Bioactivity Screening:
The added value in veterinary control

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The added value in veterinary control

I) YEAST BASED BIOASSAYS FOR THE DETECTION OF STEROID HORMONES

II) VALIDATION OF THE GR-CALUX® FOR SCREENING (GLUCO)CORTICO-STEROÏD ACTIVITY IN CATTLE FEED

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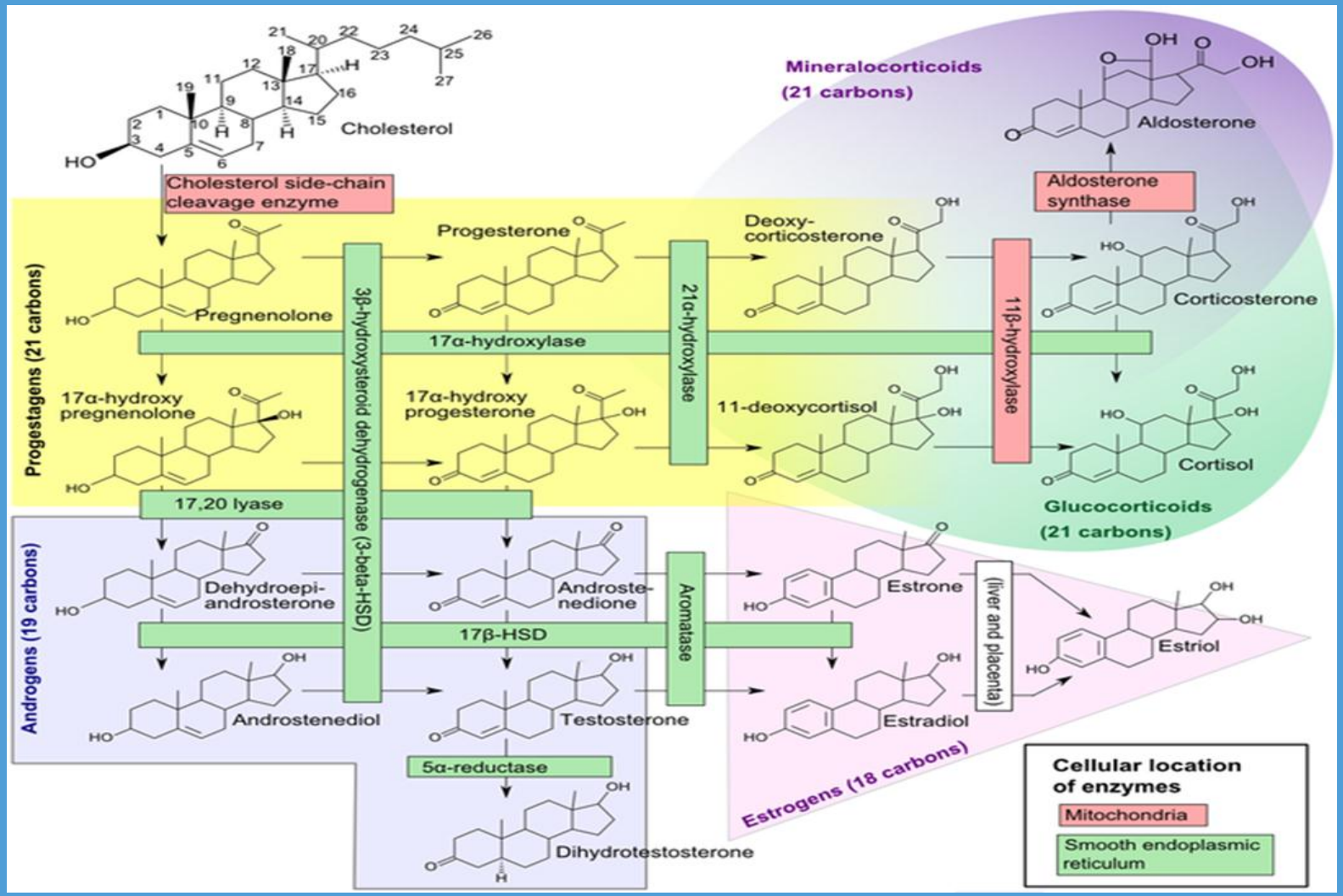
Steroid synthesis (steroidogenesis)



Steroids and other hormone active substances

- Natural steroids and their metabolites and conjugates, e.g. OH-metabolites and glucuronidated or sulphated conjugates
- Synthetic steroids, e.g. EE2, mestranol, hexestrol, boldenone, trenbolone, dexamethasone
- Hormoonesters, i.e. manmade esters from both natural and synthetic steroids
- Phytoestrogens (isoflavonoids)
- Chemicals, e.g. PCBs, pesticides, surfactants, plastics etc.
- Etc.

EU regulations I

- 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

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?
- ?

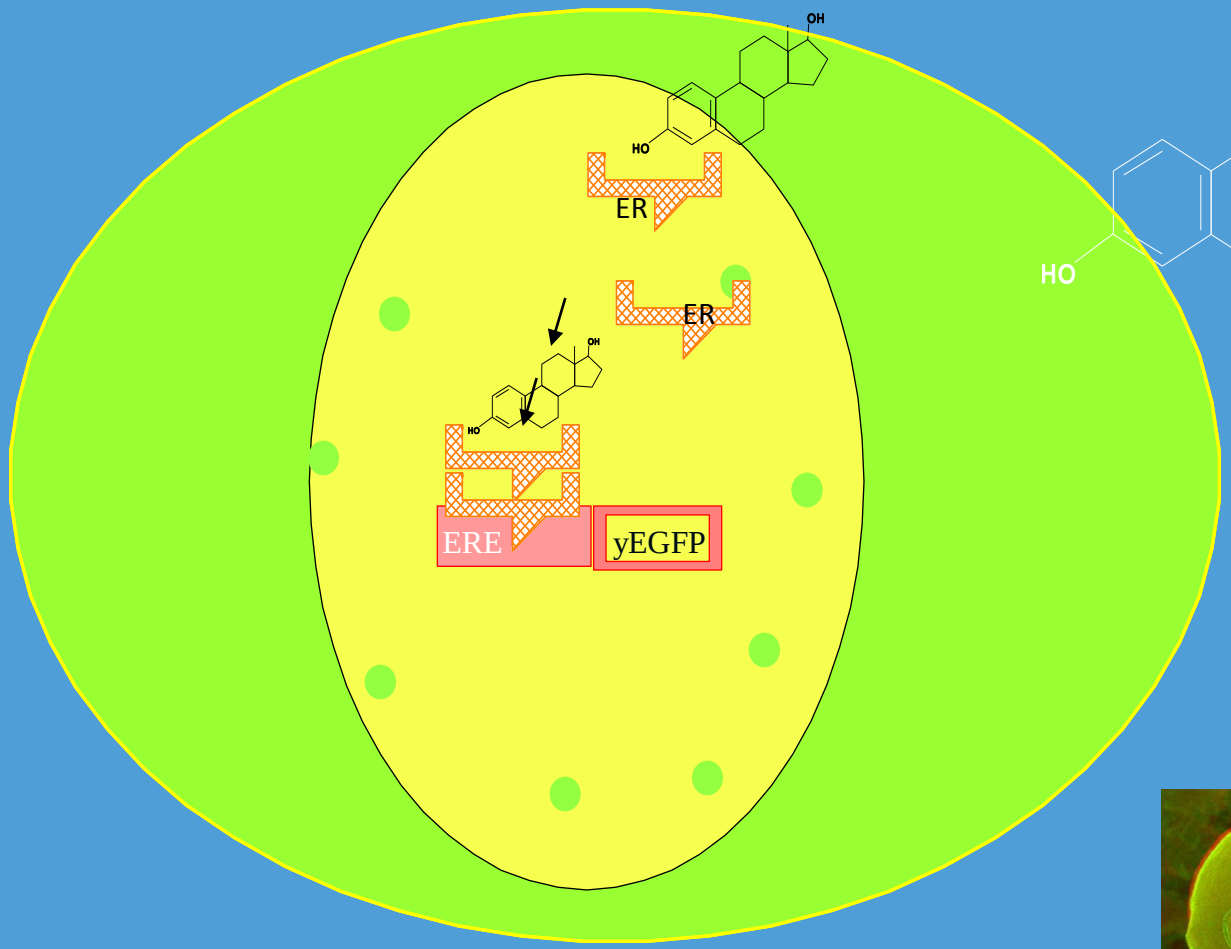
Bioactivity measurements

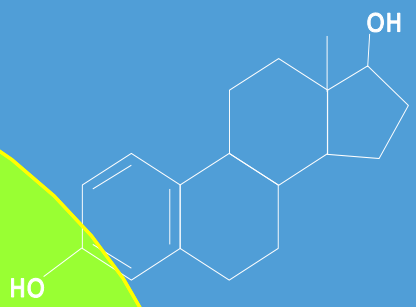
Transcriptional Activation (TA) bioassays (yeast or mammalian cell based)

- 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 receptor, they fulfil Directive 96/22/EC that prohibits all substances having hormonal action

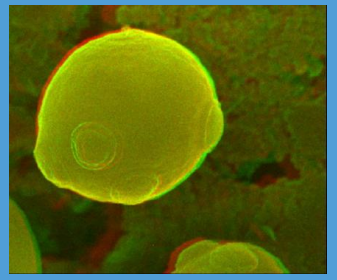
- Moreover, they are:
  - Sensitive and specific
  - Quick, simple and robust
  - Applicable to urine, feed and preparations

