Feed/Food safety by DR CALUX bioanalysis for dioxins/dl-PCBs

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&
Prof. Bram Brouwer

BioDetection Systems BV (BDS),
Science Park, Amsterdam, NL
BioDetection Systems B.V. (BDS) is a
- Amsterdam, NL and Rancagua, Chile
- based ISO 17025 accredited service laboratory.
- approved by MAPA for dioxins/dl-PCBs analysis in food/feed both for commercial and official purposes
- accepted by China/Russia for customs clearance
- dioxins feed/food crisis experienced
- providing bioanalysis, such as the innovative CALUX® bioassays for e.g. dioxins/dl-PCBs in e.g. food/feed, soil and blood
History of DR CALUX & Brazil

- 1991: DR CALUX (WUR) First DIOXIN paper
- 1993: Approval RIKILT Brazil CPP (RIKILT)
- 1995: BDS Amsterdam Approval in EU (EC/69)
- 2001: Begin testing in Chile
- 2004: CETESB (Sao Paulo) German crisis 3000 samples
- 2005: BDS Chile Approval MAPA
- 2010: Approval of DR CALUX at LANAGRO
- 2016: >3500 samples tested from Brazil
- 2016: 50 licenses in 20 countries
- 2016: 8000 CALUX s/yr
- 2016: Approved DR CALUX

CETESB (environment)
LANAGRO (food/feed)

Meeting - ©2016 BDS bv all rights reserved
**Principle CALUX bioassay**

- **Ligand Binding**: The ligand interacts with the chemical receptor.
- **Transport Protein**: The transport protein facilitates the movement of the ligand into the nucleus.
- **Chemical Responsive Element (CRE)**: Once in the nucleus, the ligand activates the CRE, leading to transcription.
- **Transcription**: The CRE stimulates transcription of specific genes.
- **Add Substrate (Luciferine)**: The substrate luciferine is added to initiate the luminescence reaction.
- **Luciferase**: Luciferase converts the substrate into light, generating a measurable signal.

The CALUX bioassay principle employs the measurement of light production as an indicator of chemical activity, making it a valuable tool in toxicological and environmental assessments.
High throughput extraction and clean-up

**Extraction:**
- capacity 30 samples/person in 4 hrs

**Clean-up:**
- fast: (15 samples/hr )
- low costs: <10 Euro pP
Robotic dioxin/dl-PCB analysis by DR CALUX

Benefits of DR CALUX
- Costs (e.g. materials < 20 €/sample)
- High capacity (e.g. 40 samples/person/week)
- Hands on sample 1hr/sample (set of 10 samples)
- Easy to learn and use (training in 2 weeks)
Typical standard lab equipment: < 50 kEuro

- Shaker
- Autoclave
- Mikroscope, N2 dewar
- Microvial Evaporator
- CO₂ Incubator
- Luminometer
BDS Chile in Rancagua:...
a typical DR CALUX laboratory..

Cell culture room

Luminometer measurement

Extraction/clean-up room
EU- Strategy Dioxins and dl-PCBs in Feed and Food since 2002

COMMISSION DIRECTIVE 2002/69/EC
of 26 July 2002
laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstuffs
(Text with EEA relevance)

(7) A screening method of analysis with proven, widely acceptable validation and high throughput could be used to select the samples with significant levels of dioxins. The levels of dioxins in these samples need to be determined by a confirmatory method of analysis. It is therefore appropriate to establish strict requirements for the confirmatory methods of analysis and minimum requirements for the screening method.
COMMISSION REGULATION (EC) No 1883/2006
of 19 December 2006

laying down methods of sampling and analysis for the official control of levels of dioxins and
dioxin-like PCBs in certain foodstuffs
(Text with EEA relevance)

(5) A screening method of analysis with proven widely
acceptable validation and high throughput should
be used to select the samples with significant levels of
dioxins and dioxin-like PCBs. The levels of dioxins and
dioxin-like PCBs in these samples need to be determined
by a confirmatory method of analysis. It is therefore
appropriate to establish strict requirements for the con-
firmatory methods of analysis and minimum
requirements for the screening method.

