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Simple and rapid determination of dioxin in fish and sea food using a highly sensitive reporter cell line, CBG 2.8D

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ABSTRACT

Food, especially animal origin food is the main source of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), and dioxin-like polychlorinated biphenyls (dl-PCBs) for human exposure. So, a simple, rapid and cheap bioassay method is needed for determination of dioxins in food samples. In this study, we used a new highly sensitive reporter cell line to determine the concentration of dioxins in 33 fish and seafood samples. The samples were extracted by shaking with water/isopropanol (1:1 *v/v*) and hexane and cleaned-up by a multi layered silica gel column and an alumina column, then analyzed using CBG 2.8D cell line. We compared the results obtained from the CBG 2.8D cell assay to those obtained from conventional High-Resolution Gas Chromatography–High Resolution Mass Spectrometry (HRGC–HRMS) analysis. Good correlations were observed between these two methods (r^2=0.93). While the slope of regression line was 1.76, the bioanalytical equivalent (BEQ) values were 1.76 folds higher than WHO-TEQ values and the conversion coefficient was 0.568 (the reciprocal of 1.76). In conclusion, CBG 2.8D cell assay was an applicable method to determine dioxins levels in fish and sea food samples.

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dl-PCBs) are ubiquitous, bio-accumulative and toxic persistent pollutant that easily reach to human food chain. These pollutants negatively affect normal physiologic functions of liver, skin, immune and reproductive system and exert teratogenic, endocrine disrupting and carcinogenic effects that are mediated mainly through the aryl hydrocarbon receptor (AhR) signaling pathway (Tian et al., 2015). It

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has been reported that over 90% of total human exposure to PCDD /Fs and dl-PCBs comes from the food of animal origin (Bocio and Domingo, 2005; Chobtang et al., 2011), especially fish and sea food (Wang et al., 2017; Zhang et al., 2015). In China, especially in coastal areas, aquatic food is the main contributor to the dietary intake of PCDD/Fs and dl-PCBs (Wang et al., 2017; Zhang et al., 2015).

High resolution gas chromatography - high resolution mass spectrometry (HRGC–HRMS) is a "gold standard" method for detection and quantification of individual PCDD/F congeners and dioxin-like compounds. However, HRGC–HRMS requires expensive equipment, highly trained technicians and consumes considerable time limiting the applicability of HRGC–HRMS especially for the large-scale testing. Therefore, the development of a simple, rapid and low-cost bioassay screening method is needed.

AhR reporter assays are engineered cell lines that express AhR gene and a reporter construct expressing a luciferase protein under the control of an AhR-regulated dioxin-responsive element (DRE). AhR reporter assays that utilize native DRE from *CYP1A1* gene promoter have been widely applied to measure dioxin concentrations in different matrices, including environmental samples such as soils (Lin et al., 2014), flue gas and fly ash (Zhou et al., 2014; Zhang et al., 2018) and biological samples, such as fish (Kojima et al., 2011), breast milk (Leng et al., 2009) and blood serum (Croes et al., 2011). In addition, bioassay method could also use for estimating the overall potency of compounds that induce effects through interactions with AhR.

Previous reports indicated that increased copy number of DRE and specific direction of their placement on DNA can improve the precision and sensitivity of in vitro AhR reporter gene assays (Takeuchi et al., 2008). Our lab developed a new highly sensitive AhR activity reporter gene assay cell line named CBG 2.8D (Zhang et al., 2018). The present study was aimed to verify the applicability of CBG 2.8 D reporter assay for determination of the levels of PCDD/Fs and dl-PCBs in fish and sea food samples, as a prescreening step to follow up by the HRGC–HRMS method if needed. To validate the screening utility of CBG 2.8D assay, thirty-three fish and sea food samples were acquired from the supermarkets and determined for dioxin levels using both CBG 2.8D cell assay and HRGC–HRMS analysis. We found that the results obtained using CBG 2.8D reporter cell assay were highly concordant with the results of the gold standard HRGC–HRMS method. Thus, our study has confirmed the utility of CBG 2.8D cell assay as an inexpensive and expedited screening tool for evaluating the levels of PCDD/Fs and dl-PCBs in fish and sea food. The new CBG 2.8D reporter cell assay has a potential to prevent AhR-mediated negative health effects on consumers when applied for fish and sea food screening before it reaches the market.