Development of a yeast estrogen bioassay

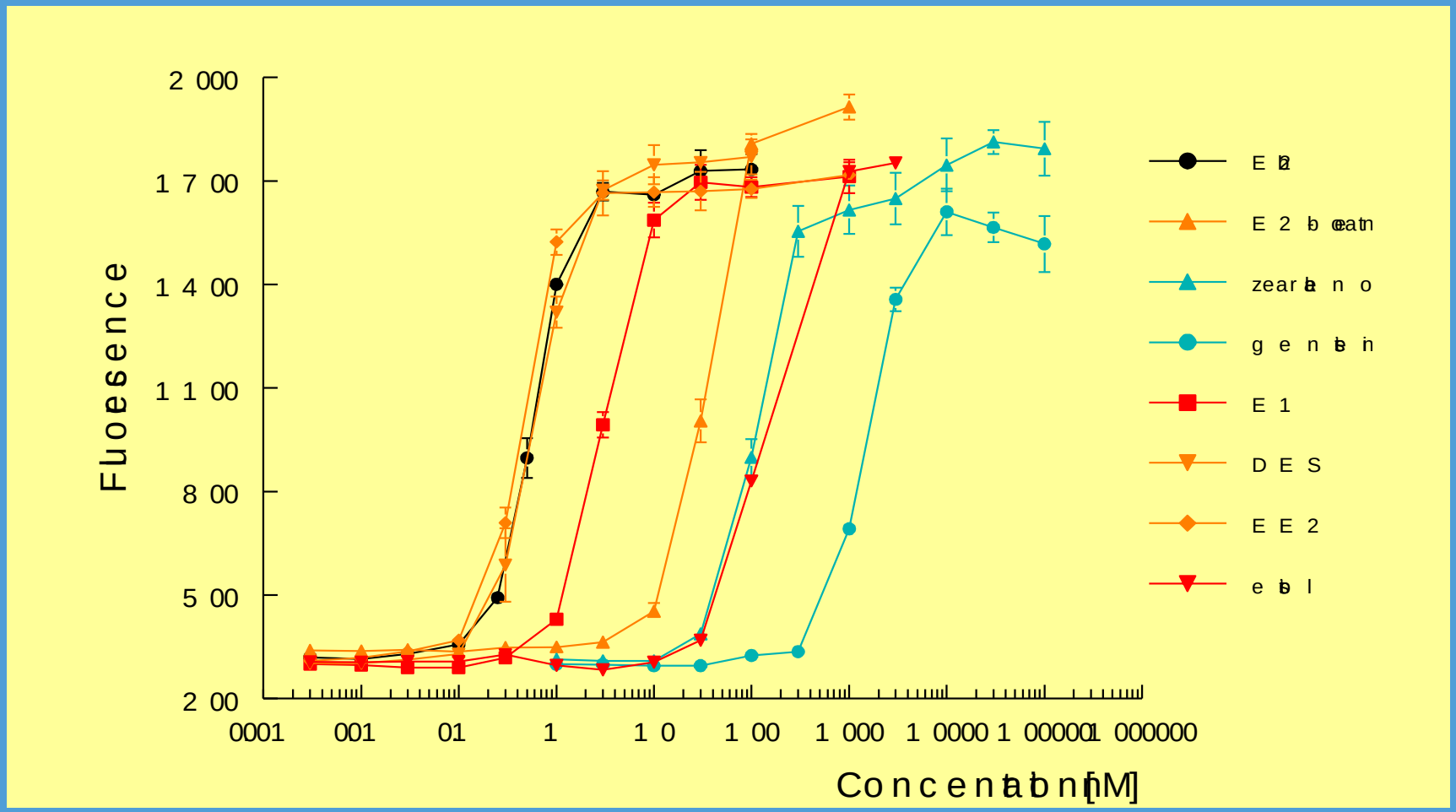




17β-estradiol



The yeast estrogen bioassay



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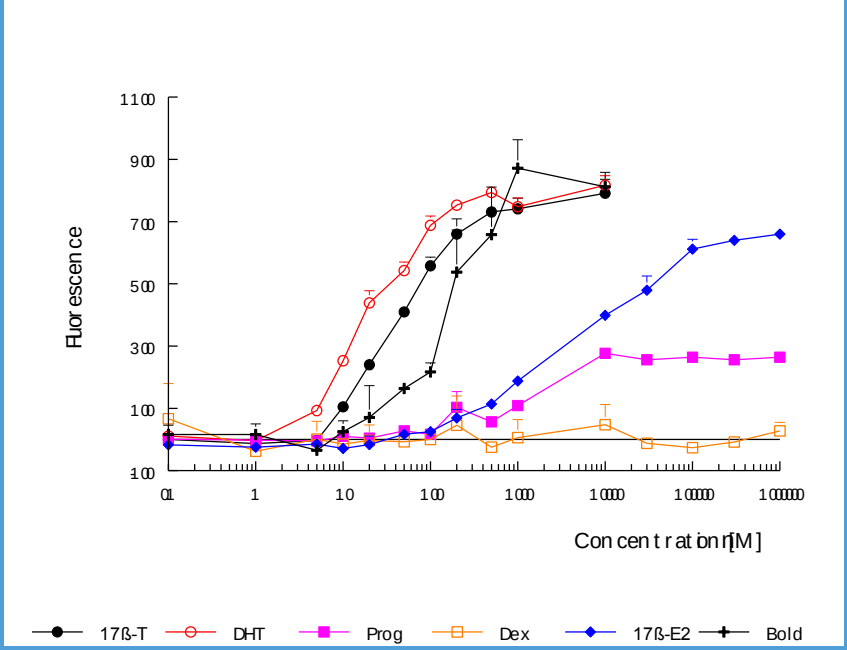
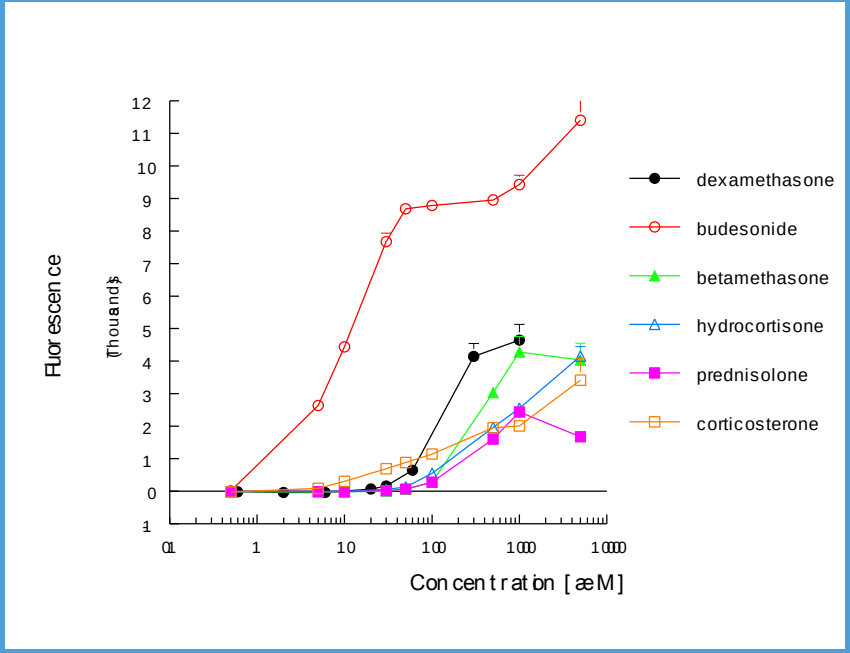
Bovee et al., *Gene* **325** (2004) 187-200
Bovee et al., *JSBMB* **91** (2004) 99-109

Similarly we developed a yeast androgen bioassay and yeast corticoid bioassay





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

I am not going to show you that:

- SERMs and SARMs show their specific responses in these yeast hormone bioassays too
- Both the yeast estrogen and androgen bioassay were fully validated for both the screening of feed and calf urine samples (according to Directive 2002/657/EC and accredited ISO 17025)
- The yeast estrogen bioassay performed well in an inter-laboratory ring test with calf urine samples
- Was shown a cheap alternative for real practise: estrogen bioassay screening calf urine samples vs GC-MS analysis

Bovee et al., *JSBMB* **118** (2010) 85-92
Bovee et al., *ACA* **529** (2005) 57-64
Bovee et al., *FAC* **23** (2006) 556-568
Bovee et al., *ACA* **637** (2009) 225-234
Bovee et al., *ACA* **637** (2009) 265-272
Nielen et al., *FAC* **23** (2006) 1123-1131

I am also not going to show you that:

- A 'natural' herbal supplement for prostate problems, causing gynaecomastia in a 67 year old man, was screened with the yeast estrogen bioassay and that it turned out that the supplement contained DES (Geldrop Hospital, The Netherlands)

- The yeast estrogen and androgen bioassays specifically indicated the anti-androgenic potential of the printing ink compound 2-isopropylthioxanthone (ITX), which was confirmed *in vivo* (rat) (food packaging)

- Was validated by Waternet/Waterproef Laboratorium in The Netherlands for screening estrogens in water samples

Tooriaans et al., *FAC* **27** (2010) 917-925
Peijnenburg et al., *TiV* **24** (2010) 1619-1628
Nguyen et al., *TiV* **25** (2011) 2003-2009

But I am going to show you:

- A study with dietary supplements screened in the yeast androgen bioassay: a comparison with a liquid chromatography tandem mass spectrometry (LC-MS/MS) method

