Rapid methods for dioxin and dioxin-like PCBs in food and feedingstuffs: State of the art

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Abstract

The increasing number of local dioxin crises since 2002 becoming more and more apparent due to stricter controls of feed and food in the European Union and the globally increasing number of countries applying similar guidelines make it necessary to establish reliable, time and cost-effective screening methods for the dioxin intake through nutritional pathways. Five years after the last overview presentation about all kinds of different bio-analytical detection methods (BDMs)[1-3] and the establishment of quality guidelines for screening methods[4-7], time has come to include as well the improvements in the chemical methods to speed up the analysis. This review gives an overview about the state-of-the-art improvements and gives a future outlook for both methods, chemical and bio-analytical approach for rapid analyses of dioxins and dioxin-like compounds. Now several new ways of improvement are currently in the pipeline of research and testing, such as PCR[8], proteomic biomarkers[9-10] and in case of the clean-up ASE[11], PowerPrep[11] and different detection methods as well as different other ways of indicators for dioxins (e.g. correlations to fatty acids[12]).

Dioxin crises

In the last few years the list of dioxin crises in feed/food has steadily increased. A variety of sources in different countries has occurred, such as ball clay in USA/1996; illegal disposal of capacitor fluid in Belgium/1999; use of industrial waste like contaminated lime for citrus pellets from Brazil/1998; improper drying of feed ingredients leading to contamination via dioxin-containing fumes e.g. green feed from two feed producers in Germany/1999 or German bakery waste/2003 or eggs and rabbit meat from Luxemburg/2001; sewage sludge in feed in France/1999, zinc oxide, copper oxide in feed premixes (several countries 2000-2002), PCP contaminated saw-dust/choline-chloride case in Spain/2000, Carbosan

copper in France and USA/2002, farm-raised fishes/fish feed in several countries (2003/4) or eggs from free-ranging chicken in the Netherlands, Switzerland, UK, Sweden, Belgium and France (2003/4). In addition, large fire accidents involving higher amounts of chlorine-containing materials (e.g. PVC) are known to cause local dioxin contaminations. Therefore, methods have to be established which are able to give reliable results for all of these different matrices/crisis situations.

European guidelines for dioxins in feed and food

Primarily following the Belgian dioxin crisis in 1999, the EU has developed further strategies to reduce the current exposure of its citizens,. Measures include strict limits for dioxins in various food and feed stuffs. At the end of 2004 the EU will expand the definition of dioxins and this group will then include 29 compounds that produce similar biochemical effects in test animals: 7 polychlorinated dibenzodioxins (dioxins, or PCDDs), 10 polychlorinated dibenzofurans (furans, or PCDFs) and 12 polychlorinated biphenyls (PCBs). These compounds are collectively referred to as dioxin-like compounds. At the same time most of the European countries agreed to accept the Stockholm Convention of Persistent Organic Pollutants (POPs). Therefore, reducing dioxins, PCBs and other POPs for both consumer and environment protection reasons will be one of the major goals of this international agreement in the next few years. Bearing in mind that several new member states of the European Union have so far only few experiences with dioxin-like compounds, it seems to be necessary to establish cheaper, easier and faster methods for these compound classes to ensure the safety of food and feed for human health.Revision of the limits in 2004 to integrate some dioxin-like PCBs, would again change the extraction and clean-up strategies for both the screening and GC/MS methods. Furthermore, it is foreseen that by the end of 2006 the maximum levels will be revised, aiming at a further reduction and thus, even more sensitive methods will soon be required. It is planned to reduce the human exposure to dioxins by at least 25% until the end of year 2006.

Modern extraction and clean-up procedures

In the last few years faster extraction and clean-up procedures have been established and evaluated to speed up the sample pre-treatment[11]. In case of chemical analyses, 50-75% of the time a complete analysis takes are needed for the extraction and clean-up. Sample extraction times can be reduced from about 12-24 hours by soxhlet to about 20-30 min by using Accelerated Solvent Extraction (ASE) and the traditional sample clean-up can be speeded up from about 6 hours to about 1.5 hours by using an automated clean-up system called Power Prep (Table 1). From Table 1 it is evident that the major gain in terms of

time lies in the handling of large sample amounts, as is a requirement during a crisis.

Table 1: Rough estimate of the possible fastest Turn-Around Time (TAT).

