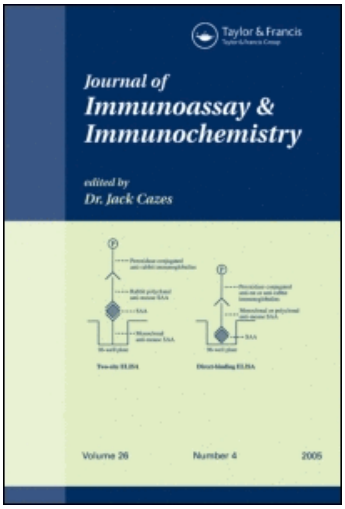


Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

IMPROVEMENT OF THE LONG-TERM STABILITY FOR DIOXIN TOXICITY EVALUATION METHOD BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Y. Sugawara[a]; M. Ishizuka[a]; K. Saito[b]; H. Nakazawa[b]
[a] Cosmo Oil Co., Ltd., Minato-ku, Tokyo, Japan [b] Department of Analytical Chemistry, Hoshi University, Ebara, Shinagawa-ku, Tokyo, Japan

Online publication date: 23 March 2010

To cite this Article Sugawara, Y. , Ishizuka, M. , Saito, K. and Nakazawa, H.(2010) 'IMPROVEMENT OF THE LONG-TERM STABILITY FOR DIOXIN TOXICITY EVALUATION METHOD BY ENZYME-LINKED IMMUNOSORBENT ASSAY', Journal of Immunoassay and Immunochemistry, 31: 2, 111 — 119
To link to this Article: DOI: 10.1080/15321811003617339
URL: http://dx.doi.org/10.1080/15321811003617339

Journal of Immunoassay and Immunochemistry, 31:111–119, 2010

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321811003617339

Taylor & Francis
Taylor & Francis Group

IMPROVEMENT OF THE LONG-TERM STABILITY FOR DIOXIN TOXICITY EVALUATION METHOD BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Y. Sugawara,[1] M. Ishizuka,[1] K. Saito,[2] and H. Nakazawa[2]

[1]*Cosmo Oil Co., Ltd., Minato-ku, Tokyo, Japan*
[2]*Department of Analytical Chemistry, Hoshi University, Ebara, Shinagawa-ku, Tokyo, Japan*

□ *A dioxin enzyme immunoassay (EIA) is one of the methods that may satisfy the requirements to reduce the cost and turn around time for the dioxin analysis. We developed a dioxin enzyme-linked immunosorbent assay (ELISA) to rapidly analyze for trace levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in human milk. In this study, to aim to supply the stable assay quality, the development of long-term stable coated plates for the ELISA system was reported. To the conventional coated plate (wet plate), the dry plate ELISA indicated the stability to be able to store for 1.5 years at 11° C. The IC_{50} of this ELISA was 17± 4 pg/well. The standard curve showed almost the same as that of the wet plate. A fairly good correlation between cross-reactivity of the ELISA and WHO-TEF was achieved for environmental matrices. This ELISA should be more practical for environmental sample monitoring.*

Keywords GC-MS, immunoassay, PCDDS, PCDFS, stability, TCDD

INTRODUCTION

In spite of a high demand for monitoring dioxins from the government and the public, the cost and time required for analysis often severely limits the scope and thoroughness of a sampling effort. However, a traditional method using high-resolution gas chromatography/mass spectrometry (HR-GC/MS) requires a complicated sample cleanup, special equipment and a highly trained analyst. This analytical technique is expensive and time consuming.[1] To meet the demand for monitoring dioxins, some rapid dioxin screening methods by biological assay were authorized by the some governments.[2–4] These methods include the human reporter gene system assay and the enzyme-linked immunosorbent assay (ELISA) with the

Address correspondence to Y. Sugawara, Cosmo Oil Co., Ltd., 1-1, Shibaura 1-chome, Minato-ku, Tokyo 105-8528, Japan; Tel.: +81-3-3798-3212; Fax: +81-3-3798-3523. E-mail: yukio_sugawara@cosmo-oil.co.jp

