Bioassay screening and bioassay-directed identification of known and unknown hormone active substances

RIKILT INSTITUTE OF FOOD SAFETY WAGENINGEN UR

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Outline

- Introduction: the hormone residue challenge
- Reporter gene estrogen bioassay
- Validation data for calf urine and feed samples
- Bioassay versus GC/MS/MS screening
- Bioassay-directed identification: LC/bioassay/QTOFMS
- Reporter gene androgen bioassay
- Bioassay-directed identification:LC/bioassay/QTOFMS
- Other hormone bioassays under development
- Conclusions

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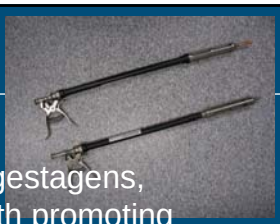
Hormone abuse in food production



- Abuse of steroids (estrogens, androgens, gestagens, corticosteroids) and beta-agonists as growth promoting agents in food producing animals
- EU ban since 1988: *…prohibit...substances having **hormonal action**...and **beta-agonists**…* (96/22/EC)
- Thousands of substances might be relevant…

  …but in current residue monitoring: only limited number of target compounds…unable to detect new or outdated ones
- Target level: between zero and MRPL (≤ 1-2 ng/g)

→ Unrealistic to enforce EU ban with analyte-list approach !

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- Thousands of substances might be relevant…

  …but in current residue monitoring: only limited number of target compounds…unable to detect new or outdated ones
- *Solution:* **Yeast screening methods based on hormonal activity !**
  - simple
  - robust
  - fast
  - applicable to urine, feed, illegal preparations, water and environmental samples

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In vivo bioassays have some disadvantages...

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...and perhaps *in vitro* bioassays should be preferred

Not applicable anno 2006 ?

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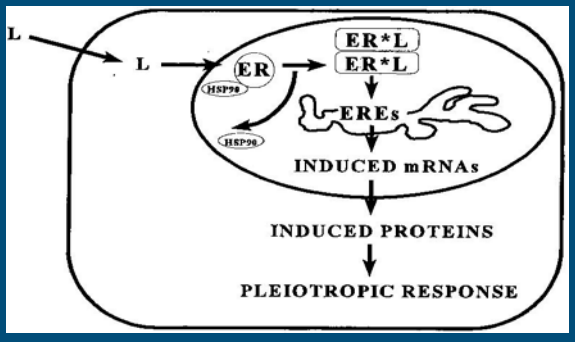
How to design such an *in vitro* bioassay ?

- Biological action of estrogens



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Rikilt yeast Estrogen Assay (REA)

- Genetically (stable) modified **yeast**
  - That expresses the human estrogen alpha receptor: cDNA of the ERalpha behind the strong GPD-promoter (glyceraldehyde-3-phosfate-dehydrogenase)
  - That contains a reporter construct that enables the yeast to produce a green fluorescent protein (yEGFP) following binding of an estrogen to the receptor: two consensus ERE-sequences in a truncated CYC1-promoter (not active cytochrome-c oxidase promoter, due to deletion of UAS1 and UAS2: no induction from sugars, oxygen and iron)

T.F.H. Bovee et al., Gene, 325 (2004) 187-200

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Rikilt yeast Estrogen Assay (REA)

- Genetically (stable) modified yeast
  - expresses the human estrogen alpha receptor
  - contains a reporter construct: the yeast produces a green fluorescent protein (yEGFP) following binding of an estrogen to the receptor
- High sensitivity
  - EC50 of 30 picogram estradiol per well
- Fast and easy
  - only 4 or 24 hours
  - no cell wall disruption, no addition of a substrate

