APPLICATION OF THE CALUX™ ASSAY TO THE ANALYSIS OF DXNS IN FISH (THE SECOND REPORT)

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Introduction

Food has been generally recognized as the main source of human intake of dioxins (DXNs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and dioxin-like coplanar polychlorinated biphenyls (Co-PCBs), so it is very important to secure the supply of food.

Recently, a total diet study in Japan revealed that main source of the dietary intake of DXNs is likely to come from the intake of fish and shellfish. Therefore, monitoring the levels of DXNs in fish and shellfish would provide important information for risk assessment and management, especially in Japan. Traditionally, DXNs in food and environmental samples have been analyzed by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/MS). However, HRGC/MS requires the use of expensive equipment and the sample preparation procedures are often time consuming and costly. Thus, the use of HRGC/MS is not entirely suited to the task of rapid and frequent monitoring of large numbers of samples. In order to execute the management, it is necessary to develop a rapid and inexpensive screening method.

In DXN2001[3], we reported preliminary data of CALUX™ (Chemically Activated Luciferase Expressio) assay for the screening of DXNs retailed fish. In this study, we examined for the application in detail.

Materials and Methods

Fish samples

Twenty two fish samples (3 yellow tail, 3 mackerel, 2 cod, 4 tuna, 2 salmon. 2 bonito, 2 sea bass and a flatfish) purchased at the market in Japan were analyzed in the comparative studies with HRGC/MS analysis and CALUX™ assay. A commercially available certified reference sample of carp was also analyzed to examine extraction efficiency of DXNs by shaking extract for CALUX™ (Wellington Laboratories, Guelph, Canada).

Sample extraction and clean-up procedures

1. Grind sample and aliquot 10 grams of sample.
2. Add 15 milliliters of acetone to sample aliquot.
3. Add 10 milliliters of dichloromethane/hexane (1:2) and mix.
4. Centrifuge the mixture at 500 rpm for five minutes to separate the phases.
5. Apply the dichloromethane/hexane layer to the extraction column.
6. Repeat step 3 through 5 two times.
7. Wash the column with 10 milliliters of dichloromethane/hexane (1:2).
8. Following concentration, the sample extract was cleaned up and separated into a fraction containing PCDDs/DFs and a fraction containing Co-PCBs.

BIOANALYSIS

CALUX™ Assay

The CALUX™ assay uses a patented recombinant mouse cell line that contains the luciferase reporter gene under control of dioxin responsive elements[4]. When these cells are exposed to environmental ligands such as DXNs, luciferase protein is synthesized. The amount of light produced by the luciferase protein is directly related to DXNs-TEQ. The CALUX™ assay method used has been described previously. Briefly, the cells were grown in the 96-well view plates and exposed to fish sample extracts and 2,3,7,8-TCDD standards (250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, 0.9, 0.5 ppt), using DMSO as the vehicle (final DMSO concentration 1 % in cell culture medium). The plates were incubated at 37 °C and 5 % CO_2 for 20 hours to produce optimal expression of luciferase activity. And then, the medium was removed and the cells were lysed. Luciferase activity was determined using a luminometer (Lucy 1 produced by Anthos Corp.). Luciferase activity was determined as relative light units (RLU).