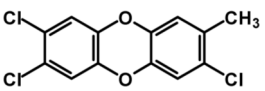
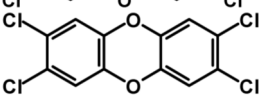
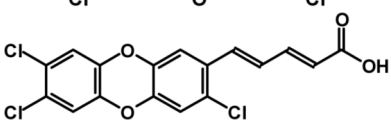
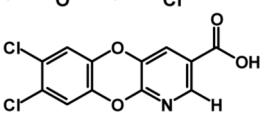
anti-dioxin antibodies. The purpose of this research is establishing ELISA for measuring dioxins in various environmental samples, which is simple, rapid, and highly sensitive to perform, and contribute to monitor of investigations for pollution and actual conditions by dioxins. We reported the development of dioxin toxicity evaluation method by ELISA previously. The samples were not only human milk but also various environmental matrices such as exhaust gas, fly ash, soil and household dust.[5–7] In order to put in practical use our ELISA system, it is very important that this system obtains the long-term conservative stability. To realize it, we focused on the development of the dry plate ELISA and reported its basic performance.

MATERIALS AND METHODS

Chemicals and Immunoreagents

The surrogate standard for ELISA, 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized by Sanborn et al.[8] Preliminary data indicated these compound responds similarly to 2,3,7,8-TCDD in an antiserum based ELISA.[9] Any other chemicals and immunoreagents including coating hapten III (7,8-dichlorobenzo[5,6][1,4]dioxino[2,3-b]pyridine-3-carboxylic acid) coupled to BSA for 2,3,7,8-TCDD and Antiserum #7598 for this ELISA (raised against an immunizing hapten I (5-[3,7,8-trichlorodibenzo-*p*-dioxin-2-yl]-trans,trans-penta-2,4-dienoic acid) were described in previous report.[10] Table 1 shows the structures of hapten I and III. Goat anti-rabbit antibody coupled to horseradish peroxidase (goat anti-rabbit HRP conjugate) and 3,3′,5,5′-tetra-methylbenzidine (TMB) were purchased from Sigma-Aldrich Corp. (St. Louis, USA).

TABLE 1 Structures of TCDD, Surrogate Standard TMDD and Dioxin Hapten I & III

Compound	Structure
TMDD	
TCDD	
Hapten I	
Hapten III	

Preparation of the Dry Plate and the Wet Plate

Microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with the optimized concentration (0.5 μg/mL, 100 μL/well) of coating antigens (hapten III BSA conjugate)[10] in carbonate-bicarbonate coating buffer (pH 9.6). They were incubated overnight at 4°C. The following day, the coated plates were washed 5 times with 0.05% (v/v) Tween 20 in PBS (PBST) and were incubated for 30 min at room temperature with 300 μL of 0.5% (w/v) BSA in PBS with 5% sucrose (blocking solution) per well. After the removal of the blocking solution, whereas the wet plates were substituted with PBS and stored at 4°C, the dry plates were dried *in vacuo* for 4 hours at 25°C. Then the dry plates were put into the aluminum bags respectively, and were packed *in vacuo*. These wet and dry plates were tested the stability periodically at 2–11°C (regular stability test), and at 37°C (accelerated stability test).

ELISA

Standards were prepared in 1:1:2 (v:v:v) DMSO–MeOH with 100 ppm Triton X-100 phosphate buffered saline (PBS, pH 7.5) containing 2 mg/mL bovine serum albumin (PBSB). After an initial blocking step with blocking solution, and a wash step, 50 μL of standards were added into standard wells in a microtiter plate (dry plate or wet plate). Next, 50 μL of the antiserum diluted in PBSB was added to each well. The final ratio of DMSO–MeOH to PBSB was 1:3. The plates were incubated for 90 min. Following a wash step, 100 μL of goat anti-rabbit HRP conjugate was added (diluted in PBST). After a 60-min incubation period, the plates were washed with wash buffer, and 100 μL of enzyme substrate containing TMB was added to each well. After 20 min, the color reaction was stopped by addition of 50 μL of 2 M sulfuric acid. The resultant color was measured at 450 nm with a Model 550 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA) in single-wavelength mode, and dioxin levels in the environmental matrices including human milk sample were calculated on the basis of a standard curve derived from a fit of absorbance versus the logarithm of concentration.

