THE RAPID ANALYSIS FOR DIOXIN DERIVED FROM AGENT ORANGE IN SOIL Ⅲ - CALUX ASSAY FOR DEVELOPING COUNTRY

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Introduction

Defoliants were used in central and southern Vietnam through a program codenamed Operation Ranch Hand between 1961 and 1971 during the Vietnam War by the U.S. military to destroy forest cover and food crops [1)]. The primary defoliant was "Agent Orange" which was one of herbicides and contaminated with the highly toxic of chlorinated dioxins, especially 2,3,7,8-tetrachlorodibenzo- *p*-dioxin (2,3,7,8-TeCDD). Therefore, the ubiquitous environmental pollution by 2,3,7,8-TeCDD might have been caused in the southern Vietnam for the past 50 years. In 2011, the JICA / JST (Japan Science and Technology Agency/Japan International Cooperation Agency) has launched a project of bio-diesel fuel production by means of plantation of trees to produce oil in the land where have been contaminated with Agent Orange, for the purpose of the regeneration of these devastated land in Vietnam[2]. Therefore, first of all, an investigation on current contamination levels of dioxin in the devastated land should be conducted for oil production without the dioxin pollution. However, regarding dioxin analysis in soil, conventional methods for dioxin analysis using Soxhlet extraction and column chromatograph cleanup are very complex, labor-intensive, and time-consuming for a large number of soil samples for measuring and monitoring dioxin in the devastated land.

In this study, we attempt to develop a simple and rapid analytical method for dioxin derived from "Agent Orange", i.e., 2,3,7,8-TeCDD, in soil using ultrasonic extraction[3] and a semi-automated cleanup device[4,5]. Detection methods of dioxin were also applied bioassay methods such as KinExA (Kinetic Exclusion Assay) and CALUX (Chemical Activated Luciferase Gene Expression) assay in addition to HRGC/HRMS. We tried to evaluate integrity of this method by conducting a cross-check of same soil samples in two laboratories between Japan and Vietnam. In this paper, the results of cross-check by the developed analytical method with CALUX detection are described and evaluation of this method is also discussed.

Materials and methods

1) Standard solution: Native PCDDs/DFs, calibration curve were purchased from Wellington Laboratories (Canada).

2) Soil sample:
(1) Samples for preliminary evaluation: Japanese soil and sediment with concentration between 1.9 to 5500pg-TEQ/g (n=10) were used. In addition, 2,3,7,8-TeCDD concentrations in pseudo soil expected to be 0pg/g, 10pg/g, 100pg/g, 500pg/g, 1000pg/g, 3000pg/g, respectively were used. Soxhlet extraction was performed for the preliminary evaluation.
(2)Samples introduced by Vietnam trainees: Japanese low concentration soil with 2,3,7,8-TCDD added at Ehime University were used (n=3).
(3.1) Pseudo soil: prepared mixed nicely the soil containing negligible concentration of 2,3,7,8-TeCDD and Celite (Wako, Japan) absorbed 10,000pg/g of 2,3,7,8-TeCDD. 2,3,7,8-TeCDD concentrations in pseudo soil are expected to be 0pg/g, 100pg/g, 250pg/g, 500pg/g and 1000pg/g, respectively.
(3.2) Contaminated soil by 2,3,7,8-TeCDD: collected at a polluted area by Agent Orange in Vietnam (n=2)

3) Apparatus:
(1) Ultrasonic apparatus: UT-206H (Sharp, Japan)

(2) Semi-automated cleanup device: SZ-DX-PT050 (Seeds Tec, Japan)
(3) Lumino-meter: Ensipre (Perlin Elmer, Japan), CALUX (Xenobiotic Detection System, USA)

4) Methods

(1) Ultrasonic extraction

Figure 1 shows the flow diagram of dioxin extraction from soil using an ultrasonic extraction apparatus. In GC-MS analysis, internal standard for cleanup spike was added to the acetone extract.

