Hidden cocktails uncovered

Screening of bioactive pollutants in food and food contact materials

Foto: Wolfgang Wessel

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Are our food, feed and food contact materials safe regarding complex mixtures of many kinds of different endocrine disruptive bioactive pollutants?

Theoretically this should be the case, because we are protected by guidelines i.e., a) the European directive 93/23/EC which bans all hormonal actions in stockfarming; b) EC Directive 96/22/EC which prohibits all substances having hormonal action in stockfarming as well as EC regulations 178/2002 and EC 882/2004 which oblige the member states to identify emerging risks and use validated and accredited methods for control analysis as well as for food contact materials by EC Framework Regulation 1935/2004 (migrants must not endanger human health). Now, to analyze for all kinds of hormonal actions such as (anti)-estrogens, (anti)-androgens, (anti)-thyroids, (anti)-progestin's or (anti)-glucocorticoids, caused by single, or complex mixtures of food contaminants, the only way forward is by using effect-based analysis tools – which in reality is rarely done. So far, most testings for hormone active contaminants using bioactive screening tools have been convened to assess the possible impact of food contact materials on drinking water, whereby until now the focus has been mostly on estrogenic effects of e.g., Bisphenol A and phthalates measured by methods such as ER CALUX. However, so far there are no critical limit values determined in regulations except for a trigger value of 7 ng estradiol equivalents/l water (Vergouwen et al 2011). It is urgently needed that the other hormonal pathways are further tested for water as well as for other foods and food contact materials.

How can we handle complex mixtures of bioactive pollutants in food and food contact materials?

Many chemicals and complex mixtures of chemicals are part of the feed/food chain which may exert their potentially harmful effects through several different toxicologically relevant pathways . There are however many more chemicals that are used nowadays in a variety of applications and products, for which the toxic profiles are not well established. The REACH regulation, and Water Framework Directives demand that a toxicity profile is determined for all high production volume chemicals. It is anticipated that several of those HPV chemicals may show potentially harmful effects and thus may enter the regulations for food and materials safety in the near future, thereby expanding the demand for safety analysis for a much wider range of food contaminants than presently known. In order to manage safety monitoring of food and contact materials a paradigm shift from chemical-by-chemical analysis to integrated effect assessment is urgently needed. Toxicity testing to assess human and environmental exposure faces similar challenges and regulatory toxicology particularly lacks efficient non-animal tools to measure the impact of complex mixtures of contaminants that may typically be present in food and contact materials. Because of research stimulated by many EU projects, we are now able to introduce reliable quantitative effect-based reporter gene assays for high throughput toxicity screening of complex mixtures of chemicals /pharmaceuticals such as obesogens, hormones, persistent organic pollutants (POPs), per/polyfluorinated compounds (PFAAs/PFTs), flame retardants (PBDEs), veterinary drugs (cortisol-like compounds) and anabolic steroids in the food and food contact materials chain. The instrumental basis of such bioassays (e.g. CALUX for dioxins/PCBs) tests are already established since 20 years and have been accepted for more than a decade in European, American and Asian countries' national and international standards (EC/252/2012. JIS 463, USA-EPA 4435). These new bio-analytical tools will help to close the gap of many health issues without know-how of relevant food/feed impacts and to further understand reasons for several increases in feed/food related sicknesses (such as obesity, cancer or miscarriages).

It is becoming evident that not only acutely toxic chemicals are posing human- and ecotoxicological risks but that chronic exposure even to low concentrations of mixtures of known and mosty unknown pollutants can have profound effects as well. In particular, pollutants mimicking endogenous sex steroid hormones have been shown to have strong effects on food issues such as feminized male fishes (many endocrine disrupting chemicals), or effects of plastic additives to drinking water (such as polycarbonate bottles -bishenol A issue-) even at ng/l concentrations. For the problem of identifying risks of mixtures of chemicals in food and food contact materials more comprehensive methods such as bioassays are needed to assess the risk of chemicals and chemical mixtures. Bioassays, measuring the biological response rather than the exact nature of chemicals is the bridge between the chemical analysis of a limited number of already known chemicals and the animal testing of yet unknown effects. Such high-through-put robotic screening (HTPS) bio-analytical tools are relatively simple to learn/operate to allow for cost-effective operation, straightforward data interpretation and are therefore aiming at wide-spread global distribution.