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

Dietary supplements

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

2 supplements show androgenic activity in the yeast androgen bioassay

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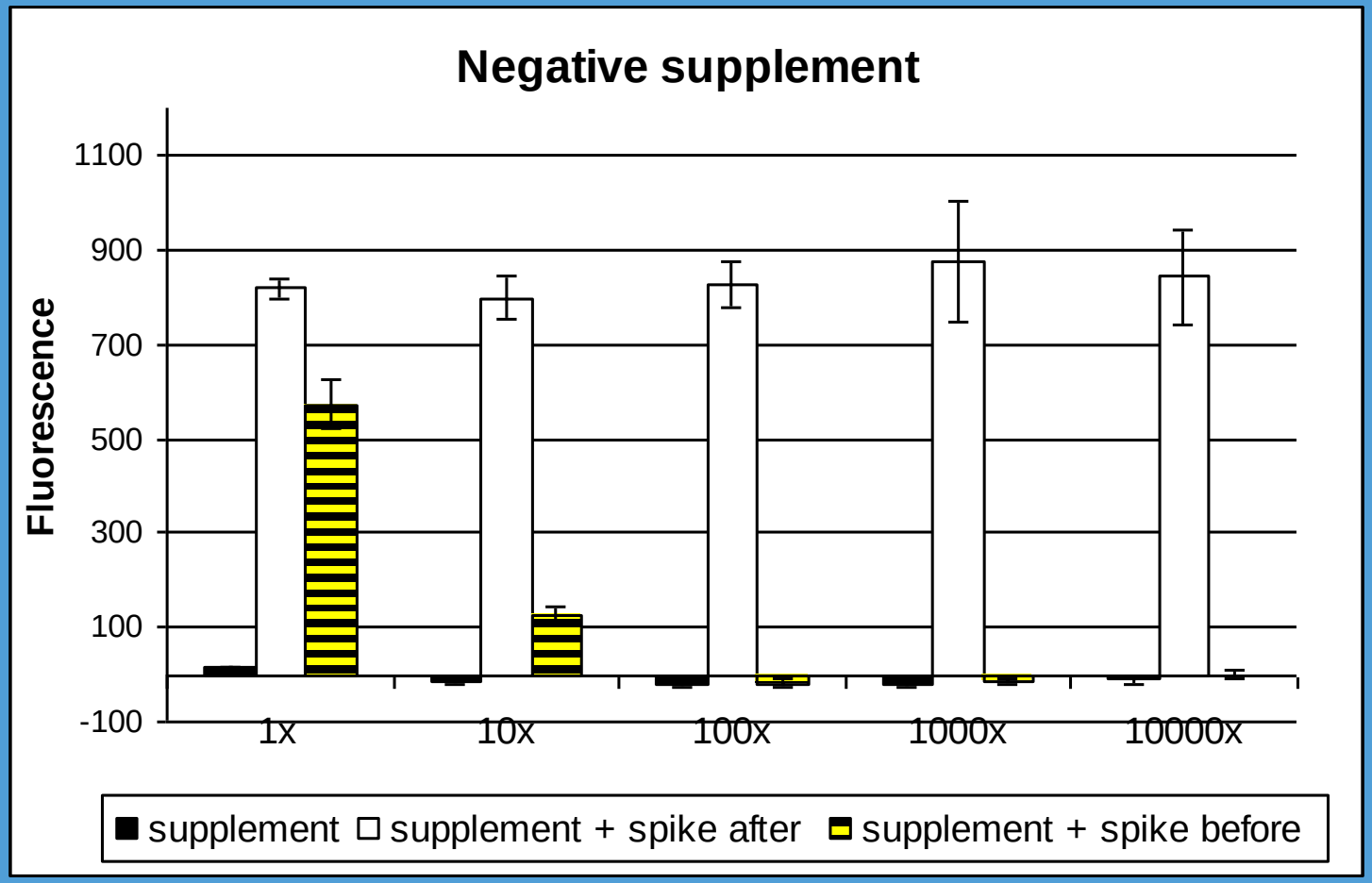
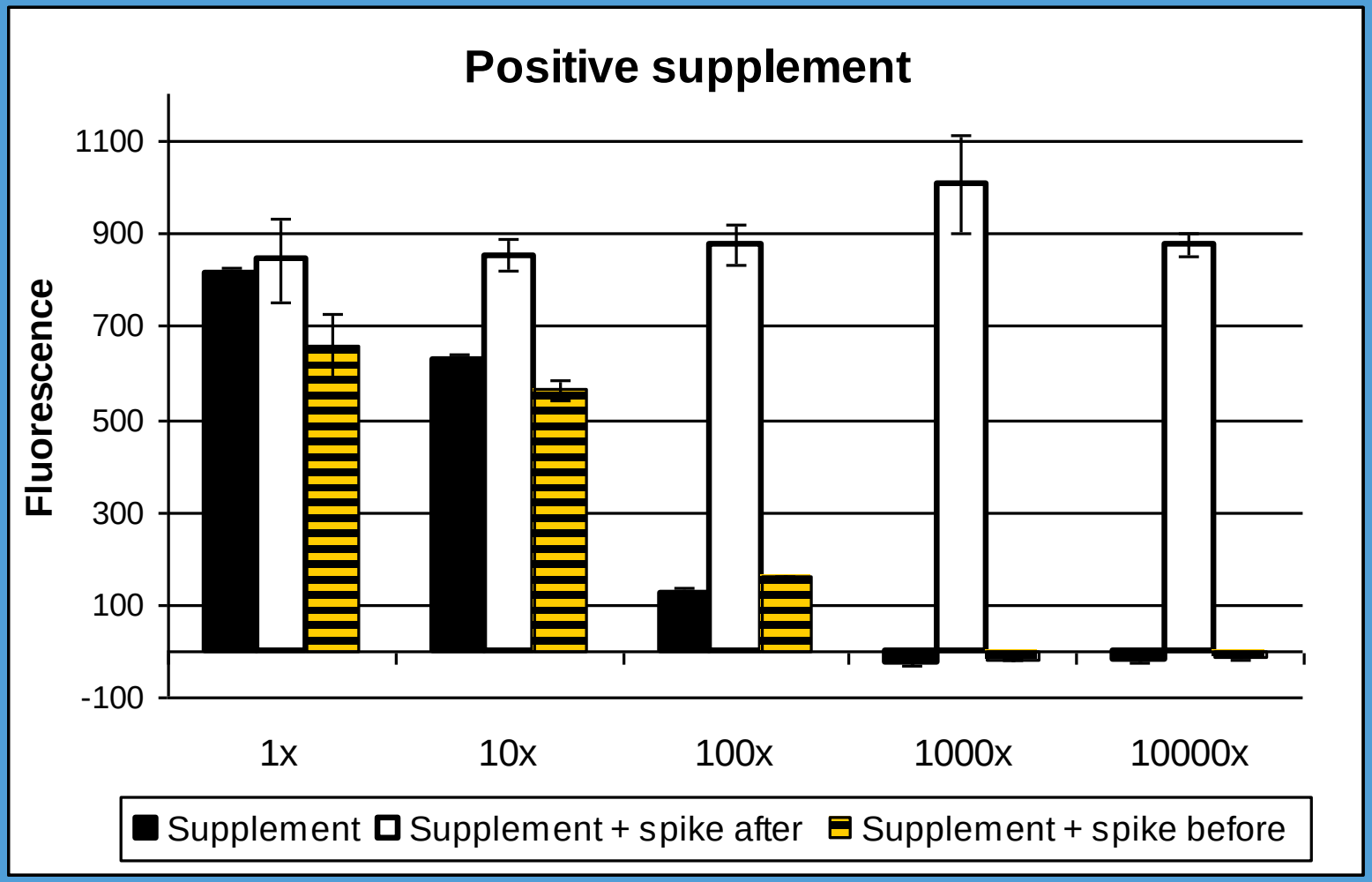
Van Poucke et al., *ACA* **586** (2007) 35-42

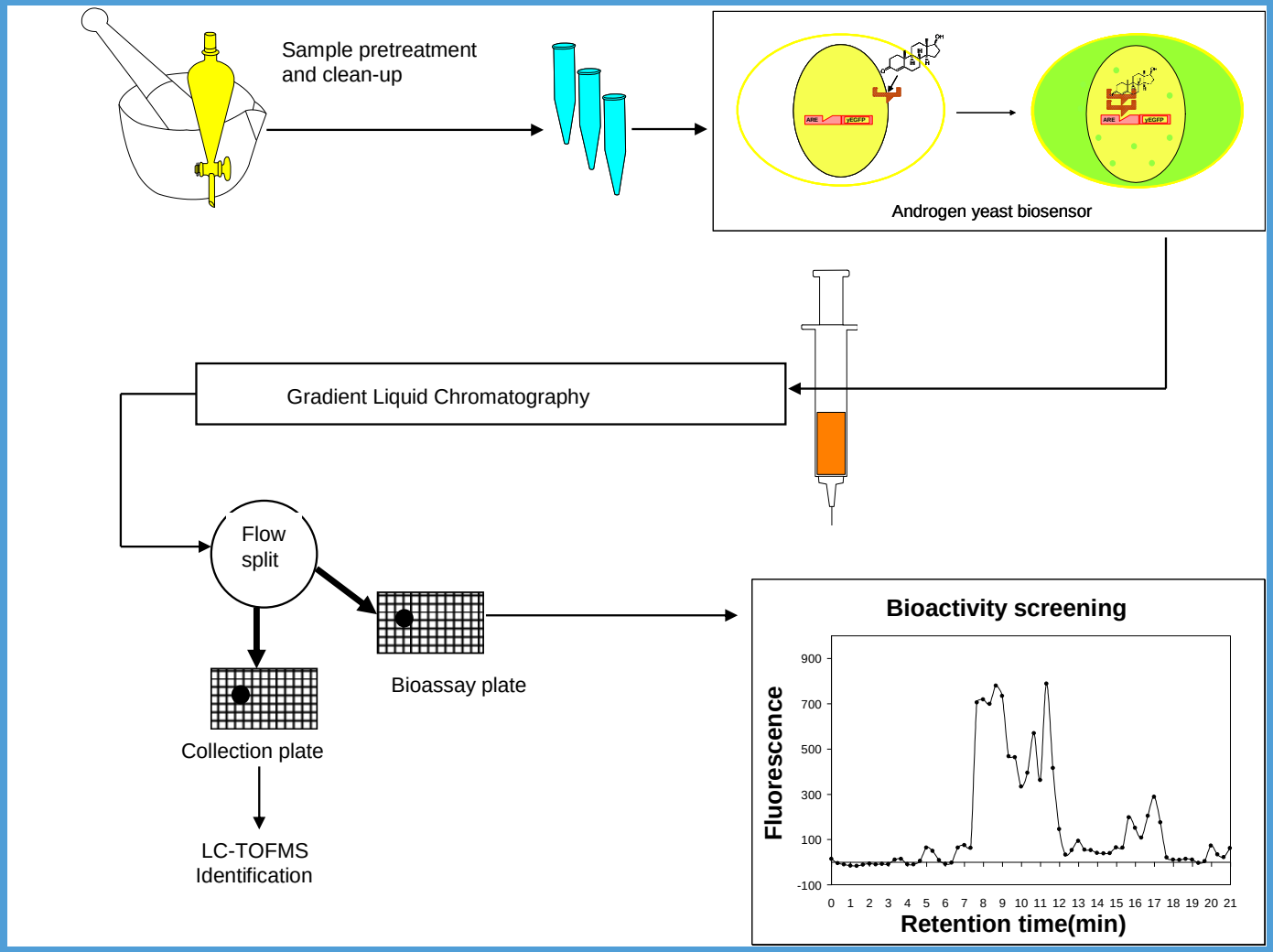
Results - Negative supplement



Results – Suspect supplement (negative LC-MS/MS)