Monitoring for the presence of dioxins in foodstuffs may be performed by a strategy involving a screening method
in order to select those samples with levels of dioxins and dioxin-like PCBs that are less than 25% below or exceed
the maximum level. The concentration of dioxins and sum of dioxins and dioxin-like PCBs in those samples with
significant levels needs to be determined, confirmed by a confirmatory method.
Ministério da Agricultura, Pecuária e Abastecimento

BIODETECTION SYSTEMS
Nome Empresarial: Biodetection Systems
TRN: NL809623626B01
Endereço: Science Park, nº 406
ZIP Code: 1098 XH
Cidade: Amsterdam/ Holanda
Fone/Fax: ( )

Responsável pela Direção do Laboratório:
E-mail:
Portaria: nº 87, de 04.11.2015
D.O.U.: nº 212, de 06.11.2015, Seção 1, pág.: 5.

PORTARIA Nº 87, DE 04 DE NOVEMBRO DE 2015.

O SECRETÁRIO DE DEFESA AGROPECUÁRIA DO MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, no uso das atribuições que lhe conferem os arts. 13 e 45 do Anexo I do Decreto nº 8.492, de 13 de julho de 2015, tendo em vista o disposto na Instrução Normativa nº 57, de 11 de dezembro de 2013, na Instrução Normativa nº 41, de 29 de outubro de 2015, e o que consta do Processo nº 21000.007421/2015-26, resolve:


Art. 2º Estabelecer que o escopo do credenciamento ficará disponível no site eletrônico do MAPA, por área de atuação.

Art. 3º Esta Portaria entra em vigor na data de sua publicação.

DÉCIO COUTINHO
Performance criteria for cell- and receptor-assays: 
Directive 2014/589 (food) and 2014/709 (feed)

Bioanalytical screening methods:

- Semi-quantitative approach: result of screening shall be expressed as compliant or suspect to be non-compliant.
- Result for PCDD/F and/or dl-PCBs expressed in Bioanalytical Equivalents (BEQ)
- BEQ level to 2/3 serve of maximal level serve as cut-off
- requirements:
  - Good comparison GC/HRMS - bioassay results
  - Low rate of false negatives
  - Performance in PT studies
  - Suspected samples confirmed by GC-HRMS
  - ISO 17025 accredited lab
Feed/raw materials:
NO false negative and < 5% false compliant rate
**Chicken meat:** DR CALUX (BDS Chile) vs HC/HRMS

- All samples compliant by DR CALUX and GC/HRMS: no false +/-
- (Cut-off DR CALUX: 2.0, Action-limit EU: 2.0)

**Pork meat:** DR CALUX (BDS Chile) vs GC/HRMS

- DR CALUX: 2/46 false + (4.3%), no false -
- (Cut-off DR CALUX: 0.8, Action-limit EU: 1.25)

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**Food:**

- NO false negative and < 5% false compliant rate
# Frequently asked questions

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Used sample amount</th>
<th>LOQ PCDD/F/dl-PCB</th>
<th>Cut-off PCDD/F/dl-PCB (EC 589/2014 and 709/2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td></td>
<td>2/3 max EC value</td>
<td></td>
</tr>
<tr>
<td>Plant-origin</td>
<td>9 gr</td>
<td>0,11</td>
<td>1,0</td>
</tr>
<tr>
<td>Plant solid</td>
<td>9 gr</td>
<td>0,11</td>
<td>0,83</td>
</tr>
<tr>
<td>Minerals</td>
<td>9 gr</td>
<td>0,11</td>
<td>0,67</td>
</tr>
<tr>
<td>Food</td>
<td>2/3 max EC value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken meat</td>
<td>3 gr fat</td>
<td>0,33</td>
<td>2,0</td>
</tr>
<tr>
<td>Cows meat</td>
<td>2 gr fat</td>
<td>0,50</td>
<td>2,7</td>
</tr>
<tr>
<td>Eggs</td>
<td>3 gr fat (20 ml)</td>
<td>0,33</td>
<td>3,3</td>
</tr>
<tr>
<td>Milk</td>
<td>3 gr fat (30 ml)</td>
<td>0,33</td>
<td>3,7</td>
</tr>
</tbody>
</table>
Further QA/QC - summary

