DISCREPANCIES BETWEEN BIO-ANALYTICAL AND CHEMO-ANALYTICAL RESULTS HAVE A NON-NEGLIGIBLE MESSAGE

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Introduction

Worldwide, the number of chemical contamination events is increasing; hence, all means to keep them at bay deserve our scientific attention. Many man-made xenobiotic chemicals form a global burden, since they have toxic properties. Moreover, they often exhibit pronounced lipophilicity and resistance to metabolic degradation, leading to accumulation and biomagnification in biota and in the food chain. The halogenated aromatic hydrocarbons [HAHs] such as dioxins and furans are well known examples.

Today, humans are exposed to an extremely complex ñcocktailò of multifold and highly different chemicals. Several of these contaminants persist in the environment for decades, with some of them being studied already for a very long time. Examples are inorganic and organic heavy metal compounds, pesticides, polychlorinated biphenyls [PCBs], dibenzo-p-dioxins [PCDDs] and dibenzofurans [PCDFs]. Another category are the emergent chemical contaminants; there is still a relative dearth in understanding their properties, exposure routes as well as adverse effects on human health. Examples are plastic and ink ingredients such as plasticizers, flame retardants, photoinitiators and others; preservative agents for food, cosmetics, drugs or packaging materials; both human and veterinary drug residues; mycotoxins, phytotoxins and phycotoxins. Exposure science has a key role in the evaluation of recently introduced chemicals, some of them being the newest replacements of formerly used contaminating agents [1-3].

Since humans are exposed to a plethora of both man-made and natural chemicals, a shift from primarily in vivo animal studies to in vitro assays, in vivo assays with lower organisms, and computational modeling for toxicity assessments is now suggested. For legislative control the combination of chemical analyses with high-throughput screening analyses is the better and scientifically sound means to tackle food safety issues [4-7].

The present paper illustrates the diverging results obtained by conventional GC-HRMS and CALUX analyses of dioxins and discusses the message provided by the obvious discrepancies. Observed discrepancies should not be understood as absence of success in the initial efforts but rather as encouragements with positive effects on the pace of further development.

Materials and methods

TEQ-principle - ñDioxinsò is a generic name for 75 PCDDs and 135 PCDFs. There is general agreement that adverse effects of PCDDs, PCDFs and dioxin-like PCBs proceed through the activation of the aryl hydrocarbon receptor [AhR] pathway. This common action mechanism supports the application of the toxic equivalency factor [TEF] concept [8]. Usually the reporting units are toxic equivalent concentrations or TEQ values, which are defined as the summed products of congener concentrations and their corresponding TEFs.

Here it must be mentioned that several candidate dioxin-like compounds do not have an assigned TEF value, however, even when evidence was provided for their capacity to activate the AhR. Additionally, dioxins and dioxin-like chemicals are generally not found as individual congeners but as complex mixtures with highly variable relative

and absolute congener concentrations. The molecular mechanism for their biological effects is at the basis of a bioanalytical approach.

Detection methods - Capillary gas chromatography in combination with high-resolution mass spectroscopy [GC-HRMS] is currently considered to be the reference technique for the determination of PCDDs, PCDFs and dioxin-like PCBs. It is characterized by good selectivity, measurement precision and accuracy.

The Chemical Activated LUciferase gene eXpression [CALUX] in vitro cell bioassay is a mechanistically based technique that detects all AhR agonists [9-10]. The method is applicable to food and feed matrices [11-17], human fluids [18 ï 19] and environmental samples such as marine biological matrices and sediments [20- 23]. A detailed examination of the critical methodological parameters and aspects of the CALUX assay, that can affect the quality and accuracy of the analyses, has already been published [24]. Since CALUX and GC-HRMS results have a different meaning, CALUX results are referred to as bioassay equivalent concentrations or BEQs rather than TEQs.