Sample set	**ASE**	**Evapora t-ion**	**Power-Prep**	**Turbovap**	**Measurement [hrs]**	**TAT [hrs]**
1	20 min	30 min	90 min	15 min	HRMS: 1* CALUX: 24 ELISA: 4-5	HRMS: 4 CALUX: 30 ELISA: 7-8
6	120 min	60 min	90 min	45 min	HRMS: 6* CALUX: 24 ELISA: 4-5	HRMS: 11-12 CALUX: 30 ELISA: 7-8
100	4 days	2.5 days	6 days	1.5 days	HRMS: 144* CALUX: 24 ELISA: 8-10	HRMS: 7-14 days CALUX: 3-6 days ELISA: 4-6 days

Assumption: Only machine steps and cell culture; * runtime doubled when including dl-PCBs

Quality and performance criteria from the EU Commission Directives 2002/69 and 70/EC from July 26, 2002 for the testing of foodstuffs and feed

In the last few years several studies showed that bioassays are able to fulfil the quality guidelines set by the European Union in 2002[13-16]. Nevertheless, only few studies could demonstrate the low number of false negative results required by the EU guidelines, since this requires testing of large amounts of samples analysed simultaneously by HRGC/HRMS. Additionally, most of the screening methods participating in the limited number of international intercalibration studies haven't demonstrated a sufficient reliability and comparability to each other or to the chemical analysis, although the tests perform well in a small number of laboratories.

Sensitivity

One critical issue about using screening methods is the sensitivity of the method. In several studies the CALUX assay was claimed to have a sensitivity level of 1/5th of the level of interest set by the European Union in 2002 for several kinds of feed/food. However, in most cases these claims remain to be accompanied by a demonstration of this performance with incurred samples around these low levels.

Table 2: Matrix-dependent comparison of reported LOQs, WHO-TEQs and CALUX-TEQs

Matrix	EU-Limit	CALUX LOQ	CALUX-TEQ/WHO-TEQ	Correlation CALUX to HRMS	Reference
Fish	4.0	0.1	1.7	0.89	17
Pork	1	0.7	1.5	0.85	18
Food	0.75-4.0	0.1-0.7	Max 3-fold	0.92	19
Feed	0.75	0.1	2	0.75	18
Cow milk	3.0	1.1	1.6		20
Mother milk			Max 2 fold		21

Originally, TEQ levels were calculated from a TCDD-calibration curve. In the last few years, the CALUX bioassays changed the TEQ calculation by adding recovery corrections[22] and correlation factors[23] to give more reliable results compared to the confirmatory method. An alternative is the inclusion of control samples in a test series for evaluating the response obtained with the test samples (screening approach).

Other bio-analyses and chemical-analyses technologies for rapid analyses of dioxins and dioxin-like PCB in feed/food

a) EROD bioassay:

In the past few decades the EROD bioassay testing has been by far the most extensively used cell bioassay system for the detection of dioxin-like compounds[24-25]. The assay is rapid, reproducible, several cell lines and reagents are readily available and no known legal restrictions or limitations to free use exist for these cells and the bioassay system. Limitations of the H4IIE cell bioassay are that it is less reliable and sensitive than the CALUX bioassay. It has also been observed that many of the EROD-inducing chemicals are substrates or inhibitors for CYP4501A and can in theory inhibit EROD activity, which could than result in underestimation of the dioxin TEQ. So far, only limited results of feed/food samples measured by EROD bioassays according to the EU guidelines have been reported.

b) ELISA technology

Kit-based bioassays commonly used in the feed/food testing are currently established and tested in several laboratories for dioxin measurements in feed/food. They offer the advantages of a cell-free system which reflects the TEQ-values. A disadvantage is that antibodies don't obey the TEQ-principle, but this is

overcome by developing antibodies that recognize the Ah-receptor/ligand complex. These kit-based systems are simple to use and the levels are measured by a plate reader in around 5 hours assay time. Similar to bioassays, the main problems to be solved at the moment are the different matrix-dependent extraction methods, clean-up systems and finally, the sensitivity of the antibody for dioxin-like compounds. The advantage of these kit-based bioassays is the possibility of a cheaper and faster method than CALUX due to a shorter incubation time and no time-consuming cell culture, requiring special facilities. However, 24 h incubation time in the CALUX assay is used to allow the cells to metabolise certain interfering compounds and this is not possible with immunoassays. ELISA technologies are widespread and standardised. So far, they are only successfully applied on highly contaminated matrices with sufficient results (false negatives for soils = 1%; false positives = 3%). Standard curve between 1-64 pg TCDD-TEQ/well (r^2 = 0.997) have been reported. The drawback of these methods is the lack of sensitivity and so far not enough TEQ data compared to HRMS measurements for all kinds of different feed and food matrices. Lack of extraction/clean-up methods for all kinds of feed/food are also currently under investigation. In most studies a consistent overestimation (~10-fold) depending on the sample type is reported.

c) PCR technology

Since a few years further researches are made with the Polymerase Chain Reaction (PCR) analysis, the so called AhPCR kit[8]. Dioxins bind to the Ah receptor in a specific manner and subsequently to a specific piece of DNA (DRE)., Following removal of the non-bound DREs part of the remaining DRE is amplified and detected by using real-time PCR. With regard to kit-based bioassays, PCR technology or proteomic tools (d), it is recognized that no evidence has yet been submitted of commercially available assays having sufficient sensitivity and reliability for the screening of dioxins at the required levels in samples of food/feedingstuffs.

d) Proteomic biomarker analyses

Research with proteomic biomarkers tries to make correlations between enzymes (e.g. superoxide dismutase), stress proteins (e.g. hsp60), receptors or cytosketal proteins (e.g. myosin)[9, 10] and dioxin-like compounds, following exposure of to these compounds.

e) Rapid methods for dioxin-like compounds based on chemical methods

Alternative chemical methods for dioxin analyses are the so-called comprehensive multidimensional gas-chromatography (GCxGC or CMDGC)[26] and less expensive MS-techniques such as the ion-trap MS/MS[27-28]. These techniques are currently evaluated within the EU-DIFFERENCE project, that will end in 2005.