T.F.H. Bovee et al., Gene, 325 (2004) 187-200

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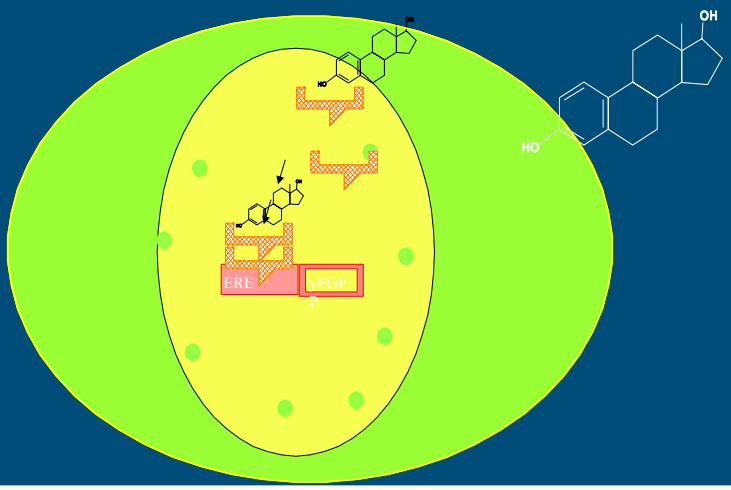
REA yeast bioassay



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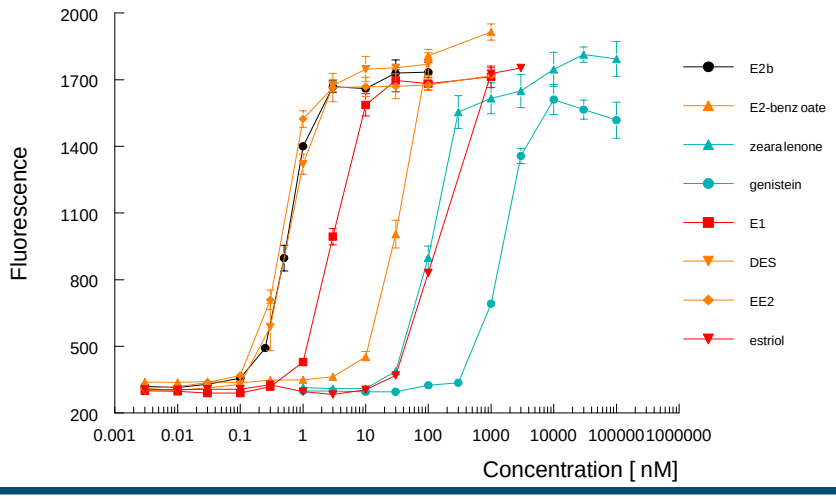
Response of estrogens in the REA bioassay



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REP: Relative Estrogenic Potency

estrogen	REP (ERα)	REP (ERβ)
17β-estradiol	1.0	1.0
ethynylestradiol	1.2	1.0
diethylstilbestrol	1.0	2.0
hexestrol	0.4	0.09
dienestrol	0.6	0.09
mestranol	0.1	1.0×10^{-4}
estrone	0.2	0.1
17α-estradiol	0.09	0.02
zearalanol	0.03	0.02
genistein	5.0×10^{-4}	0.01
8-prenylnaringenin	0. 01	3.9×10^{-3}

T.F.H. Bovee et al., J. Steroids Biochem. & Molec. Biol. 91 (2004) 99-109

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With this REA bioassay you see

- That all kind of different estrogenic compounds give a response and this response corresponds to the estrogenic potency of that compound.
- Only estrogenic compounds. Negligible response to testosterone, progesterone, dexamethasone etc.
- You will detect known and unknown substances that have estrogenic properties.

T.F.H. Bovee et al., JSBMB 91 (2004) 99-109

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Sample clean-up

- Calf urine 2 ml, adjust pH 4.8 and incubate o/n at 37 °C with β-glucuronidase/arylsulfatase
- Add 2 ml 0.25 M sodium acetate buffer pH 4.8 and subject to SPE on a C18 column (elute with 4 ml acetonitrile)
- The eluate was applied to an NH2-column and the eluate thus obtained was evaporated to 2 ml under nitrogen

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Sample clean-up

- 100 µL in triplicate for bioassay, remaining 1700 µL for identification (suspect samples only) by LC/bioassay/MS ;
- add yeast suspension ; read fluorescence (485/530 nm) and calculate t_{24}-t_0 values ;
- report suspect (> CCα) or compliant (negative): " on / off "

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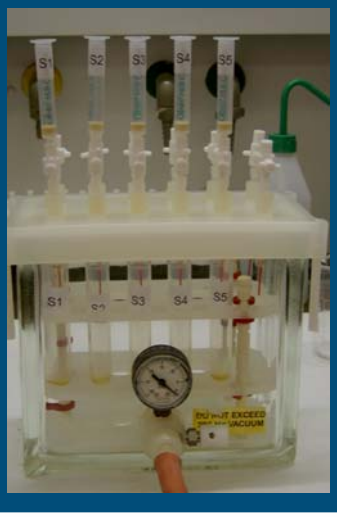
Extract the sample using the generic SPE...