1. Materials and methods

1.1. Chemicals and cell culture materials

Acetone, n-hexane and dichloromethane were purchased from J. T Baker, Co., Ltd. (USA). Isopropanol, HPLC water, Dimethyl sulfoxide (DMSO) and Silica gel for multi-layer column chromatography were get from Sigma Aldrich, Co., Ltd. (USA). Alpha Modified Minimum Essential Medium (α-MEM), fetal bovine serum (FBS), penicillin-streptomycin (antibiotics) solutions and 0.25% trypsin with 0.02% ethylene diamine tetra-acetic acid (EDTA) were purchased from GIBCO (USA). Luciferase Reporter Gene Assay Kit was purchased from Promega (USA). The PCDD/F and dl-PCBs standards were bought from Wellington Laboratories Inc. (Canada). HRGC–HRMS (DFS, Thermo, USA/ AutoSpec, Waters, USA).

1.2. Collection, extraction, and cleanup of fish and sea food samples for bioassay

Fish and sea food samples were collected from supermarkets in 2014, Beijing, China. The samples (muscular part) were homogenized using a food blender and stored at −20 °C until analysis. The natively contaminated fish reference material (WMF-01) was purchased from Wellington Laboratories Inc. (Ontario, Canada).

Approximately 10 g of homogenized samples for CBG2.8 D cell assay were extracted by shaking with hexane after sample was mixed with water/isopropanol (1:1). Repeat the extraction three times. The extraction solvent was collected and concentrated to 1 mL in rotary evaporator. Then, the concentrate purified using the multi-layered column including two layers of 5 g 33%, 5 g 20% sulfuric acid silica and 1 cm of activated anhydrous sodium sulfate. The dioxin compounds were eluted with 40 mL of n-hexane, evaporated and re-suspended in 50 μL dimethyl sulfoxide (DMSO) for bioassay, further purified using an alumina column (5 g) and the fraction containing all dl-PCBs was eluted with 15 mL toluene and the other fraction contains PCDD/Fs was eluted by 40 ml of hexane/ dichloromethane (1:1 *v*/*v*).

1.3. Cell culture and CBG2.8 D bioassay

The recombinant CBG 2.8D cells developed from a mouse Hepatoma cell line were cultured in α-MEM medium supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1% antibiotics at 37°C, 5% CO_2 and 100% humidity (Zhang et al., 2018).

The cell-based bioassay was described by Zhang (Zhang et al., 2018). Briefly, seeding plates, incubated for 24 hr in the CO_2 incubator to reach confluence. Then, 100 μL growth medium containing 2, 3, 7, 8-TCDD standards, DMSO or reference sample or samples were added to the wells in triplicate (0.8% DMSO). The calibration standard curve was built based on different dilutions of 2, 3, 7, 8-TCDD and used for quantification of samples BEQ concentrations. After 24 hr, remove the medium and rinsed with PBS then lysed in 100 μL of the cell lysis buffer. The luciferase activity was measured by adding 100 μL of Luciferase Assay Reagent used Glomax Multi-detection system (Promega) with automatic injection. The concentrations of dioxins were calculated using the quantitative regression line of the standard curve for 2, 3, 7, 8-TCDD and expressed as BEQ values.

1.4. HRGC–HRMS analysis

Sample extraction and cleanup for HRGC–HRMS test was described everywhere (Zhang et al., 2011; Wang et al., 2017). The

seventeen PCDD/Fs congeners and twelve dl-PCBs congeners were analyzed by HRGC–HRMS which was equipped with DB-5 MS capillary column (60 m × 0.25 mm i.d. × 0.25 μm). The temperature program for the analysis of PCDD/Fs started with 3 min at 150°C, raised to 235°C at 25°C/min holding for 8 min, and then to 275°C at 2°C/min holding 5 min and from 275°C to 290°C at 1.0°C/min holding 1 min, lastly, ultimately to 310°C at 7°C/min holding for 2 min. When analyzed dl-PCBs, the temperature program was started at 100°C and held 3 min, then to 210°C at 30°C/min holding 1 min, further to 270°C at 2°C/min and held for 3 min, finally to 310°C at 30°C/min holding for 2 min. In addition, the ionization energy was 45 eV.

