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Application of the AhR reporter gene assay for the determination of PCDD/Fs and DL-PCBs in feed samples

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Abstract

Introduction: Polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (DL-PCBs) belong to a well-known group of pollutants. Present in feedstuffs, they bioaccumulate in tissues of food-producing animals. Food is the source of over 90% of human PCDD/Fs and DL-PCBs intake. Thus, feed control is one of the pillars of the EU strategy and a mean of reducing human exposure. The article presents AhR based reporter gene bioassay method for PCDD/Fs and DL-PCBs analysis in feed and its validation results. **Material and Methods:** Analytes were extracted from samples with fat. Subsequently, fat and other interferences were removed from extract using sulphuric acid modified silica. Extract was further cleaned and PCDD/Fs separated from DL-PCBs using carbon column. Contaminants detection was performed using H1L6.1c3 cell line, which produces luciferase in response to AhR ligands present in extract. **Results:** Performance characteristics (repeatability, reproducibility, and apparent recovery) fulfil the requirements of Regulation 2017/771/EU. The positive correlation between bioassay and reference HRGC-HRMS method was confirmed. Moreover, the role of screening method used in connection with the confirmatory HRGC-HRMS method in providing feed and food safety has been discussed. **Conclusion:** Bioassay is a useful method for dioxin and DL-PCBs analysis, allowing cost reduction of monitoring programmes with minimal risk of false negative results.

Keywords: feedstuffs, dioxins, dioxin-like polychlorinated biphenyls, bioassay.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCB) constitute a group of toxicants that may contribute to food toxicity *via* contaminated feeds. Therefore, feed safety is an essential element of food safety. The Stockholm Convention and, consequently, European Commission focus on eliminating and reducing production and release, control, removal and use of those toxic chemicals. Only 17 out of 210 PCDD/Fs congeners are of toxicological concern, and the analysis of dioxins is based on the combined concentration of these 17 dioxin and furan congeners. PCBs differ from PCDDs and PCDFs in that they are industrial products, while dioxins and furans are unintentionally produced by-products. Twelve of the 209 PCB congeners, due to their chemical structure and biological activity, are considered to be dioxin-like compounds (DL-PCBs).

Feeds that make complete feed products derive from a variety of plant, animal, and mineral origin materials. Dioxins and PCBs can contaminate plant-based animal feeds through a diversity of pathways, including the airborne deposition onto plant and soil. The contamination may be a result of local pollutant emission, industrial and non-industrial activities, contamination during production and transport, carelessness of management production, or may result from illegal practices (2, 13, 17, 24). Compound feed may also be contaminated by components of animal origin such as animal fat, fish meals, and oil, or minerals. Lipophilic contaminants consumed by food-producing animals are transferred to animal tissues and deposited in adipose tissue and muscles, and can be passed into milk and eggs. Food is the source of over

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90% of human PCDD/Fs and PCBs intake, so reducing human intake of dioxins is highly dependent on minimisation of the feed material contamination (4). Nevertheless, isolated cases of contamination might still happen (18, 19).

Dioxins and PCBs, due to their toxic properties (immunosuppression, hormonal disorders, carcinogenicity), are subjected to stringent legislation in all EU countries (4, 21). Maximum levels of these compounds in food and feed have been set by successive regulations of the European Parliament and of the European Commission (7). The first maximum levels for dioxins in feed were established by the European legislation by Directive 2002/32/EC. Since November 2006, the maximum DL-PCBs content is also regulated (Directive 2006/13/EC). The EU policy requires that the member states conduct mandatory monitoring of feed and food to evaluate the existing levels of dioxins and related compounds, and characterisation of the background levels in EU countries. Due to very low concentrations of dioxins and PCBs in the feed (ng kg^{-1}), their determination requires highly specific and sensitive analytical methods based on modern chemical and biological techniques. For the quantitative determination of 29 dioxin and DL-PCB congeners in feedstuffs, EU recommends application of the most sensitive technique, namely high-resolution gas chromatography coupled with high-resolution mass spectrometry (HRGC/HRMS). Very high costs limit the availability of this method, and are one of the main reasons for a limited number of research on the dioxin and DL-PCB profiles in feed materials.