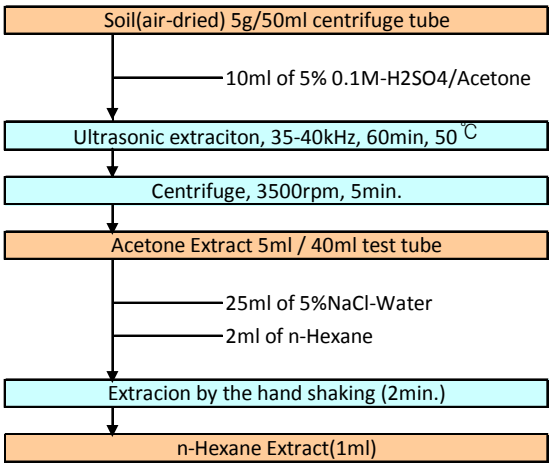

Figure 1. Flow diagram of dioxin extraction from soil based on ultrasonic extraction method.

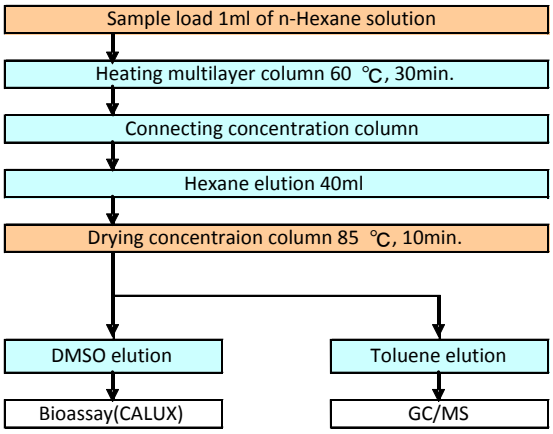

Figure 2. Flow diagram of purification and concentration of the sample extract.

(2) Semi-automated cleanup

One ml of n-hexane extract was used for cleanup using a semi-automated cleanup device as shown in Figure 2. In CALUX analysis.

(3) CALUX Assay determination

After substituting into DMSO solution, cell culture media were added and applied to CALUX assay2. Since there is no facility for cell cultivation or subculture in Vietnam, it will be difficult to do such process. Therefore, plates were prepared, sealed, and put in a box (1400 CASE PERICAN® with purge valve) in Japan, and shipped to Vietnam and measured dioxins using portable plate system. By using a water bath, this box could also be used as a simple CO_2 incubator. CO_2 gas was blown for ten seconds and the plate was reactivated for 1 to 2 hours. Then the cell was dosed and cultivated in the simple incubator and the media was removed. Finally, the luciferase activity (RLU) induced by luciferase assay system was measured using a luminometer[6].

(4) Dioxin analysis in Soil samples for evaluation of the method

Each pseudo and contaminated soil sample was extracted dioxins with acetone by ultra-sonication (Figure 1). The same acetone extract was used for analysis of dioxin by two laboratories, i.e., Ehime University of Japan and CEM of Vietnam, independently, for cross check study. In this study, target compound is only 2,3,7,8-TeCDD because that this compound was dominant in the environmental and biota samples collected in the areas polluted by “Agent Orange”

Results and discussion

1) Preliminary evaluation samples：CALUX results of each soil and sediment samples showed 2.7 to 3.8 times more than GC/MS results, except for a low concentration sample, which result was 1.9pg-TEQ/g. From its correlation figure, using slope value of y=ax, 3.1593 as FACTOR, we have estimated GC/MS estimated value by dividing CALUX value by FACTOR(3.1593). Most of the calculated value, called as GC/MS ratio showed good results as between 0.84 to 1.19 except for low concentration sample of 1.9pg-TEQ/g. In the meantime, the

results of Agent Orenge contamination simulalted soil samples, which added TCDD standard, showed 0.95 to 1.5 times more than actual concentration added. From its correlation figure, the slope value of y=ax was 1.2833(FACTOR). Since the value was low enough that we were able to gain good results without the FACTOR, 0.95 to 1.5, for TCDD contaminated soil, we have decided to do comparison without any conversion.