1.5. *CBG 2.8D bioassay validation and quality control*

As CBG 2.8D was validated for determination the BEQ concentration of dioxins in food, as follows. The laboratory method blanks were tested with each batch samples. Certified fish reference material(CRM) WMF-01 (Wellington Laboratories) was analyzed as a positive control and 2,3,7,8-TCDD solution standard (3.0pM/well, the middle of the quantifiable range) was analyzed for each plate used for method validation. Since the regression line of the standard curve was from 0.4 pM/well to 8.0 pM/well, the limit of quantification (LOQ) was 0.08 pg TEQ/g ww for 10 g sample dissolved in 50 μl DMSO. When the sample was not within the linear range of the standard curve, the CBG 2.8D cell bioassay was re-done with a diluted or concentrated sample, correspondingly.

2. Results

2.1. *Standard curve of CBG 2.8D cell assay*

The MDL and EC_{50} of CBG 2.8D bioassay were test previously (Zhang et al., 2018). To make the CBG 2.8 D cell assay more applicable to determine low concentration samples, such as food and feed samples (Wang et al., 2017). We developed another standard curve. As shown in Fig. 1, the ranged of this standards curve was from 0.4 pM/well to 8.0 pM/well 2,3,7,8-TCDD and the residual errors were small (−1.9~22.6%) and lower coefficients of variations (CVs) were observed (6.8~19.7%). To determine the accuracy and precision of the quantification, six concentrations of 2,3,7,8-TCDD were assayed in triplicate on three different days.

2.2. *Recovery, repeatability of CBG2.8 D bioassay*

The recovery test of PCDD/Fs mixture and 3,3′,4,4′,5-PeCB (PCB 126) were done to evaluate the potential interference of fish and sea food in CBG 2.8 D cell assay. Four prepared samples were spiked with 0, 0.5 and 1.3 pg BEQ/g ww PCDD/Fs mixture or 0, 0.4 and 1.9 pg BEQ/g ww 3,3′,4,4′,5-PeCB. Then they were extracted, cleaned up and determined by CBG 2.8 D cell assay. The results are presented in Table 1. The recovery of samples spiked with the PCDD/Fs mixture was from 102% to 116%, for samples spiked with 3,3′,4,4′,5-PeCB was 82%~121%. These recovery levels were quite satisfactory. The results of recovery assessment indicated that the extraction and clean-up procedure meet the requirements of the recovery for PCDD/Fs and dl-PCBs from fish and sea food.

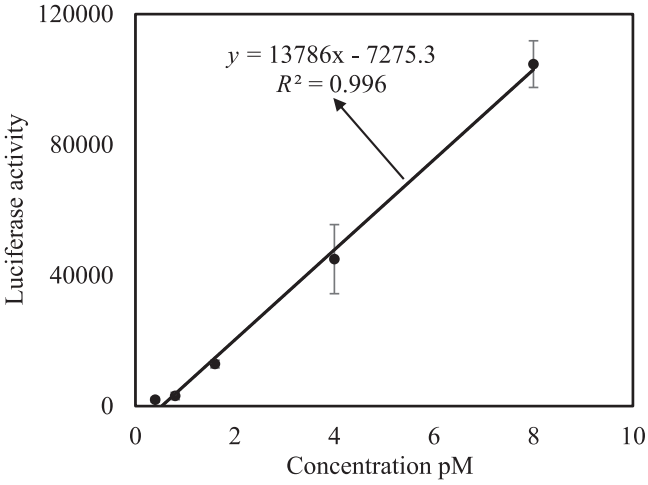

Fig. 1 – Quantitative standard curve of 2,3,7,8-TCDD. CBG 2.8D cells were treated with 0.4 pM, 0.8 pM, 1.6 pM, 4.0 pM, 8.0 pM 2,3,7,8-TCDD, after 24 hr incubation, luciferase activity was determined. Results presented mean±SD (*n* = 6).