Bioanalytical methods are the second group of methods authorised by the EC for dioxin testing in feed materials. These methods are more rapid and much cheaper. They use cell-based assays, receptor assays, or immunoassays (8, Regulation 2017/771/EU). CALUX bioassay (Chemically Activated LUciferase gene eXpression), which belongs to that group, has been shown to be a sensitive tool to detect dioxins and dioxin-like chemicals by measuring AhR-dependent gene induction (1, 5). Seven PCDD, ten PCDF, and twelve DL-PCB congeners (four non-*ortho* and eight mono-*ortho)* exhibit high binding affinity to an intracellular aryl hydrocarbon receptor (AhR), a transcription factor that regulates expression of multiple genes important in development, physiological function, and toxicity (4, 21). While in the chemical method toxicity of total dioxins present in sample (a mixture of present toxic congeners) is calculated as the sum of the concentrations of individual congeners multiplied by corresponding toxic equivalency factors (TEF), in bioassay method, a direct measurement of BEQ (TEQ) value of the sample is done (26).

The present research was intended to develop a method for semi-quantitative detection of PCDD/Fs and DL-PCBs in feed at ppt levels, for which maximum level (MLs) and action levels (ALs) have been set in the EU legislation (Regulation 277/2012/EU). The study aimed to verify the suitability of the XDS-CALUX® bioassay in a plant, animal, and mineral feed ingredients and compound feed. The method involved rapid and simple extraction and clean-up procedure and PCDD/PCDFs and DL-PCBs detection and quantification using bioassay with luminescence measurement. The bioassay performance was verified by validation and congener-specific chemical analysis with HRGC/HRMS method. Correlations between BEQ and WHO-TEQ have been demonstrated as well. The method meets the requirements of the currently valid recommendations of the European Commission (2017/771/EU) and was accredited by the Polish Centre for Accreditation - PCA (certificate no 957).

Material and Methods

Reagents and chemicals. All chemicals and reagents were of high grade purity. N-hexane, toluene, methanol, acetone, and ethyl acetate were of HPLC grade obtained from POCH (Poland). Silica gel 60 (Fluka, Germany) and anhydrous sodium sulphate (J.T. Baker, USA) were ACS grade. Sulphuric acid 95%−98% (POCH, Poland) was of analytical grade. Active carbon and Celite 545 were from Merck (Germany). Each batch of reagents was checked for interferences in CALUX bioassay. All solvents used in the sample preparation and analysis were checked for suitability by testing each lot of solvent for activity in the bioassay prior to use.

Cell culture reagents. RPMI 1640 advanced medium, Glutamax, Pen/Strep (500 µg/mL/5000 IU), trypsin, and PBS, pH 7.4, buffers were purchased from Gibco (UK). Foetal bovine serum was obtained from Biosera (USA). Luciferase Assay System and Luciferase Cell Culture Lyses Reagent were from Promega (Germany) and DMSO (ACS grade) from Aldrich (Germany).

Standards of PCDD/Fs and PCBs. Standards of 17 PCDD/F congeners with established WHO-TEF values in toluene or nonane, purity >98%, concentration 50 µg/mL, were obtained from Wellington Laboratories (Canada) or Accustandard, Inc (USA). 2, 3, 7, 8-TCDD (50 µg/ml in DMSO, 99% purity) was purchased from Cerilliant (USA), and PCB-126 in isooctane (35 µg/mL, 100% purity) from Accustandard, Inc. Both of them were used to prepare calibration solutions. The standards were stored in accordance with certified recommendations.

Reference materials. Home-made reference materials (HRM) were used. The materials were naturally contaminated at the level of around the maximum level (fishmeal, compound feed) or spiked with 17 PCDD/F congeners and PCB-126 (vegetable oil, animal fat, fish oil, mineral materials). Their concentrations were confirmed by HRGC/HRMS analysis. Reference materials were used to correct for apparent recovery.

Cell line. The bioassay was carried out using a recombinant H1L6.1 cell line, which was mouse hepatoma cells (Hepa1c1c7) stably co-transfected with the plasmid pGudLuc6.1 (5, 10). Cells H1L6.1 were grown at 37°C in 5% CO_2 and 97%−99% humidity in cell culture flask containing advanced RPMI 1640 with 8% foetal calf serum (FCS), 1% Glutamax, and 1% penicillin/streptomycin. After trypsinising, the cells were counted in Naubauer haemocytometer. Cells were cryo-preserved at 1−2 × 10^6 cell per ml in cryogenic vials. Freezing medium was RPMI with 8% FCS, 1% penicillin, and 8% DMSO, and after freezing for 24 h at –70°C, the cells were transferred to liquid nitrogen for long storage. After thawing, the cells were grown in 25 cm^2 and then 75 cm^2 culture flasks to 80% confluence.