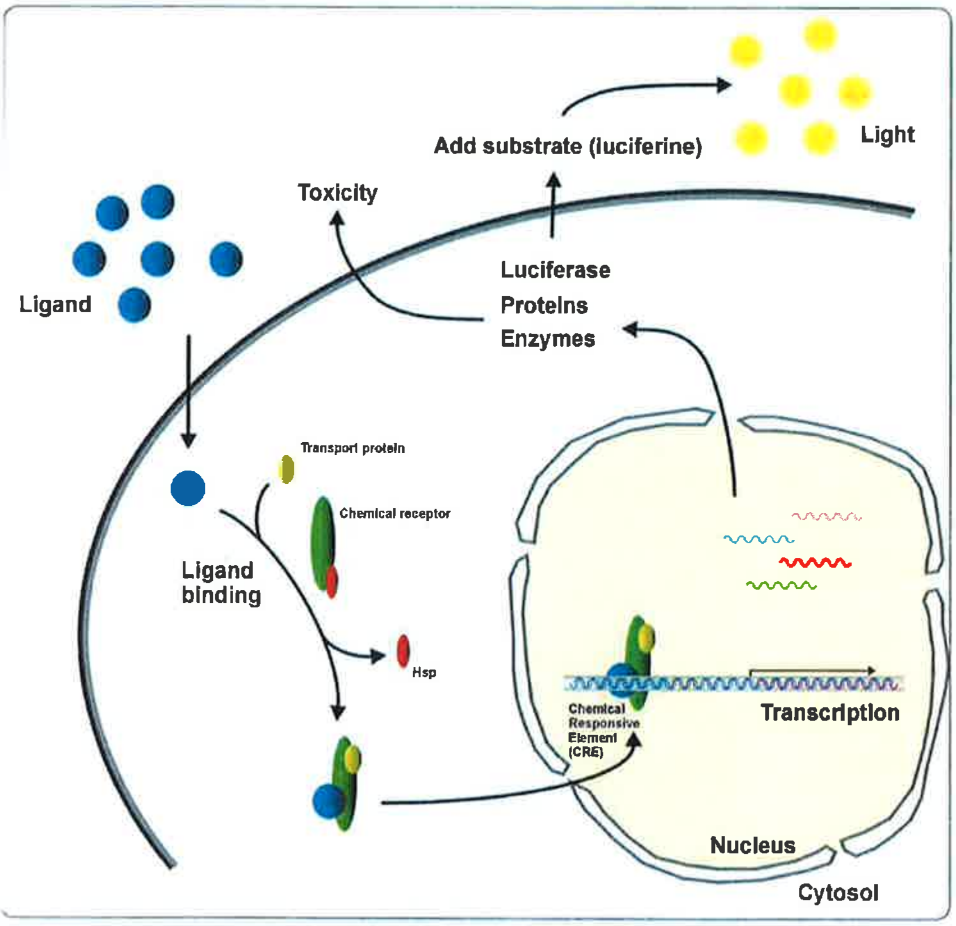

Fig. 1: Principle of the CALUX reporter gene assay

Principle of CALUX® reporter gene assays to measure major toxicity pathways in the food and food contact materials chain

