State-of-the-art in POPs Analysis: Outcomes of the DIFFERENCE and DIAC projects

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Dioxins in Food and Feed-Reference Methods and New Certified Reference Materials

“DIFFERENCE”

Dioxin Analysis by Comprehensive Multi-Dimensional Gas Chromatography (GCxGC)

“DIAC”

COMPETITIVE AND SUSTAINABLE GROWTH (GROWTH) PROGRAMME
Background and Societal Needs

- Belgian chicken incident 1999
- Need for dioxin analysis capacity
- Need for cheap and reliable screening and confirmatory methods
- New EU MRLs, 1 July 2002
Objectives DIAC

- Optimisation of GCxGC-ECD system for dioxin analysis
- Selection of ‘best’ modulator
- Optimisation of quantification comparison with HRMS
- Test of alternative detection method: ToF-MS
- Simplification of extraction and clean-up
WP 1, task 1
Mini-workshop

WP 1, task 2
Optimisation of GCxGC

WP 2, task 1
Quantification standard solution/cleaned sample

WP 3, tasks 1, 2
Simplification extraction/clean-up methods

WP 2, task 2
Quantification real-life samples GCxGC vs. GC-HRMS

WP 4
Alternative MS detection method

WP 5
Dissemination of results
Workshop for dioxin users

Final report
<table>
<thead>
<tr>
<th>DIFFERENCE Objectives</th>
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<tr>
<td>Selection of relevant food and feed matrices</td>
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<td>Preparation of candidate CRMs</td>
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<td>Feasibility of certification</td>
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<tr>
<td>Optimisation of bio-analytical and chemical methods for dioxin analysis</td>
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<td>Validation and standardisation</td>
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<tr>
<td>Optimisation of extraction and clean-up</td>
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<tr>
<td>Standardised protocols for use in Europe</td>
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</table>
### WHO Dioxins, Furans and dioxin-like PCBs

<table>
<thead>
<tr>
<th>PCDDs</th>
<th>PCDFs</th>
<th>Dioxin-like PCBs (+IUPAC nos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-TCDD</td>
<td>2,3,7,8-TCDF</td>
<td>3,3',4,4'-TCB (77)</td>
</tr>
<tr>
<td>1,2,3,7,8-PcCDD</td>
<td>1,2,3,7,8-PcCDF</td>
<td>3,4,4',5-TCB (81)</td>
</tr>
<tr>
<td>1,2,3,4,7,8-HxCDD</td>
<td>2,3,4,7,8-PcCDF</td>
<td>3,3',4,4',5-PeCB (126)</td>
</tr>
<tr>
<td>1,2,3,6,7,8-HxCDD</td>
<td>1,2,3,4,7,8-HxCDF</td>
<td>3,3',4,4',5,5'-HxCB (169)</td>
</tr>
<tr>
<td>1,2,3,7,8,9-HxCDD</td>
<td>1,2,3,6,7,8-HxCDF</td>
<td>2,3,3',4,4'-PeCB (105)</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-HpCDD</td>
<td>2,3,4,6,7,8-HxCDF</td>
<td>2,3,4',5-PeCB (114)</td>
</tr>
<tr>
<td>OCDD</td>
<td>1,2,3,7,8,9-HxCDF</td>
<td>2,3',4,4',5-PeCB (118)</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-HpCDF</td>
<td>2',3,4,4',5-PeCB</td>
<td>123</td>
</tr>
<tr>
<td>1,2,3,4,7,8,9-HpCDF</td>
<td>2,3,3',4,4',5-HxCB</td>
<td>156</td>
</tr>
<tr>
<td>OCDF</td>
<td>2,3,3',4,4',5-HxCB</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>2,3',4,4',5,5'-HxCB</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>2,3',4,4',5,5'-HpCB</td>
<td>189</td>
</tr>
</tbody>
</table>
## EU Requirements for Dioxin and dl- PCB Analysis

<table>
<thead>
<tr>
<th></th>
<th>Screening Methods</th>
<th>Confirmatory Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>False Negative Rate</td>
<td>&lt;1%</td>
<td></td>
</tr>
<tr>
<td>Trueness</td>
<td></td>
<td>-20 to +20%</td>
</tr>
<tr>
<td>CV</td>
<td>&lt;30%</td>
<td>&lt;15%</td>
</tr>
</tbody>
</table>
## Quality Criteria (2002/69/EC)