Effect of Sucrose in the Blocking Solution

The effect of sucrose was tested by dissolving the blocking solution to prepare the dry plate. Concentrations tested were blank, 0.5, and 5% in the blocking solution. A part of those plates were utilized the assay immediately, and then stored at 37°C. The plates stored were compared the

performance of the standard curve periodically (the accelerated stability test). Other assay conditions were as described in the ELISA section.

Effect of FBS in the Goat Anti-Rabbit HRP Conjugate

To characterize the effect of fetal bovine serum (FBS) and BSA for the stability of the goat anti-rabbit HRP conjugate (the second antibody solution) in PBST, 10% FBS and 0.5% BSA in PBST were tested. Each portion of those solutions was utilized to measure the blank standard immediately, whereas the rest was stored at 37°C and then tested periodically.

Determination of Cross-Reactivities

The cross-reactivity of the ELISA was evaluated about 27 kinds of dioxin congeners, which contribute 17 PCDD/F congeners and 3 co-PCB congeners to the world health organization-toxicity equivalency factor (WHO-TEF 2006), and 7 congeners with less than four chlorine atoms, using the dry plates. The condition and procedure for the validation of those congeners were described in previous report.[7] The cross-reactivities (CR) were calculated relative to the concentration producing 50% inhibition (IC_{50}) by TMDD. The data were obtained from standard curves of the related compounds and calculated according to the following formula: $\%CR = (IC_{50}$ of TMDD/IC_{50} of the cross-reacting compound) $\times$ 100.

Validation for Human Milk Samples, Sample Clean Up and GC/MS

The condition and procedure for validation for human milk samples, sample clean up and GC/MS were described in previous report.[10]

RESULTS AND DISCUSSION

Performance of the Dry Plate

To supply a simple rapid screening assay for dioxins, the kits for the assay should be ready to use ideally. Thus it is necessary the ELISA system that we developed obtains the long-term stability. To realize it, the stability of the coated plates is crucial especially. Figure 1 shows the effect of sucrose to the dry plates. In the research, Suzuki and others reported that some freeze-dried proteins with sugar had thermal stabilizing effect.[11] To evaluate the long-term stability of the dry plates at low temperature, we tested it under accelerative atmosphere at high temperature (37°C). While the

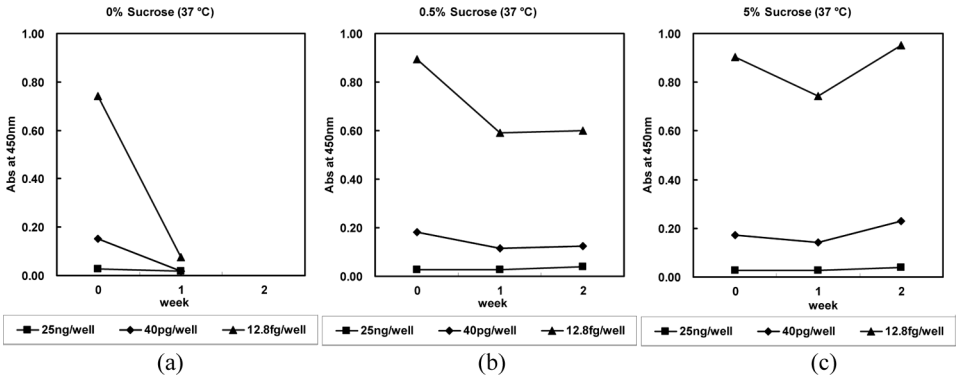


FIGURE 1 Stability of the dry plate using various concentrations of sucrose.

maximum absorbance in 5% sucrose showed almost the same, the maximum absorbances in blank and 0.5% sucrose decreased sharply after one week since the start of the accelerated stability test. Therefore 5% sucrose was considered the most suitable of the additives tested to lead the long-term stability for the dry plates.

