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

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
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 unable to detect new or outdated ones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 !

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

Not applicable anno 2006?

How to design such an *in vitro* bioassay?

- Biological action of estrogens
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-phosphate-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)


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

REA yeast bioassay

Response of estrogens in the REA bioassay
### REP: Relative Estrogenic Potency

<table>
<thead>
<tr>
<th>estrogen</th>
<th>REP (ERα)</th>
<th>REP (ERβ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ethynylestradiol</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>hexestrol</td>
<td>0.4</td>
<td>0.09</td>
</tr>
<tr>
<td>dienestrol</td>
<td>0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>mestranol</td>
<td>0.1</td>
<td>1.0 x10^-4</td>
</tr>
<tr>
<td>estrone</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>17α-estradiol</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>zearalanol</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>genistein</td>
<td>5.0 x10^-4</td>
<td>0.01</td>
</tr>
<tr>
<td>8-prenylnaringenin</td>
<td>0.01</td>
<td>3.9 x10^-3</td>
</tr>
</tbody>
</table>


### 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*
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

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 (> CC_{α}) or compliant (negative): "on / off"
Extract the sample using the generic SPE...

…put the urine extract into the well...
...add the yeast cell suspension...

...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

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*
Long term performance of the estrogen bioassay on animal feed

Feed control chart

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")

Predicted bioassay performance

- Bioassay sensitivity is based on hormonal activity:
- if the relative estrogenic potency of 17α-estradiol = 0.09,
- if CCα17β-E2 corresponds with 0.22, CCβcalc.,17β-E2 with 0.44, and CCβexp.,17β-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...
Bioassay versus GC/MS/MS screening

![Bar chart showing the comparison of GC/MS/MS and bioassay for 17a-E2 concentration](chart.png)

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

LC/bioassay/QTOF: 1 ng estrogens standard

RIC
Spiked calf urine: 2 ng/ml

TIC intensity is 1400X higher...

...than estrogens!

(Un)known estrogens in calf urine

enterolactone m/z 297

bioactive m/z 241

inactive m/z 271

TIC

biogram
Unknown estrogens in calf urine

Identified estrogens in calf urines

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

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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
Response of androgens in the RAA bioassay

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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 \mu L in triplicate for bioassay, remaining 1400 \mu L for identification (suspect samples only) by LC/bioassay/MS;
- add yeast suspension

**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
Spiked reagent blank

LC / Androgen Bioassay detection

AR response

βT

βBol

THG

Male urine

LC / Androgen Bioassay detection

AR response

Well #
Male urine, spiked with THG

LC / Androgen Bioassay detection

![Graph showing AR response vs well#]

LC/TOFMS human urine sample

Androgenic well #34: $\text{C}_{21}\text{H}_{28}\text{O}_2$ database search

<table>
<thead>
<tr>
<th>search engine</th>
<th>options</th>
<th>comments</th>
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<tbody>
<tr>
<td>Merck Index</td>
<td>5</td>
<td>gestagens</td>
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<tr>
<td>Steraloids</td>
<td>8</td>
<td>gestagens</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>44</td>
<td>9 steroids, gestagens</td>
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<tr>
<td>SciFinder</td>
<td>1626</td>
<td>39 commercial, 17 steroids</td>
</tr>
</tbody>
</table>

Other yeast bioassays in the pipeline

- Progesterone
- Glucocorticosteroids

Conclusions

- A robust bioassay has been developed, validated and accredited 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
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.

**Invitation**

**Acknowledgements**

- Michel Nielen - RIKILT - LC-bioassay-MS
- Ron Hoogenboom - RIKILT - bioassays
- Richard Helsdingen - RIKILT - bioassays
- Gerrit Bor - RIKILT - bioassays
- Astrid Hamers - RIKILT - bioassays
- Elsa Antunes Fernandes - RIKILT - bioassays
- Henri Heskamp - RIKILT - LC-bioassay-MS
- Eric van Bennekom - RIKILT - LC-bioassay-MS
- Paula Balzer-Rutgers - RIKILT - LC-bioassay-MS
- Dutch Ministry of Agriculture, Nature and Food Quality - financial support
- UK DEFRA (Hormone Radar project) - financial support
- World anti-doping agency (WADA) - financial support
Thank you for your attention