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BIOTEST AS AN INDICATOR OF DIOXIN-LIKE PCBs PRESENCE

ZASTOSOWANIE BIOTESTU JAKO INDYKATORA OBECNOŚCI DIOKSYNOPODOBNYCH PCB

Abstract: Dioxins (*polychlorinated dibenzo-p-dioxins, PCDDs, polychlorinated dibenzofurans*, PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) are toxic compounds which are commonly present in the food chain. According to the European Union regulations the presence of these compounds in food may be determined by complementary and screening and methods considered confirmatory. The combination of the two methods allows a relatively fast selection of food and feed samples containing dioxins at concentrations exceeding acceptable levels and permits an unequivocal confirmation of tested compounds. The aim of the study was to evaluate the usefulness of the biotest based on genetically modified cell lines sensitive to PCDDs and PCDFs for the simultaneous detection and quantitative determination of dl-PCBs in food samples. Mouse hepatoma cell lines (Hepa1L6.1c3) with the luciferase reporter gene were used. The principle of the biotest involving the dioxin mechanism of action includes the presence of receptor Ah agonist Hepa1L6.1c3 cell line synthesize luciferase at concentrations proportional to the agonist dose. The measurement of luciferase activity for various 2,3,7,8-TCDD concentrations (calibration curve) permits quantitative measurements of the Ah receptor antagonist content in examined food samples. The extraction of dl-PCBs from matrixes (salmon, herring and sprat muscles) was performed using the extraction and purification procedure for PCDDs/PCDFs analysis. Separation of dl-PCB from PCDD/PCDF was done by a column chromatography. The concentration of dl-PCBs was determined using the biotest. The results involving the same samples were compared with those obtained by the HRGC/HRMS method regarded as confirmatory. The results confirm the biotest as a useful method for dl-PCBs determinations; the results obtained by the two methods are parallel and meets the criteria defined by the Commission Regulation (EC) No. 1881/2006.

Keywords: dioxins, dioxin-like PCB, bioassay, detection method

Polychlorinated biphenyls consist of 209 congeners with a varied number and position of chlorine atoms in the aromatic rings. These compounds found numerous industrial applications including flame retardants, hydraulic fluids and transformer heat exchangers. Many reports concerning toxicity of technical PCB mixtures involved the implementation of regulations that limit or ban production in majority of developed countries [1].

Investigations concerning the mechanism of toxic action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD and PCDF) indicate that 12 PCB congeners (IUPAC numbers 77, 81, 105, 114, 118, 126, 156, 157, 167, 169 and 189) are able to activate AhR and cause dioxin-like toxic effects (dioxin-like PCBs, dl-PCBs). The most toxic congener among dl-PCBs is PCB-126 (WHO-TEF equal 0.1) [2].

AhR is a cytoplasmic receptor, which after binding the agonist forms a complex with the protein AhR nuclear translocator (ARNT) and then subsequently migrates to the nucleus. AhR:ligand complex can specifically bind to dioxin responsive elements (DRE) in nuclear DNA sequences present in the promoter regions of numerous genes including P450 1A1 and 1A2 cytochromes, cytosol aldehyde dehydrogenase 3, NAD(P)H:quinine oxidase, and other enzymes required for xenobiotic metabolism.

The knowledge of the dioxin mechanism of action mediated by AhR allowed the development of many bioassays used for detection of dioxins and dioxin-like substances.

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In vitro cell cultures were transformed using plasmids containing a reporter gene under the transcriptional control of AhR. The activation of the receptor in the presence of AhR agionists involved the activation of transcription and translation of AhR-dependent genes including the reporter gene. The XDS-CALUX (Chemically Activated Luciferase Expression made by Xenobiotic Detection Systems, Inc., Durham, USA) is a biotest which utilizes the luciferase reporter gene in mouse hepatoma cell line (H1L6.1c3). This test has been used in the National Veterinary Research Institute in Pulawy (Poland) for the determination of PCDD and PCDF content in food and feedstuffs since 2005.