Figure 2 shows the representative standard curves to TMDD using the dry plate and the conventional wet plate. The standard curve of the dry plate was showed almost the same as that of the wet plate. Between those plates, the maximum absorbances showed little variation (0.93–1.00).

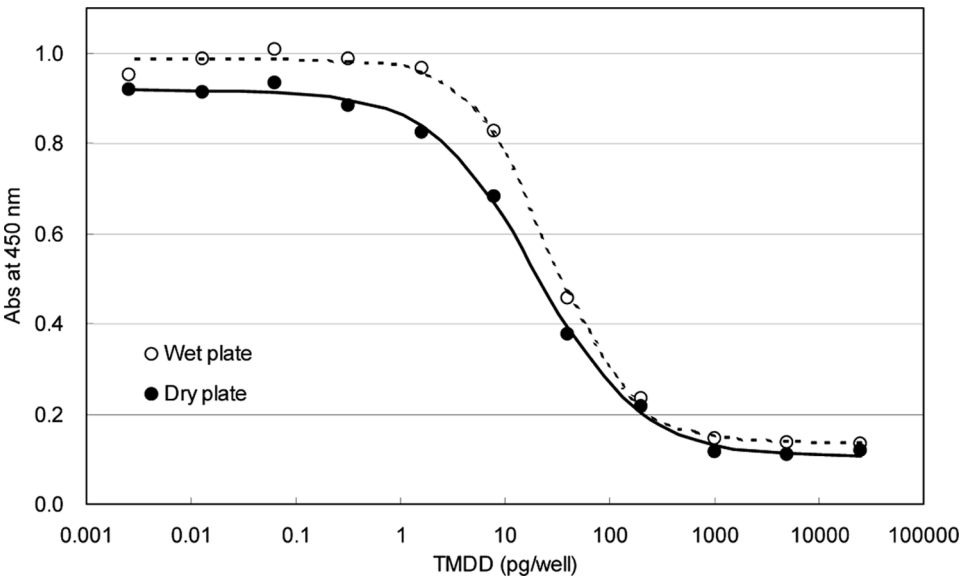


FIGURE 2 Standard curves to TMDD using the dry plate and the wet plate. The IC_{50} of the wet plate is 27.1 pg/well, whereas the IC_{50} of the dry plate is 19.8 pg/well.

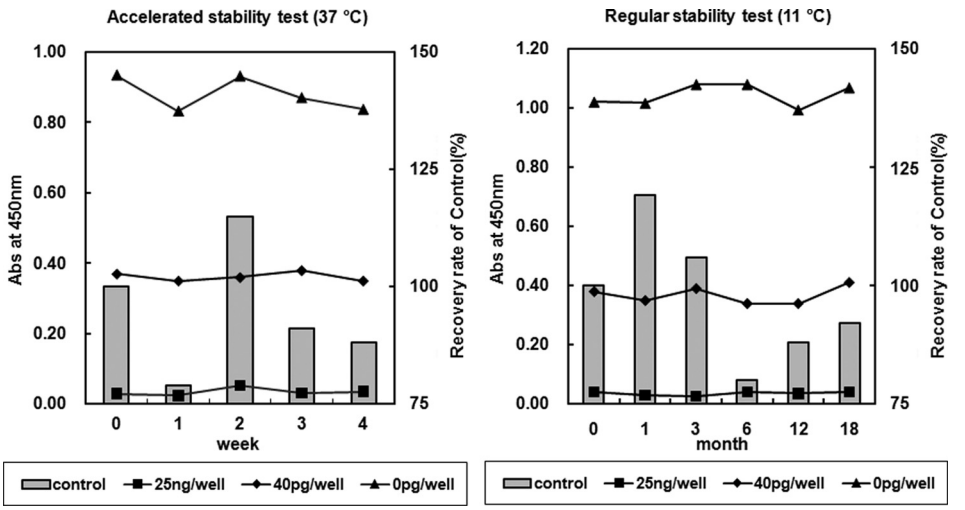


FIGURE 3 Comparison of the stability at 37°C and 11°C with the dry plate. *The recovery rate of the control concentration (15 pg/well) was calculated by the following formula: (Measuring value/15) × 100.