Equipment. The following equipment was used: laboratory grinders and blenders, 1 mm sieves, analytical and moisture balances, horizontal shaker, biological safety hood, microscope, centrifuge, automatic pipettes, CO_2 incubator, and luminometer.

Analytical method protocol. The method included chemical and biological stages. In the chemical stage (extraction and clean-up), target analytes were isolated together with fat by a proper mixture of organic solvents. Removal of interfering substances and separation PCDD/Fs from DL-PCBs was done on chromatographic columns. PCDD/Fs and DL-PCBs concentrations were analysed *in vitro* after reaction of enzyme (luciferase) with the substrate (luciferin). Luciferase was produced by cells in the presence of PCDD/Fs and DL-PCBs in the time- and dose-dependent manners.

Sample preparation for use with this method is described below. Fat and oil samples were melted if necessary and mixed thoughtfully. Other solid samples were ground, mixed, and screened through 1 mm sieve. After homogenisation, the samples were split into A and B subsamples. Subsample A was used for analysis and subsample B was stored for confirmatory analysis**.** Fat and oil samples were dissolved in n-hexane and dried *via* anhydrous sodium sulphate. Other samples were dried in laboratory oven at 80°C for 24 h and moisture content was measured by weight.

Extraction and purification of the samples differ depending on the matrix. The corresponding mass of ground samples was taken for extraction. Optimised sample size, extraction time, solvents, extraction mixture, and its volume are shown in Table 1. Depending on the sample fat content different columns were used as shown in Fig. 1. After eluting with a solvent, the sample extract was concentrated to near dryness in vacuum centrifuge concentrator. Extract dissolved in n-hexane was cleaned up on silica acid gel and on activated carbon column. The extract was passed through acid silica gel column directly to active carbon. The active carbon column is an affinity column that binds chlorinated dioxins, furans, and biphenyls and these compounds can be differentially eluted. PCBs with the mixture of n-hexane, toluene, and ethyl acetate, and next PCDD/F fraction is eluted with toluene after active carbon column inversion. All eluates were concentrated under vacuum and then brought up in n-hexane for bioassay. The flowchart is shown on Fig. 2. The principle of the XDS-CALUX bioassay is as follows. It is the reporter gene assay, which uses a recombinant mouse hepatoma cell line (H1L6.1c3), stable transfected with an AhR-responsive firefly luciferase reporter gene. Treatment of these cells with 2, 3, 7, 8-TCDD or other AhR agonist activates the Ah receptor, which binds to the dioxin response elements (DREs) on DNA and induces expression of the adjacent luciferase reporter gen resulting an increase in cytosolic luciferase protein (10). Luciferase activity is easily measured in a cell lysate after substrate addition (luciferin), which upon cleavage by luciferase produces luminescence. The resulting light production is quantitated as relative light units (RLU) and the increase in RLUs is related to the amount of enzyme and to amount of dioxin-like compounds in the sample extracts. This bioassay coupled with the specific sample extraction and clean-up procedure allows for measurement of the sum of PCDD/Fs and sum of DL-PCBs present in sample extracts. While in the chemical method a total sample dioxin toxicity expressed as WHO-TEQ (the mixture of present toxic congeners) is calculated as the sum of the concentrations of individual congeners multiplied by the corresponding toxicity equivalent factors (WHO-TEF), in bioassay direct measurement of BEQ (TEQ) is done by comparing to a calibration curve.

