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Development of a Fast Immunosorbent Assay for Site-Screening Dioxin Contamination in Vietnam

(Pembangunan Asai Imunosorben Pantas untuk Penyaringan Tapak Pelumusan Dioksin di Vietnam)

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ABSTRACT

Dioxins are a group of chemical compounds that cause environmental pollution and many harmful effects on human health. High-Resolution Gas Chromatography/High-Resolution Mass Spectrometry (HRGC/HRMS) is the standard method for determining dioxin concentrations in soil samples and provides the most accurate results. However, this method is time-consuming, costly, and requires modern equipment. Currently, competitive ELISA is a reliable method used for dioxin detection analysis, offering fast implementation time and low cost. Vietnam is a global hotspot for dioxin contamination, with a high number of dioxin samples for analysis. Therefore, it is essential to optimize this reliable, fast, and low-cost ELISA method for it to be applicable and replace the expensive and complex HRGC/HRMS method currently in use in Vietnam. This study presented optimized conditions for ELISA method using commercial antibodies to detect dioxin. The optimal dilution for the anti-dioxin antibody and the conjugated antibody is 1:2000 and 1:1000, respectively. The reconstitution buffer consists of 50% DMSO/H_2O, with the addition of 0.05% Triton X-100. The incubation time for anti-dioxin antibody incubated with dioxin is 60 min, while the incubation time for Horseradish Peroxidase (HRP) conjugated polyclonal antibody incubated with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate is 10 min. The quenching time for the enzyme-substrate reaction is 5 min. The half-maximal inhibitory concentration (IC_{50}) of this method is 8500 pg/well and the limit of detection (LOD) is 2.02 pg/well. Although there is a difference between the analytical results of the two methods, the well-correlated results demonstrate the potential of the ELISA method for detecting and screening dioxin contamination before performing confirmatory analysis with HRGC/HRMS. These results serve as the basis for the development of a rapid dioxin detection kit, providing a new and efficient method for detecting and screening dioxin contamination in Vietnam.

Keywords: Dioxin; ELISA; fast detection; HRGC/HRMS

ABSTRAK

Dioksin ialah sekumpulan sebatian kimia yang menyebabkan pencemaran alam sekitar dan memberi kesan berbahaya kepada kesihatan manusia. HRGC/HRMS ialah kaedah piawai untuk penentuan kepekatan dioksin dalam sampel tanah dan dapat memberi keputusan yang paling tepat. Walau bagaimanapun, kaedah ini memakan masa, kos yang tinggi dan memerlukan peralatan moden. Pada masa ini, ELISA yang kompetitif ialah kaedah yang boleh dipercayai yang digunakan untuk analisis pengesanan dioksin dengan masa pelaksanaan yang cepat dan kos yang rendah. Vietnam ialah tempat yang tinggi pencemaran dioksin di dunia dengan bilangan sampel dioksin yang tinggi untuk dianalisis, oleh itu adalah perlu untuk mengoptimumkan kaedah ELISA yang boleh dipercayai, cepat dan kos rendah ini supaya dapat digunakan dan menggantikan HRGC/HRMS yang digunakan di Vietnam. Kajian ini membentangkan keadaan optimum untuk kaedah ELISA menggunakan antibodi komersial untuk mengesan dioksin.

Pencairan optimum untuk antibodi anti-dioksin dan antibodi terkonjugasi masing-masing adalah 1:2000 dan 1:1000, kemudian penambahan penimbal ialah 50% DMSO/H_2O ditambah dengan 0.05% Triton X-100. Masa untuk antibodi anti-dioksin diinkubasi dengan dioksin ialah 60 min, masa untuk antibodi poliklonal terkonjugasi HRP yang diinkubasi dengan substrat TMB ialah 10 min dan masa untuk pelindapkejutan tindak balas enzim-substrat ialah 5 min. IC_{50} kaedah ini ialah 8500 pg/well dan had pengesanan ialah 2.02 pg/well. Walaupun terdapat perbezaan antara keputusan analisis kedua-dua kaedah, keputusan berkorelasi dengan baik menunjukkan potensi kaedah ELISA dalam mengesan dan menapis pencemaran dioksin sebelum melakukan analisis pengesahan dengan HRGC/HRMS. Keputusan ini adalah asas untuk pembangunan kit pengesanan dioksin pantas, menyediakan kaedah baharu dan cekap untuk mengesan dan menyaring pencemaran dioksin di Vietnam.