<table>
<thead>
<tr>
<th>Performance of a method</th>
<th>1 – 8 pg TEQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ (confirmatory method): range 1/5 level of interest</td>
<td>LOQ at 1pgTEQ/g fat</td>
</tr>
<tr>
<td>High sensitivity and low limits of detection</td>
<td>Up to 1pgTEQ</td>
</tr>
<tr>
<td>High selectivity (specificity)</td>
<td>Interferences PCN, PCB, PCDE</td>
</tr>
<tr>
<td>High accuracy (trueness and precision)</td>
<td>r&amp;R</td>
</tr>
</tbody>
</table>
EU: sensitivity requirements for food (pg diox./g fat)

- Pork
- Ruminants
- Milk
- Liver
- Veg. oil
- Poultry
- Eggs
- Fish: 4 pg dioxins/g product

Fish: 4 pg dioxins/g product
## GCxGC studies

- GCxGC-ECD
- GCxGC-ToF-MS
- Various first and second column combinations
- Comparison five “modulator” types

<table>
<thead>
<tr>
<th>RIVO, The Netherlands</th>
<th>Free University, The Netherlands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacob de Boer, Peter Korytár, Pim Leonards, Stefan van Leeuwen</td>
<td>Udo Brinkman, Maria Kristenson, René Vreuls</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Umeå University, Sweden</th>
<th>University of Bordeaux, France</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conny Danielsson, Peter Haglund, Mikael Harju, Karin Wiberg</td>
<td>Hélène Budzinski, Ana Blanc</td>
</tr>
</tbody>
</table>

| IQS, Barcelona, Spain | |
|----------------------||
| Jordi Díaz-Ferrero | |
Principles of GCxGC

Selection of proper column combination

Selection of modulator
- 5 different modulators:
  - SWEEPER
  - LMCS
  - Quad N\textsubscript{2}(l) jet
  - Dual CO\textsubscript{2} jet
  - Loop CO\textsubscript{2}

1\textsuperscript{st} column

Modulator
Cryogenic modulation

1. Trapping

2. Release

3. Trapping and Separation

carrier gas

CO₂

1st column

2nd column

stationary phase 1

stationary phase 2
How does GCxGC work?
How does GCxGC work?
How does GCxGC work?
How does GCxGC work?
2D plots
Cod liver: WHO-PCB separation

DB1 x HT-8

1st dimension retention time [min]

2nd dimension retention time [s]
Milk: PCB fraction (DB-XLB x LC-50)
Milk: Dioxins (DB-XLB x LC-50)

2nd dimension retention time [s]

1st dimension retention time [min]
Improved clean-up and solvent grade
Sewage sludge with improved clean-up

1st dimension retention time [min]

2nd dimension retention time [s]
Modulator comparison and column selection

- Cryogenic modulation with CO$_2$ had best performance
- Most suitable column combinations:
  - DB-XLB x LC50
  - DB-1 x 90% cyanopropyl
  - HT5 x BPX 50
- Other phases less suitable because of:
  - high background levels (bleeding of column)
  - not all critical congener pairs (with different TEF values) could be separated from each other
Integration and identification example: 23478-PeCDF

Standard

Sample
Accuracy

Deviation from GC-HRMS

DIAC 1

DIAC 2

DIAC 3

DIFF

CERT

-70% -60% -50% -40% -30% -20% -10% 0% 10% 20% 30%

Pork fat  Hake  Salmon  Tuna  Trout  Cod  Spiked milk  Spiked milk 2  Fly ash  Milk  Feedingstuff  Feedingstuff 2  Sewage sludge 2  Sediment  Herring oil  Spiked milk  Vegetable oil  Eel  Compound feed  Fish oil
Conclusions GCxGC-ECD

- High selectivity
- High sensitivity but:
  - Multi clean-up/fractionation steps are needed
- Integration of peaks is time-consuming
  - various retention time markers in GCxGC plane
- Improved software requirement
GC-LRMS/MS

- GC-ITMS/MS (GCQ/Polaris)
- MS/MS mode
- Electron impact (EI)

Department of Analytical Chemistry
University of Barcelona
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Mass Spectrometry-Dioxin Laboratory
Department of Ecotechnologies
IIQAB-CSIC, Barcelona (Spain)

M. Ábalos
E. Abad
J. Rivera
Clean Fish Extract: Dioxins

**m/z 339.9 → m/z 274.9+276.9**

1,2,3,7,8-PeCDF

**m/z 351.9 → m/z 285.9+287.9**

13C12-1,2,3,7,8-PeCDF

**m/z 355.9 → m/z 290.9+292.9**

1,2,3,7,8-PeCDD

**m/z 367.9 → m/z 301.9+303.9**

13C12-1,2,3,7,8-PeCDD
Fish oil: HRMS vs. LRMS/MS

PCDD/Fs

Concentration (pg/g)

0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

Fish oil (Herring)

