“Best Practices for Bioassay Testing of Food and other Complex Mixtures”

Summary of Amsterdam Workshop

Dr. Thomas Gude

AOAC Europe Section

- **Our Mission**
  - AOAC Europe brings together government, industry, and academia to establish standard methods of analysis that ensure the safety and integrity of foods and other products that impact public health around the world
- **What you do not know**
  - Start in 1983
  - As a member of AOAC International you are automatically a member of AOAC Europe section – when living/working in Europe
- **What you should know**
  - We are organizing regularly meetings together with other European Societies
  - We are organizing workshops:
    - Nov 2023: Bioassay
    - Q3 2024: Non-Target Methods
- **Please check: https://aoaceurope.com/**

The Amsterdam Meeting

What we like to achieve:

- In Amsterdam:

- **Awareness on the topic**
- **Commitment for fruitful discussions**
- **An initial working plan, which topics needs to be addressed**

- Today:

- **Working group(s) covering sub-questions**
- **Harmonized Guideline/Method on “Best Practices for Bioassay Testing of Food and other Complex Mixtures”**

Today's Program

05:00 PM General Introduction by AOAC Europe and Workshop details

05:10 PM Summary of Talks given in Amsterdam

1: Prof. Beate Escher – Presenter Georg Braun
Title presentation: QA/QC during applications of *in vitro* assay for food monitoring

2: Prof. Bennard van Ravenzwaay – Presenter Maricel Marin-Kuan
Title presentation: The use of metabolomics as a New Approach Methodology (NAM) in the context of the transition from *in vivo* to *in vitro* methods

3: Dr. Ir. Toine Bovee – Presenter Maricel Marin-Kuan
Title presentation: Development, validation and application of bioassays: their added value.

4: Dr. Peter A. Behnisch – Presenter Peter Behnisch
Title presentation: Plastic and plastic additives testing by effect-based bioanalysis for endocrine disrupting chemicals – a CRO perspectives

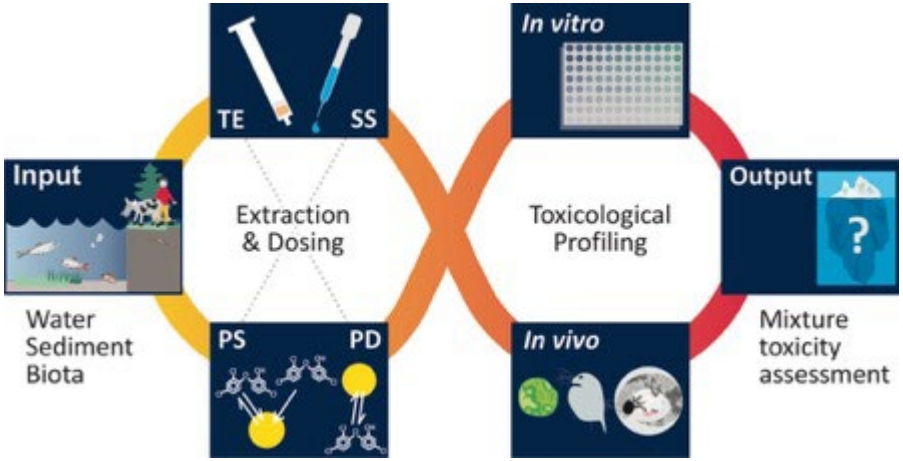
05:30 PM Summary of Break Out Groups Amsterdam meeting– Presenter Thomas Gude

05:45 PM AOAC Bioassays Working group proposal: Guidelines on Best Practices for Bioassay Testing of Food and other Complex Mixtures (Presenter Maricel Marin-Kuan)

06:15 PM AOAC International and Bioassays working group meeting (Presenter Kate Mastovska)

06:30 PM End

QA/QC during applications of *in vitro* assay for food monitoring



Beate I. Escher and Georg Braun

Cell Toxicology, Helmholtz Centre for Environmental Research – UFZ, Germany
Eberhard Karls University Tübingen, Germany

Jahnke, A., Mayer, P., Schaefer, S., Witt, G., Haase, N. and Escher, B.I. (2016). Strategies for Transferring Mixtures of Organic Contaminants from Aquatic Environments into Bioassays. *ES&T*, **50:** 5424-5431

QA/QC for application for bioassays for food monitoring

Not all bioassays that work for single chemicals are **amendable** to testing of **extracts**

- **Coextracted matrix and endogenous compounds** might interfere with the bioassay
    - **Controls:** Processing blanks, effect recovery of spiked samples
- The more difficult the sample the better must be the bioassay quality
- **Extraction methods crucial** (poor sample extract – poor bioassay result)