Kata kunci: Dioksin; ELISA; HRGC/HRMS; pengesanan cepat

Introduction

Dioxins are a group of chlorinated organic compounds with structures consisting of two benzene rings linked together by a pair of oxygen atoms or a C-C bond, of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered as the most toxic congener. Due to their hydrophobic, non-polar, lipophilic, and chemically stable properties, dioxins persist in the environment for extended periods and exhibit a high bioaccumulation capacity. Dioxin is an undesirable by-product of various industrial processes, including pesticide and herbicide production, pulp bleaching, and waste incineration, and it is also present in incinerator emissions and diesel engines (Prashant 2019). Moreover, dioxin is in the composition of Agent Orange - a chemical herbicide used during the defoliation campaign by the US military during the Vietnam War. To date, dioxin concentrations in most environmental samples in southern Vietnam have remained below the threshold for classifying soils as dioxin-contaminated (Vietnam Ministry of Natural Resources and Environment 2012). However, some military airbases, such as Bien Hoa and Phu Cat where used to be the storage and transshipment sites of Agent Orange, are still contaminated hot spots with very high levels of dioxin concentrations in soils, sediments and aquatic species. This contamination has severe repercussions for the environment, ecosystems and the health of people residing in the affected areas (Vietnam Ministry of National Defence 2018).

Presently, the standard method for quantitative analysis of dioxin is high-resolution gas chromatography/mass spectrometry (HRGC/HRMS). While this method offers sensitive and accurate quantitative measurements of dioxin concentration and toxic equivalency quantitative (TEQ) for the 17 most toxic congeners of dioxin (U.S. Environmental Protection Agency 1994), it is costly, involves complex pretreatments, time-consuming analysis, requires expensive equipment, and is not suitable for field deployment. Enzyme-linked immunosorbent assay (ELISA) is one of the practical methods that has been adopted for on-site fast screening of dioxin contamination. This assay relies on the specific binding of dioxin to its corresponding antibody, thus providing relatively high specificity. In this method, dioxin in the sample binds to the anti-dioxin antibody, which is subsequently transferred to a hapten-coated well. After removing free antigen-antibody complexes through washing, the immobilized hapten-binding antibody is detected by binding to an enzyme-conjugated secondary antibody, resulting in absorbance through a substrate-specific enzyme assay. The absorbance is inversely proportional to the amount of dioxins in the samples. By using a standard curve of known dioxin concentrations, the dioxin TEQ can be determined (Tian et al. 2012). ELISA is a simple, easily manipulated method that offers fast analysis and is cost-effective, making it ideal for on-site analysis or initial screening of a large number of samples before conducting confirmatory analysis using HRGC/HRMS equipment. Currently, in Vietnam, HRGC/HRMS, rather than the ELISA method, is employed for detecting and screening dioxin samples, incurring significant time and economic costs. Furthermore, previous studies on dioxin detection by ELISA involved the generation of antibodies in-house, suitable only for research purposes and not practical for broader applications. Developing antibodies in-house necessitates specialized equipment, expertise, and substantial costs. Conversely, commercial anti-dioxin antibodies are available, with high purity and can be obtained as needed. Therefore, in this study, we employed competitive ELISA, utilizing commercial

antibodies and optimized conditions for the assays to establish an efficient method for detecting and screening dioxin samples in Vietnam.

Dioxins are organic compounds that are nearly insoluble in water but soluble in non-polar organic solvents. Therefore, the choice of organic solvents and their ratios, compatible with both dioxin and antibodies, plays a critical role in the sensitivity of dioxin detection using ELISA. While ELISA is a straightforward assay for dioxins determination, its sensitivity is influenced by several parameters. In this paper, we optimized various factors, including the concentration of anti-dioxin antibody, HRP-conjugated polyclonal antibody, buffer compositions, incubation time for anti-dioxin antibody incubated with dioxin, HRP-conjugated polyclonal antibody incubatedwith TMB substrate, and quenching time for the enzyme-substrate reaction with 2M H_2SO_4 as well. These optimizations significantly enhanced the sensitivity and specificity of the experiment, ultimately determining the success of the dioxin detection kit developed using the ELISA method.