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…put the urine extract into the well...

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...add the yeast cell suspension...

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...read the fluorescence at t_0 and t_{24} (or t_4)

no cell lysis, no reagents, just wait and determine t_{24} - t_0

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Validation data according to 2002/657/EC (1)

- CCβ: 20 different blank **calf urines** and 5x20 calf urines spiked with 17β-estradiol (1 ng/ml), DES (1 ng/ml), ethynylestradiol (1 ng/ml), zearalanol (50 ng/ml), and mestranol (10 ng/ml): suspect
- Specificity/interferences:
  - urine spiked with 1000 ng/ml testosterone and 1000 ng/ml progesterone: compliant
  - idem, but also spiked with 17β-estradiol (1 ng/ml): suspect
- Robustness:
  - used in routine screening > 2 years: no cell toxicity, blanks always compliant, 1 ng/ml spiked samples always suspect.
- ISO 17025 accreditation

T.F.H. Bovee et al., Anal. Chim. Acta 529 (2005) 57-64

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Validation data according to 2002/657/EC (2)

- CCβ: 20 different blank **feeds** and 5x20 feeds spiked with 17β-estradiol (5 ng/g), DES (10 ng/g), ethynylestradiol (5 ng/g), zearalenone (1250 ng/g), equol (200000 ng/g): suspect
- Specificity/interferences:
  - feed spiked with 1000 ng/g testosterone and 1000 ng/g progesterone: compliant
  - idem, but also spiked with 17β-estradiol (5 ng/g): suspect
- Robustness:
  - used in routine screening > 1 year: no cell toxicity, blanks always compliant, 5 ng/g spiked samples always suspect.
- ISO 17025 accreditation pending

T.F.H. Bovee et al., Food Add. and Contam. 23 (2006) 556-568

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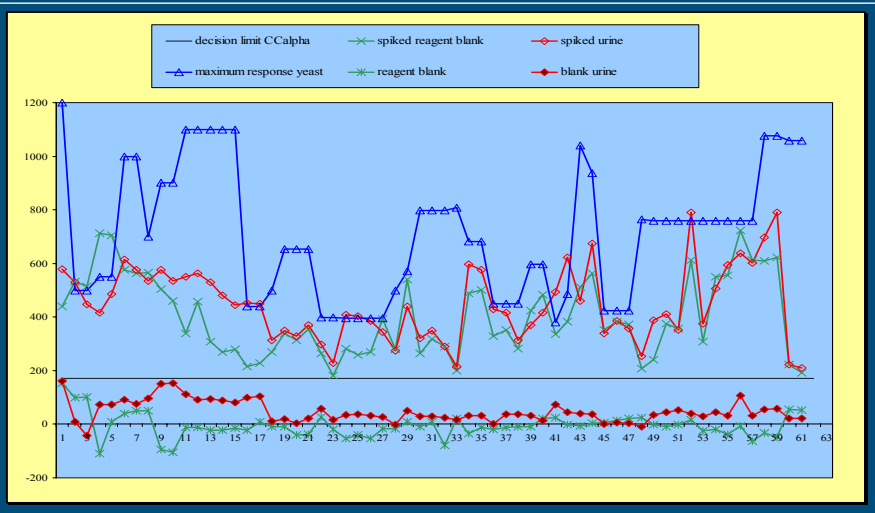
Longterm performance of the estrogen bioassay on urine