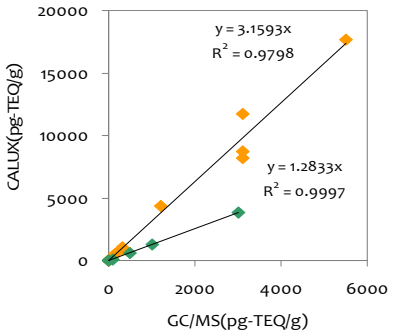

Figure 3. Correlation Figure

Table1. 10 Japanese soils and sediment samples and 6 TCDD added samples

No.		Sample	Matrix	GC/MS	Pretreatment		Factor	3.1593					
					DMSO volume	Sample volume	CALUX Result			CALUX÷GC/MS			
				pg-TEQ/g	mL	g	pg/mL	pg/g	pg-TEQ/g	(%)			
No.1	1	B170610025	Soil	**1.9**	0.240	2.85	157	**13**	4.2	221%			
No.2	1	B170960015	Sediment	**11**	0.239	6.50	1008	**37**	11.7	107%			
No.3	1	B140500035	Soil	**23**	0.242	2.89	985	**82**	26.1	113%			
No.4	1	B140780015	Soil	**40**	0.248	2.96	1474	**124**	39.1	98%			
No.5	1	B110380015	Soil	**97**	0.250	2.99	4004	**334**	105.9	109%			
No.6	1	B160840015	Soil	**150**	0.232	2.37	5452	**533**	168.8	113%	Ave.	SD.	RSD.
No.7	1	B120050025	Soil	**330**	0.243	2.99	11058	**901**	285.3	86%	313.8	29.7	9.5%
	2			**330**	0.216	2.99	13640	**985**	311.7	94%			
	3			**330**	0.229	2.99	14196	**1088**	344.5	104%			
No.8	1	B170940055	Soil	**1200**	0.245	2.85	50789	**4364**	1381.3	115%	Ave.	SD.	RSD.
No.9	1	B160290015	Soil	**3100**	0.249	2.98	97711	**8164**	2584.1	83%	3016.3	610.3	20.2%
	2			**3100**	0.236	2.98	109889	**8689**	2750.4	89%			
	3			**3100**	0.237	2.98	147613	**11735**	3714.5	120%			
No.10	1	B170940025	Soil	**5500**	0.225	2.78	219167	**17705**	5604.2	102%			

No.		Sample	Matrix	GC/MS	Pretreatment		Factor	1		
					DMSO volume	Sample volume	CALUX Result			CALUX÷GC/MS
				pg-TEQ/g	mL	g	pg/mL	pg/g	pg-TEQ/g	(%)
No.11	1	0	Soil	**0**	0.284	1.50	38	**0**	-	-
No.12	1	(+)TCDD-1	Soil	**10**	0.280	1.50	83	**15**	-	154%
No.13	1	(+)TCDD-2	Soil	**100**	0.301	1.50	481	**97**	-	97%
No.14	1	(+)TCDD-3	Soil	**500**	0.279	1.50	3203	**596**	-	119%
No.15	1	(+)TCDD-4	Soil	**1000**	0.287	1.50	6686	**1279**	-	128%
No.16	1	(+)TCDD-5	Soil	**3000**	0.254	1.50	22796	**3860**	-	129%

2) Introduction of Vietnam trainee : For TCDD added soil samples, we have compared CALUX results with GC/MS' and gained actual concentration ratio of below 1. Bioassay is an evaluation method with synergetic effect of complex isomers. However biased contamination such as by 2,3,7,8-TeCDD, bioassay actual concentration results will be almost equal to GC/MS results. Comparing KinExA results for reference, the results of both bioassay are almost equal.