- Reference standard series on each plate
- Solvent control (DMSO) on each plate
- Internal reference material (IRM (BRM-07)) on each plate
- Procedure blank and a reference samples in each test series
- Reference samples at the level of interest (GC/HRMS analyzed)
- Analysis at least 3 dilution levels
- Triplicate analysis at each dilution level
- Results expressed as BEQs
- Repeatability: coefficient of variation < 15%
- Reproducibility: coefficient of variation < 30%
- Recovery should comply to Q-Chart toleration and EC/589/2014; EC709/2014.

Approved quality criteria's according to EC/589/2014 and EC/709/2014
Typical QA/QC-data from DR CALUX from Inst. Public Health, Valencia, Spain

<table>
<thead>
<tr>
<th>REQUIREMENTS</th>
<th>INTERNAL VALIDATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROCEDURE BLANK (&lt;1PM)</td>
<td>0,6 pM</td>
</tr>
<tr>
<td>RECOVERY CERTIFIED REFERENCE MATERIAL / FORTIFIED SAMPLE.</td>
<td>91,45 % / 111,4 %</td>
</tr>
<tr>
<td>APPARENT BIOASSAY RECOVERY (30-130%)</td>
<td>83,16 %</td>
</tr>
<tr>
<td>SUPRESSION SIGNAL TEST (&gt;75%)</td>
<td>118,81 %</td>
</tr>
<tr>
<td>REPRODUCIBILITY RSDR (&lt;25%)</td>
<td>12,6 %</td>
</tr>
<tr>
<td>REPEATABILITY RSDr (&lt;20%)</td>
<td>10,33 %</td>
</tr>
<tr>
<td>FALSE COMPLIANT RATE (&lt;5%)</td>
<td>0 %</td>
</tr>
</tbody>
</table>

in courtesy of Prof. YUSA, Inst. Public Health, Valencia
Since 2004 successful yearly participation in international proficiency test
Annex to ISO/IEC 17025:2005 declaration of accreditation for registration number: L 401

of Biodetection Systems B.V. (BDS)

This annex is valid from: 24-09-2014 to 01-11-2018

Replaces annex dated: 15-01-2014

## Locations where activities are performed under accreditation

<table>
<thead>
<tr>
<th>Location</th>
<th>Abbreviation/ location code</th>
</tr>
</thead>
</table>
| BDS Amsterdam
Science Park 496
1098 XH
Amsterdam
Nederland | A’dam |
| BDS Chili Ltd
Camino Vecinal 950
Ruta-H-30
Rancagua
Chile | Chile |

<table>
<thead>
<tr>
<th>No.</th>
<th>Material or product</th>
<th>Type of activity</th>
<th>Internal reference number</th>
<th>Location</th>
</tr>
</thead>
</table>
| 1.  | Feeding stuffs, raw material for animal feeding stuffs, fats, oils, meat products, dairy products, eggs and fish products | Screening of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay | P-bds-051
conform Commission Regulation (EU) No 589/2014,
conform Commission Regulation (EU) No 278/2012 | A’dam |
| 2.  | | Screening of activity of dioxin/furans and dioxin-like PCB's; DR CALUX® bioassay and C-SPLIT | P-bds-051
conform Commission Regulation (EU) No 589/2014,
conform Commission Regulation (EU) No 278/2012 | A’dam |
| 3.  | Soil, fly-ash, blood and blood products | Determination of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay | P-bds-051
in-house method | A’dam |
| 4.  | Sludge | Determination of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay | P-bds-051
conform RIKZ protocol:
Specle'07 | A’dam |
Typical ISO 17025 accredited international feed reporting (e.g. accord. EC/709/2014)