Materials and Methods

REAGENTS

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) - 48599 was purchased from Sigma, USA. Mouse anti-dioxin antibody (CABT-L4232) was obtained from Creative Diagnostics, USA. Goat Anti-Mouse HRP-conjugated polyclonal antibody (Ab6789) was procured from Abcam, UK. TMB solution (T0440) was obtained from Sigma, USA. Hapten conjugated Bovine Serum Albumin (BSA) was synthesized according to Shan's method (Shan et al. 2001). Dioxin (TCDD) ELISA kit (PN530037) was purchased from Eurofins, USA.

Buffers. Phosphate-buffered saline (PBS); carbonate-bicarbonate buffer pH 9.6; blocking buffer: 0.5% BSA in PBS; reconstitution buffer for anti-dioxin antibody: 0.2% BSA in PBS; wash buffer and reconstitution buffer for conjugate antibody: 0.05% Tween 20 in PBS; stop solution (2M H_2SO_4). All buffers were stored at 4 °C.

METHODS

INDIRECT COMPETITIVE ELISA METHOD

The procedures for indirect competitive ELISA assays were carried out following the protocol outlined by Shan et al. (2001). Briefly, 96-well ELISA plates (Thermo Fisher, USA) were coated with 100 µL of hapten-BSA solution in 0.1 M carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. Afterward, the plate was washed five times with 250 µL of washing buffer, and then blocked with 300 µL of blocking buffer at room temperature for 30 min, followed by another washing step (3 times with the same washing buffer). Subsequently, 50 µL of sample and 50 µL of the anti-dioxin antibody were mixed and incubated at room temperature for 60 min, which were then added to the wells in triplicate. The wells were incubated at room temperature for an additional 60 min and washed 5 times. After washing step, 100 µL of the HRP-conjugated antibody solution was added and gently mixed. Following another 30 min incubation at room temperature, the wells were washed 5 times and dried by blotting. Next, 100 µL of TMB solution was added to the wells, and they were incubated at room temperature for 10 min in the dark until a blue color developed. The enzyme-substrate reaction was stopped by adding 100 µL of 2M H_2SO_4. Absorbance reading at 450 nm were then taken using an ELISA reader.

ANTIBODY TITRATION

Optimal concentrations of both the anti-dioxin and the HRP-conjugated antibodies were determined using a titration method (Crowther 2009) with some modifications. Initially, the anti-dioxin antibody solution was serially diluted in tubes, and then it was incubated for 60 min with a blank solution (a 1:1 ratio of 50% DMSO/ H_2O added 0.01% Triton X-100) before being added to the hapten-coated plates. After incubation with the anti-dioxin antibodies, the plates were washed 5 times, to which the conjugated antibody was added and diluted according to the Crowther method. Following this step, the plates were washed 5 times with the washing buffer and blotted to dry. Next, 100 µL of TMB substrate solution was added to each well, and the plate was incubated at room temperature in the dark until the color turned blue. The enzyme-substrate reaction was terminated by adding 100 µL of 2M H_2SO_4. The signal was measured at 450 nm using an ELISA plate reader. Concentrations of both the primary and secondary antibodies were selected at the point where the OD_{450} signal was the highest and remained constant at their higher concentrations.

OPTIMIZATION OF COMPOSITION OF SAMPLE RECONSTITUTION BUFFER

The ELISA plates were prepared and tested according to the procedure described in the indirect competitive ELISA

method. TCDD was reconstituted in the buffer containing either a DMSO:H_2O ratio of 1:1 or a DMSO:MeOH:H_2O ratio of 1:1:2. The ability of reconstitution buffer to dissolve dioxin was assessed based on the relative signal reduction (B/B_0), which was caculated as the ratio of the signal from the sample (TCDD in reconstitution buffer) (B) to that of its corresponding blank (reconstitution buffer without TCDD) (B_0). The formula for the sample reconstitution buffer was selected to minimize the signal reduction (B/B_0 ratio) of the TCDD sample. Subsequently, that specific organic solvent was selected for optimizing the proportion for each composition. Next, Triton X-100 surfactant was added to the buffer to enhance the solubility of dioxin. Several concentrations of Triton X-100 (0, 0.01, 0.025, 0.05, 0.075 and 0.1 %) were investigated, and the optimal concentration of the surfactant was determined. Each experiment was replicated three times, and the average OD_{450} value was recorded.

OPTIMIZATION OF INCUBATION TIME

The ELISA plates were prepared and tested according to the procedure described in the indirect competitive ELISA method above, then the relative signal reduction was calculated as previously discussed. In separate experiments, the time for antibody incubation with dioxin, substrate incubation, and reaction time after incubation were determined to verify the optimal incubation time. The time at which the lowest signal reduction in the B/B_0 ratio of the TCDD sample achieved was selected. The experiments were repeated 3 times conducted in triplicate, and the average OD_{450} value was taken.