2,3,7,8-TCDD
1,2,3,7,8-PeCDD
1,2,3,4,7,8-HxCDD
1,2,3,6,7,8-HxCDD
1,2,3,7,8,9-HxCDD
1,2,3,4,6,7,8-HpCDD
1,2,3,4,6,7,8,9-OCDD

1,2,3,7,8-PeCDF
2,3,4,7,8-PeCDF
1,2,3,4,6,7,8-HxCDF
1,2,3,6,7,8-HxCDF
1,2,3,7,8,9-HxCDF
1,2,3,4,6,7,8,9-HpCDF
1,2,3,4,7,8,9-OCDF

2,3,7,8-DF
1,2,3,7,8-PeCDF
1,2,3,4,7,8-HxCDF
1,2,3,7,8,9-HxCDF
1,2,3,4,6,7,8-HpCDF
1,2,3,4,6,7,8,9-OCDF

Concentration (pg/g)

GC-HRMS
GC-ITMS/MS
Problems with clean-up

Insufficient clean-up

Suitable clean-up

Hexa-mono-ortho-PCBs
Conclusions GC-ITMS/MS

- Low detection limits and high selectivity
- Appropriate clean-up and fractionation method needed
- Further studies needed in order to prove the general applicability of the GC-ITMS/MS for the analysis of PCDD/Fs and dioxin-like PCBs
DR-CALUX studies


- **Vrije Universiteit Brussel**, Baeyens W., Sanctorum H. and C. Schroijen

- **Université de Liège**, De Pauw E., Eppe G. and M. Scipio

- **Federal Agency for Safety of the Food Chain**, Behets S., Fontaine A. and H. Vanderperren

- **Flemish Institute for Technological Research**, Koppen G., Schoeters G. and R. Van Cleuvenbergen

- **RIKILT – Institute of Food Safety**, Bovee T., Hoogenboom R. and W. Traag

- **Xenobiotic Detection Systems**, Brown D., Chu M., Clark G. and Gordon J.
DR-CALUX assay
CALUX activity

- Cells respond to all compounds of the sample extract that activate the AhR (dioxin-like activity)

  both the solvent and the sample contaminants

- Total dioxin-like activity (Total TEQ of a sample)
- Separation of dioxins and PCBs from many other compounds
Accuracy of CALUX results

**DIOXIN fraction**

- Calux
- GC-HRMS

**PCB fraction**

- Calux
- GC-HRMS
REP ≠ TEF

DIOXIN fraction

PCB fraction

- CALUX-meas
- WHO-TEF
- CALUX-REP

0 1 2 3 4 5

0 1 2 3 4 5

10 9 8 7 6 5 4 3 2 1 0

10 9 8 7 6 5 4 3 2 1 0
DR-Calux vs GC-HRMS in fish oil

**DIOXIN fraction**

\[ y = 2.0943x + 1.6936 \]

\[ R^2 = 0.9519 \]

**PCB fraction**

\[ y = 0.0658x + 0.9952 \]

\[ R^2 = 0.7287 \]
Conclusions DR-CALUX

- High sensitivity
- Accuracy is lower
- Fast and cheap method
- Screening method
Pressurized liquid extraction (PLE)

- PLE extraction: Dionex ASE 200 and ASE 300
- Non-selective ASE with external clean-up
- Selective ASE with on-line clean-up
  - Sulphuric acid silica
  - Integrated carbon fractionation

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Geel
Belgium

Peter Haglund,
Umeå University
Umeå
Sweden

Erland Björklund
Lund University
Lund
Sweden
Selective PLE fat retainer

- \( \text{H}_2\text{SO}_4/\text{Silica gel} \)
- Florisil
- Basic alumina
- Neutral alumina
- Acidic alumina

Filter

SFE support

Matrix / Na\(_2\)SO\(_4\) / Sand

Filter

Fat Retainer

Na\(_2\)SO\(_4\)

PCBs (+ Fat)

Björklund, Müller, von Holst, Anal. Chem. 2001, 73, 4050
Sporring, Björklund, J. Chrom. A 2004, 1040, 155
<table>
<thead>
<tr>
<th>Fat retainers</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ Ratio fat/fat retainer: 0.025</td>
</tr>
<tr>
<td>■ Recovery about 100%</td>
</tr>
<tr>
<td>■ Coextracted fat: 500 mg fat, 1-3 mg fat left</td>
</tr>
<tr>
<td>■ Colour: clear florisil and sulphuric acid silica only</td>
</tr>
<tr>
<td>■ Reaction with H2SO4: No reaction with sulphuric acid silica</td>
</tr>
</tbody>
</table>

Conclusion sulphuric acid silica preferred
Clean-up of fat using selective ASE

ASE300, 34mL cells, triglycerides 0,5g (n=3, s.e.m.)