T.F.H. Bovee et al., ACA 529 (2005) 57-64

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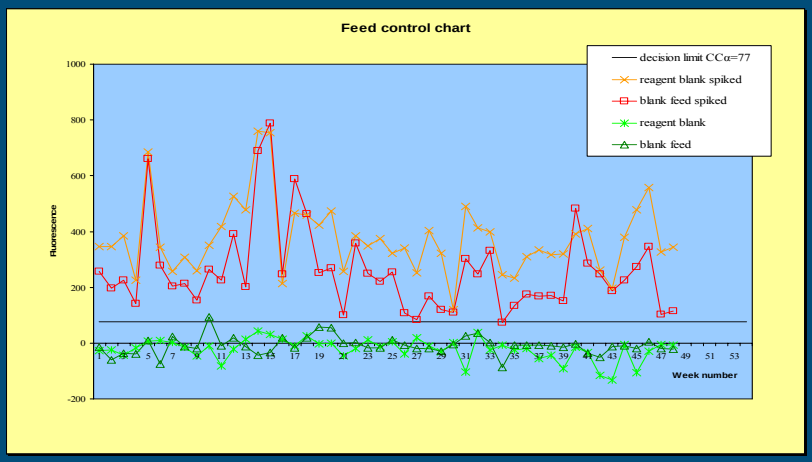
Longterm performance of the estrogen bioassay on animal feed



T.F.H. Bovee et al., FAC 23 (2006) 556-568

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Routine analysis: 126 calf urine samples

- Analysed based on estrogen activity using the bioassay
- Analysed for specific steroids (incl. stilbenes) by GC/MS/MS
- Results:
  - GC/MS/MS: 71 samples compliant (< 1 ng/ml); 55 samples contain 17α-estradiol, a few of them also estrone
  - Bioassay: 67 compliant (only 3.2 % "false suspects")

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Predicted bioassay performance

- Bioassay sensitivity is based on hormonal activity:
- if the relative estrogenic potency of 17α-estradiol = 0.09,
- if $CC\alpha_{17\beta\text{-}E2}$ corresponds with 0.22, $CC\beta_{calc.,17\beta\text{-}E2}$ with 0.44, and $CC\beta_{exp.,17\beta\text{-}E2}$ < 1.0 ng/ml (initial validation study),
- then theoretically the bioassay starts seeing 17α-estradiol from 2.4 ng/ml and the 95% detection capability will be between 4.8 and 10.9 ng/ml...

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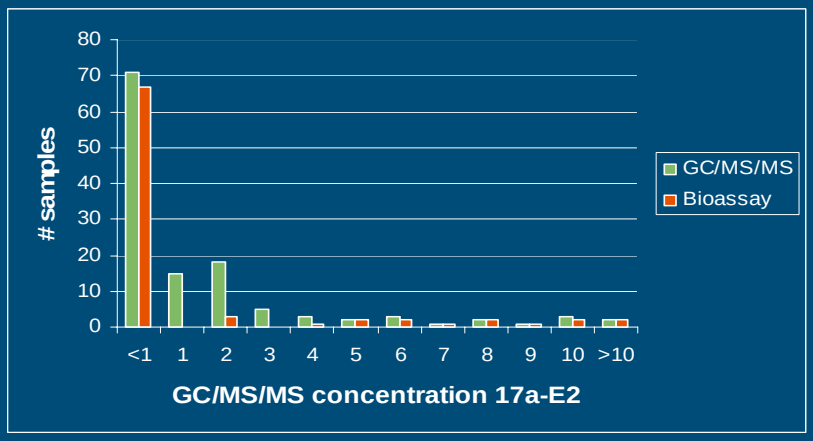
Bioassay versus GC/MS/MS screening



M.W.F. Nielen et al., Food Add. And Contam. 23 (2006) 556-568

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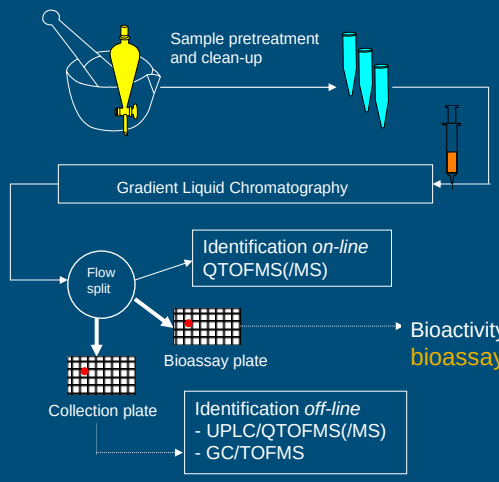
Bioassay directed identification of unknowns



Bioactivity screening:
bioassay as LC detector !