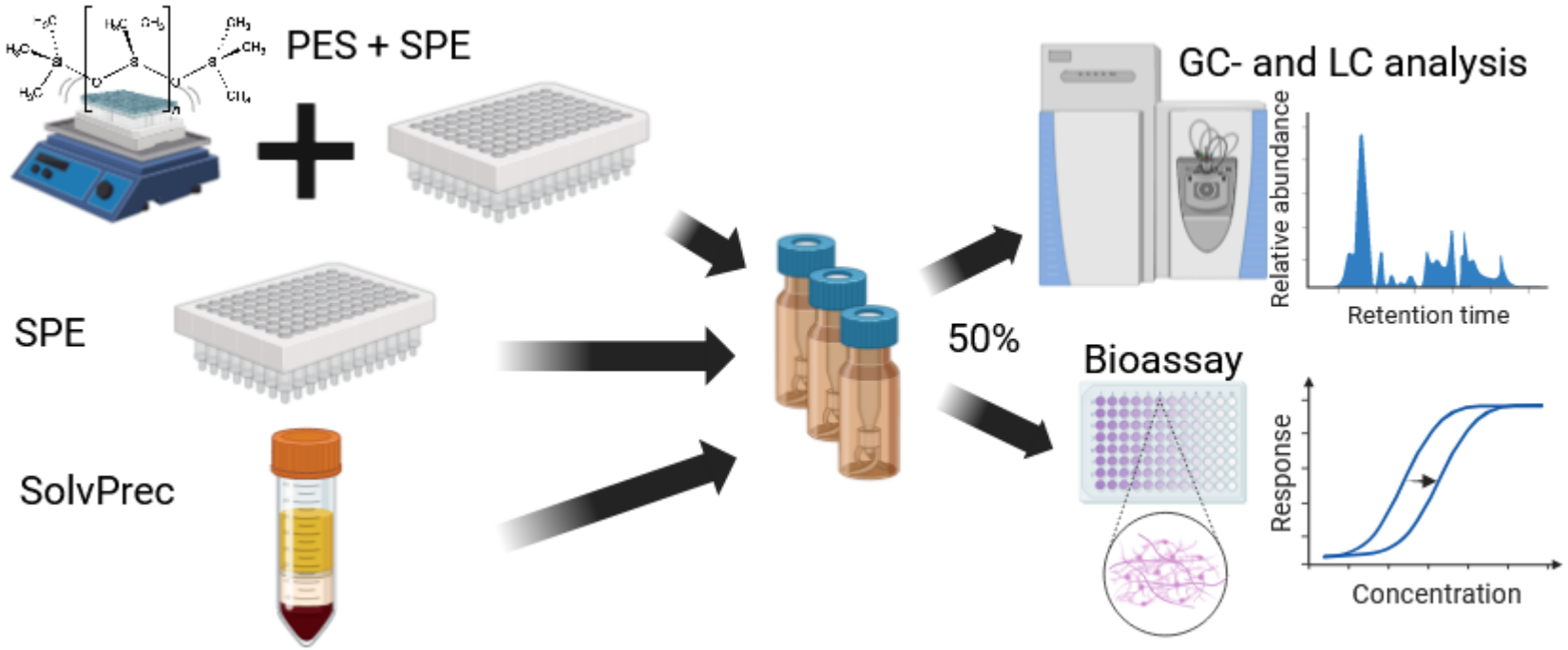
Extraction must be **unbiased and independent on the physicochemical properties** of the chemical mixtures to assure that the dosed mixture is representative of the sample

- Extraction method should show commonalities for liquid and condensed phases
- Coextracted matrix (= lipids, proteins) must be minimised
- Internal standards for extraction recovery not possible: independent recovery experiments necessary

Escher B, Neale P, Leusch F. 2021. *Bioanalytical tools in water quality assessment, second edition, www.iwapublishing.com/books/9781789061970/bioanalytical-tools-water-quality-assessment-2nd-edition*. IWA Publishing, London, UK.

Mixtures of spiked chemicals extracted from blood plasma

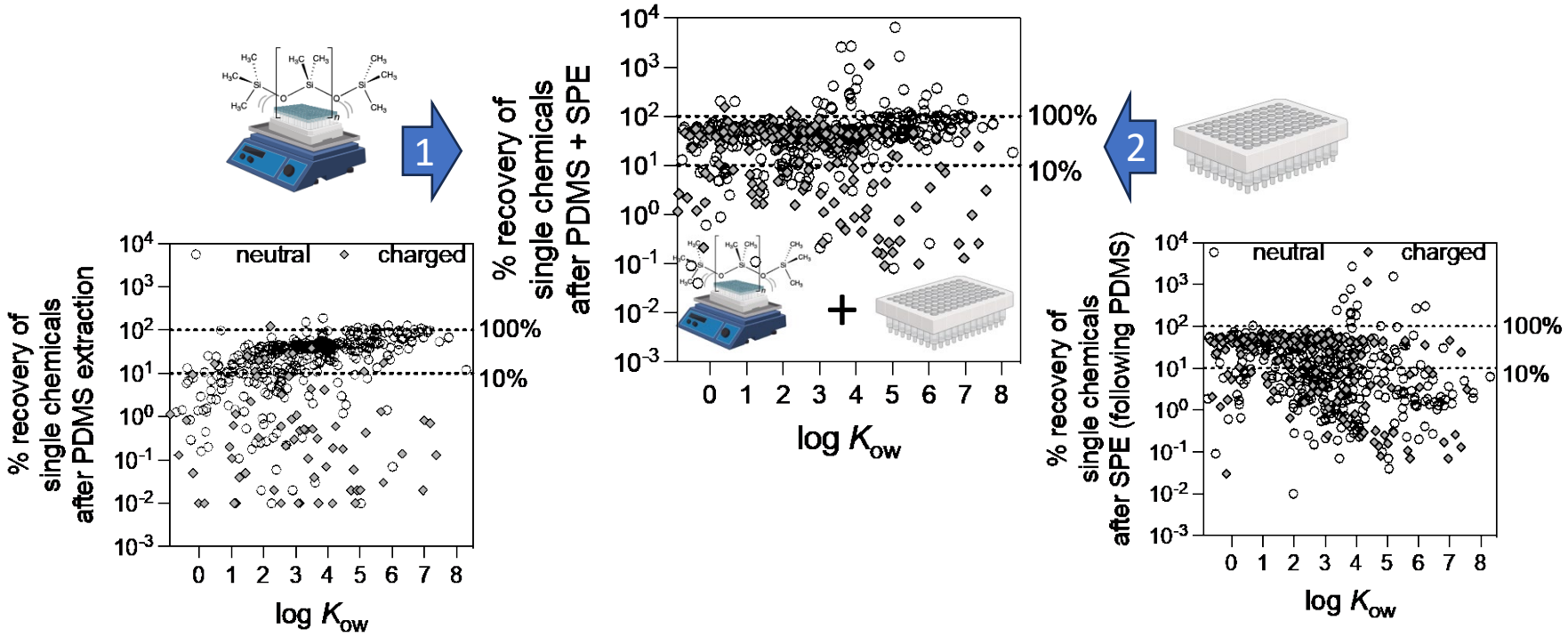
- Blood, plasma, serum, food matrices like cow milk or plant-based milk are protein-rich with water content
- Typical used solid-phase extraction neglects the highly bioactive hydrophobic chemicals
- Comparison of recovery of chemicals (n=382) between PDMS and PDMS followed by SPE: good recovery of 2-step method