CALUX® reporter gene assays are now available for major toxicity pathways (Fig.1). They are based on modes of action of toxicants. Essential target biomolecules involved in those modes of action are introduced in cells and form the very heart of the cell-based monitoring devices. The creation of such cell-based reporter assays consist of transcriptional activation by introducing cell transcription factors coupled to easily measurable reporter genes, which can be activated by toxicants and other bioactive molecules. For instance upon binding of the steroid hormone 17β-estradiol or endocrine disruptive chemicals the estrogen receptor (ER) becomes activated, and binds to recognition sequences in the promoter regions of target genes, the so-called estrogen responsive elements (EREs). To generate the first version of the so-called ER CALUX assay three of these EREs have been linked to a minimal promoter element (the TATA box) and the gene of an easily measurable protein (in this case luciferase). The thus obtained reporter gene was stably introduced in human T47D cells, that express ER endogenously. In this way the ligand-activated receptor will activate luciferase transcription, and the transcribed luciferase protein will emit light when a substrate is added. The signal will dose-dependently increase as a result of increasing concentrations of ligand. Now CALUX bioassays are available for chemical compound classes such as polyhalogenated -(x= Cl, Br, J, F) C- and N-dioxins PXDD/Fs/PXBs, hormones/anabolic steroids, glucocorticoids (cortisol-like compounds), thyroid hormones, retinoids, carcinogenic poly aromatic hydrocarbons (PAHs), peroxisome proliferator receptors PPAR (involved in obesity), NF-kappaB (involved in inflammatory response), p53 (involved in the DNA damage response), p21 (involved in cell cycle arrest), HIF1α (involved in hypoxia/angiogenesis), XBP1 (involved in the endoplasmatic reticulum stress response) or cytotoxicity (see table 1 for an overview).

Applications of panels of High through-put (HTP) screening CALUX® reporter gene assays

Applications of CALUX reporter gene assays when used alone or in panels are

Bioactive Food Pollutants	HTPS CALUX	Pathway
C- and N-Dioxins PXDD/Fs, dl-PXBs (X= Cl, Br, F, methyl)	DR CALUX	Dioxin receptor
Carcinogenic PAHs (such as Benzo(a)pyrene)	PAH CALUX	Dioxin receptor
Estrogens, EDCs, Bisphenol A, Phthalates, Pesticides, Pharmaceuticals, cosmetics	ER CALUX	Estrogen receptor mix
Androgens, EDCs, Bisphenol A, Pesticides, Pharmaceuticals	AR CALUX	Androgen receptor
Progestins, EDCs, Anti-babypill, Pesticides, Pharmaceuticals	PR CALUX	Progesterone receptor
Glucocorticoids, EDCs, Asthma spray, Immune-suppressive agents	GR CALUX	Glucocorticoid receptor
Thyroid hormones, EDCs, Brominated flame retardants	TR CALUX	Thyroid receptor
Retinoids, Pesticides, Pharmaceuticals	RAR CALUX	Retinoic acid receptor
Obesogens, fluorinated compounds PFAAs, Anti-diabetic pharmaceuticals	PPARgamma CALUX	Peroxisome proliferatorY1 receptor
Obesogens, fluorinated compounds PFAAs, Anti-diabetic pharmaceuticals	PPARalpha CALUX	Peroxisome proliferatorα receptor
Pro-inflammatory cytokines	NFkappaB CALUX	NFkappaB activation
Cytotoxic/static agents, Genotoxic compounds like PAHs, Pharmaceuticals, dyes	p21 CALUX	p21 activation
Cytotoxic/static agents, Genotoxic compounds like PAHs, Pharmaceuticals, dyes	p53 CALUX	p53 transcriptional activity
Electrophiles, oxidative stress, heavy metals	Nrf2 CALUX	Nrf2 transcriptional activity
β-Catenin/ involved in development and carcinogenesis	TCF	TCF transcriptional activity
Carcinogens, UV	AP1 CALUX	AP1 transcriptional activity
Hypoxia-mediated angiogenesis	HIF1alpha CALUX	HIF1α transcriptional activity
Endoplasmatic reticulum stressors	ER stress CALUX	XBP1 transcriptional activity
Cytotoxic agents, Non-specific luciferase modulators	Cytox CALUX	Constitutive transcriptional activity

Table 1: Currently available HTPS CALUX assays

multiple and range from toxicity screening of chemicals and pharmaceutical to sport doping, food- and environmental monitoring, water quality assessment, green chemistry, epidemiology, clinical research, and the determination of health risks but also benefits of functional foods. These assays can very accurately and quantitatively measure the combined effect exerted by all kinds of chemicals present in complex mixtures (see Table 1).

Flow-Chart of food sample analysis

A robotic liquid handling system (such as the Hamilton Starlet system) with an incubator for automated handling and incubation of 96 or 384 well plates is used for cell culturing. The luciferase measurement is than done by a luminometer with automated injection systems (e.g. stacker).