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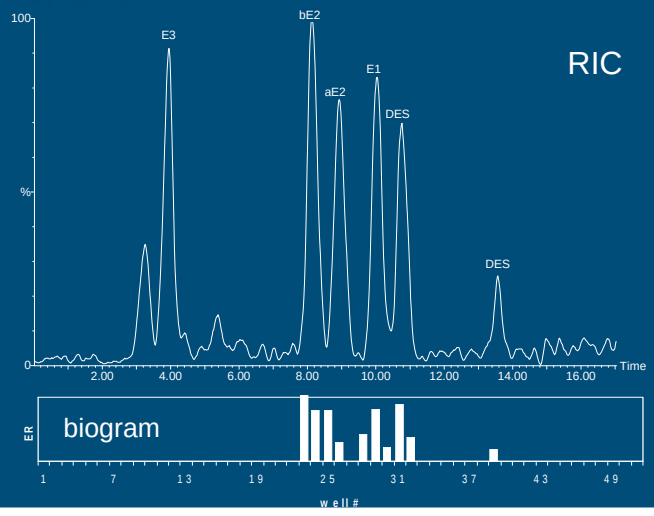
LC/bioassay/QTOF: 1 ng estrogens standard



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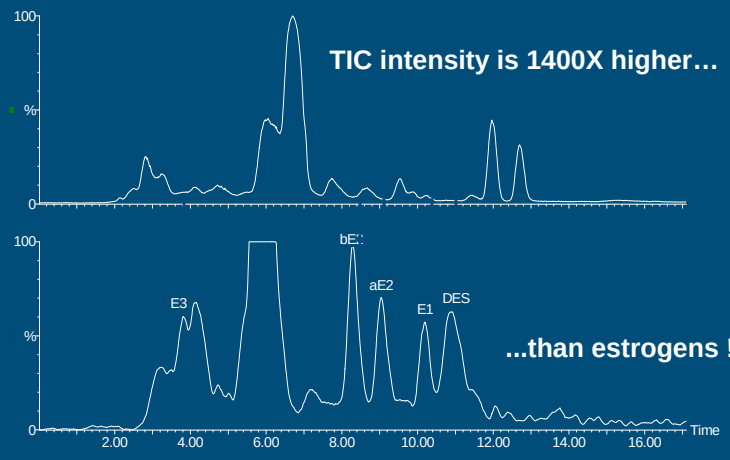
Spiked calf urine: 2 ng/ml



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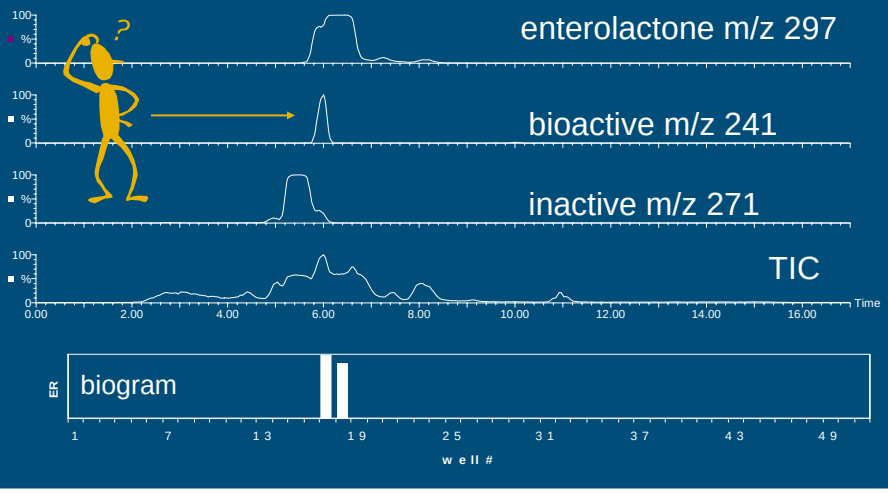
(Un)known estrogens in calf urine