The goal of this work was to adopt the XDS-CALUX bioassay to determine dioxin-like PCBs in fish tissues.

Material and methods

Principle of dl-PCBs content determination method. The method involved both chemical and biological steps. The chemical step included coextraction of dl-PCBs and lipid from a sample. The extract is cleaned using H_2SO_4-modified silica and activated carbon columns followed by elution of dl-PCBs from the carbon column with hexane:toluene:ethyl acetate (8:1:1, V:V:V). After evaporation, the dl-PCBs content is determined by the XDS-CALUX. The activation of AhR by dl-PCBs present in the extract triggers luciferase expression in a dose-dependent manner. The measurement of luciferase activity according to a 2,3,7,8-TCDD calibration curve allows the determination of dl-PCBs content and expressing the result in WHO-TEQ [2].

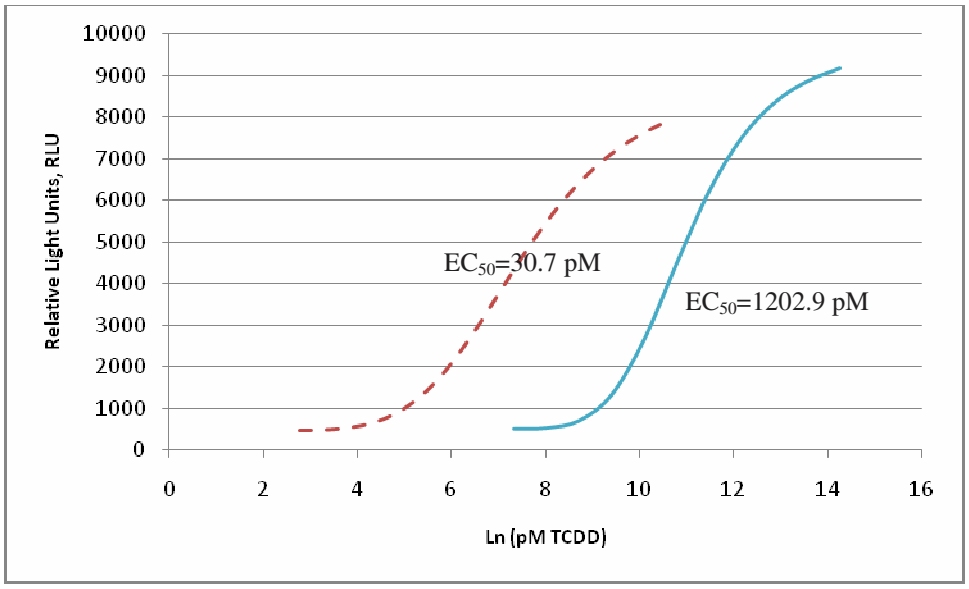

Fig. 1. Dose-response curves of 2,3,7,8-TCDD (dashed line) and PCB-126 (solid line). Cells were exposed to TCDD at a concentration of 0.8 to 776.4 pM and PCB-126 at a concentration of 14.1 to 30674.8 pM. Measured luminescence (in RLU, *Relative Light Units*) of reporter gene product (luciferase) is drawn on the *y* axis, and the natural logarithm of PCB-126 and 2,3,7,8-TCDD molar concentrations are drawn on the *x* axis

Adaptation of the CALUX bioassay for dl-PCBs detection. PCB-126 has the highest biological activity among all dl-PCBs congeners and is responsible for about 90% of dl-PCB-derived toxicity of food and feed. However, this congener is a weaker AhR activator in comparison with the most potent 2,3,7,8-TCDD dioxin. Dose response curves for both 2,3,7,8-TCDD and PCB-126 are shown in Figure 1. For each dl-PCB congener, the relative potency factor (REP) expressing its activity related to 2,3,7,8-TCDD in given biological setup can be calculated. The REP is considered as the ratio of the EC_{50} of 2,3,7,8-TCDD to the EC_{50} of an examined congener (PCB-126).