Braun et al. 2023, ES&T 10.1021/acs.est.3c05962

Recovery of mixtures of chemicals extracted from blood plasma

Two-step method achieves best recovery for all chemicals with exception of cations



Braun et al. 2023, ES&T, 10.1021/acs.est.3c05962

Metabolomics from in vivo to in vitro

Prof. Dr. Bennard van Ravenzwaay

Mode of action determination

> 110 MoA patterns established

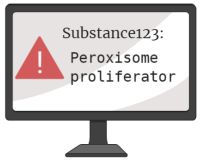
Compound Metabolome Comparison

Ranking of (new) compound's metabolome against metabolome of 1000 substances

Biochemical pathway analysis

Identify molecular mechanisms of toxicity

Predictivity rate: 80%

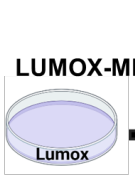


Sensitivity: as classical toxicology

MetaMap®Tox: an in vivo success story

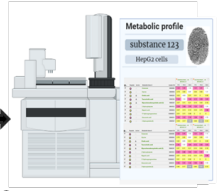
van Ravenzwaay et al Tox Letters, 172, 2007

Metabolomics *in vitro works….* *Ramirez, T. et al. 2018, Archives of Toxicology, 92 ….* ***but***

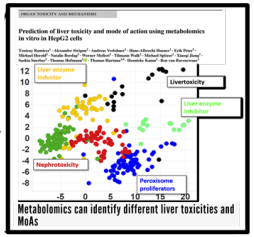


HepG2 cells

Substance exposure

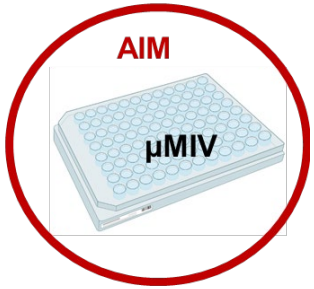


Metabolic profile



MoA prediction

Not economically feasible



Miniaturization of Metabolomics in vitro

Prof. Dr. Bennard van Ravenzwaay

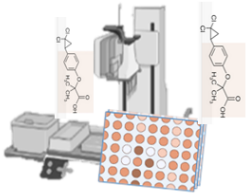
Cell seeding density

15000 HepG2 cells/well
Robust metabolic signal

Cytotoxicity Testing

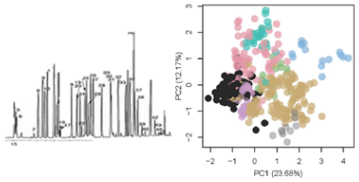
Multiplexed: ATP +
Membrane integrity

Quenching and Extraction



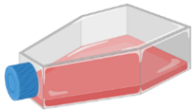
Directly in the 96 well-plate
Minimal perturbations

Data Analysis



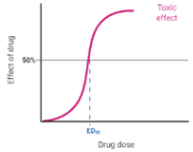
QA process and statistics

Passage Number



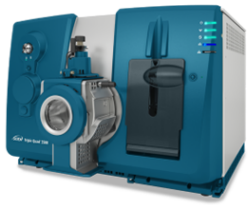
From 5 to 9
Flexibility + Robustness

Dose Setting



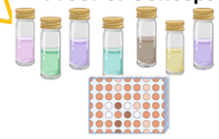
5 dose levels : EC_1,EC_5,EC_{15}, EC_{50}, EC_{85}
Metabolomics dose response

Analytical Method



Target LC-MS/MS
Good metabolite coverage

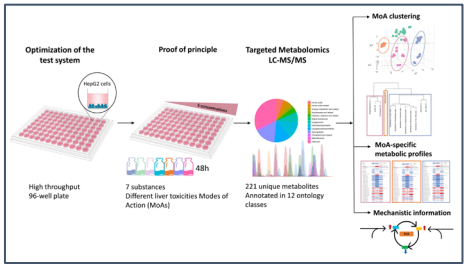
Proof of Concept



Different Liver Toxicity MoA

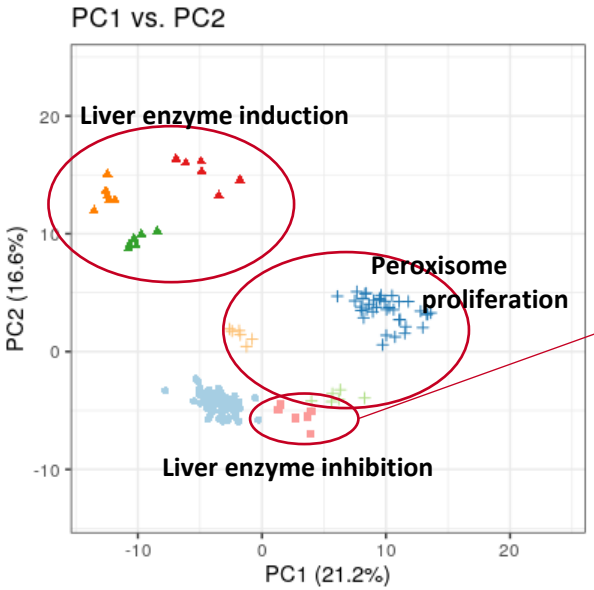
Proof of principle of the µMIV platform – µMIV distinguishs different Mode of Actions