While the IC_{50} in the standard curve of the wet plate was 27.1 pg/well, whereas the IC_{50} in that of the dry plate was 19.8 pg/well, and the sensitivity of the dry plate was slightly higher than that of the wet plate. The average IC_{50} of the four standard curves was 17 ± 4 pg/well.

Regarding stability, degradation of the dry plate was not observed for one month by accelerated stability test at 37°C (Figure 3). The absorbance range of 0 pg/well of TMDD that is the most sensitive to the degradation showed between 0.80 and 1.00. The absorbance of 25 and 40 pg/well also showed almost flat variation. The recovery rate of 15 pg/well that is the representative concentration for the IC_{50} demonstrated 80 to 120%. These data suggested the ability to keep this stability for more than one year at 4°C. Actually, the result of the regular stability test exhibited no degradation for eighteen month at 11°C. It turns out that the dry plate can be used in practical.

In addition, Figure 4 shows the results of the stability designed to test the effect of FBS in the anti-rabbit HRP conjugate. One important factor controlling stability is the storage buffer that should contain some protein, typically as used in a blocking solution.[12] Thus we focused on BSA and FBS known as one of the typical serum proteins. Under the accelerated atmosphere of the stability, the absorbance of the conjugate in PBST plus 2.5 fold BSA to PBSB decreased sharply at one week, however, the absorbance of the conjugate in PBST plus 10% FBS kept almost the same during four weeks. This indicated the PBST plus 10% FBS was suitable to the anti-rabbit HRP conjugate for long-term stability at 4°C.

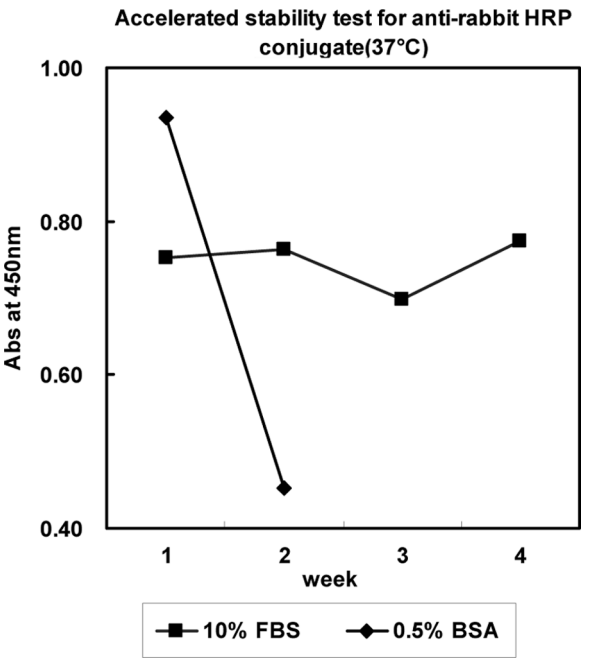


FIGURE 4 The stability of the anti-rabbit HRP conjugate with FBS.

Cross-Reactivity

For determination of cross-reactivity, the IC_{50} of TMDD was assigned a value of 100%, and the cross-reactivities for other compounds were reported according to their IC_{50}'s relative to this value. 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD have higher cross-reactivities than other compounds with both plates.

Moreover, good correlation was acquired by plotting cross-reactivity obtained for each compound on the vertical (Y) axis versus the corresponding WHO-TEF on the horizontal (X) axis (Figure 5). As this result, it has shown the antibody used by this study had not only the excellent specificity against 2,3,7,8-TCDD, but the practicability as a method of screening for evaluating the toxicity based on TEQ.