In this study, EC_{50} values were 30.7 pM and 1202.9 pM for 2,3,7,8-TCDD and PCB-126, respectively. The calculated $REP_{PCB-126}$ value was 0.026 indicating that PCB-126 is about 39 times less potent than 2,3,7,8-TCDD in the XDS-CALUX, thus the dl-PCB results should be corrected by this factor in the case of using the 2,3,7,8-TCDD calibration curve for dl-PCB determination. Moreover, it was necessary to increase the sample amount to achieve a low detection limit of the method. On the basis of analysis of numerous dose-response curves, we assessed the lowest detectable amount of PCB-126 at a level of about 3.5 pg (ie 0.35 pg WHO-TEQ). To obtain the limit of detection (about 0.5 pg WHO-TEQ/g matrix) it was necessary to use 20 g of sample (fish muscles). An increased amount of matrix caused an enlarged extracted fat and needed to modify the clean-up process. Routine analyses involved a column with modified silica (33% H_2SO_4). This method produced high background values, so we performed experiment to check the efficiency of 22, 33, 44, 55 and 66% H_2SO_4 modified silica in clean-up of 5 g fish oil. The 44% H_2SO_4 modified silica was used for further experiments, because it gave both sufficient clean-up and failed to clog columns.

Method of dl-PCB determination in fish muscles. Fat is extracted from 10 g of lyophilized sample by shaking with hexane:dichloromethane (1:2, V:V). The extract is evaporated to dryness and the obtained fat is weighted. After adding 44% H_2SO_4 modified silica analytes are extracted three times with hexane (30, 10 and 10 cm^3) and spread to two columns, the first with 44% H_2SO_4 modified silica and the second with a mixture of activated carbon:celite 545 (1:99, w:w). dl-PCBs are eluted with hexane:toluene:ethyl acetate (8:1:1, V:V:V), evaporated to dryness and measured using XDS-CALUX bioassay.

Bioassay. The cells are grown in RPMI 1640 with L-glutamine medium enriched with 8% of fetal calf serum and 1% of penicillin/streptomycin in a temperature of 37°C, 5% of CO_2 concentration and relative humidity higher than 97%. Before exposition cells are transferred to a 96-well plate, 1.5 x 10^5 cells/well. After 20 to 24 h incubation, cells are exposed to extracts or standard solutions and once again incubated for 20 to 24 h. After incubation, the cells are lysed and the luciferase activity in the lysate is measured in the presence of luciferine. Concomitant measurements of a 2,3,7,8-TCDD dose-response relationship permitted the quantitative evaluation of dl-PCB presence in examined extract.

Checking the bioassay ability to analyze dl-PCB content. The method was validated according to the 1881/2006/EC [3]. All parameters obtained fulfilled the requirements of the methods used in dioxins and dl-PCB analysis. This method was used to analyze dl-PCB content in fish muscle samples, among them 11 herring, 11 sprat, and 6 salmon samples. Results were compared with those obtained by the HRGC/HRMS method considered the golden standard in dioxin and dioxin-like compounds analysis [4].

Results and discussion

Results of dl-PCB content in fish samples obtained by the bioassay and instrumental HRGC/HRMS method are shown in Table 1. The closest agreement of the bioassay (an average of 2.54) and HRMS (2.29 pg WHO-PCB-TEQ/g; $t_{observed} = 5.74$, $t_{critical\ 0.001} = 4.59$) results were found in herring samples. In the case of sprat and salmon the values were 2.29 and 3.33 pg WHO-PCB-TEQ/g ($t_{observed} = 17.25$, $t_{critical\ 0.001} = 4.59$) and 3.80 and 5.79 pg WHO-PCB-TEQ/g ($t_{observed} = 6.17$, $t_{critical\ 0.001} = 5.41$), respectively. The bioassay results were underestimated with regard to the dl-PCB content by 30% on average. Differences between the means obtained by the two methods are statistically significant for all matrices. One of possible explanations of this finding is that the HRGC/HRMS method determines concentrations of only 12 congeners of dl-PCB, and the final result (in WHO-TEQ, Toxic Equivalent) is obtained by adding quotients of congener concentrations and congener WHO-TEF. The CALUX bioassay measures overall activity of all dioxin-like compounds present in the matrix. Disagreement between WHO-TEF and REP values can be to some extent responsible for differences in results.

