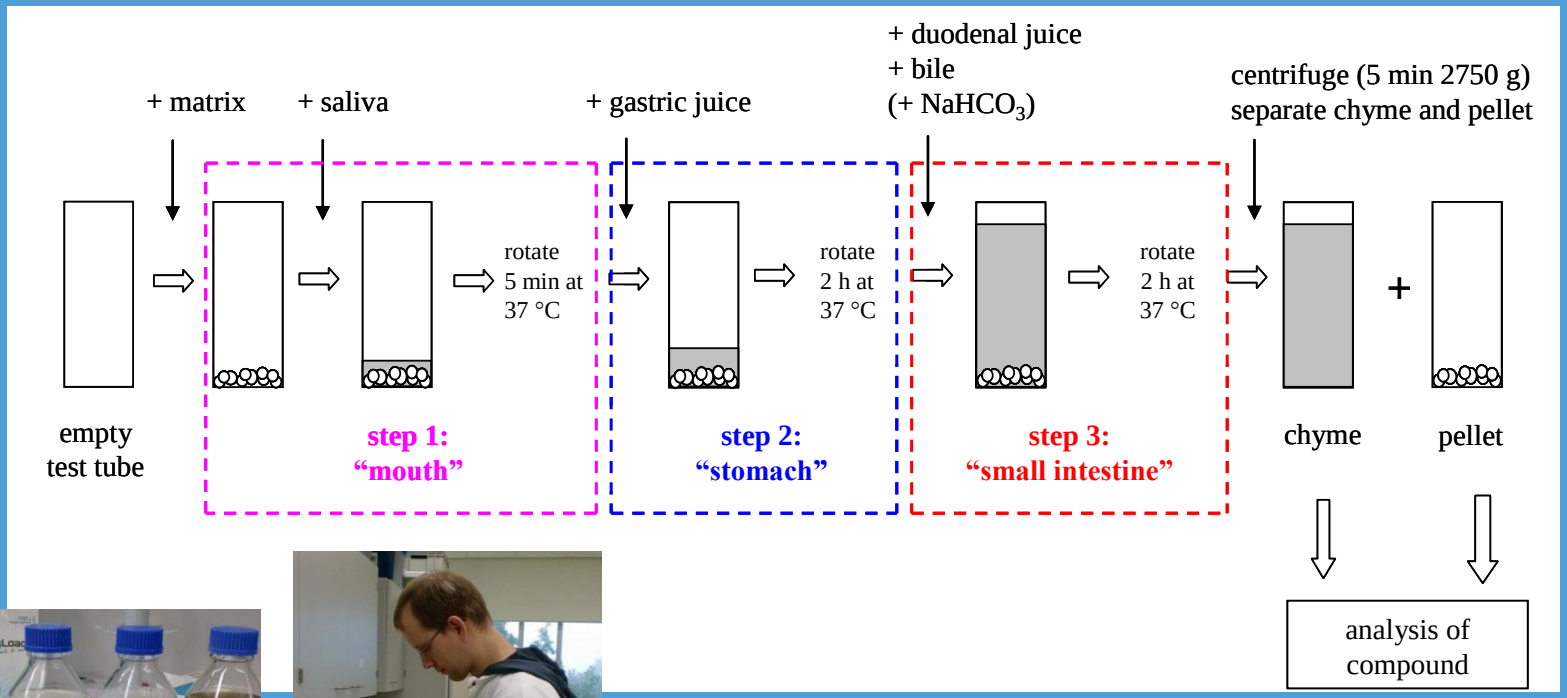
THIS PRESENTATION

- **A Bioassays**: effect based screening assays for the detection of hormones and EDCs, e.g. in veterinary control

- **B In vitro models for digestion, metabolism and bioavailability**

In vitro digestion

Static in vitro model of the human digestive tract



E.g. used at RIKILT to study stability of silver nano particles and conversion of marine toxin esters