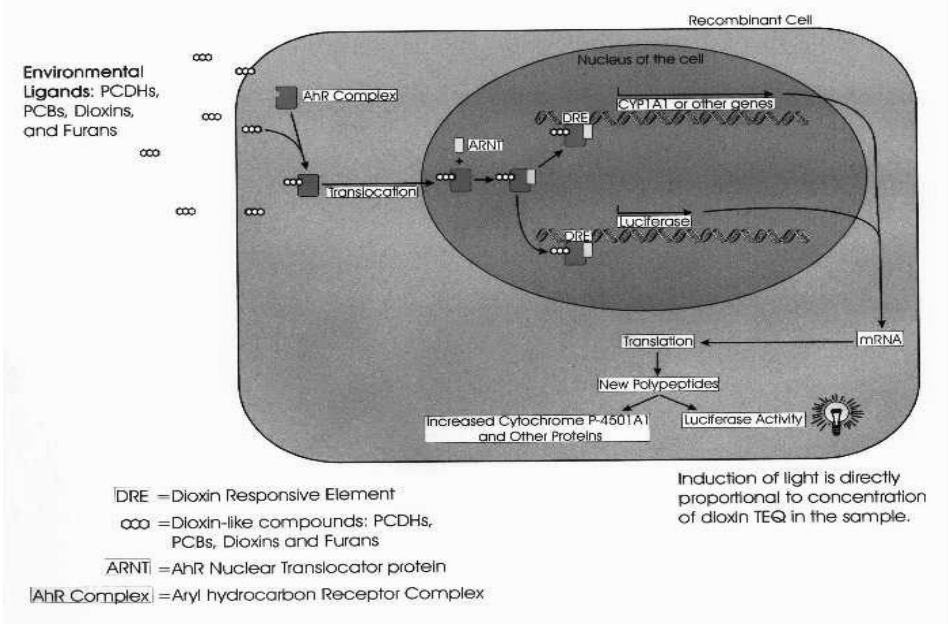

Figure 1. Mechanism of CALUX Cell HRGC/MS analysis

The extraction and cleanup of samples for HRGC/MS followed previously published protocols[5]. The analysis of the 17 active PCDDs/Fs and 12 Co-PCBs (non-ortho and mono-ortho PCBs) were performed by HRGC/MS using an HP6890 plus gas chromatograph coupled to a JMS-700 mass spectrometer (JEOL Ltd., Japan). The TEQ concentrations were calculated using the WHO-TEFs (1997).

Results and Discussion

Recovery of DXNs from by shaking extraction in CALUX™

To examine recovery rate DXNs from fish by shaking extraction in , carp sample was extracted by shaking extraction or alkaline digestion, and then analyzed HRGC/MS. Table1. shows the result of recovery of DXNs by the extraction, which was represented as relative values alkaline digestion. The recovery rate of DXNs was 70-80%, indicating acceptance in screening method.

Correlation between CALUX™ Assay and HRGC/MS analysis

Figure 2 shows the correlation of total PCDDs/DFs and Co-PCBs. The correlation coefficient of PCDDs/DFs was very high (R=0.887) and the value analyzed by CALUX™ Assay was about 1.7 time that by HRGC/MS. The correlation coefficient of Co-PCBs was also very high (R=0.911) but the value analyzed by CALUX™ Assay was about 0.22 times that by HRGC/MS.

Table1. Recovery of shaking ext.

	Compound	Recovery (%)
	2378-TCDD	78.2
PCDD	12378-PeCDD	79.6
PCDFs	23478-PeCDF	78.0
Co-PCBs	33'44'5-PeCB(#126)	74.7
	Total PCDFs	74.0
	Total PCDFs	74.5
	Total PCBs	75.9
	Total DXNs	74.8

*recovery was represented as relative values to alkaline digestion

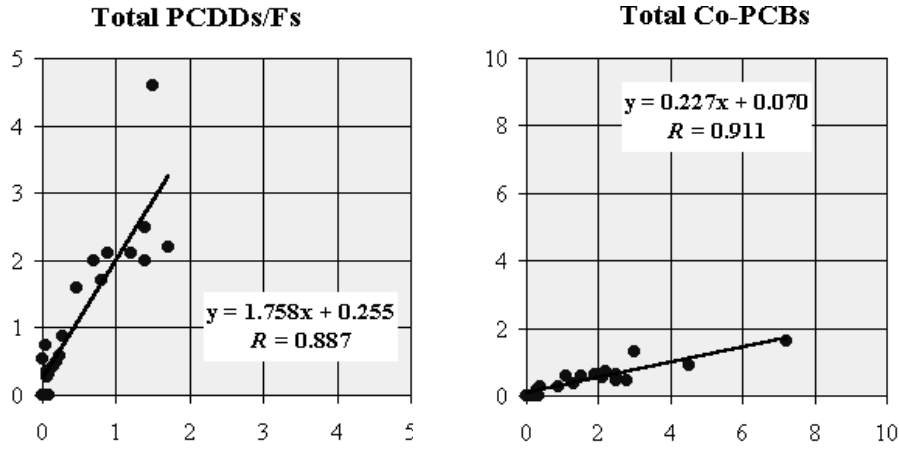

Figure 2. Correlation between CALUX Assay and HRGC/MS (PCDDs/DFs and Co-PCB, x axis ; HRGC/MS(pgTEQ/gfat) y axis ; CALUX (pgTEQ/gfat))

Figure 3 shows the correlation of total DXNs (Separate ; total PCDDs/DFs and total Co-PCBs were analyzed separately and sumed) and total DXNs (Non-separate ; total PCDDs/DFs and total Co-PCBs were mixed and then analyzed). Since CALUX™ Assay was evaluated from biological response, co-existence of Co-PCBs might affect the activity of PCDDs/Fs, but the result was little affected by the co-existence. This might suggest that the distributions of Co-PCBs were restricted to a certain degree in the case of fish samples.