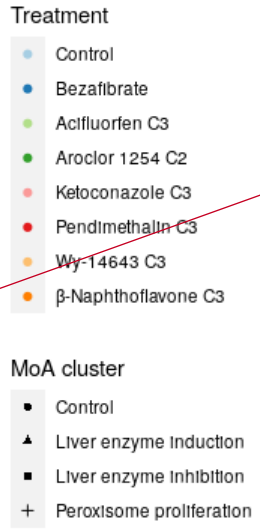
µMIV identifies different toxicological Mode of Actions

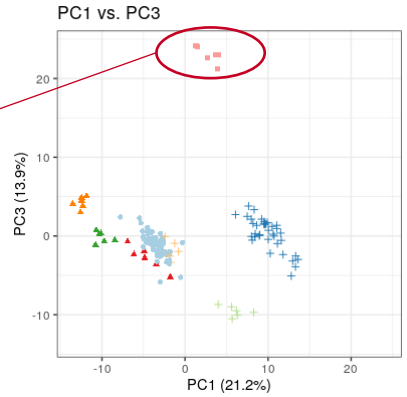


substances testing

Different MoA for liver toxicity



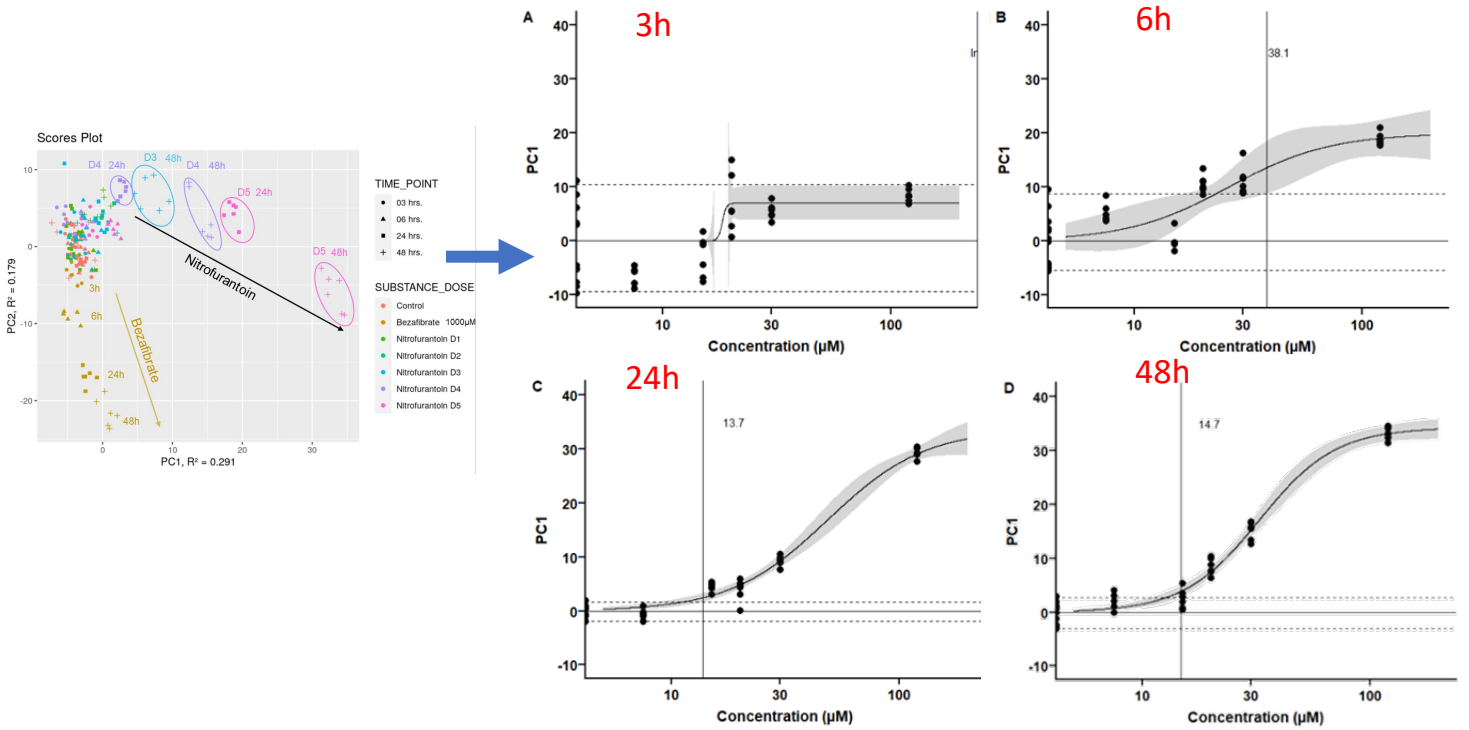




MoA = Mode of action
µMIV = miniaturization of metabolomics in vitro

Ramirez-Hincapie, S. et al. 2023, Cell biology and toxicology, open access

Point of departure (PoD) derivation from µMIV data at different time points (3h, 6h, 24h and 48h)



Ramirez-Hincapie et al. 2023, Archives of Toxicology, 97, open access

PoD = Point of departure
µMIV = miniaturization of metabolomics *in vitro*

The POD at earlier time points is higher (less „toxic“)

At 24h and 48h the POD approaches a similar value.