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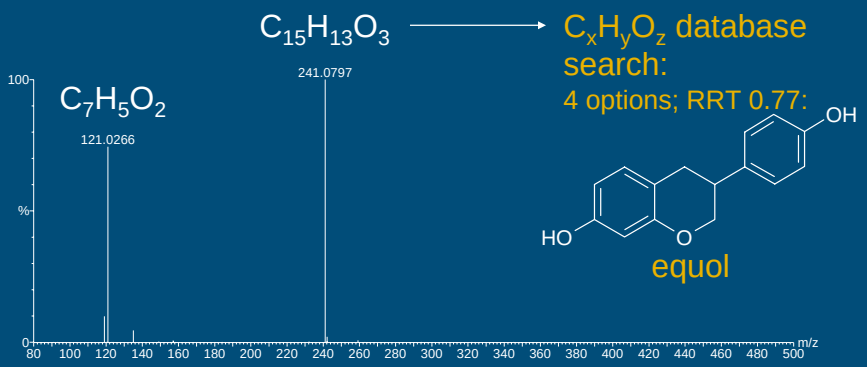
Unknown estrogens in calf urine



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Identified estrogens in calf urines

- 17α-estradiol -natural hormone
- equol -phytoestrogen metabolite
- Bisphenol-like -endocrine disrupter
- nonylphenol -endocrine disrupter

M.W.F. Nielen et al., Anal. Chem. 76 (2004) 6600-6608

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Androgen Bioassay design: like Estrogen Assay

- Genetically (stable) modified yeast
  - expresses the human androgen receptor
  - contains a reporter construct: the yeast produces a green fluorescent protein (yEGFP) following binding of an androgen to the receptor
- Highly sensitive for 17β-testosterone, DHT, boldenone, trenbolone, nortestosterone, THG, etc.
- Negligible sensitivity for estradiol, progesterone, dexamethasone etc.
- Fast and easy
  - only 4 or 24 hours
  - no cell wall disruption, no addition of a substrate

T.F.H. Bovee et al., 2006 submitted

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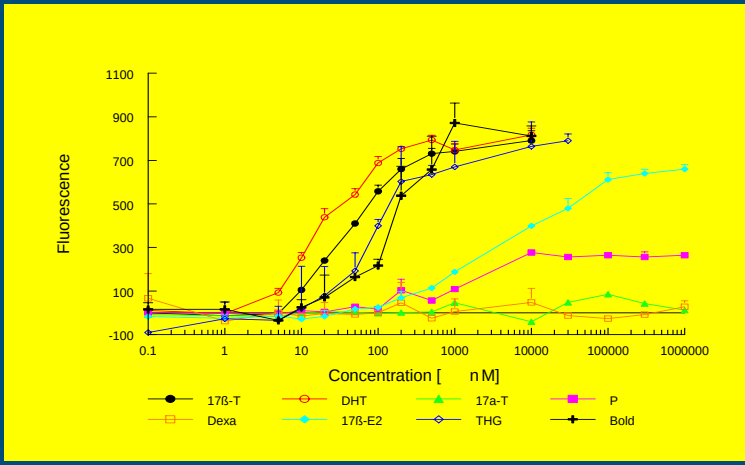
Response of androgens in the RAA bioassay



T.F.H. Bovee et al., 2006 submitted

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Androgens in human urine

Generic screening procedure

- 2 ml urine -samples, -blanks, -controls;
- enzymatic hydrolysis (Helix Pomatia);
- SPE C_{18}/NH_2, acetonitrile/water eluate, concentrate to 2 ml;
- 200 µL in triplicate for bioassay, remaining 1400 µL for identification (suspect samples only) by LC/bioassay/MS;
- add yeast suspension

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Example: androgens in human urine

- Three male and three female volunteers
  - urine samples as such
  - urine samples spiked with 5-15 ng/ml THG
- Direct Bioassay screening
  - Result: all urines suspect for androgen activity
- LC/bioassay for androgen activity detection
  - biograms confirm natural androgens via well numbers
  - biograms indicate additional bioactive well for THG

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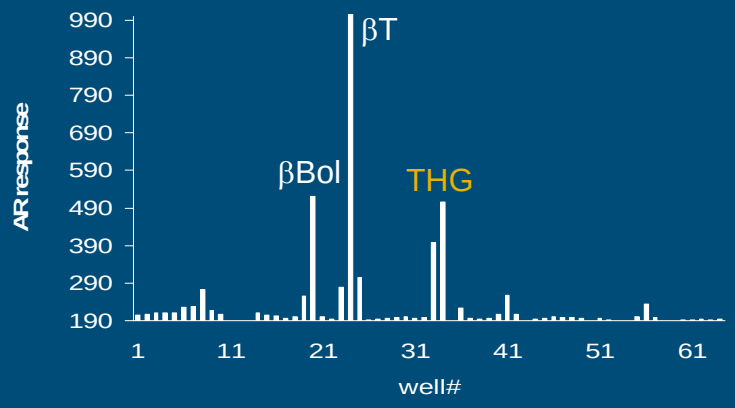
Spiked reagent blank