It is worth noting that the correlation of the bioassay and HRGC/HRMS methods should never attain 1, thus, Pearson's correlation coefficient r = 0.82 (Fig. 2) obtained in our analysis seems to be quite satisfactory and similar to the literature data involving the correlation of PCDD/PCDF results.

Summing up, the bioassay can be a useful tool for a screening analysis assessing overall toxicity of sample, but the official control required the confirmation of positive results by the HRGC/HRMS method.

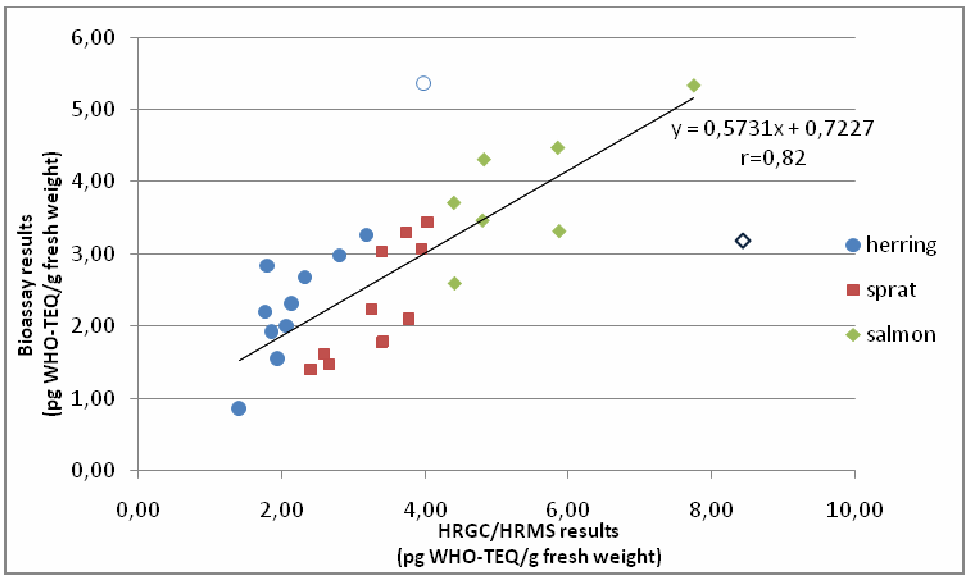

Fig. 2. Bioassay (XDS-CALUX) and chemical analysis (HRGC-HRMS) results correlation. Empty symbols are outliers not used in correlation analysis. Pearson correlation coefficient r = 0.82 (n = 26)

Table 1

Comparison of dl-PCB results obtained using bioassay and HRGC/HRMS methods

Matrix	Sample no.	Bioassay	HRMS
		[pg PCB-WHO-TEQ/g fresh weight]	
Herring	002	0.86	1.40
	005	2.84	1.79
	008	2.01	2.06
	020	2.68	2.32
	027	5.36	3.98
	028	2.31	2.13
	046	1.93	1.86
	064	2.98	2.80
	066	2.20	1.77
	073	1.55	1.94
	067	3.26	3.18
Mean ± standard deviation		**2.54 ± 1.16**	**2.29 ± 0.75**
Salmon	003	3.29	3.74
	004	1.78	3.39
	007	1.62	2.59
	023	1.80	3.41
	024	1.47	2.66
	025	1.41	2.40
	045	2.23	3.25
	065	2.10	3.77
	068	3.44	4.03
	069	3.07	3.95
	072	3.03	3.40
Mean ± standard deviation		**2.29 ± 0.77**	**3.33 ± 0.56**
Sprat	001	3.72	4.40
	006	5.34	7.75
	019	4.48	5.85
	026	3.18	8.44
	063	4.32	4.82
	070	2.60	4.41
	071	3.47	4.80
	086	3.32	5.87
Mean ± standard deviation		**3.80 ± 0.87**	**5.79 ± 1.54**