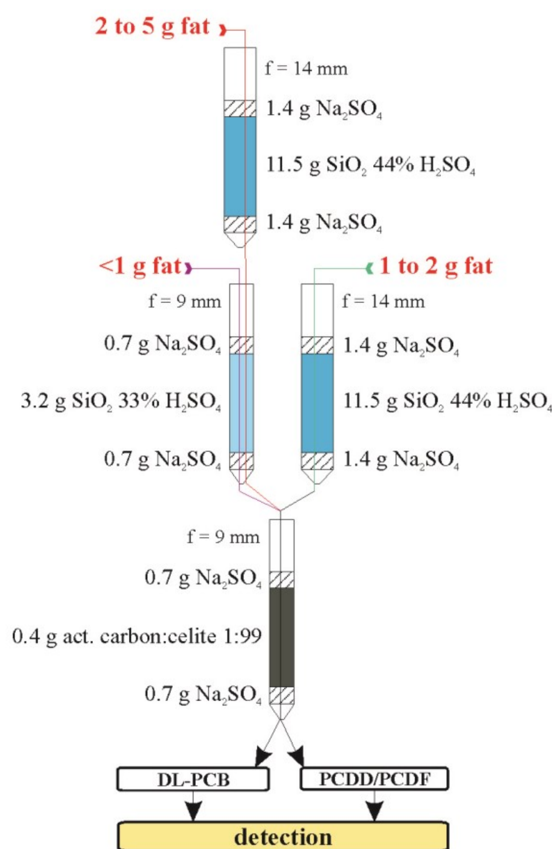

Fig. 1. Outline of clean-up and DL-PCBs and PCDD/Fs separation procedure depending on amount of fat

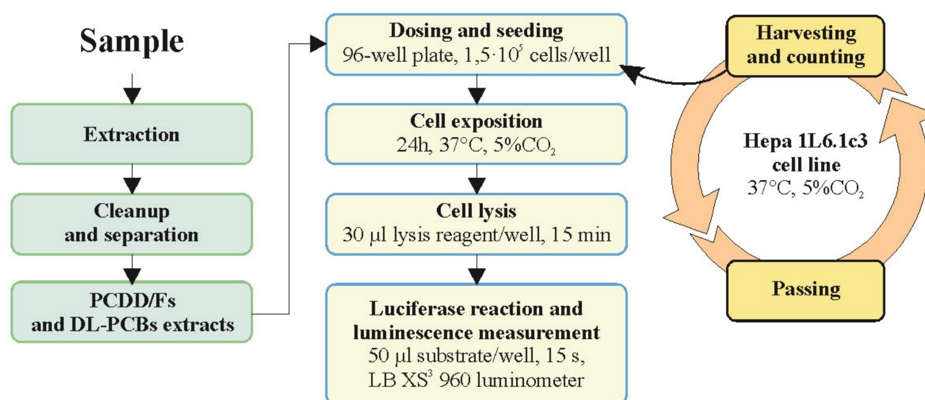

Fig. 2. Flowchart of bioassay

Sample extracts, reference materials, and method blanks were transferred to growth medium with 2% DMSO and 90 µL was put on 96-wells microplate. Subsequently, 90 µL of cell suspension (2×10^6 cells/ml) was added, giving concentration of 1.8×10^5 cells/well and 1% DMSO in the medium. In the same manner, cells were exposed to eight concentrations of 2, 3, 7, 8-TCDD in duplicate (0.18−45 pg/well), eight concentrations of PCB-126 in duplicate (3.6−1,800 pg/well), medium blank, assay blank in triplicate, and four repetitions of 2, 3, 7, 8-TCDD solution to check for assay repeatability. After 20–24 h incubation, the medium was removed and cells were washed with 50 µL of PBS. Lysis was performed by adding 30 µL of Cell Culture Lysis Reagent (Promega) and shaking for 7.5 min, followed by next 7.5 min incubation before luminescence measurement. Luciferase produced light resulting from cleavage of luciferin (luciferase substrate) was quantified as relative light units (RLU/s) using Centro XS^3 LB 960 luminometer (Berthold Technologies, Germany). LumiTestPlate (Berthold Detection Systems) was used to control luminometer performance and repeatability. Resulting data were then analysed using an Excel spreadsheet to calculate BEQ for sum of PCDD/Fs and DL-PCBs.

The results in the application of bioanalytical screening methods were expressed as bioanalytical equivalents (BEQ) with an expanded uncertainty (U) evaluated following the European law (Regulation 278/2012/EU). The expanded uncertainty was calculated using a coverage factor of 2, which gives a confidence level of 95%. For feed and feedingstuffs, the dioxin and DL-PCB levels are on a product basis with 12% moisture content.