Comparison of the Dry Plate ELISA System to GC/MS

In order to examine the practicability, we compared the dioxins in human milk between GC/MS and the dry plate ELISA system. In the conventional ELISA system with the wet plate, a good correlation between ELISA values and the total toxicity equivalency quantity (total-TEQ) values by GC/MS was observed.[10] As the results, the dry plate ELISA system also

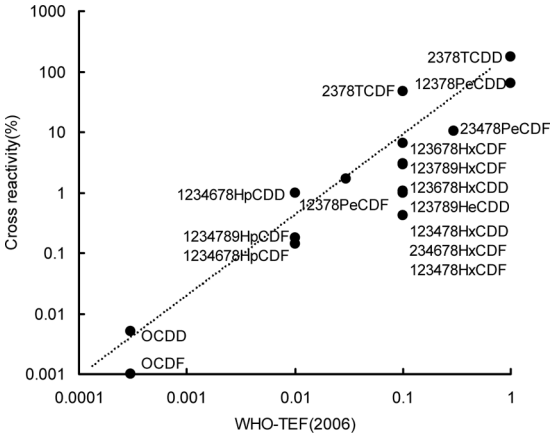


FIGURE 5 Relationship between cross-reactivity of the dry plate ELISA and WHO-TEF.

showed a strong correlation ($r = 0.976$) to the total-TEQ in human milk (Figure 6). This dry plate ELISA system also exhibited strong correlations to GC/MS values in exhaust gas, flay ash, soil and house dust samples.[7] Thus this work demonstrates the practicability of the ELISA for a new evaluation method for toxicity.

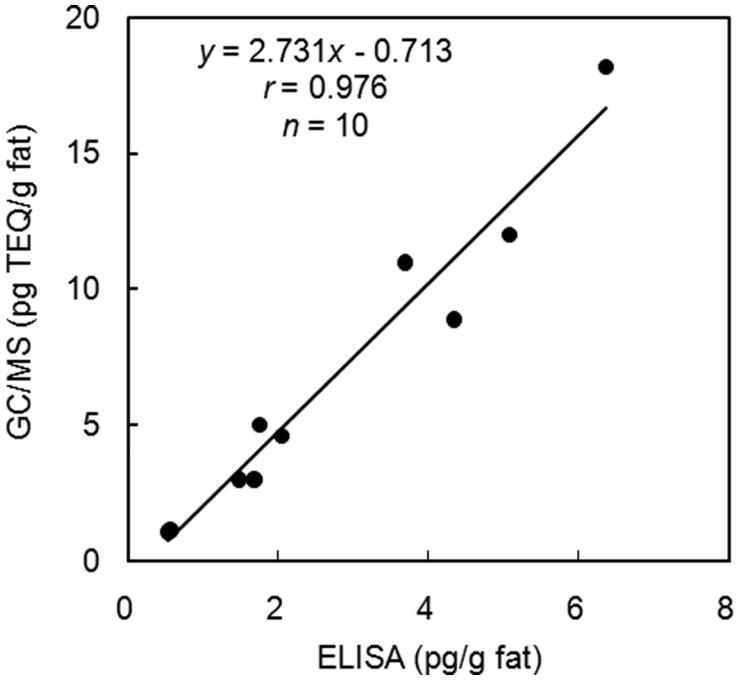


FIGURE 6 Correlation between dioxin quantities by the dry plate ELISA and GC/MS values in human milk.

CONCLUSIONS

The developed dry plate ELISA is a very practical screening tool for the determining trace levels of dioxin in environmental matrices. The stability for 18 months at 11°C indicates the thermal stabilizing effect of sucrose for the preparation of the dry plate and also a good correlation between the dry plate ELISA and TEQ values for human milk sample indicate that this assay can be used as a TEQ screening method for PCDDs and PCDFs. On the long-term stability and the correlation to the various environmental samples, this dry plate ELISA system demonstrated more useful practicability as a toxicity evaluation method for dioxins. In addition, it will be inferred that the combination of the anti-rabbit HRP conjugate FBS added and the dry plate approach the ideal ready-to-use assay.