In the case of sprat and salmon samples (Table 1), the losses of dl-PCB may take place during the evaporation stage so it needs optimization. In addition, it is believed that the cleaning-up should be improved especially in the case of the adsorption and elution of dl-PCB fraction. Modification of these processes should significantly increase the recoveries of analytes and make a closer agreement between the bioassay and HRGC/HRMS results. At present, improvements of the method are being continued.

Conclusions

1. The method used is sensitive, repeatable, and permits a relatively fast determination of dl-PCB in samples of fish muscles.
2. The method is suitable for screening analysis.
3. The adsorption and elution of dl-PCB needs optimization.

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ZASTOSOWANIE BIOTESTU JAKO INDYKATORA OBECNOŚCI DIOKSYNOPODOBNYCH PCB

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Abstrakt: Dioksyny (polichlorowane dibenzo-*p*-doksyny (PCDD) i polichlorowane dibenzofurany (PCDF)) oraz dioksynopodobne polichlorowane bifenyle (dl-PCB)) są związkami toksycznymi występującymi m.in. w łańcuchu żywieniowym. Zgodnie z przepisami Unii Europejskiej, do oznaczania ich zawartości w żywności można zastosować metody komplementarne: przesiewową i potwierdzającą. Ich połączenie pozwala na stosunkowo szybką selekcję próbek żywności i pasz zawierających dioksyny w stężeniach przekraczających dopuszczalne poziomy oraz pozwala na jednoznaczne potwierdzenie obecności badanych związków. Celem pracy było określenie przydatności biotestu bazującego na genetycznie zmodyfikowanej linii komórek wrażliwych na działanie PCDD i PCDF do równoczesnego wykrywania i ilościowego oznaczania dl-PCB w próbkach żywności. W badaniach zastosowano linię komórkową hepatomy mysiej (Hepa1L6.1c3) z wprowadzonym transgenem lucyferazy pod kontrolą receptora Ah. Zasada biotestu wykorzystującego mechanizm działania dioksyn jest następująca: w obecności agonistów receptora Ah komórki Hepa1L6.1c3 syntezują enzym lucyferazę w stężeniu proporcjonalnym do dawki agonisty. Pomiar aktywności lucyferazy wobec serii stężeń 2,3,7,8-TCDD (krzywa kalibracyjna) pozwala na ilościową ocenę zawartości agonistów receptora Ah w badanym ekstrakcie próbki żywności. Do ekstrakcji dl-PCB z matrycy (mięśnie łososia, śledzia, szprota) użyto zoptymalizowanej dla PCDD/PCDF metody ekstrakcji i oczyszczania. Na kolumnach chromatograficznych oddzielano dl-PCB od PCDD/PCDF. Zawartość dl-PCB oznaczano, stosując biotest. Wyniki uzyskane biotestem porównywano z rezultatami oznaczeń chemiczną metodą potwierdzającą HRGC/HRMS, wykonaną w tych samych próbkach. Uzyskane dane pozwalają ocenić biotest jako przydatne narzędzie w badaniach zawartości dl-PCB, ponieważ wyniki uzyskane obydwoma metodami są porównywalne, a metoda spełnia kryteria określone przepisami prawa wspólnotowego (Rozp. 1881/2006/WE).

Słowa kluczowe: dioksyny, dioksynopodobne PCB, biotest, metoda detekcji