Bioassay directed identification of unknowns



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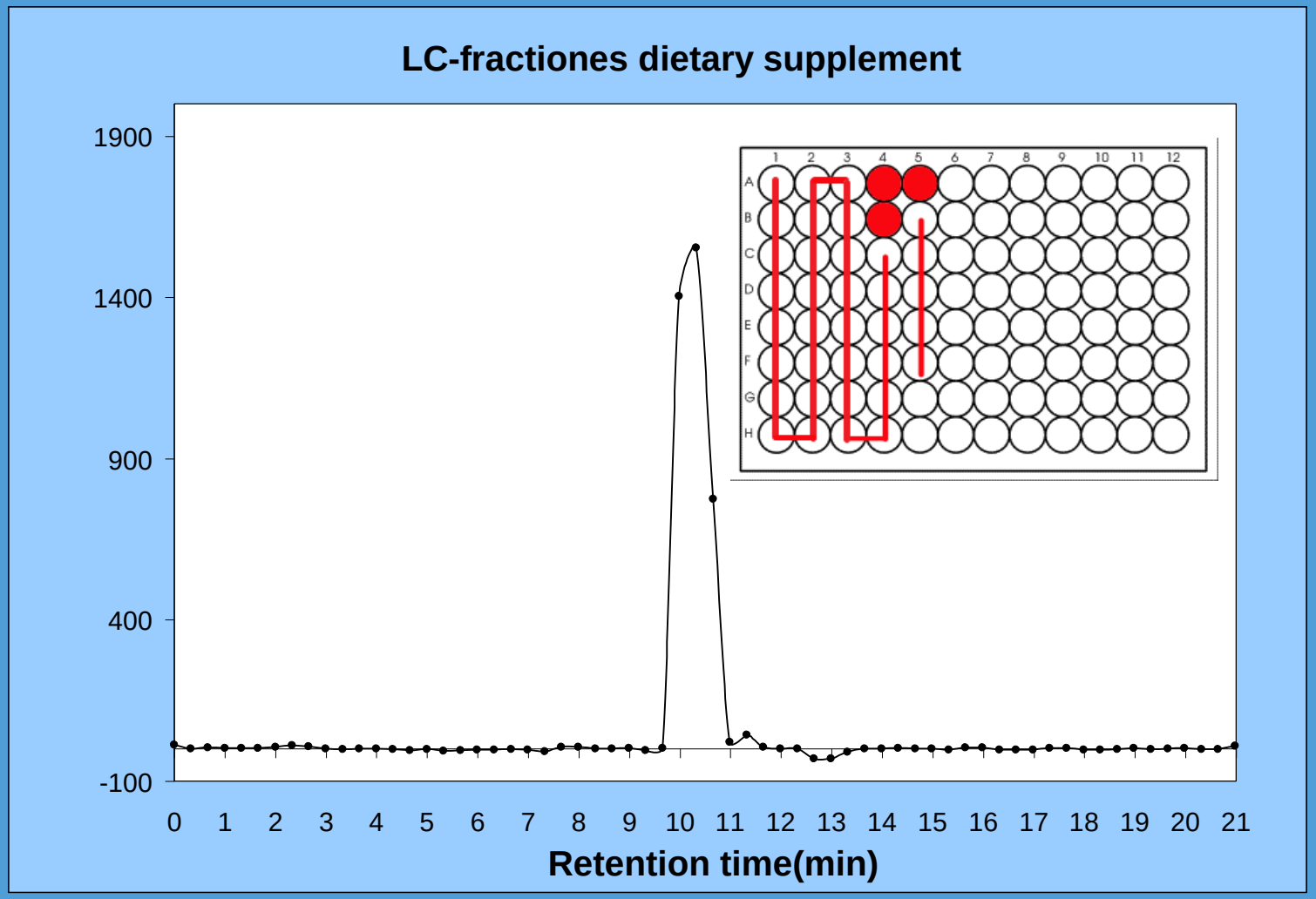
Results - Bioassay directed identification



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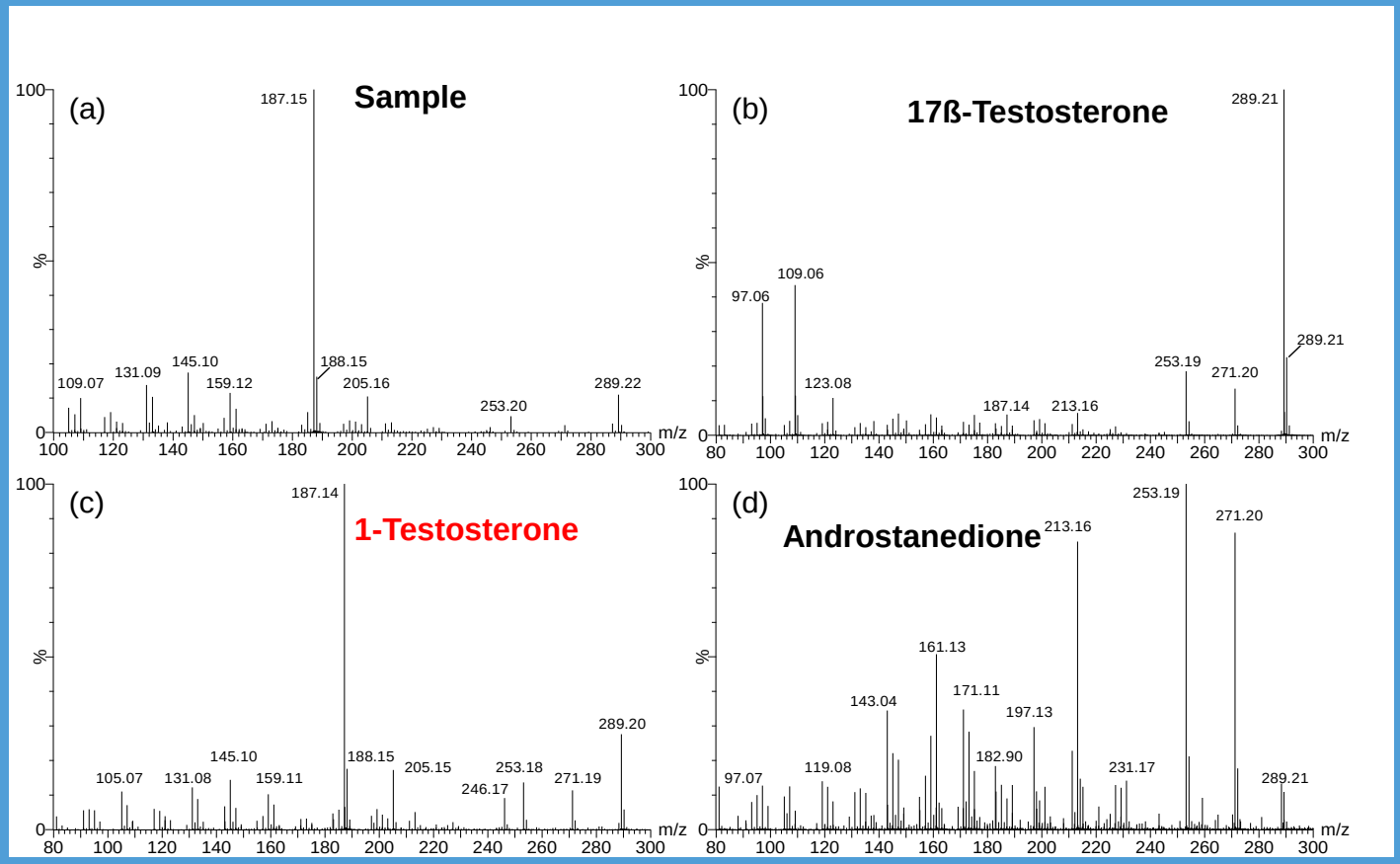
Suspect dietary supplement in yeast androgen bioassay & LC-MS/MS confirmation unknown androgen



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

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

Conclusions part 1

- Nicely validated bioassays for the detection of estrogens and androgens in calf urine and animal feed that can easily be introduced at your laboratory.

- These assays have an added value compared to analytical screening alone.

- **⇒ However, bioassays can not operate on their own. Suspected samples need confirmation. There is no vs.**
- **⇒ Bioassays and analytical methods are thus complementary !**

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- 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)

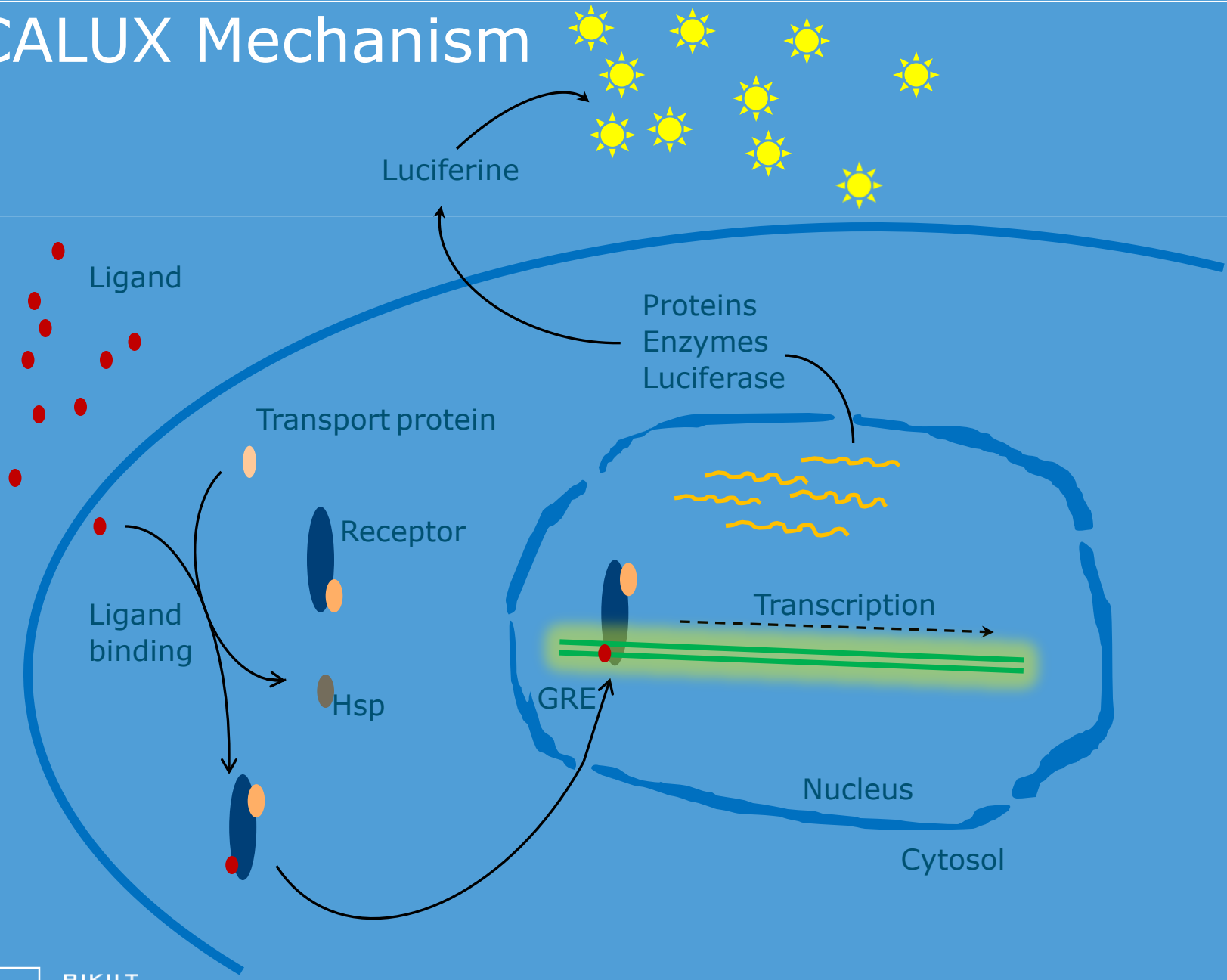
- Some initial experiments showed promising results for GR-CALUX® bioassay (BDS; U2OS cell line)

Principle of the test

- U2OS is a human osteosarcoma cell line expressing wild-type p53 (53 tumor protein is an anti-oncoprotein that is encoded by gene TP53) and Rb (retinoblastoma protein is an anti-onco protein that inhibits excessive cell growth), but no longer possesses p16. Cyclin-dependent kinase inhibitor 2A (CDKN2A or p16) is an anti-onco protein that plays an important role in regulating the cell cycle.