Expsoure time play a role for PoD derivation.

Reduction, Refinement, Replacement

Metabolomics in vivo and in vitro: an overview of applications

1) Screening: early detection of „bad“ compounds	Tox. Letters, 172, 2007
2) Identification of molecular mechanisms	Tox. Letters, 225, 2014
3) Chemical grouping and read across	Mut. Res. 746, 2012, Reg. Tox. Parm. 81, 2016
4) Smart Studies: include omics data in regulatory testing	Arch. Tox. 96, 2022
5) In vitro metabolomics (MIV)	Arch. Tox., 92, 2018
6) µMIV: hazard and MoA identification	Cell biol. Tox. 2023
7) µMIV: dose and time response, PoD	Arch. Tox., 97, 2023

Take-home messages:

- ✓ **Growing demonstration of feasibility for in vitro application**
- ✓ **Multiple mode of action can be identified with one test**
- ✓ **This technology allows for determination of point of departure**

Development, validation and application of bioassays: their added value

Toine Bovee
November 3, 2023
Amsterdam

Bioassays for food control, there are many:

1) **Cell based effect bioassays**, e.g. for the detection of antibiotics, dioxins and PCBs, steroid hormones, marine toxins, lectins, and mutagenic compounds
2) **Enzyme based "effect bioassays"**, e.g. for the detection of microcystins, statins, NSAIDs, pesticides and Viagra-like compounds
3) **Other bio-based methods**, e.g. TTR, TBG, and β2-adrenoceptor competitive binding assays, or based on a whole organism, like the DapTox test with Daphnia

- Once developed, and proven to work with academic standards, these **assays need to be made applicable for a certain matrix**. I.e., methods need to be developed for e.g. animal feed, supplements, water, milk, egg, meat, urine, mussels, fish, hair and feathers.
- After method development for a certain matrix or several matrices, these **methods need to be validated and accredited before they can be used in routine monitoring for food control purposes with legal strength**.

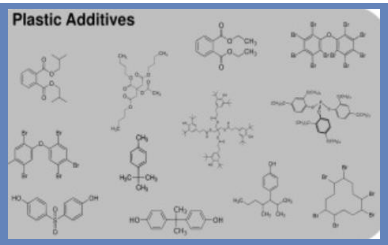
Bioassays in food control, their added value

- Mass spectrometric analysis is often targeted and of great strength for enforcement. However, even untargeted MS methods are most often not able to detect unknown bioactives. Bioassays, and especially the cell-based effect assays, detect both the known contaminants/toxins and the yet unknown toxins (unknown bio-actives). The latter is an added value for food safety control. There are many examples where bioassay analysis has shown this added value compared to MS analysis only. E.g.:
  - detection of estradiol in animal feed (with the yeast estrogen bioassay)
  - detection of DES in human supplement (with the yeast estrogen bioassay)
  - detection of 1-testosterone, 4-androstenediol and 5-androstanediol in human supplements (with the yeast androgen bioassay)
  - detection of THG (tetrahydrogestrinone) in human urine (with the yeast androgen bioassay)
  - detection of TBDF (tetrabromodibenzofuran) in animal feed, egg, and chicken meat (with the DR-CALUX bioassay)

Bioassays in food control, their added value

- detection of Cl-PAH in chlorinated paraffins (with the DR-CALUX bioassay)
- detection of dexamethasone in human supplement (with the yeast (gluco)corticoid bioassay and GR-CALUX bioassay)
- detection of ciguatoxin in fish (with the neuro-2a bioassay)
- detection of sildenafil analogues in human supplements (with the PDE-5 enzyme inhibition assay)
- detection of isopropyloctopamine in human supplement (with the competitive β-adrenoceptor binding assay)

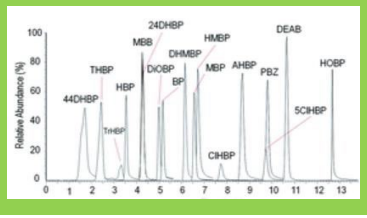
Thank you for your attention!

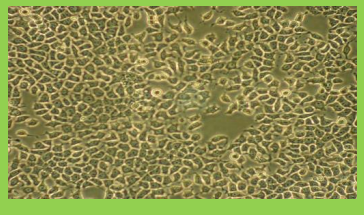


Plastic, water and chemicals testing by non-animal methods (NAMs) – a CRO's perspective

Peter A. Behnisch and Harrie Besselink

BioDetection Systems, Amsterdam, The Netherlands





Unknown amount of unknown chemicals with unknown in vitro toxicity profiling – Urgent steps needed…(UN, June 2023)

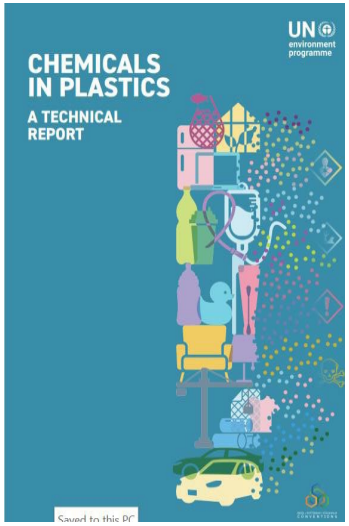
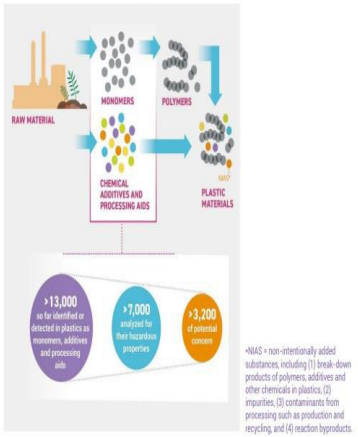


Figure 3. A simplified illustration of the production of plastics from raw materials, and an overview of chemicals that may have been used in plastic production or have been detected in plastics.