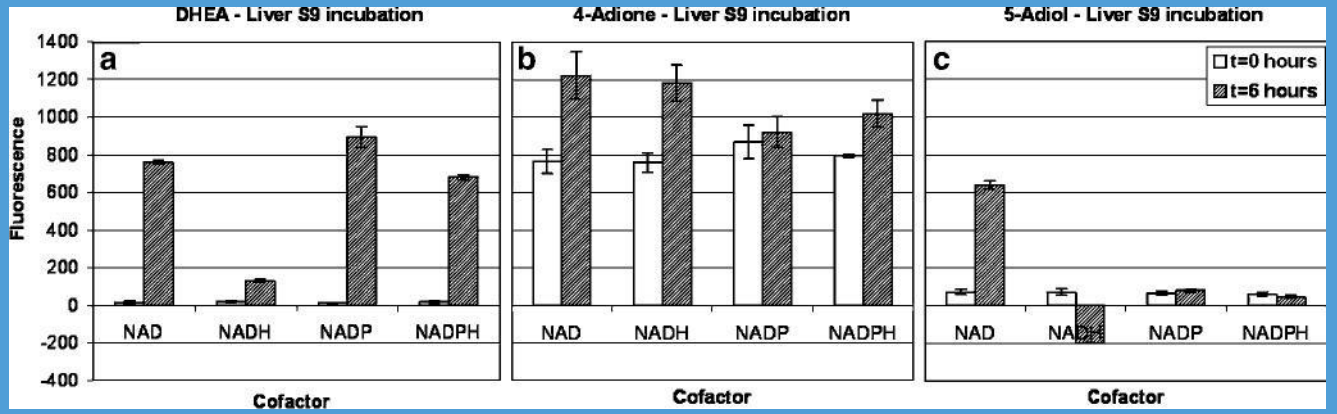
RIKILT
WAGENINGEN UR

In vitro models to study metabolism

1 Preparation of S9 liver fraction (phase I metabolism)

E.g. used at RIKILT to study the metabolism of steroid hormones, production standards of unavailable metabolites for MS

- Liver tissue was homogenized in twice their volume of Tris-HCl buffer (50 mM, pH 7.4, 1.15% KCl), using a blender.
- Homogenates were pooled and centrifuged for 25 min, 10000 g at 4°C.
- The supernatant (S9) was snap frozen in liquid nitrogen and stored at -80°C until use



3: Androgen bioassay responses of DHEA, 4-Adione and 5-Adiol, before (t=0) and after (t=6) incubation with bovine liver S9 in the presence of different cofactors. Fluorescence signals are the mean of an assay-triplicate (+/- SD) and corrected for the signal at t=0 and the reagent blank.

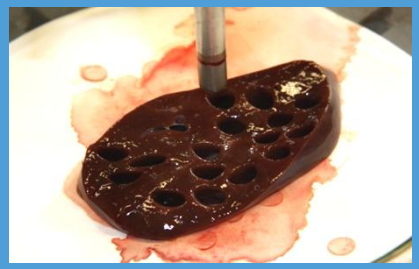
Rijk et al., *ABC* **392** (2008) 417-425

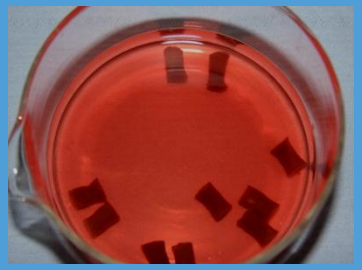
De Rijke et al., *FAC* **30** (2013) 1517-1526