Validation. The developed method for validation and performance characteristics was carried out according to the European Regulation 2017/771/EU, concerning the sampling, methods of analysis for the official control of dioxins in feedstuffs, method performance criteria, and interpretation of results. Performance characteristic describing functional quality and attributes of the tested method, such as linearity, detection level, repeatability, reproducibility, and recovery were tested. Validation experiments were performed using feedstuffs matrix fortified at 1/4, 1/2, 1× and 2× of maximum level. Six repetitions were performed for each level, together with six solvent blank samples and six matrix blank samples. The samples were split into two sets containing three repetitions at each level, and sets were analysed in reproducibility conditions (by different analyst and on a different day). Limit of detection (LoD) was calculated for each measurement as average and three standard deviations of DMSO blank on the basis of at least four repetitions of DMSO blank samples. Limit of quantification (LoQ, reporting limit) was calculated by six repetitions of matrix fortified at assumed LoQ level (usually 1/4 of maximum level) in repeatability conditions. If results fulfilled the performance criteria (repeatability, reproducibility, apparent recovery), spike level was considered to be LoQ. Calibration curve working range (linearity), between LoD and EC70, based on two repetitions of the calibration curve, was performed in each measurement. All calibration points in working range had CV < 15%. Repeatability (RSD) as the coefficient of variation between three repetitions (concentration after blank and recovery correction) obtained by an analyst in one series was calculated for each spike level. Intra-laboratory reproducibility (RSD_R), coefficient of variation between six repetitions (concentration after blank and recovery correction) performed by different analysts on different days, was calculated for each spike level. Apparent recovery was calculated as bioassay result from 2, 3, 7, 8-TCDD and PCB-126 calibration curve (in BEQ) corrected for blank and then divided by TEQ level determined by HRGC/HRMS method. The cut-off value was calculated using the lower band of the 95% prediction interval at the HRGC/HRMS decision limit. Method working range was between LoQ and the highest level investigated during validation (2× maximum level).

Quality assurance and quality control. The CALUX bioassay uses QA/QC procedure, including solvent blanks and reference materials, at a frequency of one per 20 samples. It also employs

separate positive controls for PCDD/Fs and DL-PCBs, and negative controls at a frequency of one per 96-well assay plate. Quality control charts are maintained for all reference materials as well as for the standard solution of PCB-126, congener mixture of PCDD/Fs and 2, 3, 7, 8-TCDD. They were applied on each plate. The method employs the use of diluted concentrations of 2, 3, 7, 8-TCDD and PCB-126 for the standard curve and quality control solutions containing PCB-126, and a lotion comprised of all toxic 2, 3, 7, 8 dioxin and furan congeners and 12 DL-PCBs for which WHO has established TEF values (26). Each sample was analysed in duplicate (full analysis) and accompanied by the method's blank and reference material. Each plate contained eight 2, 3, 7, 8-TCDD calibration points and eight PCB-126 calibration points, both in duplicate. Moreover, medium blank and three assay blanks (DMSO) were added. Four repetitions of low level 2, 3, 7, 8-TCDD solution were used on each plate to check for repeatability. Four-parameters Hill equation was used to transform cell response (luminescence) into concentrations. Standard curves are modelled to sigmoid curves described by four-variable Hill equation using least squares best fit. Apparent recoveries, bioassay repeatability, and Hill equation k parameter (ln EC50) were control. About 10% of compliant samples were analysed with HRGC/HRMS method to check false negative results. All suspected samples were confirmed by HRGC/ HRMS method proficiency test (PT study). Positive participation in proficiency testing accounted for external QC.

Results

Optimisation of extraction and purification conditions has involved comparing the various extraction techniques, extraction mixtures, adsorbents for column chromatography cleaning, and elution mixtures. The optimised extraction step for different feed material is shown in Table 1. Separation of PCDD/Fs from DL-PCBs by a variety of chromatographic column packing materials is shown in Fig. 1.

The obtained results revealed that combining different extraction mixture with optimal extraction time and with an acid silica plus activated carbon clean-up provides reliable, reproducible measurements with acceptable recovery and sensitivity at the required ppt range. A summary of the results of the validation process for PCDD/Fs and the sum of PCDD/Fs and DL-PCBs detection with AhR reporter gene assay are presented in Table 2.