Quality control and validation of high throughput methods.

Obviously proper validation and quality assurance is one of the main requirements for any analytical method. In the case of high throughput methods this is not different and the methods are essentially the same as for any method, but some aspects need specific attention. When automating the assay panel the whole panel can be run with the same readout, because this avoids disrupting the automated workflow. Therefore several quality control standards (e.g. reference samples, positive control, cytotoxicity, CV standard, stable EC50 range, induction factor higher than 6) have been integrated in the testing scheme.

Some examples: Polycarbonate bottles and bisphenol A – can bioassays measure here any hormone and endocrine disrupting effects?

The issue of plastic additives causing hormone-like activity tested by bio-analysis have been reported last year several times for several plastic migration issues (e.g. impact from PET and polycarbonate bottles to drinking water). It is well known that plastic additives such as bisphenol A or phthalates are weak estrogenic and anti-androgenic compounds. Therefore such activities are measurable via sensitive reporter gene assays such as ER and anti-AR CALUX down to levels of a few pg Estradiol equivalents (EEQs) per liter water or plastic migration solution. In the study of Brueschweiler and Kunz (4 April 2011) measuring drinking water samples in PET and glass Bottles only very low estrogenic effects could be measured by ER CALUX.

Is BABY FOOD safe in plastic packaging regarding hormone-like activities?

In case of migration of plastic materials to baby food we could show that no significant effects have been found by using several hormone and PAH CALUX bioassays for such plastic packaging compared to the classical glass packaging (WDR Markt 2009). »

Fig. 2: Cell incubator in combination with robot for the cell culture and pipetting of samples for a panel of CALUX bioassays

Fig. 3: Pipetting & plate handling desk of the High through-put (HTP) robot

Is our daily morning egg and our fish at diner safe of polyhalogenated C- and N-dioxins and other dioxin-like compounds?

Many eggs have been recalled in the last few months in several EU countries due to polychlorinated dioxins/PCBs. Several new scientific investigations show that also polybrominated dioxins/PBBs and polyhalogenated N-dioxins are relevant for all kinds of foods. These compounds are currently only cost-efficiently measurable by the DR CALUX screening method giving an quantitative result for the additional impact of these more than 10.000 congeners (the current EU policy only regulates 29 of these pollutants). These new dioxin-like compounds cause false negative results in the confirmative HRGC/HRMS analysis, because they are not routinely measured.

Effects of different eating behaviours to dioxins and dioxin-like PCBs for mothers and their new born babies (Bio human monitoring)?

In a study by Nelson et al (2008) on pooled samples of 250 mothers similar results from DR CALUX screening and the confirmative analysis in a double blind study have been obtained. Highest levels have been found in mothers with high fish food intake. In the New Generis project more than 1200 pairs of mothers and their newborn babies have been investigated and first new health impacts to the new born babies are currently in discussions (fetal growth and infant development).

Can per/polyfluorinated chemicals (such as PFOA / PFOS) cause effects in the OBESITY process?

Several studies using in vitro reportergene assays with peroxisome proliferator-receptors (PPARα and PPARγ) showed already that the most active PPARα-agonist is perfluorooctanoic acid (PFOA), while PFOS is the most active PPARγ-agonist (Wolf et al, 2012; Behnisch et al. in press, ToxCast 21). The PPAR receptors are thought to regulate physiological processes that influence e.g. lipid homeostasis, peroxisome proliferation, adipogenisis and reproduction.

Are unknown effects in smoked meat relevant for our health? Is our summer BARBECUE still safe...

Polycyclic aromatic hydrocarbons (PAHs), which are generated by heat treatment and smoke curing of meat, pose a risk to human health. In a study of Kuhn et al (2009) custom-made meat samples produced under defined conditions with different PAH levels were analysed using the DR CALUX bioassay. It was found that hot (65 degrees C) and longer smoking times caused a considerable increase in both the DR CALUX bioassay response (up to ca. 1300 ng dioxin TCDD equivalents/kg) and the levels of 31 individually determined PAHs. In this study it has been concluded that the DR CALUX to be sensitive in a range much lower than the European threshold value for B[a]P of 5 μg/kg smoked meat and also lower than the former German threshold value of 1 μg/kg.