- The principle is based on the production of luciferase enzyme after binding of a receptor / agonist complex at the GRE site on the DNA in the U2OS cells. The cells are exposed to (gluco)corticosteroids. The luciferase activity is a measure for the amount of agonist and is determined by bioluminescence.

CALUX Mechanism



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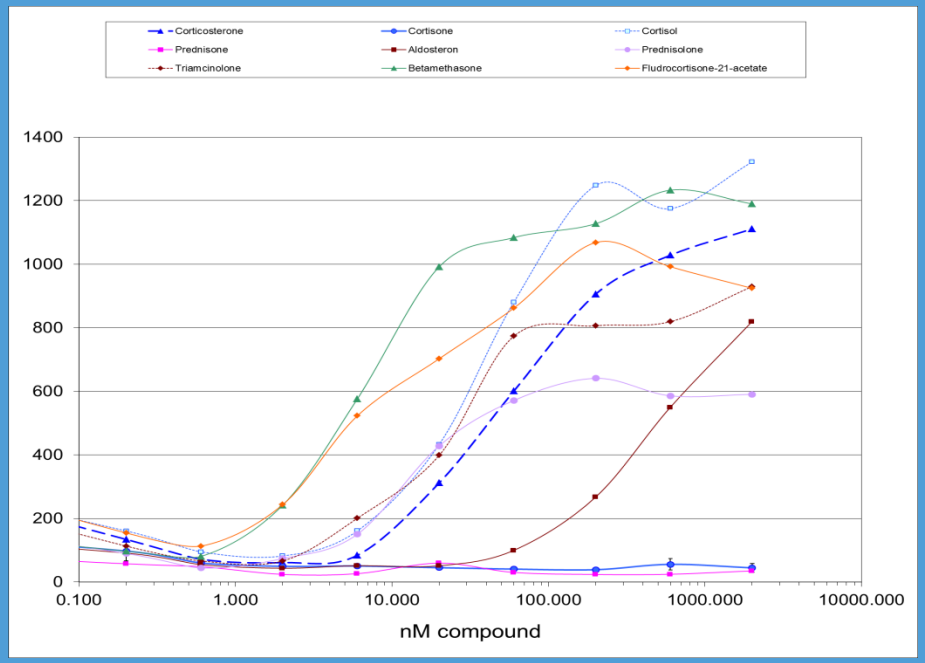
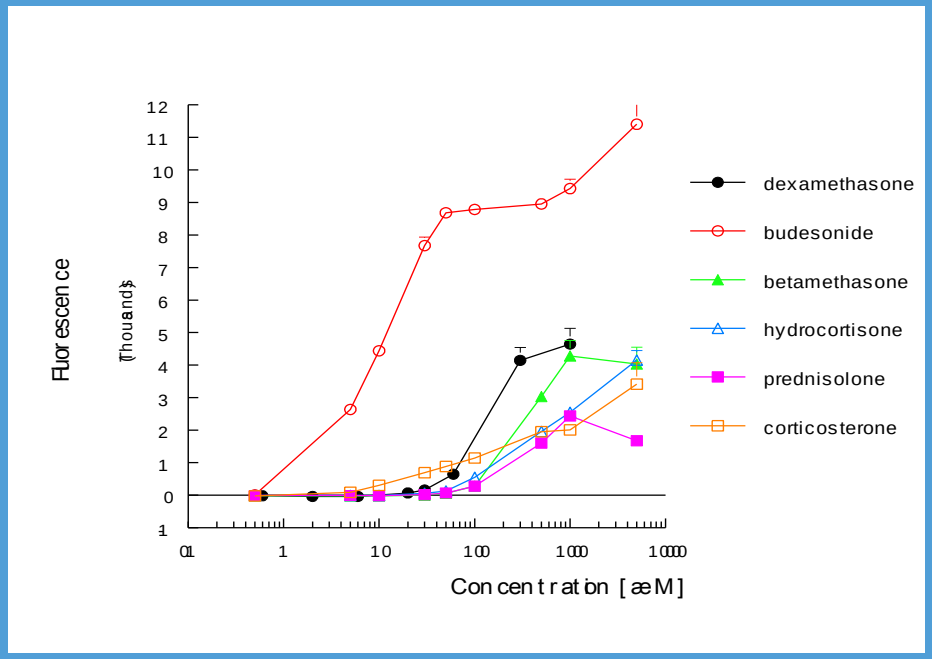
Sensitivity GR-CALUX® vs yeast glucocorticosteroid bioassay





Sensitivity nM vs µM; thus very promising

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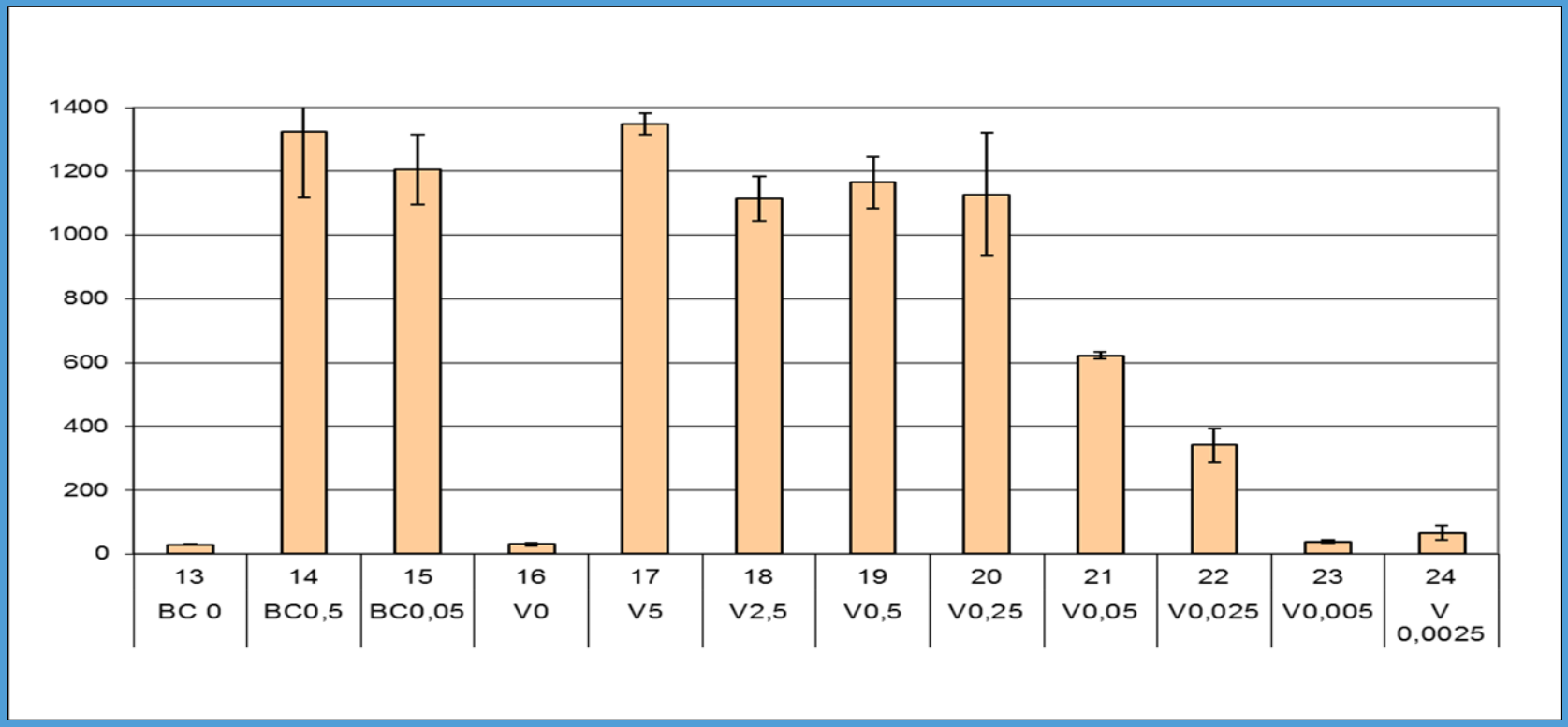
Results for GR-CALUX®
Dexamethasone spiked cattle feed



Sensitivity < 0.1 µg DEX/g; thus promising

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Decided to go for a validation of the GR-CALUX bioassay for the screening of feed

- Validation is always a balance act between costs, risks and technical possibilities
- Validation : the determination of the performance characteristics of a method of analysis
- Validation Dossier
    - Validation Plan
        - **I Classification of the method**
        - **II Selecting the parameters to determine**
        - **III Criteria and determination of parameters**
        - **IV Procedure for determination of parameters and fulfil criteria**
    - SOP
    - For each parameter
        - Results
        - Discussion
        - Conclusions
    - General Conclusions
    - Raw Data

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I Classification - Documents of interest

- ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories
- 96/23/EC Council Directive of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EC, 86/469/EC, 89/187/EC and 91/664/EC
- 2002/657/EC Commission Decision to implement the Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results
- RIKILT KHB (RIKILT Quality Manual)
- SOP's A0906, A907, A0400 and F0052 (validation)
- NEN 7777 (environmental)
- ENGL (European Network of GMO Laboratories)
- SANCO/825 – SANCO/10684 (pesticides)