Table 1. Extraction parameters

Parameters	Compound feed	Fishmeal	Animal origin	Mineral origin
Sample weight	10 g	5 g	10 g	10 g
Extraction	platform shaking	platform shaking	manual shaking	manual shaking
Time	45 min+10 min	45 min+10 min	4 × 2 min	4 × 2 min
Extraction solvents	1. toluene: methanol (4:1 v/v, 30 mL) 2. n-hexane 15 mL	1. toluene: methanol (4:1 v/v, 15 mL) 2. n-hexane 7.5 mL	1. acetone + n-hexane (10 + 10, v/v, ml) 2–3. n- hexane 10 mL	acetone+toluene 1. (20 + 10, v/v, mL) 2–4. (10 + 10, v/v, mL
Desiccation	anh. Na_2SO_4 3 g, n-hexane 2 × 10 mL	anh. Na_2SO_4 3 g, n-hexane 2 × 10 mL	anh. Na_2SO_4 3 g, n-hexane 2 × 10 mL	anh. Na_2SO_4 3 g, toluene 2 × 10 mL

Table 2. A summary of validation of PCDD/Fs and PCDD/F/DL-PCBs determination in feed materials

Matrix	Repeatability (RSD_r)	Reproducibility (RSD_R)	Apparent recovery	Reporting limit
	(%)	(%)	(%)	ng BEQ/kg feed with a moisture content of 12%
		PCDD/Fs		
Compound feed	<17.3	<13.2	80.6–93.5	0.2
Fishmeal	<19.2	<17.7	100.0–104.3	0.31
Fish oil	<16.0	<14.3	82.2–97.7	0.44
Animal fat	<18.3	<22.9	62.2–91.2	0.3
Vegetable oil	<15.8	<18.7	78.3–90.5	0.2
Feed materials of mineral origin	<16.9	<13.7	74.2–80.5	0.2
Analytical criteria	<20	<25	50–130	–
PCDD/F/DL-PCBs				
Compound feed	<15.9	<15.2	64.6–67.9	0.4
Fishmeal	<18.7	17.7	51.5–66.1	1.54
Fish oil	<11.2	<9.4	53.5–60.7	1.5
Animal fat	<16.7	<19.9	53.7–82.7	0.36
Vegetable oil	<13.3	<13.9	61.3–72.6	0.39
Feed materials of mineral origin	<19.4	<18.9	69.2–75.7	0.26
Analytical criteria	<20	<25	30–130	–

The developed method was characterised by a good apparent recovery and precision. Apparent recovery corrected using the internal standard was between 62% – 104% for the sum of PCDD/F congeners and 52% – 83% for the sum of all three groups of tested analytes. These values were in accordance with the requirements of the EU regulation. Repeatability expressed as relative standard deviation did not exceed 20%, and within-laboratory reproducibility was below 23%, for the entire range of the method. For each feedstuff category, cut-off value was established. These values were also in accordance with the requirements of Regulation 2017/771/ EU.

The precision of the method adapted for testing various matrices was acceptable and in compliance with RSD_R <25%, recommended for cell-based bioassay in the Commission Regulation 2017/771/EU. LoD values were below 0.5 pg/well for TCDD and about 1 pg BEQ/well for PCB-126. The upper limit of working range was about 10 pg/well and 25 pg/well, respectively. Reporting limit was in most cases 1/4 of the respective maximum level. Cut-off values for PCDD/Fs were between 0.56 ng BEQ kg^{-1} in compound feed, feed materials of plant and animal origin, and 3.75 ng BEQ kg^{-1} for fish oil. Cut-off values for the sum of PCDD/F/DL-PCB were in the range from 0.81 to 15.0 ng BEQ kg^{-1} feed.

In summary, the developed screening method of PCDD/F and DL-PCB analysis with the AhR reporter gene bioassay fulfils all performance criteria for screening methods specified in regulations 278/2012/EU, 709/2014/EU, and 2017/771/EU, and is fit for PCDD/Fs and PCDD/F/DL-PCBs determination in feedstuffs.

The correlation between screening and HRGC/HRMS method results is shown in Fig. 3. These are the results of comparative studies of feed materials made in 2006−2016 (15). All feed results exceeding cut-off value were subject to HRGC/HRMS confirmation analysis. Also, 2% to 10% of results below cut-off value were confirmed by HRGC/HRMS to assess bioassay false negative results ratio. So far, no false negative result has been found.