Table2. Results of 3 Pseudo soil samples

Pretreatment	Trainee1			Trainee2			GC/MS
mesurement/Technique	Trainee1/CALUX	Hiyoshi/CALUX	Trainee1/KinExA	Trainee2/CALUX	Hiyoshi/CALUX	Trainee2/KinExA	
Sample	pg-D48/g			pg-D48/g			pg-D48/g
VN1	0	0	0	0	0	0	0
VN5-1	421	628	423	513	700	718	1000
VN5-2	313	620	440	763	969	620	1000

3) Pseudo soil & Contaminated soil by 2,3,7,8-TeCDD : For the cross-check soil samples, we have compared GC/MS results with actual measured CALUX results(actual concentration) without correction by conversion factor (FACTOR) or recoverly ratio. Also to confirm validity of kit-cell, we have also cross-checked with normal incubated cell. Overall, all the results' GC/MS ratio (CALUX divided by GC/MS; toxicity at each institute) without FACTOR were within 0.5 to 2.0. However coefficient of varience (c.v.) of triplet repeated test exceeded 20% at both Hiyoshi and CEM, showing large variation. Kit-cell activity is unstable and since term of validity is about 3 weeks, it is not suitable for continuous use. Considering condtion of developing country, such as difficulty to provide cell through licensing agreement or perform appropriate cell culture, we have used kit-cell. However considering various kit-cell characteristic, we plan to consider for use of freezed cell which can be used for longer period. In addition, we need to improve pretreatment loss and continue manual training to control variety in pretreatment and measurement process.

Table3. Results of 6 cross-check samples

No.	Hiyoshi(Normal cell)					Hiyoshi(Kit cell)					CEM(Kit cell)				
	Ave. (n=3)	S.D.	C.V.	Ehime Univ.	GCMS Ratio	Ave. (n=3)	S.D.	C.V.	Ehime Univ.	GCMS Ratio	Ave. (n=3)	S.D.	C.V.	CEM GCMS	GCMS Ratio
VN-1	0	-	-	0	-	0	-	-	0	-	43	-	-	0	#DIV/0!
VN-2	88	13.8	15.8	91	0.96	99	28.7	29.1	91	1.08	91	14.3	15.6	97	0.94
VN-3	188	21.4	11.4	210	0.90	211	77.5	36.8	210	1.00	200	94.0	47.0	244	0.82
VN-4	294	8.3	2.8	330	0.89	276	61.1	22.1	330	0.84	403	129.3	32.1	354	1.14
VN-5	939	144.7	15.4	987	0.95	872	206.3	23.7	987	0.88	576	159.3	27.6	1159	0.50
QC-4	1754	122.1	7.0	1640	1.07	1485	155.4	10.5	1640	0.91	1540	434.6	28.2	1896	0.81
QC-5	751	114.9	15.3	761	0.99	670	182.9	27.3	761	0.88	665	169.8	25.5	887	0.75

pg-TEQ/g

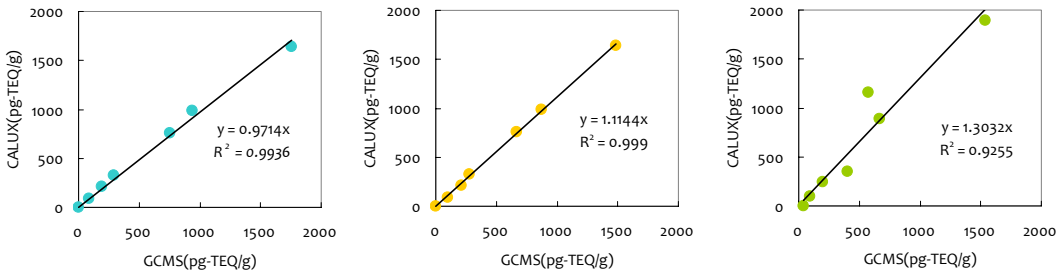

Figure 4. Correlation Figure(From right: Normal cell(Hiyoshi), Kit cell(Hiyoshi), Kit cell(CEM))

Once cross-check confirmation is completed, we will continue the project so that survey on soil contamination by Agent Orange can be done at laboratory in Vietnam by Vietnamese people's own hand.

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