Conclusions

The limit of quantification by CALUX™ Assay was 0.16 pgTEQ/g wet. The original extraction and clean-up method can be substituted for alkali extraction. The correlation between HRGC/MS and CALUX™ was very high in any conditions. CALUX™ Assay has a tendency to overestimate total TEQ compared with HRGC/MS. So it can be concluded that CALUX™ Assay is useful method for screening and monitoring DXNs in fish samples.

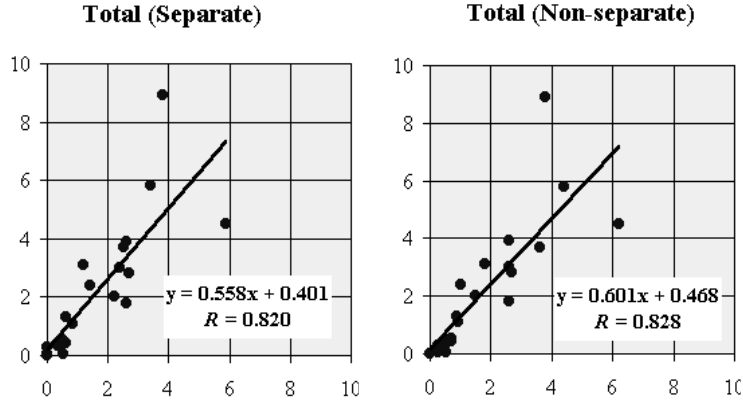

Figure 3. Comparison of CALUX™ Assay and HRGC/MS (Total DXNs;Separate and Non-Separate) (x axis ; HRGC/MS (pgTEQ/gfat) y axis ; CALUX (pgTEQ/gfat))

In order to execute the risk management for DXNs in the foods such as fish, it is indispensable to develop a rapid and inexpensive screening method to handle a great deal of samples. CALUX™ Assay meets these conditions (within 5 days and about 1/10 cost) and we confirmed the reliability of this screening tool for fish samples. The Belgian Federal Ministries of Public Health and Agriculture has already analyzed fat samples of chicken, egg and pork by CALUX™ Assay[6]. But in Japan, it is very important to secure fish since people eat many fish. For the risk management, further frequent examination of DXNs levels in fish are needed and study on the applicability of CALUX™ Assay as screening and monitoring tool for fish.

References

1. Basic Guidelines of Japan for the Promotion of Measures against Dioxins(1999)
2. Law concerning Special Measures Against Dioxins(1999)
3. APPLICATION OF THE CALUX ASSAY TO THE ANALISIS OF DXNS IN FISH (THE FIRST REPORT) Junko Fujino, Tomoaki Tsutsumi, Yoshiaki Amakura, Masafumi Nakamura, Hiroko Kitagawa,Tsukasa Yamamoto, Kumiko Sasaki, Masatake Toyoda (2001) Organohalogen Compounds51,348
4. Denison,M., Brouwer,A.and Clark,G.(1998). U.S.patent # 5,854,010
5. Masatake Toyoda,Takako Iida,Tsuguhide Hori,Toshihiko Yanagi,Youichi Kono and Hiroyasu Uchibe:J. Food Hyg. Soc. Japan, 40,111?121(1999)
6. A COMPARATIVE STUDY OF GC-HRMS AND CALUX™ TEQ DETERMINATIONS IN FOOD SAMPLES BY THE BELGIAN FEDERAL MINISTRIES OF PUBLIC HEALTH AND AGRICULTURE, Ilse Van Overmeire, Leo Goeyens, Hedwig Beernaert, Sarah Srebrnik, Geert De Poorter, Willy Baeyens, George Clark, Michael Chu, Andrew Chu, David Chu, Richard Morris, and David Brown,(2000) ,Organohalogen Compounds45,200