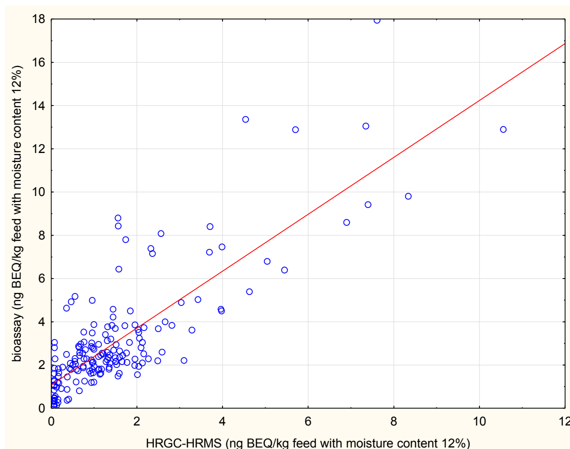

Fig. 3. Correlation between HRGC/HRMS and bioassay results. Regression equation was y = 1.079+1.316x. Pearson correlation coefficient was r = 0.816 (p = 0.000)

Discussion

PCDD/Fs and PCBs in feed may originate from various sources (13, 14). They penetrate into tissues of animals fed contaminated feed, and consequently, they get to food of animal origin, posing a health concern, and thus resulting in detailed regulations of their levels by governments and international organisations (7). Therefore, analysis of chemical contaminants is an essential part of food safety testing programmes to ensure compliance with regulatory limits and consumer safety. Low concentrations (ppts) present in feeds make analysis quite challenging, however, modern analytical techniques are up to this task. The basic analytical approach involves an extraction using a suitable solvent, clean-up to remove interfering compounds, chromatographic separation, and a selective detection. The maximum levels of PCDD/Fs and DL-PCBs, the first pillar of the EU strategy, have been set for feedingstuffs and foodstuffs of animal origin, with only some exceptions. Limits for feed are expressed on a product basis with 12% moisture content. The maximum levels of dioxins were described as the TEQ value of 7 PCDDs and 10 PCDFs with assigned TEFs and have been applicable since July 1, 2002 (Directive 2002/70/EC). To examine the dioxins below 10^{-12} g concentration, appropriate analytical methods were needed. EU strategy recommended screening and confirmatory methods in analysing this group of pollutants. Possibilities to use both methods were described for the first time by the EC Directive 2002/70/EC (7, 14). Since November 2006, DL-PCB maximum content has also been regulated (2006/13/EC). The EU strategy also requires conducting mandatory monitoring of feed and food by the member states, to evaluate the existing levels of dioxins and related compounds. Monitoring, which has to focus on high numbers of randomly taken samples that usually show background contamination levels, requires inexpensive methods. The gold standard in dioxin analysis is gas chromatography coupled with mass spectrometry (HRGC/HRMS). This quite expensive and laborious method gave rise to the development of more affordable bioassays based on cell cultures. The EU law established first performance criteria for screening and confirmatory analytical methods used in the official control of feed in 2002 (Directive 2002/70/EC).

Dioxins and DL-PCBs that exert their biological effects by a molecular mechanism create a basis for every type of bioanalytical approach. Gene expression mediated by a receptor AhR induction can be obtained with the techniques of genetic engineering and molecular biology, by placing DRE elements before the promoter region. This feature was used to create recombinant cell lines reacting to the dioxin presence with induction of a reporter gene, and allowed for development of several biological assays for dioxin determination, from which two are most currently used:

the XDS-CALUX and DR-CALUX. Most frequently the bioassays use luciferase as a reporter gene and mouse cell line Hepa1c1c7 (XDS-CALUX) or rat hepatoma line H4IIe (DR-CALUX). The detected signals represent relative potencies of the contaminants' mixtures, but they do not provide congener specific information (1, 5). The CALUX bioassay has been widely used for the screening of environmental samples and food and feed (1, 3, 6, 11, 12, 15, 23, 27–29). A high correlation has been found between dioxin levels measured using the *in vitro* AhR reporter gene assay and the chemical methods (5, 8, 11, 15, 16).

The goal of the bioassay is to select samples with levels of PCDD/Fs and DL-PCBs exceeding the maximum levels (MLs) or the action thresholds (ALs). The method should ensure cost-effective high sample-throughput, thus increasing the chance to discover new incidents with high exposure levels. In compliance with the EU regulations, results of bioanalytical screening methods are expressed as bioanalytical equivalents (BEQ) and results of physico-chemical methods (HRGC/HRMS) as toxic equivalents (TEQ). BEQ is a measure of concentration of all AhR ligands present in sample extract and their relative potencies (REP) in specific biological system, compared to the potency of 2, 3, 7, 8-TCDD. TEQ is a sum of each of 17 PCDD/F and 12 DL-PCB congener concentration in sample multiplied by corresponding TEF values. In line with the EU policy, the method selects potential positive samples. Subsequently, the concentrations need to be confirmed by a confirmatory method. HRGC/HRMS method provides full information, allowing individual dioxins and dioxin-like PCBs to be identified and quantified at the level of interest (8).