Results

Human fluids - Van Wouwe et al. [18] compared GC-HRMS and CALUX results for dioxin levels in 341 human plasma samples. The GC-HRMS values ranged from 1.8 to 93.2 pg TEQ/g fat [median: 22.8], whereas the CALUX results ranged from 5.6 to 103.1 pg TEQ/g fat [median : 40.6]. The least square linear regression was CALUX = 0.74 GC-HRMS + 22.69 [Figure 1]. A significant but fairly low correlation coefficient of 0.64 referred to considerable data scattering, however. Additionally, a CALUX over GC-HRMS ratio versus GC-HRMS plot illustrated the importance of the CALUX excess values at the lowest contamination levels.

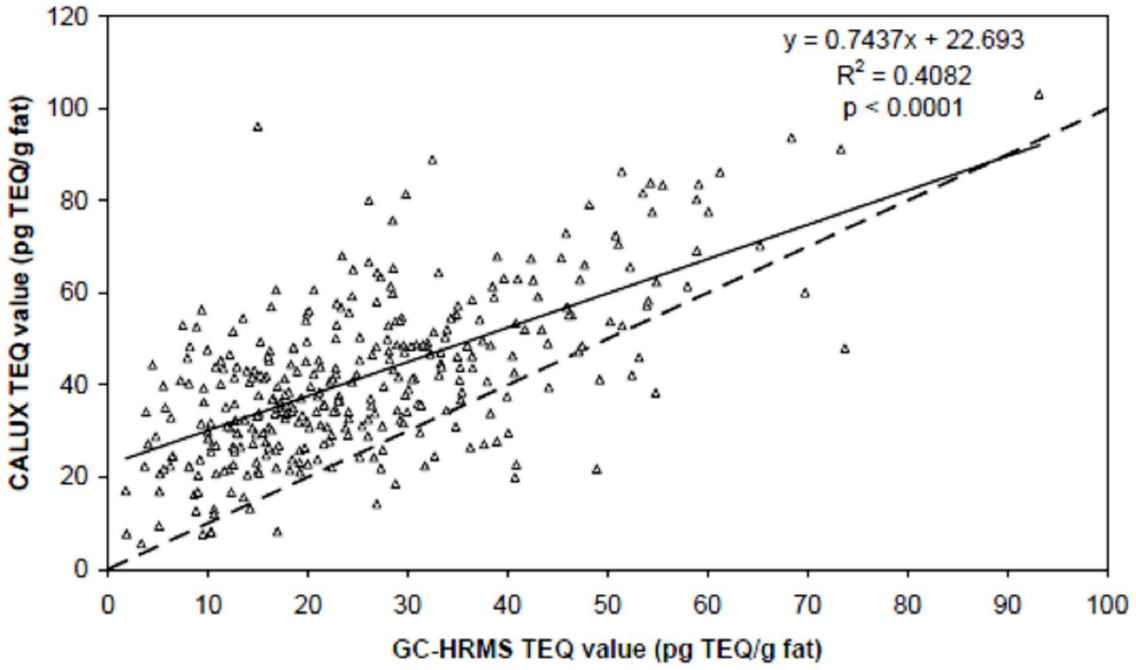

Figure 1. CALUX versus GC-HRMS data for human plasma samples, shown in the study by Van Wouwe et al. [18]; originally the term CALUX TEQ was used, it should now read CALUX BEQ

Three factors affecting the discrepancy between both types of data were investigated [18]. For undetected congeners, the concentration moiety in the TEQ calculation can either be zero [lowerbound], half the limit of quantification [medianbound] or equal to it [upperbound]. When the concentrations are set to zero, they do not contribute to the GC-HRMS TEQ value and this leads to an underestimation at low concentration levels. The CALUX measurement, on the other hand, gives one overall response for all congeners. Therefore, differences

between CALUX and GC-HRMS results will increase with increasing numbers of undetected congeners. Using lowerbound values instead of upperbound values reduced the differences or discrepancies by 6.9 pg TEQ/g fat or less, with highest reductions occurring for the lowest concentrations.