The repeatability of CBG 2.8D cell assay in fish and sea food was assessed by replicate analyses of the same cleaned up extract in a single run or two separate runs on different days. As shown in Table 2, the CVs of repeatability were 2.2%~6.1%. We also test the repeatability of CBG 2.8D in combination with the extraction and clean-up procedures by replicate pretreatment and analyses (2 times) of the same fish sample and the CV was from 18.0% to 23.3% (Table 2). These results suggest that the repeatability of CBG 2.8D cell assay used to measure dioxins in fish and sea food is relatively good.

2.3. *Comparison of CBG 2.8D cell assay with HRGC-HRMS analysis*

The results of fish and sea food samples obtained from CBG 2.8D bioassay and HRGC–HRMS were compared (Fig. 2). Thirty-three samples were determined without fractionation. Among them, 10 samples were further fractionated to PCDD/Fs and dl-PCBs. For PCDD/Fs, the BEQ concentrations obtained by CBG 2.8D cell assay were almost three times as high as the TEQ values from HRGC–HRMS analysis and the correlation coefficients were 0.88 (Fig. 2**a**). For dl-PCBs, the values tested by cell assay were 0.77 folds of those from HRGC–HRMS analysis and the correlation coefficients between the two methods were 0.92 (Fig. 2**b**). For sum of PCDD/Fs and dl-PCBs, the correlation coefficients between HRGC–HRMS analysis and cell assay were 0.93 (Fig. 2**c**). Additionally, CBG 2.8D cell assay also performed well in the analysis of dioxins in fish and sea food without fractionation and the slope of the regression line was 1.76 for fish and sea food samples, also a high correlation coefficient (r^2 = 0.93, n = 33) was obtained between the two methods. As show in Fig. 2**c** and **d**, there was no significant difference in correlation coefficients between these two methods with or without fractionation of the samples. These

Table 1 – Recovery of dioxins from retail fish spiked with a mixture of 17 PCDD/Fs (precision and recovery) or 3,3′,4,4′,5-PeCB.

Samples	Spiked levels (pg BEQ/g ww)		Concentrations (pg BEQ/g ww)	Recovery (%)
Shrimp	PCDD/Fs	0	1.0	/
		0.5	1.6	112
		1.3	2.4	107
Snapper	PCDD/Fs	0	0.7	/
		0.5	1.3	102
		1.3	2.2	116
Saury	PCB126	0	0.2	/
		0.4	0.5	82
		1.9	2.3	113
Grass carp	PCB126	0	0.2	/
		0.4	0.6	107
		1.9	2.6	121

BEQ: bioanalytical equivalent; PCDD/Fs: polychlorinated dibenzo-p-dioxins and dibenzofurans; PCB: polychlorinated biphenyls.

Table 2 – Repeatability of CBG 2.8D cell assay.

Samples	Same pretreatment		Two separate pretreatment	
	Repeatability pg BEQ/g ($n = 3$)		Repeatability pg BEQ/g ($n = 3$)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Mackerel-1	0.97 ± 0.06	6.1	1.13 ± 0.26	23.3
Mackerel-3	1.43 ± 0.08	5.3	1.47 ± 0.28	18.7
Trout	2.53 ± 0.05	2.2	2.8 ± 0.5	18.0

BEQ: bioanalytical equivalent; SD: Standard Deviation; CV: Coefficient of Variation.

observed good linear relationships between the two methods suggested that CBG 2.8D cell assay would be a good screening method to prescreening dioxins levels in fish and sea food.

Since CBG 2.8D BEQs were higher than HRGC–HRMS results. To confirm whether this discrepancy was due to the differences between the WHO-TEF and CBG 2.8D REP values as described previously. We re-calculated the TEQ values of HRGC–HRMS by multiplying the concentrations of congeners determined by HRGC–HRMS analysis (data not shown) by their relative potency values (REP) in the CBG 2.8D assay instead of WHO-TEF, and compared this obtained REP-TEQ values with the BEQ concentrations (Fig. 2**e**). It showed a high correlation coefficient ($r^2 = 0.93$, $n = 33$), and the slope of the regression line was decreased from 1.76 to 1.47. It indicates that the differences between the WHO-TEF and CBG 2.8D REP values were the main reason caused the discrepancy in TEQ and BEQ values. Since the BEQ values were always higher than TEQ values, there must exist other compounds act as AhR agonists except the target dioxins in fish and sea food samples.