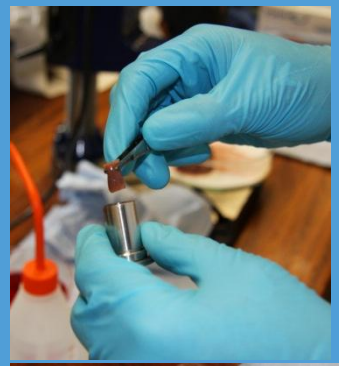
RIKILT WAGENINGEN UR

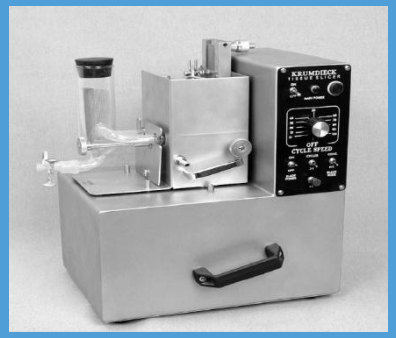
In vitro models to study metabolism

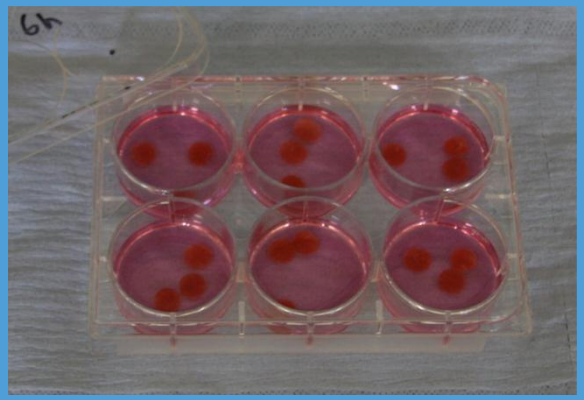
2 Preparation of precision liver cut slices