I Classification of the method

- Conform or equivalent to e.g. NEN
- Specific national / international regulations: **Group A**/B substances
- **Target matrix** / compound
- GMO / Pesticides / Metals and Arsenic / Radioactive compounds
- Quantitative or **Qualitative**
- **Screening**, Confirmation or Combined

II Selecting parameters for validation

Parameter	Qualitative method		Quantitative method	
	Screening	Confirmation	Screening	Confirmation
Accuracy / Recovery	-	-	-	+
Repeatability	-	-	+	+
Interlab Reproducibility	-	-	+	+
CCα (decision threshold)	-	+	-	+
CCß (detection capability)	+	+	+	+
Specificity	+	+	+	+
Robustness	+	+	+	+
Stability	+	+	+	+
Calibration curves	-	-	+	+

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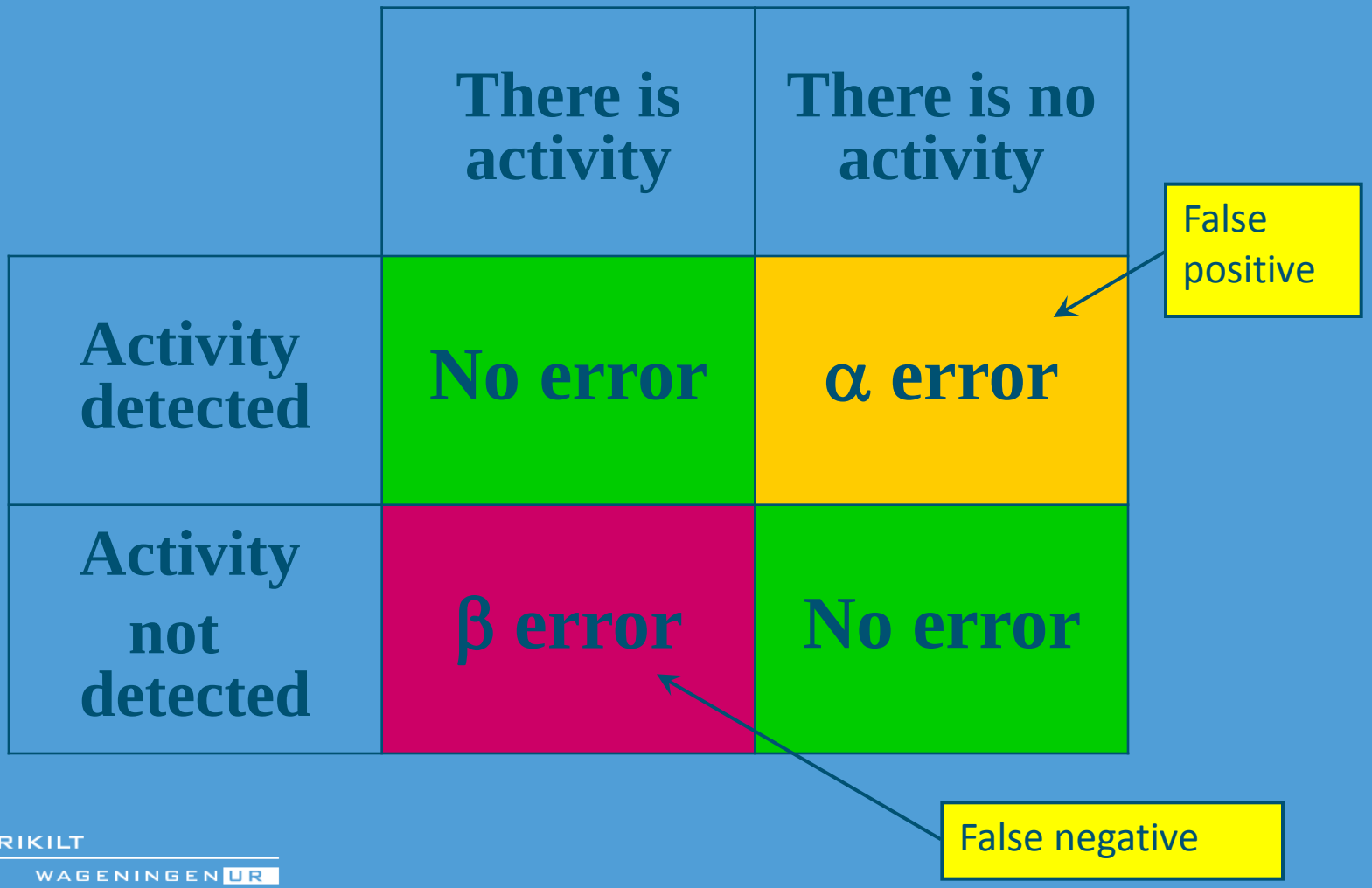
II Selecting parameters: The α and β errors



III Determination of CCα and CCβ

- RIKILT approach for qualitative screening methods:
  - determine CCα, CCβ using min. 20 matrix blanks

$$CC\alpha = \overline{X}_b + 3 * Sb$$

$$CC\beta = CC\alpha + 1.64 * Ssp$$

  - estimate relevant concentration in matrix, based on EC50 in the GR-CALUX® (response curves)
  - spike at least 20 blanks at estimated concentration
  - check if $\overline{X}\ sp > CC\beta = CC\alpha + 1.64 * Ssp$

IV Procedure: 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

IV 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

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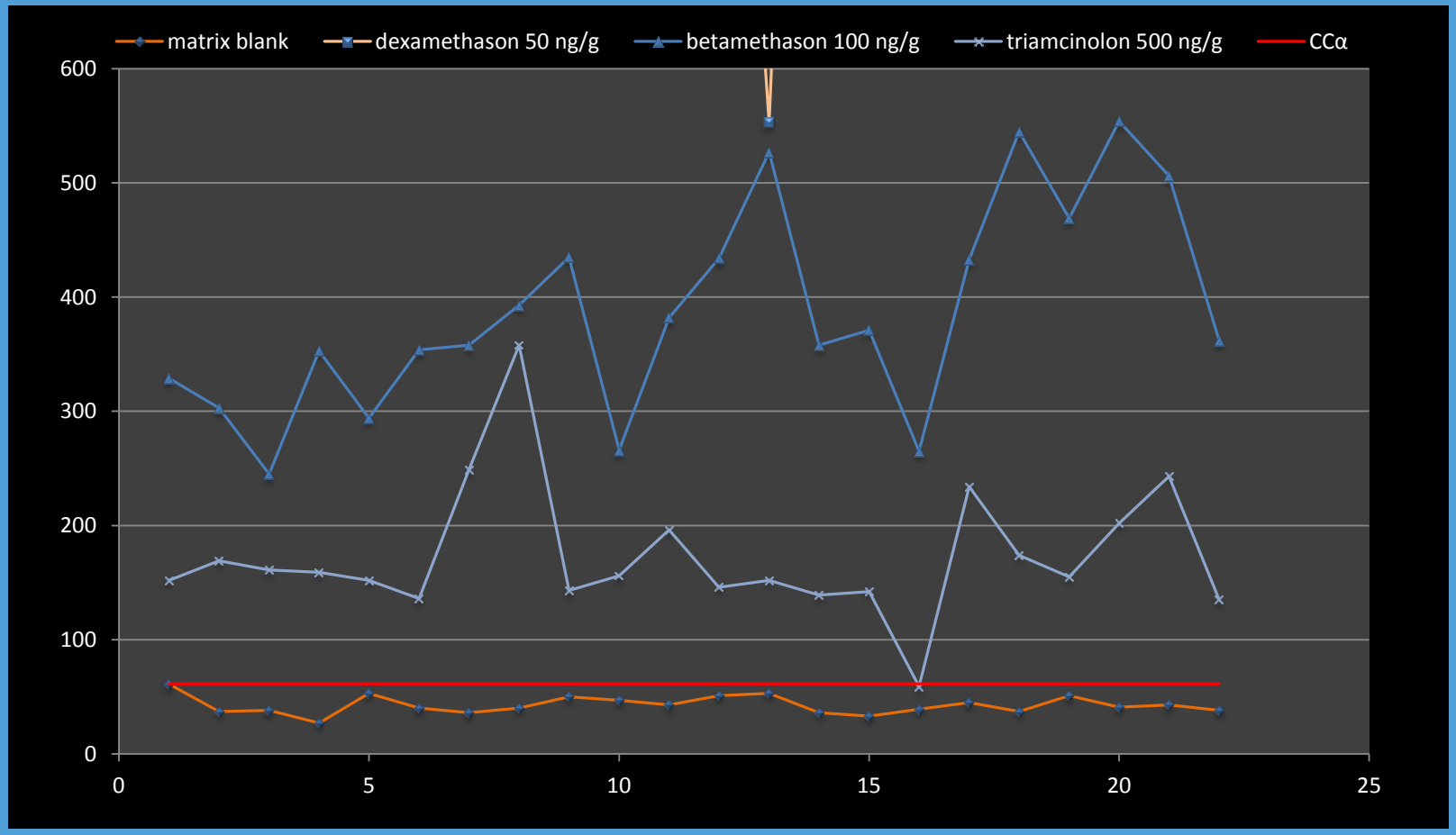
Results: CCα and CCβ criterion checks