Recommendations and regulations of the EU legislation in the period up to 2017 caused a number of changes and modifications in the biotest already used in analytical laboratories. In our study, we investigated the applicability of the cell-based bioassay as a pre-screening step to the HRGC/HRMS analysis, to the most recent requirements for methods of PCDD/Fs and DL-PCBs determination in feedstuffs samples (Regulation 2017/771/EU). Sample preparation for dioxins and DL-PCBs content analysis is the important step in halogenated compound analysis. A procedure should be able to remove compounds causing false non-compliant results or compounds that may decrease the response, causing false compliant results. Other AhR active ligands should be removed by appropriate extraction and clean-up procedures. Chemical fractionation enables to isolate the desired PCDD/Fs and DL-PCBs from sample extracts. A variety of isolation method and clean-up procedures have been tested that minimise background activity from sample extracts but still allow quantitative extraction of target compounds (PCDD/Fs and DL-PCBs) from feed matrices (Table 1). Addition of the sulphuric acid silica gel column to the clean-up step facilitates removal of lipid from the sample extract, but also separates dioxins/furans from PAHs and other unwanted AhR active compounds present in the extract (6, 22, 25, 27).

Method performance parameters were verified by validation and confirmatory HRGC/HRMS method. Also, quality control was the key issue in providing specific criteria for the method. Blank controls, analysis of control samples, as well as external quality control (PT study) were successfully performed. The losses of PCDD/Fs and DL-PCBs as a result of agonistic and antagonistic effects, or differences between the TEF and REP values were corrected by determination of bioassay apparent recovery. The presented method was optimised and characterised by parallel analysis with HRGC/HRMS.

The AhR reporter gene assay is routinely used for national feed survey programme (9, 15, 16, 20). The development of a method was a necessary measure for an assessment of the initial situation of feed control, allowing the user to describe the starting point and to track changes over time. Feed survey was carried out for the first time in 2004 to gain a representative overview of the actual dioxin levels in feed materials. Being the appropriate tool for a description of the situation, it has been continued in subsequent years. The samples were collected in a representative way and analysed in accordance with the sampling and analysis regulations being in force in the given time (2002/70/EC, 1883/2006/EC, 152/2009/EC, 278/2012/EU, 709/2014/EU, 2017/771/EU). In the meantime, bioassay has undergone modifications and improvements. Several years of monitoring studies, conducted with the developed methodology and covering the full range of national feed ingredients, were in accordance with the EU law, and allowed for an exhaustive assessment of the dioxins and DL-PCBs status in feed materials. The results of the official feed surveillance and the results of comprehensive monitoring programmes, which were carried out on the basis of EU recommendation 2004/704, were available for competent authorities. From 2004−2016, over two thousand feed samples were analysed with bioassay method and only about 10%–12% of suspected samples had to be confirmed by the HRGC/HRMS method. This strategy greatly reduces the cost of monitoring. So far, no false negative samples have been found and false positive ratio is below 1%. Bioassay-based method of BEQ determination in feedstuffs is particularly useful for screening large numbers of samples. The obtained data have shown the usefulness of this bioassay as complementary for HRGC/HRMS method. Data correlation with the HRGC-HRMS chemical method, a small number of false positive results, relatively fast and cost-effective method allow determining BEQ levels in different feed matrices. Validated XDS-CALUX bioassays fulfil the new requirement criteria (2017/711/UE) and hence may serve as a valuable tool for pre-selection of samples suspected to exceed the respective levels of interest.

The described bioanalytical screening method for detection of PCDD/Fs and DL-PCBs in feed at ppt levels is accredited for compliance with ISO 17025, and is routinely used for national feed survey programmes.

In summary, due to the low cost and high throughput, the AhR reporter gene assay bioassay can be used as a pre-screening tool to select and prioritise samples for subsequent analysis by HRGC/HRMS. Although the bioassay does not specify the identity of individual congeners, it serves as a very useful tool for the evaluation of sample contamination. It is also very convenient for evaluating feed and food chain safety. Bioassay method can be routinely used in feed monitoring, in emergencies, and in situations of increased risk of crisis.

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