LC / Androgen Bioassay detection



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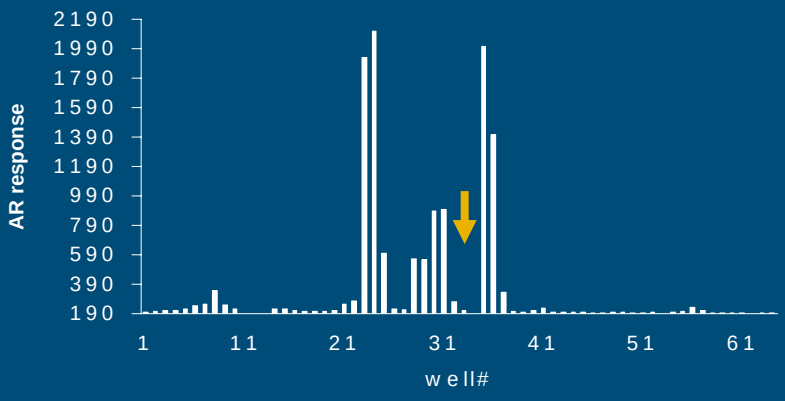
Male urine

LC / Androgen Bioassay detection



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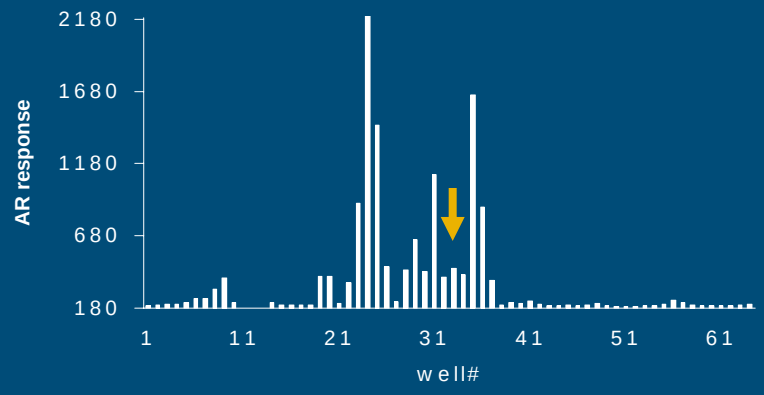
Male urine, spiked with THG

LC / Androgen Bioassay detection



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LC/TOFMS human urine sample

Androgenic well #34: $C_{21}H_{28}O_2$ database search

search engine	options	comments
Merck Index	5	gestagens
Steraloids	8	gestagens
Sigma-Aldrich	44	9 steroids, gestagens
SciFinder	1626	39 commercial, 17 steroids

M.W.F. Nielen et al., Anal. Chem. 78 (2006) 424-431

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Other yeast bioassays in the pipeline

- Progesterone
- Glucocorticosteroids

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Conclusions

- A robust bioassay has been developed, validated and accreditated for estrogens in calf urine and feeds; the androgen version is on track for achievement
- Bioassay screening is addressing the 96/22/EC ban on substances *having hormonal action*
- Substances having weaker bioactivity are less sensitive and might not comply with a chemical MRPL (for example zeranol)
- Only *suspect* bioassay screening results must be identified: either by conventional confirmatory GC/MS methods, or using LC/bioassay/QTOFMS approaches

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Invitation

- The RIKILT estrogen bioassay has been given to veterinary control laboratories in the UK (Queens University-Belfast), Italy (University of Turin) and France (Laberca-Nantes) and to several environmental laboratories active in endocrine disruptors (Germany: GSF and TU-Dresden, Netherlands: Aquasense, Belgium: VITO)

- The latest laboratories are:

•KFRI - Korean Food Research Institute

•IRAS-UU – University Utrecht – Institute for Risk Assessment Sciences

•Royal Chulabhorn Research Institute in Bangkok

•Gent University, FFW – Faculty Pharmaceutical Sciences

- You can try it also and use it, on a co-operation basis.

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Thank you for your attention

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