Chemicals of concern in plastics are found across various sectors and products value chains

Table 4. **A selection of *in vitro* bioassays that have been used in NIAS research** (Koster *et al.* 2016).

Potential endocrine activity	Cytotoxicity	Genotoxicity/potential carcinogenicity
Oestrogen receptor (ER) redistribution Androgen receptor (AR) redistribution	Cell Organelle Health (COH); end points: DNA content, cytochrome C, mitochondrial membrane potential, RNA synthesis kinetic inhibition.	Indicator assays for genotoxicity (PARP, GADD45,...), Comet-FPG assay
Transcriptional activation assay Oestrogen receptor (ER) (anti) Androgen receptor (AR) Glucocorticoid receptor (GR) Progesterone receptor (PR) Thyroid recepted (TR) Peroxisome Proliferator Activated Receptor (PPARγ)	Cell Proliferation and Cell Death (CPD); end points: apoptosis – caspase3, p53; DNA content, DNA proliferation – BrdU	Mutagenicity test (Ames test, mammalian cell gene mutation tests, micronucleus (MN) test) Potential carcinogenicity Cell Transformation Assay (detection of both geno- and non-genotoxic carcinogens)
H295R Steroidogenesis assay (changes in hormone production)		

SOLUTIONS to move FORWARD

Update regulatory testing guidelines, for instance by including rapid and cost-effective approaches such as bioassays and computational tools. This serves as a prerequisite for research to fill existing knowledge gaps, e.g., regarding the mixture toxicity of leachates from plastics or the toxicity and bioaccumulation potential of hydrophobic chemicals.

One option to assess the overall toxicity of chemicals released from plastic products is to test the overall extract/leachate using *in vitro* bioassays, which are rapid and cost-effective (Groh and Muncke 2017), and use other screening technologies that measure relevant toxicological effects such as cytotoxicity, genotoxicity and endocrine effects (Table 4) (Koster et al. 2016). The AOP approach (see section 4.1.2) can

Known vs Unknown Chemicals – Urgent steps needed…

Muncke et al. Environmental Health (2020) 19:25
https://doi.org/10.1186/s12940-020-0572-5