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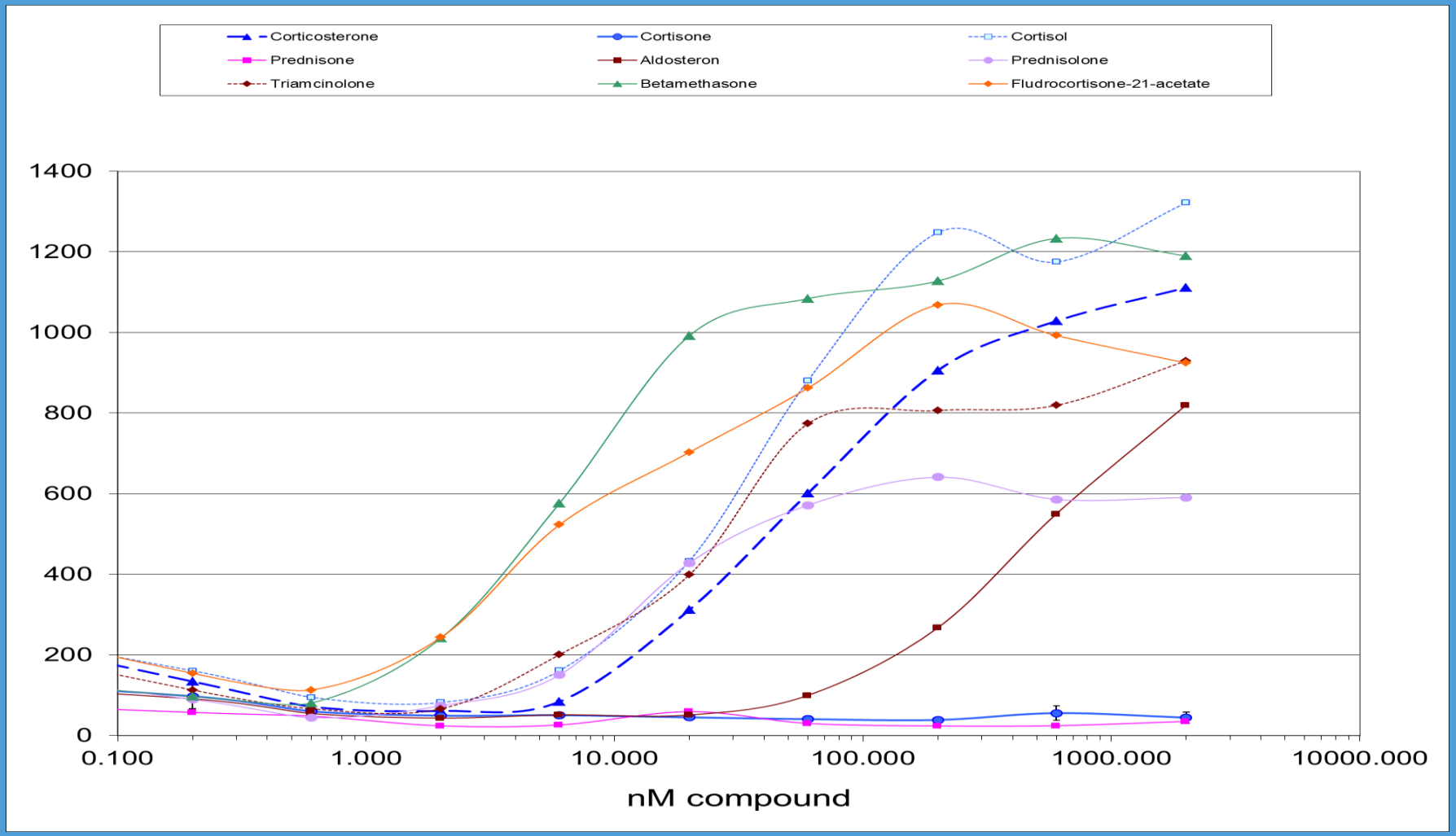
Results specificity testing



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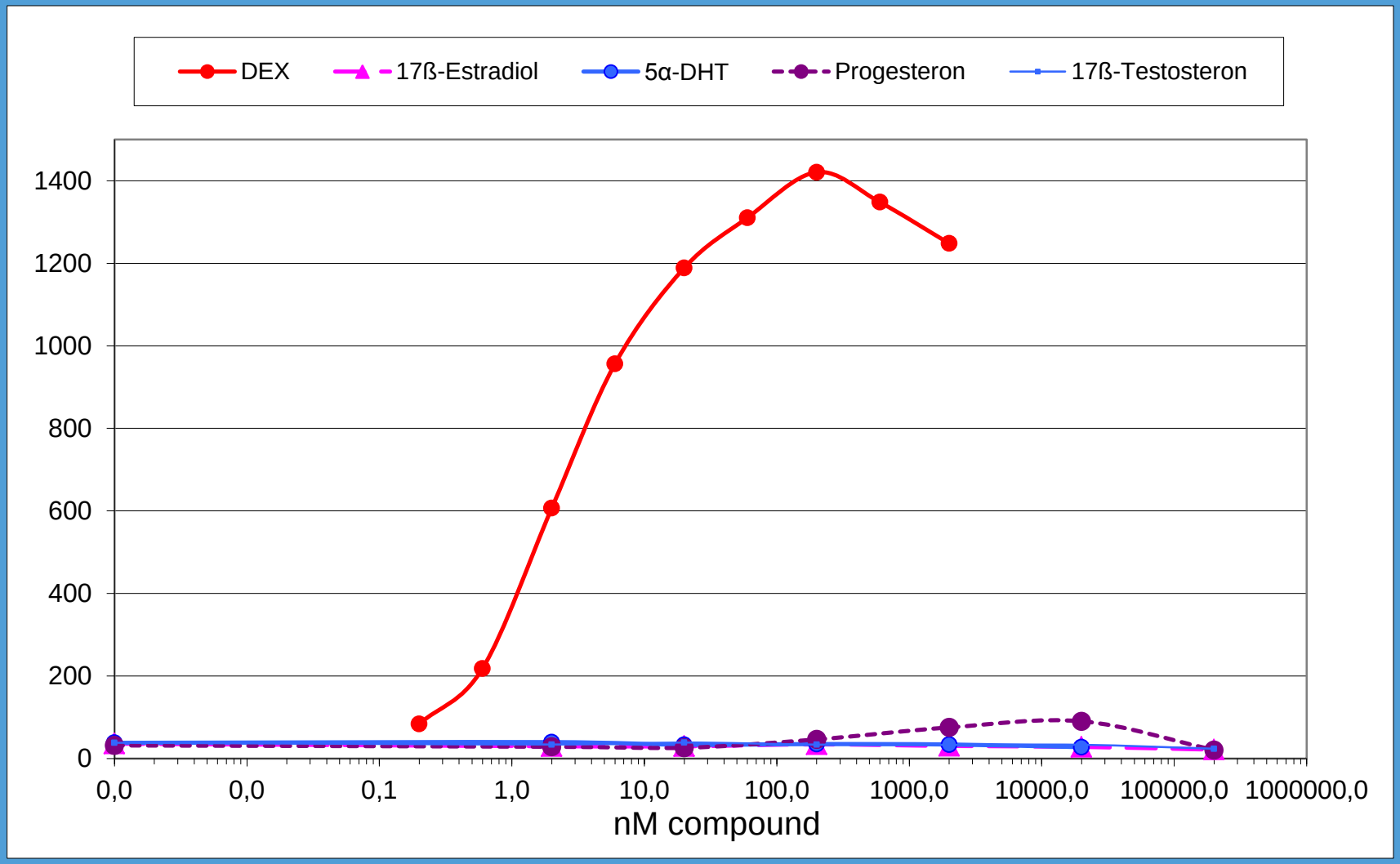
Results specificity testing



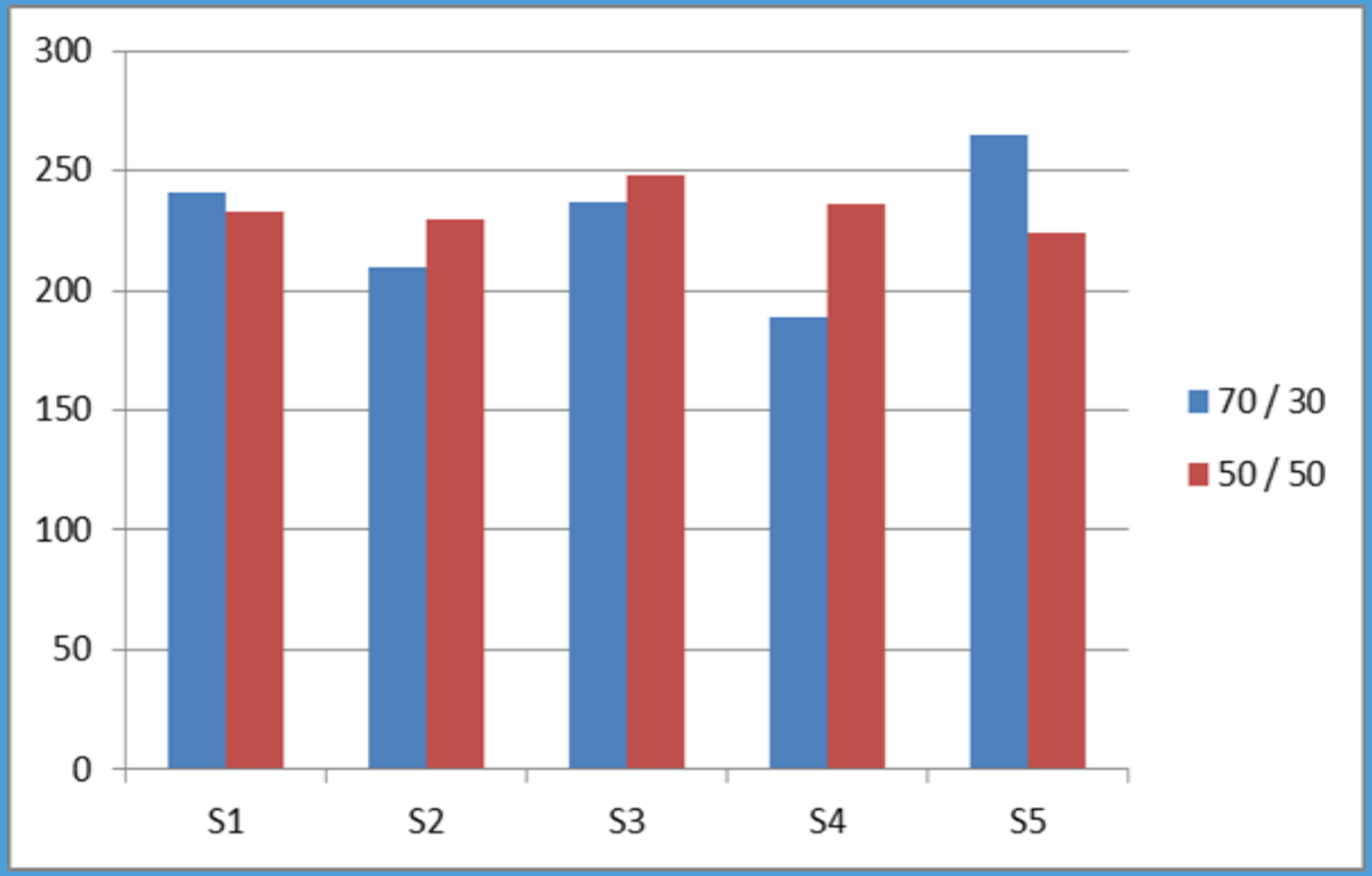
ROBUSTNESS

If robustness = R then $\lim_{R \to \infty} (R)$ = studentproof

- Definition: robustness is the degree of insensitivity of an analytical method for changes in the test conditions
- How to determine? The answer is in the definition: change the test conditions and see what happens. All experiments in triplicate (3 different feed samples used)
  - SPE: composition of washing-buffer: 70/30 or 50/50
  - Confluency cells on exposure: 60-70% / > 80%
  - pH luciferase assay mix: 7.6 / 7.8 / 8.0