In CALUX applications differences between relative potency [REP] and TEF values must also be considered. To evaluate the differences, concentrations measured with GC-HRMS were multiplied with their corresponding REP values. The absolute differences between TEF-based and REP-based results amounted to 6.7 or less, whereby largest differences were observed for the higher concentrations. The combination of both the LOQ effect and the REP effect explained a decrease of approximately 40% [relative value], leaving room for additional effects such as the occurrence of many other AhR agonists.

According to Ericson Jogsten et al. [25] the levels of AhR-active polybrominated dibenzo-p-dioxins [PBDDs] and dibenzofurans PBDFs found in human adipose tissue samples can contribute for 14% to the TEQ value. The use of brominated flame retardants is likely to be a source for increased human and wildlife exposure to PBDDs and PBDFs [26]. Moreover, these compounds were shown to have similar potencies as their chlorinated counterparts by both enzyme immunoassay and CALUX bioassay [27]. They are unintentionally formed as impurities during the synthesis of polybrominated diphenylethers and are confirmed to occur in many different matrices.

Detection of brominated dioxins in choline chloride - Reports about false non-compliant CALUX-results ï the samples were suspicious since their BEQ values were very high - with the widely used feed ingredient choline chloride led to an increased monitoring in the Netherlands.

Samples were tested with CALUX and some showed increased responses, corresponding to an estimated BEQ level of approximately 5 ng BEQ/kg. Analysis with GC-HRMS did not show any PCDD/Fs or dl-PCBs, confirming the previously observed false non-compliant results. Subsequent analysis of these samples by GC/TOF-MS revealed the presence of various flame retardants like polybrominated diphenylethers [PBDEs], tribromophenol and even a new octabrominated compound, called OBIND, in the positive samples. However, none of these are AhR agonists and, hence, they can not explain a CALUX response. The presence of brominated compounds and especially tribromophenol pointed to the contamination with PBDDs and PBDFs. This was confirmed at the European Union Reference Laboratory [28]. This second case study evidences that the combined use of a bioassay and a performant chemo-analytical method results in the detection as well as identification of potential novel risks. Of course, intensive monitoring programmes with screening assays and follow-up of false-positive samples is required.

Discussion and conclusion

Earlier studies have evidenced the AhR activity of multifold chemical compounds [24, 29]. A significant amount of information on planar aromatic hydrocarbon AhR ligands is already available but the actual spectrum of chemicals that can bind to and activate the AhR pathway encompasses many more chemicals, both synthetic and naturally occurring ones [29, 30]. Within the realm of the synthetic chemicals and their inherent impurities, that appear during the manufacturing procedures, multifold AhR agonists surface. As mentioned, they were found in human fluids as well as in food items. Widespread environmental contamination by these chemicals exists with increasing concentrations in air, water, soil, sediment, wildlife and humans.

The application of screening approaches such as CALUX measurements should, therefore, have two predominant objectives. On the one hand, screening approaches have a red-light-green-light function. This means that they can be of significant help during the control by the inspection authorities. Several authors [4-7] pleaded already for intensive screening to identify the suspected samples that must be sent to confirmatory GC-HRMS analysis. On the other hand, the extremely complex chemical mixtures that contaminate food and feed are but poorly evaluated by a GC-HRMS analysis, that is restricted to 29 chlorinated congeners. Obviously, TEQ values can never equal BEQ values. LOQ effects, REP effects and the occurrence of many AhR agonists are strong arguments for the observed differences between GC-HRMS and CALUX results. Every observed difference can be traced back to [multifold] molecules, however, and many of them might have a toxic potency. This should not go unnoticed.

The authors of the present paper strongly suggest that bioassays in combination with chemo-analytical methods should be the analysis tool par excellence for selection of non-compliant [e.g. food and feed items] and unacceptable [e.g. environment samples contaminations] as well as identification [quantitative analysis] of

innumerable suspicious chemicals, some known to produce adverse health effects, some of them not sufficiently studied and others not yet identified.

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