REFERENCES

1. Schecter, A. A selective historical review of congener-specific human tissue measurements as sensitive and specific biomarkers of exposure to dioxins and related compounds. Env. Health. Persp. **1998**, *106* (Suppl. 2), 737–742.
2. Public notice No. 92 by the Japan Ministry of Environment. The measuring methods prescribed by the Minister of the Environment under Article 2, paragraph 1, item 4 of the Ordinance for Enforcement of the Law Concerning Special Measure against Dioxins, 2005.
3. USEPA method 4425. Screening Extracts of Environmental Samples for Planar Organic Compounds (PAHs, PCBs, PCDDs/PCDFs) by a Reporter Gene on a Human Cell Line, 2000.
4. USEPA method 4025. Screening for Polychlorinated Dibenzodioxins and Polychlorinated Dibenzofurans (PCDD/Fs) by Immunoassay, 2002.
5. Sugawara, Y.; Saito, K.; Ogawa, M.; Kobayashi, S.; Shan, G.; Sanborn, J.R.; Hammock, B.D.; Nakazawa, H.; Matsuki, Y. Development of dioxin toxicity evaluation method in human milk by enzyme-linked immunosorbent assay—assay validation for human milk—. Chemosphere **2002**, *46*, 1471–1476.
6. Saito, K.; Takekuma, M.; Ogawa, M.; Kobayashi, S.; Sugawara, S.; Ishizuka, M.; Nakazawa, H.; Matsuki, Y. Enzyme-linked immunosorbent assay toxicity evaluation method for dioxin in human milk. Bull. Environ. Contam. Toxicol. **2003**, *70*, 636–643.
7. Saito, K.; Ishizuka, M.; Hosono, S.; Sugawara, Y.; Iwasaki, Y.; Ito, R.; Nakazawa, H. Development of screening method of dioxins in environmental samples by enzyme-linked immunosorbent assay. Bunseki Kagaku **2006**, *55* (12), 993–997.
8. Sanborn, J.R.; Gee, S.J.; Gilman, S.D.; Sugawara, Y.; Jones, A.D.; Rogers, J.; Szurdoki, F.; Stanker, L.H.; Stoutamire, D.W.; Hammock, B.D. Hapten synthesis and antibody development for polychlorinated dibenzo-*p*-dioxin immunoassays. J. Agric. Food. Chem. **1998**, *46*, 2407–2416.
9. Sugawara, Y.; Gee, S.J.; Sanborn, J.R.; Gilman, S.D.; Hammock, B.D. Development of a highly sensitive enzyme-linked immnosorbent assay based on polyclonal antibodies for the detection of polychlorinated dibenzo-*p*-dioxins. Anal. Chem. **1998**, *70*, 1092–1099.
10. Sugawara, Y.; Saito, K.; Ogawa, M.; Kobayashi, S.; Shan, G.; Sanborn, J.R.; Hammock, B.D.; Nakazawa, H.; Matsuki, Y. Development of dioxin toxicity evaluation method in human milk by enzyme-linked immunosorbent assay—assay validation for human milk. Chemosphere **2002**, *46*, 1471–1476.
11. Suzuki, T.; Imamura, K.; Fujimoto, H.; Okazaki, M. Role of sucrose-LDH hydrogen bond for thermal stabilizing effect of sucrose on freeze-dried LDH. Drying Technology **1999**, *17* (7&8), 1429–1439.
12. Law, B.; Malone, M.D.; Biddlecombe, R.A. Enzyme-linked immunosorbent assay (ELISA) development and optimization. In *Immunoassay A Practical Guide*; Law, B.; Ed.; Taylor & Francis: London, 1996; Ch. 7, 127–149.