2.4. *Correction of values obtained from the cell assay to values from the HRGC–HRMS analysis*

The requirement of CBG 2.8D cell assay method as a prescreening tool was the BEQ values close to or over the TEQ values from HRGC–HRMS analyses. We multiplied the BEQ values by the conversion coefficient 0.568, which is the reciprocal of the slope of the regression line (1.76). Then we compared these converted values with the values from instrumental analysis (Fig. 2**f**). The converted concentrations were close to the concentrations from HRGC–HRMS (the slope was 0.99). It means that the CBG 2.8D cell assay could predict values of dioxins in fish and sea food by application of the conversion coefficient 0.568.

2.5. *Prescreening test of dioxins in 8 fish and sea food samples using CBG 2.8D cell assay*

We carried out a small application of this screening method in 8 fish and sea food samples collected from a supermarket in Beijing, China. The concentrations were ranged from 0.29 to 1.40 pg BEQ/g fresh weight. We next verified these dioxins levels in eight samples using the HRGC–HRMS method, then calculate the TEQ values using WHO-TEF. As shown in Fig. 3, the BEQ values were as twice as high as the TEQ values from the HRGC–HRMS analyses. Then the values from the cell assay were converted using the conversion coefficient 0.568 and the values similar with the results obtained from the HRGC–HRMS analyses.

