Bioassay screening and bioassay-directed identification of (un)known hormone residues

michel.nielen@wur.nl
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
- Conclusion
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  ...but in current residue monitoring: only limited number of target compounds, unable to detect new or outdated ones.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 (\( \leq 1-2 \text{ ng/g} \))

→ **Unrealistic to enforce EU ban with analyte-list approach !**
Hormone abuse in food production

- 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  ...but in current residue monitoring: only limited number of target compounds, unable to detect new or outdated ones.

**solution**: screening methods based on hormonal activity!
  - simple
  - robust
  - fast
  - applicable to urine, feed and illegal preparations
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How to design an *in vitro* bioassay?

- Biological action of estrogens
Rikilt 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*
<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>
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<td>dienestrol</td>
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<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>
</tbody>
</table>
**Estrogens: generic screening procedure**

- 2 ml urine -samples, -blanks, -controls (spiked with 1 ng/ml 17β-estradiol): enzymatic hydrolysis (Helix Pomatia);
- SPE C$_{18}$/NH$_2$, acetonitrile eluate, concentrate to 2 ml;
- 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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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 (10 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

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routine analysis: 126 calf urine samples

- analysis based on estrogen activity using the bioassay
- analysis 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 5.6 % "false suspects").
predicted bioassay performance

- Bioassay sensitivity is based on hormonal activity:
- if the relative estrogenic potency of $17\alpha$-estradiol = 0.09,
- if $CC_\alpha_{17\beta-E_2}$ corresponds with 0.22, $CC_\beta_{calc.,17\beta-E_2}$ with 0.44, and $CC_\beta_{exp.,17\beta-E_2} < 1.0$ ng/ml (initial validation study),
- then theoretically the bioassay starts seeing $17\alpha$-estradiol from 2.4 ng/ml and the 95% detection capability will be between 5 and < 11 ng/ml....
bioassay versus GC/MS/MS screening

GC/MS/MS concentration 17α-E2

# samples

<1 1 2 3 4 5 6 7 8 9 10 >10

GC/MS/MS

Bioassay
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The bioassay and LC/bioassay/QTOFMS concept

start

Generic sample preparation

Include in routine targeted LC/MS/MS or GC/MS method

Bioassay

LC/Bioassay + TOFMS

check in CxHyOz databases

check against reference

(New) bioactive substance found

end

y

n

y

n

Structure elucidation unknown using QTOFMS/MS

y

n

Chemical synthesis postulated unknown

end

Bioassay

2

Bioassay

1

y

n
Bioassay directed identification of unknowns

Sample pretreatment and clean-up

Gradient Liquid Chromatography

Identification on-line QTOFMS(/MS)

Bioactivity screening:
  bioassay as LC detector

Bioassay plate

Identification off-line
  - UPLC/QTOFMS(/MS)
  - GC/TOFMS

Collection plate

Flow split
(un)known estrogens in calf urine

enterolactone m/z 297

bioactive m/z 241

inactive m/z 271

TIC

ER biogram
unknown estrogens in calf urine

C\textsubscript{15}H\textsubscript{13}O\textsubscript{3} → C\textsubscript{x}H\textsubscript{y}O\textsubscript{z} database search:
4 options; RRT 0.77:
equol
identified estrogens in calf urines

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

Spiked reagent blank

LC / Androgen Bioassay detection

AR response

βT

βBol

THG

well#
Female urine

LC / Androgen Bioassay detection
Female urine, spiked with THG

LC / Androgen Bioassay detection
Conclusion

- 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 and Italy, and to several environmental laboratories active in endocrine disruptors. You can try it also and use it, on a co-operation basis.

michel.nielen@wur.nl
# Acknowledgements

- Toine Bovee - RIKILT bioassays
- Ron Hoogenboom - RIKILT bioassays
- Henri Heskamp - RIKILT LC-bioassay-MS
- Eric van Bennekom - RIKILT LC-bioassay-MS
- Hans van Rhijn - RIKILT LC-bioassay-MS
- Sara Stead - CSL York Hormone Radar
- Chris Elliott - VSD Belfast Hormone Radar

Dutch Ministry of Agriculture, Nature and Food Quality financial support
UK DEFRA (Hormone Radar project) financial support
World anti-doping agency (WADA) financial support