<table>
<thead>
<tr>
<th>Client Code</th>
<th>Method</th>
<th>Parameter</th>
<th>Result</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed 1</td>
<td>DR CALUX</td>
<td>PCDD/F (BEQ)</td>
<td>Compliant (0,20)</td>
<td>ng BEQ/kg</td>
</tr>
<tr>
<td>Feed 1</td>
<td>DR CALUX</td>
<td>PCDD/F and dl-PCB (BEQ)</td>
<td>Suspect (1,4)</td>
<td>ng BEQ/kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BDS Code</th>
<th>Matrix</th>
<th>ISO 17025</th>
<th>Date arrival</th>
</tr>
</thead>
<tbody>
<tr>
<td>12345</td>
<td>Feed, plant origin</td>
<td>yes</td>
<td>12-12-2016</td>
</tr>
</tbody>
</table>

Reporting according current EC guidelines
Approved by all us known authorities (e.g. EU, MAPA, CAIQ, Rosselkhoznadzor)
## Feed reporting according German feed law (§44)

<table>
<thead>
<tr>
<th>Client Code</th>
<th>Parameter</th>
<th>Unit</th>
<th>Dry mass</th>
<th>Value</th>
<th>Exceedance Max. value</th>
<th>Compliance</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed 1</td>
<td>BEQ-PCDD/F</td>
<td>ng/kg</td>
<td>88%</td>
<td>1,5</td>
<td>&gt; Cut-off</td>
<td>Suspect (screening)</td>
<td>EC/709 (2014)</td>
</tr>
<tr>
<td>Feed 1</td>
<td>BEQ-DL-PCB</td>
<td>ng/kg</td>
<td>88%</td>
<td>0,2</td>
<td>&lt; LOQ</td>
<td>Comform (screening)</td>
<td>EC/709 (2014)</td>
</tr>
<tr>
<td>Feed 1</td>
<td>Sum BEQ-PCDD/F and dl-PCB</td>
<td>ng/kg</td>
<td>88%</td>
<td>1,7</td>
<td>&gt; Cut-off</td>
<td>Suspect (screening)</td>
<td>EC/709 (2014)</td>
</tr>
</tbody>
</table>
### BDS acceptance and usage at international feed/food quality systems

<table>
<thead>
<tr>
<th>Maximal limit</th>
<th>Action limit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCDD/F/dl-PCBs:</strong></td>
<td><strong>PCDD/F/dl-PCBs:</strong></td>
</tr>
<tr>
<td><strong>Used by</strong></td>
<td><strong>Used by</strong></td>
</tr>
<tr>
<td>- egg associations (e.g. KAT, IKB)</td>
<td>- Q&amp;S Quality System</td>
</tr>
<tr>
<td>- German § 44a of federal law on food and feed</td>
<td>- GMP⁺</td>
</tr>
<tr>
<td>- feed associations (e.g. Fediol)</td>
<td></td>
</tr>
</tbody>
</table>
Chile 2008 – mineral premix from industrial production reported with dioxin levels

- Since summer Asia banned pigs/beef from Chile due to higher dioxin levels
- The source seems to be from the ZnO mineral premix for animal feed
Follow-up Food/Feed Monitoring (Chile, 2009): Minerals

Max EU limit PCDD/F-PCB = 1.5 ng TEQ/kg product

5.4% above max EU PCDD/F-PCB limit

Max EU limit PCDD/F = 0.75 ng TEQ/kg product

7.6% above max EU PCDD/F limit

75% max EU PCDD/F limit

38 ng TEQ/kg product

mineral clay

zinc oxide
National Food/Feed Monitoring Plan (Chile, 2009): Meat, feed and waste oils