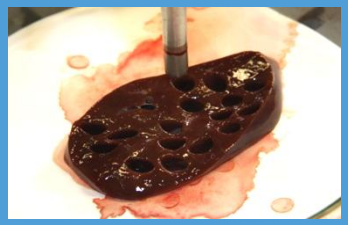


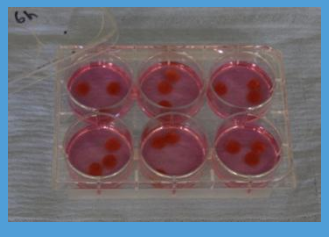


RIKILT
WAGENINGEN UR

In vitro models to study metabolism

2 Preparation of precision liver cut slices (phase I and II metabolism)





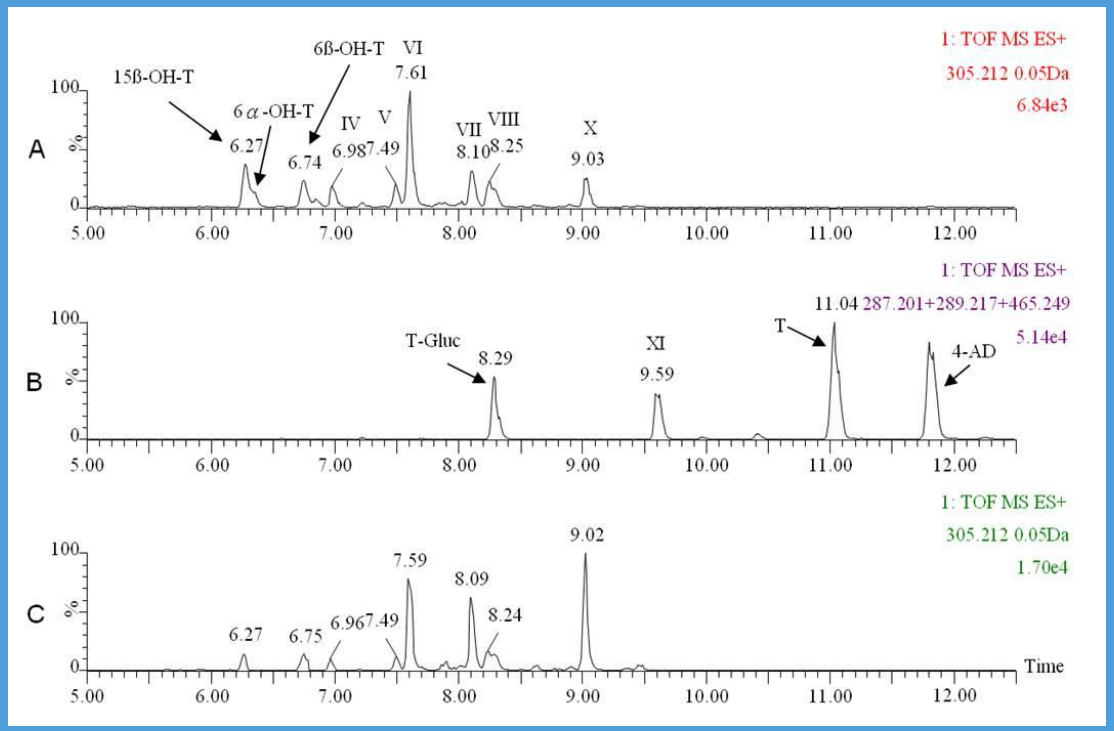


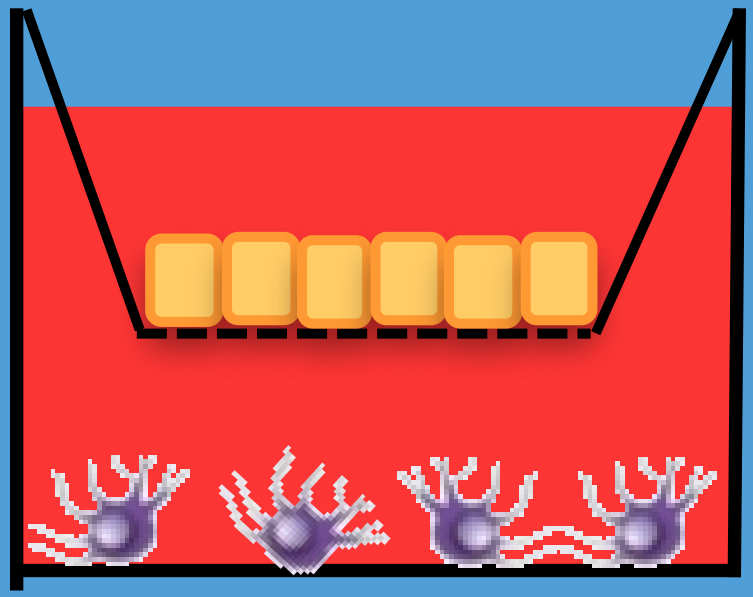
Figure 3. UPLC-TOFMS mass chromatograms. UPLC-TOFMS reconstructed accurate mass chromatograms for (A) *m/z* 305.2117 (B) *m/z* 287.2011 + *m/z* 289.2168 + *m/z* 465.2488 of bovine liver slice incubations with 100 µM T for 6 hours and for (C) *m/z* 305.2117 after co-exposure of liver slices with 100 µM T and 50 µg/ml cycloheximide.

RIKILT
WAGENINGEN UR

Rijk et al., *TiV* **26** (2012) 1014-1021

In vitro models to study metabolism and bioavailability

1 Trans well system with human intestine Caco-2 cells



E.g. used at RIKILT to study the metabolism and transport of flavonoids, marine toxin esters, silver nano particles, bioactives in onions etc.

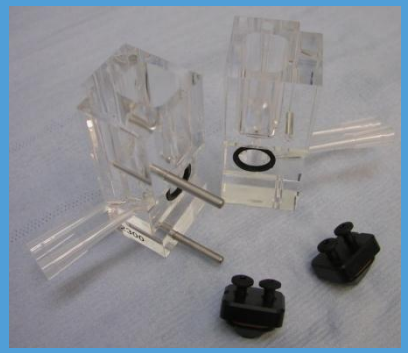
Bovee et al., in preparation

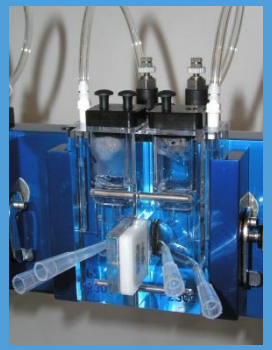
RIKILT
WAGENINGEN UR

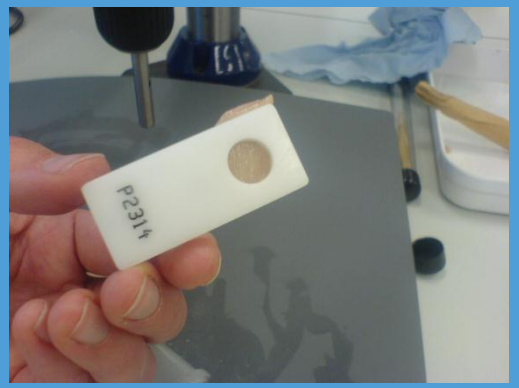
In vitro models to study metabolism and bioavailability

2 The Ussing chamber model

Set up and currently optimised at RIKILT







Questions?

RIKILT
WAGENINGEN UR