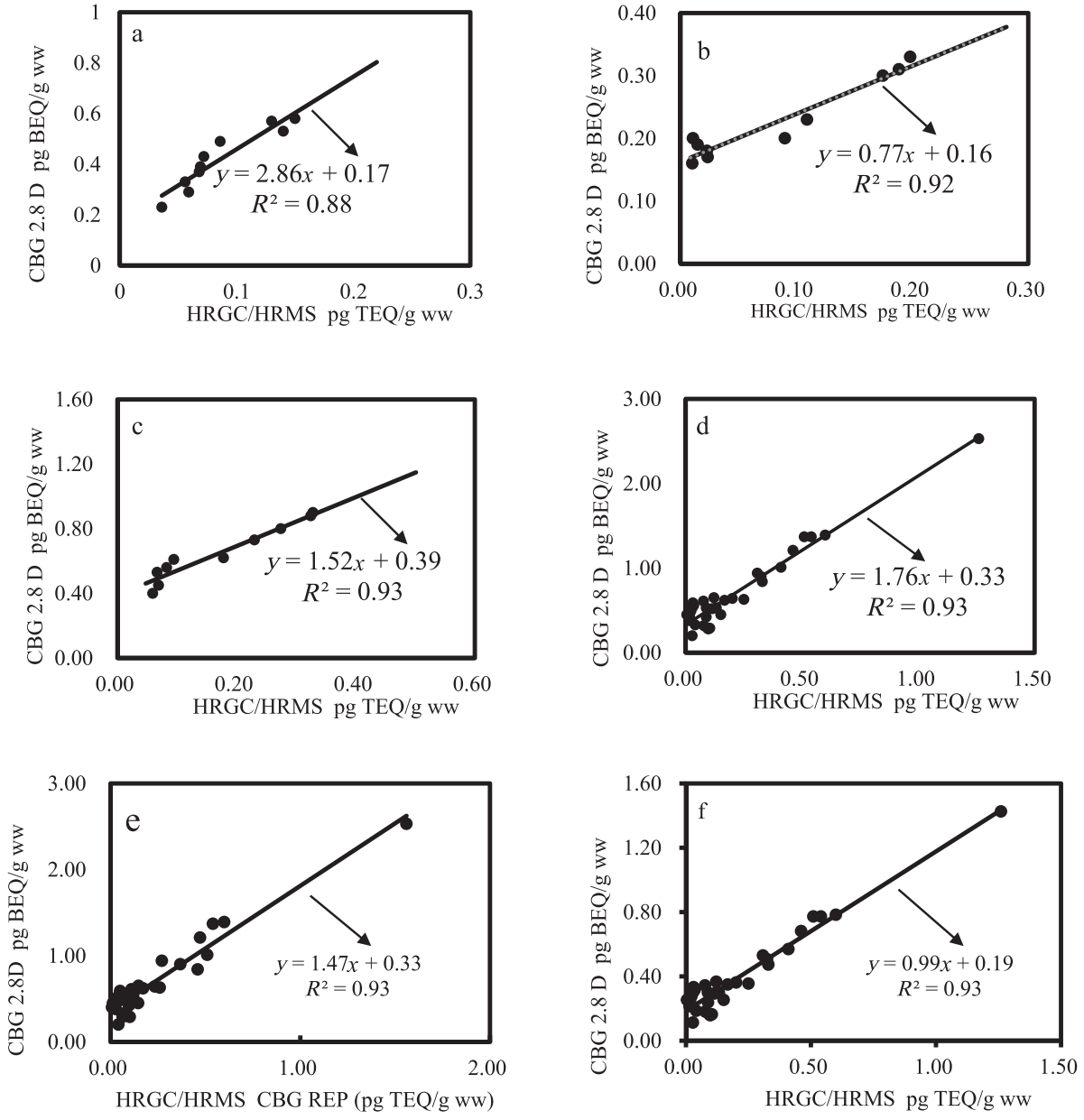

Fig. 2 – Correlation between the results obtained from CBG 2.8D cell assay and concentrations from HRGC–HRMS analysis for the determination of dioxins in fish and seafood samples. Each sample extraction was aliquoted in to two. One for CBG 2.8D cell assay, the other was for HRGC/HRMS. After further clean up, chemical analysis CBG 2.8D bioassay was done for TEQ or BEQ. The correlation between BEQ and TEQ was statistic by scatter plot, correlation ecoefficiency and regression analysis. (a) and (b) Fractionated PCDD/Fs and dioxin-like PCBs (n = 10); (c) sum of PCDD/Fs and dioxin-like PCBs; (d) total BEQ of dioxins in 33 samples; (e) values obtained from HRGC–HRMS analysis re-calculated by using CBG 2.8 D REP and then compared with CBG 2.8D values; (f) CBG 2.8 D cell assay results multiply by conversion coefficient 0.568 and then compared with TEQ values.

3. Discussion

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor involved in xenobiotic metabolism and clearance, cellular proliferation, differentiation and immune system development and potentially on other physiologic processes (Chiba et al., 2012). Here, we used a stable AhR bioassay cell line CBG 2.8D which engineered by introduction of a highly sensitive AhR-mediated reporter in mouse AhR expressing Hepa cells to test dioxins concentration in fish and sea food. Our previously results pointed that this bioassay cell line has provided a sensitive screening method (Zhang et al., 2018). The REP of CBG 2.8D, the MDL, EC50 and the repeatability of this system has been determined previously (Zhang et al., 2018). Since 0.1 pM of 2,3,7,8-TCDD could induce luciferase activity to levels significantly above background, the sensitivity of this bioassay is superior to the previously reported screening methods (He et al., 2011). Moreover, high sensitivity is necessary in monitoring of foodstuff and feedstuffs, since the relatively low concentrations of dioxins in food and feed were reported (Wang et al., 2017). This cell assay also presented

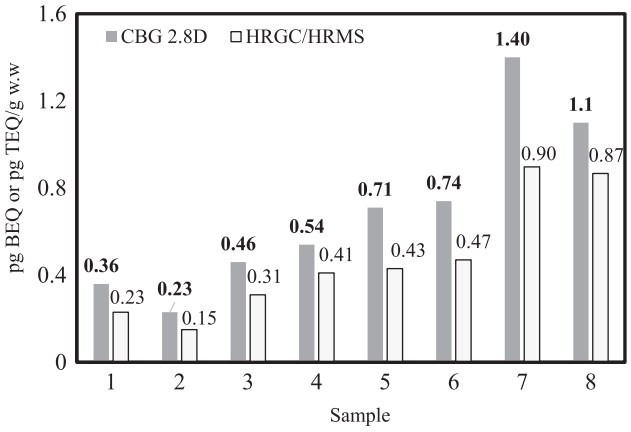

Fig. 3 – Pre-screening test of dioxin contents in 8 fish and seafood samples using CBG 2.8D cell assay and verified using HRGC–HRMS analysis.

good accuracy (recovery 82%~121%) and repeatability (CV below 30%). In addition, the good correlation between CBG 2.8D cell assay and HRGC–HRMS analysis enabled this method to predict WHO-TEQ values in fish and sea food. These characteristics make CBG 2.8D cell assay become an applicable screening method for dioxins in fish and sea food, also it can use as screening method for dioxins in other food and even feed samples.