Environmental Health

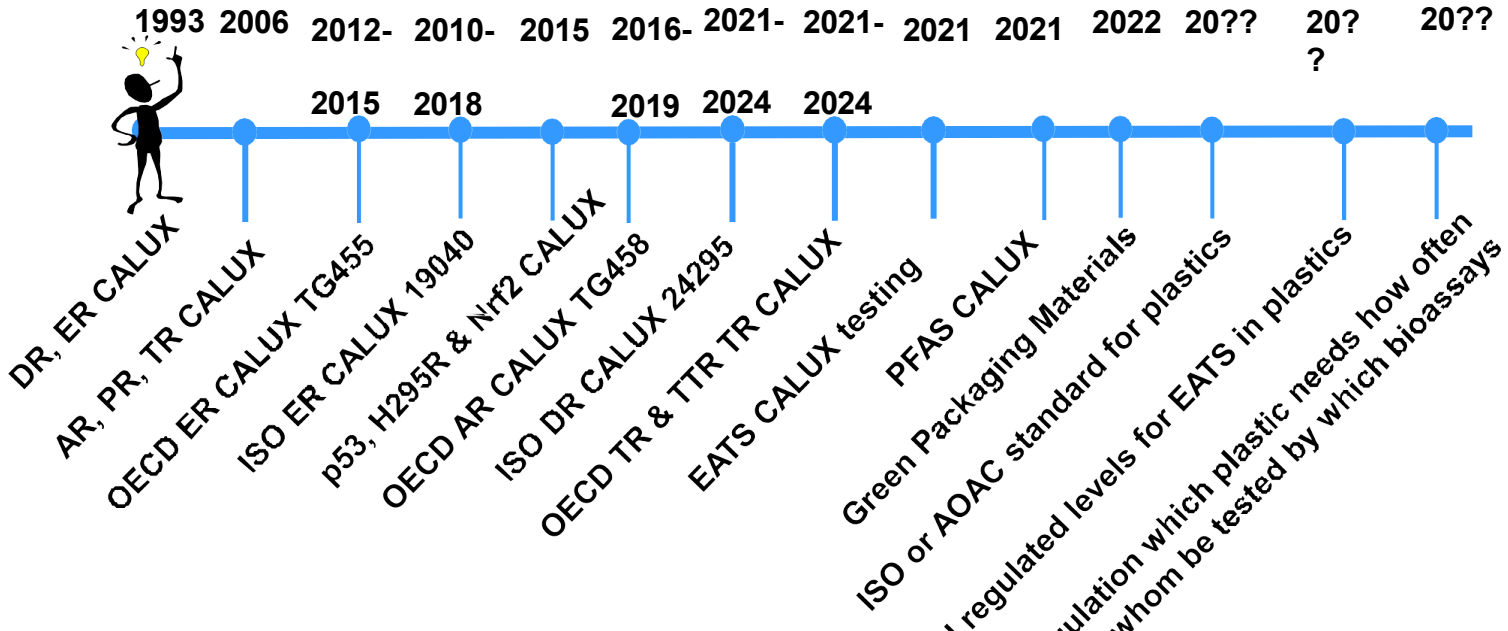
COMMENTARY Open Access

Impacts of food contact chemicals on human health: a consensus statement

Jane Muncke[1*], Anna-Maria Andersson[2], Thomas Backhaus[3], Justin M. Boucher[4], Bethanie Carney Almroth[3], Arturo Castillo Castillo[5], Jonathan Chevrier[6], Barbara A. Demeneix[7], Jorge A. Emmanuel[8], Jean-Baptiste Fini[7], David Gee[9], Birgit Geueke[1], Ksenia Groh[1], Jerrold J. Heindel[10], Jane Houlihan[11], Christopher D. Kassotis[12], Carol F. Kwiatkowski[13], Lisa Y. Lefferts[14], Maricel V. Maffini[15], Olwenn V. Martin[16], John Peterson Myers[17,18], Angel Nadal[19], Cristina Nerin[20], Katherine E. Pelch[13], Seth Rojello Fernández[21], Robert M. Sargis[22], Ana M. Soto[23], Leonardo Trasande[24], Laura N. Vandenberg[25], Martin Wagner[26], Changqing Wu[27], R. Thomas Zoeller[28] and Martin Scheringer[4,29]

- **Toxicity** and exposure information is available **only for few of the IAS/NIAS**
- Risk assessment **of unknown chemicals is not possible** under the current regulatory approach
- **Modernize tiered approach for screening** and prioritization
- Addressing **mixture toxicity**
- Modernizing risk assessment by including **endocrine disruption**

Key milestones of bioassays (NAMs) for developing guidance docs (e.g., for plastic)



- list of pollutants get larger & larger
- list of relevant in vitro toxicity endpoints get longer & longer
- list of the be tested materials get larger & larger

⇨ standardization process for EDCs in plastic materials needs more attention!

Endocrine Disrupting Chemicals by EATS-NAMs testing
Typical case study

Analysis of compounds suspect of endocrine disruption (EDC) on a dedicated EATS-panel:
(anti-) Estrogenicity
(anti-) Androgenicity
(anti-) Thyroidogenicity
TTR binding
Thyroid peroxidase (hTPO) inhibition
Steroidogenesis (H295R)

Read-across and new approach methodologies applied in a 10-step framework for cosmetics safety assessment – A case study with parabens

Gladys Ouedraogo [a], Camilla Alexander-White [b], Dagmar Bury [c], Harvey J. Clewell III [d], Mark Cronin [e], Tom Cull [f], Matthew Dent [f], Bertrand Desprez [g], Ann Detroyer [a], Corie Ellison [h], Stefania Giammanco [i], Eric Hack [j], Nicola J. Hewitt [k], Gerry Kenna [l], Martina Klaric [g], Reinhard Kreiling [m], Cathy Lester [n], Catherine Mahony [n], Enrico Mombelli [o], Jorge Naciff [h], John O'Brien [i], Andreas Schepky [p], Sarah Tozer [n], Bart van der Burg [q], Barbara van Vugt-Lussenburg [q], Sharon Stuard [h], Cosmetics Europe [g,*]

[q] BDS, Science Park 406, 1098XH, Amsterdam, the Netherlands

activity

Table 11
Summary of EATS testing results. PC10 (for agonistic tests)/PC20 (for antagonistic tests) values are shown in -Log M; the color indicates the potency (yellow < orange < red).

Step 7b. *Biokinetic refinement*

End point	MP		EP		PP		BP		pHBA	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Cytotoxicity	>	>	>	>	-3.5	>	-4.0	-3.0	>	>
(Anti-) estrogenic and (anti-) androgenic assays										
ER α CALUX	-4.5	-4.2	-5.5	-4.8	-6.0	-5.1	-6.0	-5.0	>	>
anti-ER α CALUX	>	>	>	>	>	>	>	>	>	>
AR CALUX	>	>	>	>	>	>	>	>	>	>
anti-AR CALUX	-4.9	-4.7	-4.7	-4.4	-4.5	-4.2	-4.9	-4.3	>	>
Thyroidogenic assays										
TR β CALUX	>	>	>	>	>	>	>	>	>	>
anti-TR β CALUX	-3.0	>	>	>	>	>	>	>	>	>
TTR	-2.7	nd	-4.8	nd	-4.5	nd	-4.8	nd	>	nd
hTPO	-2.0	nd	>	nd	>	nd	>	nd	-3.0	nd
Steroidogenesis										
H295R-E2	-5.0	nd	-5.0	nd	-5.0	nd	-5.0	nd	-3.0	nd
H295R-T	>	nd	-4.0	nd	-4.0	nd	>	nd	>	nd

Take home messages

- Since more than 30 years it is known that many plastic additives have hormone- like activities and can be easily picked-up by in vitro bioassays.
- Development of international standards for such in vitro bioassays for endocrine disrupting chemicals are very slowly and have now been finalised for compounds testing (OECD TG 455, 456, 458), for some environmental matrices (ER and DR for water), but not for any materials (such as packaging materials)
- Many published studies show that a panel of bioassays is a safe approach for a greener & sustainable future of plastics & other packaging materials.
- Our studies applied on plastics, water and food matrices shows that they can be easily adopted to many kinds of in vitro toxicity endpoints.
- Now it is urgently needed to step forward to bridge the single chemical compounds testing with the effect-based in vitro biological analysis steps of such complex mixtures of all kinds of known and unknown chemical & toxicological properties.