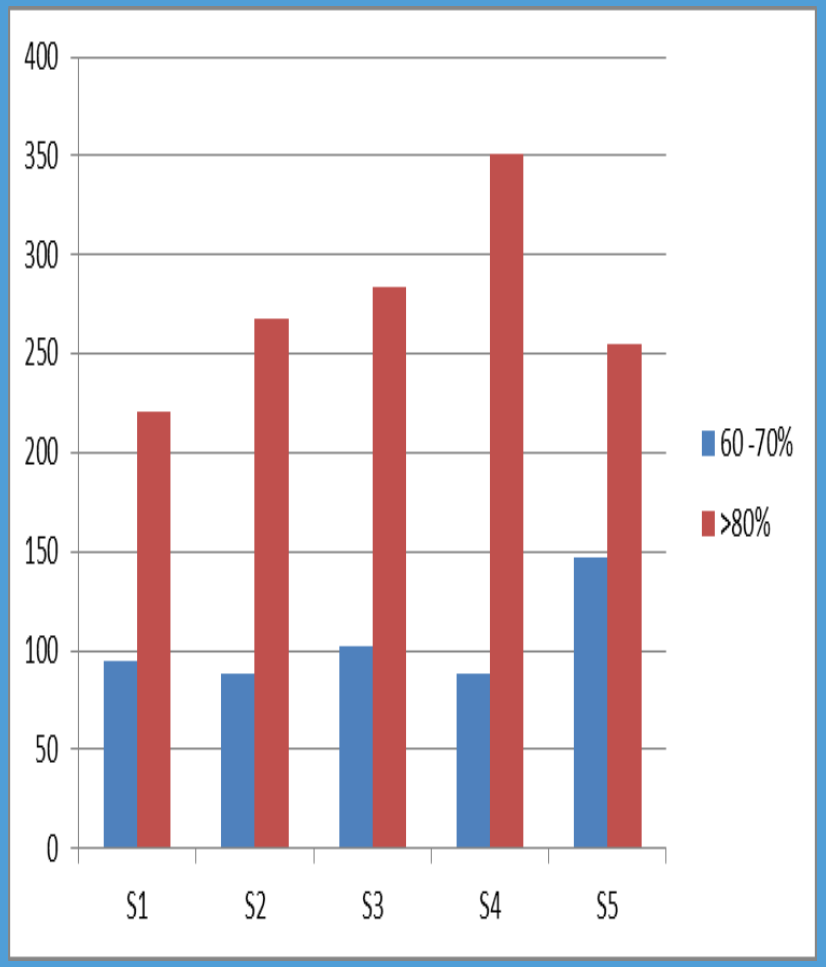
Results robustness testing

influence of washing-buffer

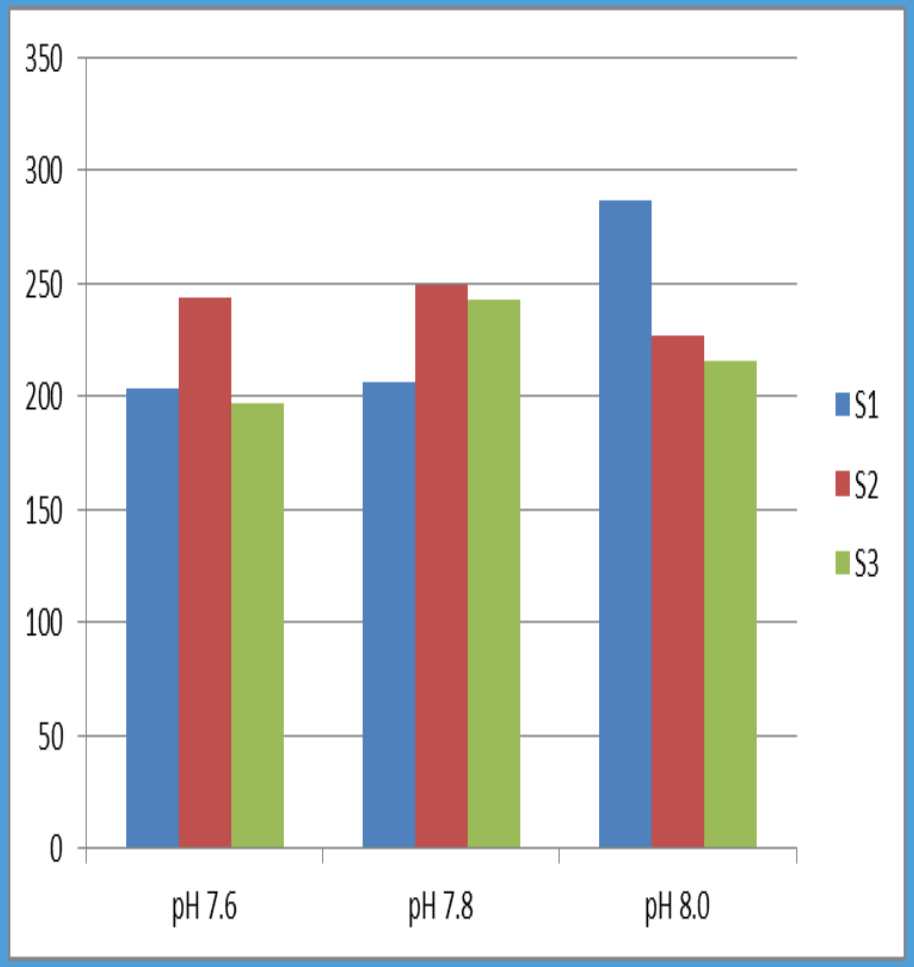


Results robustness testing

influence of confluency



influence of pH assay mix



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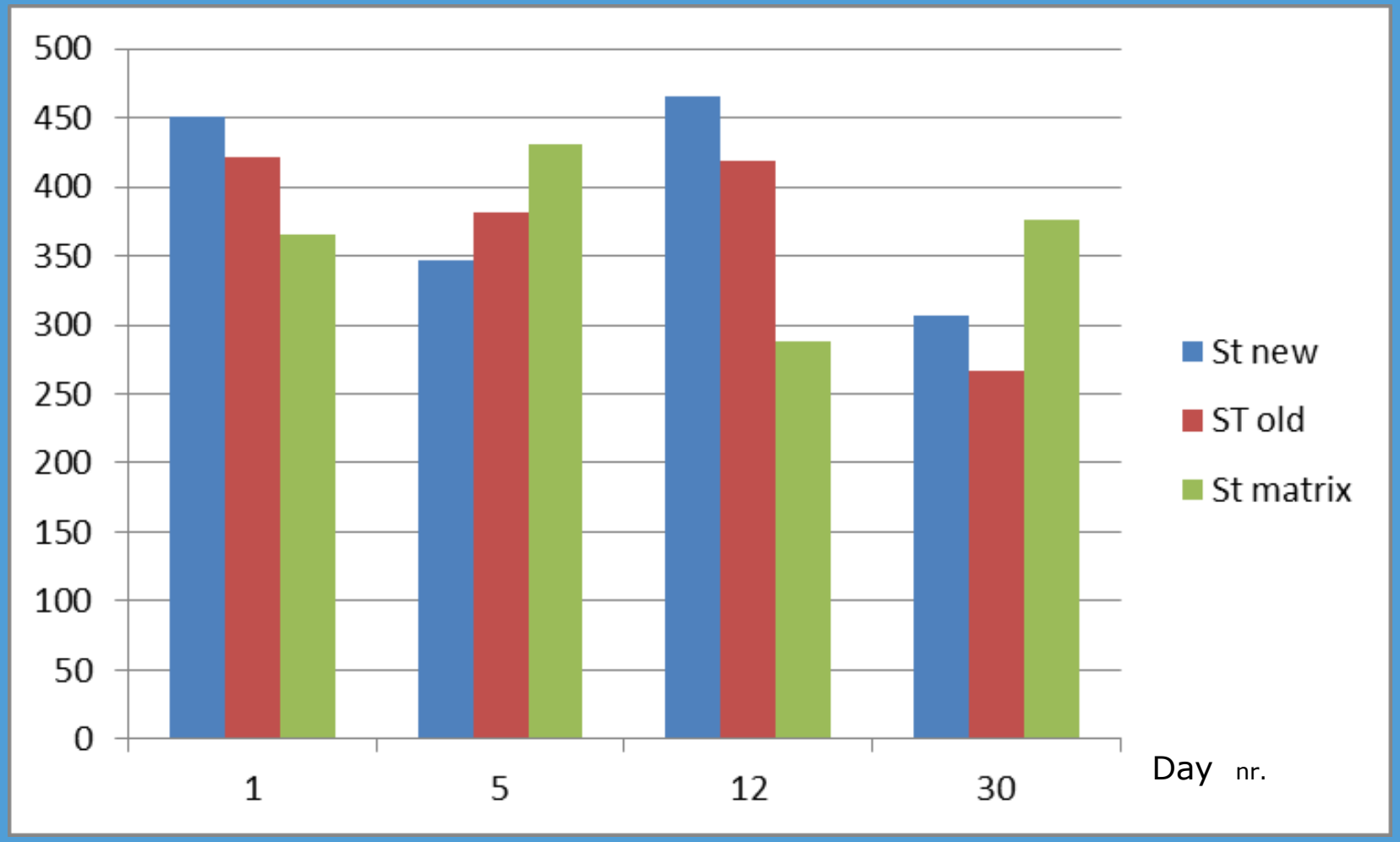
STABILITY

- DEFINITION: stability is the extent to which deviations in the analytical result can occur during storage or analysis.

- How to determine?
  - Prepare both standard-solution and standard in matrix, divide in aliquots, store under standard-conditions (room temperature) and analyse aliquots at different time intervals, check against response of a freshly prepared standard solution.

Results for stability testing (sofar)



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Conclusions validation GR-CALUX® feed

- CCα, CCβ criterions are met for all three selected substances ($\overline{X}\ sp > CC\alpha + 1.64 * Ssp$), max. 5% false negatives at relevant concentration.
- The GR-CALUX® bioassay is specific for (gluco)corticosteroids.
- As to robustness: changes in the conditions have no influence on screening result (washing and pH of the luciferase assaymix), but >60% is really critical
- Components, dissolved in DMSO and in matrix are stable for at least 30 days.

- General conclusion: method is fit for purpose

Acknowledgements

- **RIKILT**: Henri Heskamp, Astrid Hamers, Richard Helsdingen, Gerrit Bor, Jeroen Rijk, Michel Nielen
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Questions?

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