Retained fat (%)

Pentane
Hexane
Heptane

100°C    50°C 100°C    50°C 100°C    50°C100°C    50°C
5-20g silica

Fat / fat retainer ratio (FFR)

Sporring, Björklund, J. Chrom. A 2004, 1040, 155
Traditional extraction/clean-up vs. PLE

**Vegetable oil**

- Lab A: 3 pgTEQ/g Oil
- Lab B: 2 pgTEQ/g Oil
- PLE: 4 pgTEQ/g Oil

**Fish oil (n=6)**

- Lab A: 6 pgTEQ/g Oil
- Lab B: 5 pgTEQ/g Oil
- PLE: 7 pgTEQ/g Oil

Legend:
- PCDD/F
- PCB
- Total-TEQ
Individual congeners

Vegetable Oil (pg/ g oil)

Fish Oil (pg/ g oil)

Lab A  Lab B  PLE
Selective PLE with carbon

- Dionex ASE200; 33 mL cell
- 3g Fish oil mixed in Na$_2$SO$_4$
- “Normal” PLE parameters
- Three consecutive extractions (fractions)
Integrated carbon fractionation of PCBs and Dioxins

1. Heptane
2. Heptane:DCM (1:1)
3. Toluene

- Bulk PCBs
- Mono-ortho-PCBs
- Non-ortho-PCBs and PCDD/Fs
Elution profile integrated carbon fractionation

Fish oil

<table>
<thead>
<tr>
<th>Fat recovery (%)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>98.7</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.7</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

1. Heptane
2A. DCM/heptane (1:1)
2B. Acetone/heptane (2.5:1)
3. Toluene
Conclusions PLE

- Fast method
- Integrated carbon PLE cost efficient method
- Less labour intensive than traditional method
- Attractive alternative for traditional dioxin method
## Validation studies

<table>
<thead>
<tr>
<th>VITO (Flemish Institute for Technological Research) R. Van Cleuvenbergen</th>
<th>RIVO S. Van Leeuwen, J. de Boer</th>
</tr>
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<tr>
<td>Other partners from DIFFERENCE and participants outside DIFFERENCE</td>
<td></td>
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<tr>
<td>Method validation – Interlaboratory studies</td>
<td></td>
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<tr>
<td>---------------------------------------------</td>
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<tr>
<td>Verification of calibration curves</td>
<td></td>
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<tr>
<td>Verification of analytical process</td>
<td></td>
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<tr>
<td>Verification of matrix effects during quantification</td>
<td></td>
</tr>
<tr>
<td>Repeatability, within &amp; between lab reproducibility (ISO 5725)</td>
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<tr>
<td>Detection Capability</td>
<td></td>
</tr>
<tr>
<td>Selectivity</td>
<td></td>
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<tr>
<td>Ruggedness</td>
<td></td>
</tr>
<tr>
<td>Standards, quality control solution, clean fish extract</td>
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</tr>
<tr>
<td>Vegetable oils with spikes of PCBs, PCNs, PCDE’s</td>
<td></td>
</tr>
<tr>
<td>Fish oil, milk, vegetable oil, vegetable oils with spikes, cereal based feed, chicken, vegetable feed, egg, fish, pork</td>
<td></td>
</tr>
</tbody>
</table>
DIFFERENCE: (Candidate) CRMs for Dioxin Analysis
Spiked Milk

CALUX: signal suppression due to high spike of mono-ortho CB-118?

GCxGC: overestimation due to combination LOQ and reporting upperbounds
Precision (%) for spiked vegetable oil

**Within-Lab Reproducibility**

- **GC-HRMS**
- **CALUX**
- **GC-LRMS**
- **GCxGC-ECD**

Maximum residue level
---
Precision requirement
<table>
<thead>
<tr>
<th>Technique</th>
<th>Sens.</th>
<th>Accur.</th>
<th>Precis.</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-HRMS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Confirmatory method</td>
</tr>
<tr>
<td>GC-LRMS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Potential alternative for HRMS?</td>
</tr>
<tr>
<td>GCxGC-ECD</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>Improved software required</td>
</tr>
<tr>
<td>CALUX</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>Screening technique</td>
</tr>
</tbody>
</table>

## Conclusions

- GCxGC-ECD (and GCxGC-ToF-MS), and GC-ion-trap MS/MS may serve as alternative (routine) methods for dioxin analysis.

- CALUX is the alternative for times of crisis, but corrections for recovery are essential.

- The use of PLE will significantly reduce the extraction and clean-up time.
Acknowledgement

All DIFFERENCE and DIAC partners

Thank you for your attention

www.dioxins.nl