In PCDD/Fs and total dioxins analysis, the values obtained by cell assay were almost two folds higher than HRGC–HRMS analyses results (Fig. 2a and d). Previous studies have reported that experimental cell-based BEQ values very often differ from the WHO-TEQ values as the relative potencies (REP values) of the compounds are not equal to the WHO-TEF values (Croes et al., 2011; Zhang et al., 2018). After excluded the effect of REP and TEF by re-calculated the HRGC–HRMS concentration multiplied by CBG 2.8D-REPs, the cell assay values were also high. The differences between the observed values in the CBG 2.8D assay and re-calculated values suggested that there exist some other compounds, except the target dioxins, that activated AhR in the samples and influenced the BEQ values. PBDD/Fs have been reported to induce the luciferase activity in cell assay and they are considered almost equivalent to those of the corresponding chlorinated compound (Samara et al., 2009). We did not verify whether these compounds existed in the samples which was tested in this study. But if these compounds were present, it could not be removed in our clean-up procedure, and they could contribute to the BEQ values.

In the dl-PCBs analysis, the values obtained in CBG 2.8D cell assay were a little lower than those produced by HRGC–HRMS analysis. But the observed concentrations of CBG 2.8D were all higher than predicted values (Fig. 2b). One direct reason may be caused by the large intercept which is greater than all the sample values produced by HRGC–HRMS. The other reason of this difference between BEQ and TEQ values was probably due to the difference between CBG 2.8D REPs and WHO-TEFs for dl-PCBs detected in the samples. The HRGC–HRMS results revealed that PCB 126 was the predominant contributor to the WHO-TEQ of dioxin-like PCBs and the contribution to WHO-TEQ value was over 60% of all the analyzed samples (data not shown). However, the CBG 2.8D REP of PCB126 was 0.017 (Zhang et al., 2018) and it is 6 folds lower than WHO-TEF value. Therefore, the values of dl-PCBs obtained by CBG 2.8D cell assay tended to be lower than those analyzed by HRGC–HRMS analysis.

A good relationship was observed between CBG 2.8D and HRGC–HRMS without fractionation of PCDD/Fs and dl-PCBs, (Fig. 2**d**) and the values from CBG 2.8D were 1.76 folds higher than that from HRGC–HRMS. In sum of fractionated PCDD/Fs and dl-PCBs (Fig. 2**c**), we also observed good relationships between these two methods, but the slope was 1.47 lower than non-fractionated 1.76. That was because during the separation of PCDD/Fs and dl-PCBs, some of the potential AhR agonists were removed. Since the CBG 2.8D cell assay for PCDD/Fs analysis was about 2.86 folds higher than that of HRGC–HRMS analysis and just a little lower for dl-PCBs analysis. It was considered this method can carry out with or without fractionation. As the total BEQ values were always higher than TEQ, so it can avoid false negative and meet the requirement of prescreening method. Also consider about the original intension of developing this prescreening method, CBG 2.8D was more applicable in testing total dioxins concentrations.

Establish the dioxins screening method was necessary and it can use to monitor the levels of dioxins in food, feed and environment and help to establish the maximum levels of dioxins in food and feed (Lin et al., 2014; Zhou et al., 2014; Zhang et al., 2018). In 2006, European Commission published the maximum limit levels for dioxins in consumer foodstuffs and the limit for PCDD/Fs of fish is 3.5 pg WHO-TEQ/g fresh weight and for sum of PCDD/Fs and dl-PCB is 6.5 pg WHO-TEQ/g fresh weight ((EU) No 1259/2011). Eight samples which we determined using CBG 2.8D cell assay were below this maximum level and the results were high than that obtained from HRGC–HRMS. This maybe leads to relatively high ratio of false positive, but it could reduce false negative. In practical application, only when the value close to or exceed the maximum level, we should confirm the values using the HRGC–HRMS method.

4. Conclusions

In conclusion, the CBG 2.8D cell assay is a useful screening method for dioxins in fish and sea food and some other food samples. Also, it can be used to assess the potential risk of dioxins in foodstuffs.

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