Anabolic steroids in sport foods or dietary supplements – how healthy they are and what do we know about their side effects?

With such CALUX bioassays many anabolic steroids (AAS) for the identification of unknown side-effects of compounds and complex mixtures in the context of sport doping have been analyzed (Houtman et al. 2009). From this and other studies (Rijk et al 2009, Plotan et al 2011) several endocrine disrupting effects have been analyzed in sports and dietary supplements – proving again that the concept of using only chemical analysis tools cannot measure and predict such health related issues of bioactive compounds.

Pesticides – new bioactive side-effects regarding hormone- and obesity-like effects via human cell line HTPS screening panel CALUX

In the EU project ChemScreen the side-effects of several pesticides have been newly investigated with several unexpected results such as for Chlordane/Dieldrin (anti-androgenic), methoxychlor (estrogenic), methylmercury-chloride (Nrf2) or tributyltinacetate (cytotox, AP1, Nrf2, ESRE, ER, TRb, PPARg2, RAR).

Old chemicals (DDT, DDE) cause new effects (anti-androgenic CALUX) in several wildlife species

Suzuki et al (2011) demonstrated the occurrence of anti-androgenic activity to be present in Baikal seal, and using TIE methods p,p'-DDE was identified as an important causative compound for the activity, contribution to almost 60% to the anti-androgenic effect in the Baikal seal tissues.

Fig. 4: HTP 96 well plate luminometer reader (with stacker) for the measurement of a panel of CALUX bioassays

Veterinary drugs in our daily meat – is this a real issue? Are we really protected by the EC Directive 96/22/EC which prohibits all substances having hormonal action in stockfarming as well as EC regulations 178/2002 and EC 882/2004 which oblige the member states to identify emerging risks?

In livestock production glucocorticoids can be used to mask bad nursing, housekeeping and feeding of animals because of their inhibition of inflammatory, allergic, and immunological responses. In addition, glucocorticoids are very tempting for broilers as they also stimulate growth and improve feed conversion to get a higher amount of meat. In our first study we could show that the GR CALUX can measure many of these veterinary drugs (e.g. dexamethasone, flumethason, budesonide) as well as the LOQ of bovine urine is as sensitive as other analysis (ELISA, LC/MS/MS; below 2 ppb). The combinations of bioassay screening with chemical identifications allows us now to uphold all the laws (all hormonal action), while bioassays or chemicals methods alone cannot.

Menopause of women – search for alternative medicines to minimize the side effects:

In the study of Doyle et al (2009), it was demonstrated by ER CALUX bioassay that from 20 plant medicines used in markets in Costa Rica only three plants (*T. parthenium, P. major* and *P.dioica*) really had estrogenic effects and therefore maybe suitable as hormone replacement therapy for menopause. Therefore with such methods also new bioactive plant species can be investigated.

Nutraceuticals – improved bioactive health properties of food items.

Such bioassays (e.g. using tests for oxidative stress such as Nrf2 CALUX) can be also used to improve bioactive health properties of food items to have cost-efficient monitoring tools.

Bioactive compounds – the way forward!

In the EC FP7 project Plant Libra (www.plantlibra.eu) and the Dutch EcoLink projects such CALUX screening tests are intensively used to find new bioactive compounds in plant food supplements and at giving an introduction to their role and benefits for public health.

Conclusions

The innovative CALUX technology developed by BioDetection Systems is the first practical HTPS method to analyse on the same laboratory equipment many bioactive compounds, that regulated in European guidelines (e.g. dioxins/PCBs, Pesticides, Benzo(a)pyrene, bisphenol A, phthalates). But only in case of dioxins/PCBs in food the HTPS bioactive screening methods (such as CALUX bioassays) are yet integrated in the official testing procedure (such as EC/252/2012). These quantitative/qualitative bio-analytical methods are definitely easier-to-learn, cheaper as the current chemical analysis - and allow a fast HTP screening to handle source and time-efficient crisis situations of chemical cocktails in food, food contact materials and other health related issues.