Summary of Break Out Groups

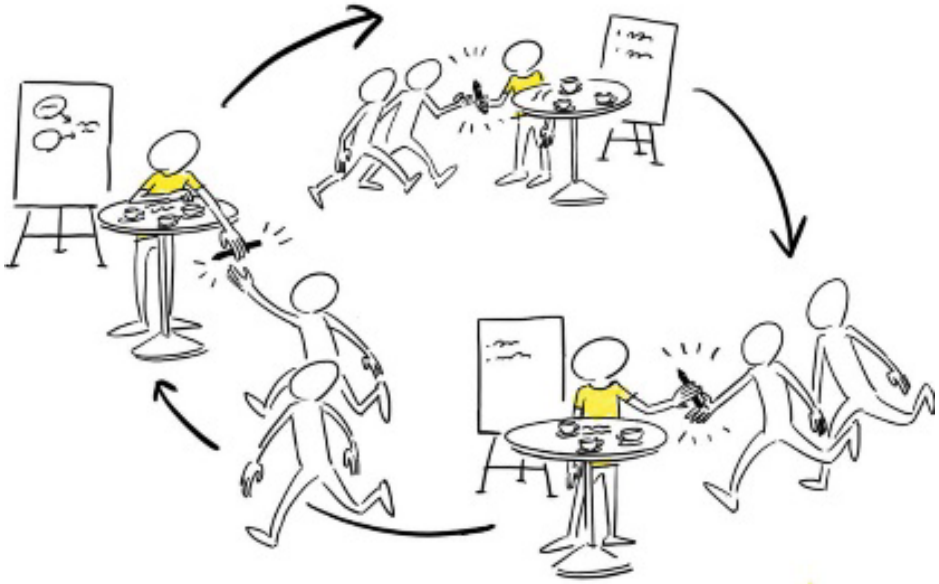
All Participants could join in the “world caffee format” the following 4 stations:

Group 1: OECD Guidance Document on Good In Vitro Method Practices (GIVIMP) use

Group 2: Current extraction practices

Group 3: Critical factors for accurate testing

Group 4: Key topics for future WGs



Group 1: OECD Guidance Document on Good In Vitro Method Practices (GIVIMP) use

Question: Do you apply the OECD Guidance Document on Good In Vitro Method Practices (GIVIMP) and do you consider those as appropriate for complex mixtures? Why are or not appropriate? Other guidelines available to recommend?

Feedback:

- Documentation, standardization and guidelines are important
- Use of control samples
- Validation is slow and costly, but important
- Before the official validation, there should be a platform, where knowledge sharing is facilitated
- Resources (financial, intellectual)
- Single compounds versus mixtures
- More complex
- Chemical analysis and extraction documentation are key

Group 2: Current extraction practices

Question: What consideration are you taking when extracting mixtures from water, food, food contact materials and other items to test with bioassays? (What is best practice and limitation of current approach?)

Feedback:

- Design of controls and blanks
- Consider the behavior of mixtures in your controls (mix instead of single compounds)
- Consider physochemical properties
- Consider solvents
- Apply different readouts with same mode of action (impact of labels)
- Co-extracts and matrix effects (high recovery vs. interfering matrix)
- Is it beneficial to combine multiple extraction methods to cover a broad range of chemicals?
- Is it beneficial/necessary to explain observed effects?
- Is your extraction compatible in both instrumental analysis and bioanalysis?

Group 3: Critical factors for accurate testing

Question: What are the most critical factors and source of errors for consideration to do accurate testing of complex undefined mixtures and extracts from water, food and related items using bioassays?

Feedback:

1) Sample :
- Type of samples
- Example food simulants for packaging (7 simulants possible)
- Matrix interference generated by the sample?
- Readout interference by sample (fluorescence or protein binding)
- Matrix calibration for bioassays
- Quality controls (spiking test as QA)

2) Extraction:
- Method of extraction (ex. Rotavapor optimization)
- Solubility issues
- Solvents used
- DMSO vs other solvents for cell exposure
- Amount sample to extract
- Concentration issues-volatiles: false-negatives
- Ready to use solvents-long term / harmonization

3) Bioassays:
- Qualified in vitro methods
- Star screening and follow with confirmations.
- Use of DMSO for cell culture exposure?
- Dosing
- Cytotoxicity-LOQ
- Reproducibility
- Concentration LOD
- Matrix effect-particles
- Data interpretation may be bias-interferences/qualification?

4) Specific and non-specific compounds

5) Dominant effect in mixture-check cells viability vs cytotox markers

6) Validation new bioassays to assess FCM

Group 4: Key topics for future WGs

Question: There is a need for development of harmonized best practices (framework) when using bioassays for undefined mixtures.

a) to your opinion, which are the topics and key procedures that should be covered?

b) do you think those topics could be addressed through AOAC working groups?

Feedback:

- Potential focus: Food contact materials
- Extraction procedures – solvents (compound extractability vs. biocompatibility with the given bioassay), pre-concentration/dilution, time, temperature
- Combination of bioassays to characterize the given effect(s)
  – Which assays to use?
- Interpretation and selection (fitness for purpose) of bioassays: Route of exposure, bioavailability of the active compounds, threshold for the effect (for risk assessment/sample prep)
- Quality control (extraction efficiency, normalization of the effect using reference compounds)
- Determination of LOD for a reference compound – to compare sensitivity
- Confirmation procedures (chemical, other bioassays, in-silico etc.)