Differences between chemical and bio-analytical methods

An important difference between the chemical and bio-analytical assays is that the latter produce a single value, typically a TEQ value or a total PCB value. Analytical methods produce information on the isomer pattern which can be used for source identification. These new biotechnologies, however, offer the possibility to screen for new dioxin-like compounds or to measure relative potencies of other known environmental pollutants. In the last few years several other potential toxic compound classes have been already tested by means of these technologies[24]. Other potent dioxin-like compounds are for example some mixed halogenated dioxins, brominated flame retardents and some hexa-chlorinated naphtalenes[24]. Furthermore, new techniques and strategies are developed to isolate and identify novel contaminants (TIE-approach).

Assuming that the chemical and bio-analytical screening assays have similar performance characteristics it is up to the user of the diagnostic information to decide which method is preferred. Additionally, recovery corrections are so far not reported for all biotechnologies measuring dioxin TEQ levels, with the exception of the use of control samples, which require a very reproducible clean-up of samples.

International intercalibration studies

So far, several round-robin studies have been finished with different results. In the first round of an international project called DIFFERENCE (**www.dioxins.nl**), vegetable oils have been tested by HRMS, GCxMSxMS and CALUX and the z-score were below 2 for all 3 technologies[30]. In another international calibration study with cod liver measured by several kinds of bioassays, 8 of 12 laboratories had values of 60-106% of the WHO-TEQ[31] demonstrating the possibilities of promising alternative rapid methods. Recently, the JRC in Geel, assisted by RIKILT en CSL, organized a ring trial for the CALUX-assay using a set of feed and fish oil samples at various levels. An important issue in these studies is the nature of the test samples. Although incurred samples should normally be preferred, it cannot be excluded that such samples may also contain other contaminants that contribute to the response in the test, thus leading to an overestimation of the dioxin and PCB level. On the other hand, differences between WHO-TEQs and the relative potencies of the different congeners in the test may also contribute to differences and this issue requires further examination with GC/MS controlled standards. In general these studies show that the clean-up of samples, which does not allow the use of internal standards, is a very critical step. This potential source of variation is similar for all the screening assays described above.

Novel dioxin-like compounds

The results from traditional HRGC/HRMS analyses have already been compared with these new biotechnologies in several studies and in most cases they respond to several classes of POPs and therefore, the results are often higher than the results from HRMS analyses (CALUX about double, kit-based bioassays are usually about one magnitude higher). Therefore, such an approach gives a risk assessment not only for the regulated polychlorinated dioxins, but it's also possible to cover the whole range of dioxin-like compounds such as additional mixed-halogenated dioxins.

Future outlook

According to new EU guidelines for feed/food, biological screening tests for dioxin-like compounds can be used, in case the tested levels are higher or lower than 30-40 % of the level of interest. The result of the ongoing research will show us in the near future which new rapid methods will be able to fulfil the EU requirements and which results of international intercalibration studies will be comparable to the confirmation method.

As described above, these assays have numerous advantages over the more costly instrumental analysis for rapid screening application, but also a variety of disadvantages. However, many of these methodologies are still in their early stages of development and given the rapid progress in biotechnology, it is certain that additional improvements in sensitivity, specificity and detection will occur in the very near future. Similar will be true for the clean-up methods.

Before these bioassays can be accepted for regulatory use, they must be subjected to full in house validation studies and must meet widely accepted performance criteria of national and international standards organizations and federal agencies (USEPA, EU). Further approval of full validation studies by national (e.g. Sterlab, NL; DAR, Germany; Beltest, Belgium) and international agencies as well as the accreditation for laboratories according to ISO 17025, will also encourage their use. Without these criteria being met and standardization in place, regulatory agencies will hesitate to accept these new methodologies. Validation and standardization of several rapid methods for measuring dioxin-like compounds are currently in progress; some have either received or are currently pending regulatory approval. To speed up dioxin analyses, especially extraction and clean-up methods have to be modified. Several modern extraction (e.g. ASE) and clean-up procedures (e.g. Power-Prep) are already available in order to increase the speed of sample preparation. The combination of bioassay-screening tools and the mass-spectrometry confirmation method has already been used

successfully as crisis-management tools in the food/feed analyses for dioxins and dioxin-like PCBs[32].

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