EC Meat Level: EC Max Dioxins 1 – 25% = 0.75 pg TEQ/g

Chile Meat Level: EC Max Dioxins 1 – 25% = 0.75 pg TEQ/g
## Alimento

<table>
<thead>
<tr>
<th>Alimento</th>
<th>Contenido Máximo de Dioxinas y PCBs coplanares</th>
<th>Unidad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carne de cerdo y productos derivados</td>
<td>2,0</td>
<td>(pg EQT/OMS/g de grasa)</td>
</tr>
<tr>
<td>Carne de ave y productos derivados</td>
<td>3,5</td>
<td>(pg EQT/OMS/g de grasa)</td>
</tr>
<tr>
<td>Carne de bovino y ovino y productos derivados</td>
<td>6,0</td>
<td>(pg EQT/OMS/g de grasa)</td>
</tr>
<tr>
<td>Pescados y productos derivados</td>
<td>2,0</td>
<td>(pg EQT/OMS/g de peso fresco)</td>
</tr>
<tr>
<td>Huevos y productos derivados</td>
<td>3,0</td>
<td>(pg EQT/OMS/g de grasa)</td>
</tr>
<tr>
<td>Leche y productos derivados</td>
<td>6,0</td>
<td>(pg EQT/OMS/g de grasa)</td>
</tr>
</tbody>
</table>
Between 2010 and 2013 at ISP Chile 771 food samples from 8 different matrixes have been tested by DR CALUX

- 13% has been below the LOQ of the DR CALUX method
- 0.8% of the samples have been above the EU maximal value
- 2% of the negative samples have been confirmed by chemical analysis to be negative ($R^2=0.66$)

(see at http://www.ispch.cl/noticia/1544/chile-unico-pais-en-latinoamerica-que-realiza-deteccion-de-dioxinas-por-bioensayo-aprob)
Results of the DR CALUX:

- > 3500 samples tested in 4 months
- All samples analysed within 2-3 days
- Only lab in Europe which analysed all samples in demanded time
Toxic Trade
The players in the dioxin scandal

Petrotech AG (Emden, Germany) manufactures biofuel from spent cooking fats. The process also produces mixed fats.

Olivet (Rotterdam, the Netherlands) buys the fats and sells them on.

Harles and Jentzsch (Uetersen and Bösel, Germany) uses the fats in the production of feed fats. It is still not clear at what point the dioxin got into the fats.

Feed producers mix the feed fats in with their animal feed.

Farmers buy the animal feed and use it to feed their hens, turkeys, and pigs.
German pig meat (start 2011)
representive 500 from 3500 samples tested in 4 months

Distribution PCDD/F-TEQ by DR CALUX for German pig meat in Jan/Feb 2011

- All samples below EU guided level of 0.75 pg TEQ/kg fat
- High dynamic range of DR CALUX (7-times above LOQ)
DR CALUX studies in Italy
- 16 from 17 sample correct compliant
- No false positive result; one false negative result
- High dynamic range of DR CALUX (6-times above LOQ)

In courtesy of B. Barbara, E. Ferretti (Poster DIOXIN 2011)
How fast can you set-up a DR CALUX lab and have the National accreditation: a case in Australia
Soils Recycled Organics & Remediation Technologies Unit,
Sydney, Australia

Dioxins: A fishy business;
the analysis of dioxins in seafood from Sydney Harbour
Sydney Harbour; all fish samples polluted
World record:
One month for lab set-up, accreditation and 200 samples tested

<table>
<thead>
<tr>
<th>Week</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>training CALUX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chain of custody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>optimalisation lab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QA/QC data set</td>
<td>18</td>
<td>25</td>
<td>35</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total TEQ testing</td>
<td>12</td>
<td>24</td>
<td>32</td>
<td>50</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>PCB TEQ testing</td>
<td>12</td>
<td>24</td>
<td>32</td>
<td>50</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>evaluation accreditation</td>
<td>12</td>
<td>24</td>
<td>32</td>
<td>50</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>fish samples done</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>analysis done</td>
<td>42</td>
<td>73</td>
<td>99</td>
<td>145</td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>
Fish samples from Sydney harbour: Comparison of DR CALUX® and GC/HRMS
Non invasive animal blood testing for dioxins/dl-PCBs: Do we have to kill an animal which did eat contaminated feed, or is it better to test at first the blood of the animal?
Latest other dioxin crisis in Europe; always ready for surprises in the feed chain

- 2010 – Maize from Ukraine (Nederlands)
- 2011 – Sugar molasses (Germany)
- 2012 – Red colorants (The Netherlands)
- 2015 – bio-eggs old & asbestos roofs (DK, NL, D)
- 2016 - QS, FEDIOL, GMP+ ongoing monitoring for
  - especially thermic treated oils/fats,
  - herbs, minerals and growth promoters
Invitation to 10th BioDetectors Workshop in Sorrento (nearby Naples), Italy on 6/7th April 2017