IC_{50} AND LIMIT OF DETECTION (LOD)

TCDD samples with increasing concentration (ranging from 10^{-2} pg/well to 10^{8} pg/well) were analyzed using the optimized indirect competitive ELISA method. The IC_{50} value representing the concentration of TCDD at which it binds to 50% of anti-dioxin antibody, was determined using the four-parameter equation (Shan et al. 2001; Sugawara et al. 1998):

$$y = \{(A - D) / [1 + (x/C)^B]\} + D$$

where A is the OD_{450} value of the blank (reconstitution buffer); D is the OD_{450} value of dioxin at infinite concentration; C is the IC_{50} value; B is the curve slope at the point of inhibition; x is the dioxin concentration and y is the OD_{450} value of dioxin concentration.

The limit of blank (LOB) (the lowest OD_{450} of blank), was calculated by the formula with a confidence level of 90% (Clinical and Laboratory Standards Institute 2004):

$$LOB = OD_{450\ blank} - x_{(\alpha/2)}.SD_{blank}$$

The limit of detection (LOD) (the lowest OD_{450} of detectable TCDD concentration), was calculated by the formula with a confidence level of 90%:

$$LOD = LOB - x_{(\alpha/2)}.SD_{low\ TCDD\ concentration\ sample}$$

where the $OD_{450\ blank}$ is the mean OD_{450} value of the blank sample; SD_{blank} is the standard deviation of the blank samples; $SD_{low\ concentration\ TCDD\ sample}$ is the standard deviation of low TCDD concentration sample; $x_{(\alpha/2)}$ is the coefficient following the normal distribution corresponding to the significance level α. With a significance level of 0.1 (or confidence level of 90%), $x_{(\alpha/2)}$ has a value of 1.645.

Based on the standard curve, the lowest detectable TCDD concentration was determined following the resulting LOD. The experiment was conducted in triplicate, and the average results were recorded.

HRGC/HRMS ANALYSIS

Analysis of dioxin was conducted following TCVN 10883:2016 method (Vietnamese Standard Method - Vietnam Ministry of Natural Resources and Environment 2016) with some modifications. HRGC/HRMS analysis was carried out on a Trace 1310 chromatograph equipped with a Thermo TG-Dioxin column (60 mm × 0.25 mm × 0.25 µm) in combination with a High Resolution Magnetic Sector MS DFS (ThermoScientific, German) mass-spectrometer (electron ionization) at a resolution R=10000. The detailed conditions was as follows: (HRGC) column temperature program, 140 °C (2 min), 140-228 °C (40 °C/min), 228-290 °C (1.6 °C/min), 290-315 °C (5 °C/min), 315 °C (7.05 min); carrier gas, Helium; split flow, 70 mL/min; carrier flow, 1.2 mL/min; injection temperature, 280 °C; ịnjection volume, 2 µL (splitless). HRMS ion source temperature, 280 °C; ionizing current, 1mA; ionizing energy, 45 eV; and accelerating voltage, 4800 V.

DIOXIN ELISA KIT ANALYSIS

The dioxin extract in reconstitution buffer was also analyzed using an ELISA kit from Eurofins, USA. In this

analysis, 125 µL of the standard solutions or samples and 125 µL antibody solution were mixed in separate tubes. The mixture was vortexed and incubated for 60 min. Afterward, 100 µL of the mixture was added to duplicate wells on the test strips. The wells were covered with tape and shaken for 30 s, followed by a 60-min incubation at room temperature. After incubation, each well was washed four times with 250 µL of diluted wash buffer and then dried by blotting on a stack of paper towels. Subsequently, 100 µL of Anti-Rabbit-HRP conjugate was added to the individual wells and incubated for 30 min at room temperature. The washing step was repeated four times. Then, 100 µL of the substrate solution was added to the wells and incubated for 20 min. To quench the reaction, 100 µL of the stop solution was added to the wells in the same sequence as for the color solution. Absorbance was measured at 450 nm within 15 min after the addition of the stop solution.

COMPARISON BETWEEN ELISA AND HRGC/HRMS METHODS

Dioxin-contaminated soil samples were extracted according to the instructions provided with the Reagen kit. Subsequently, the samples were analyzed for dioxin content using both the optimized competitive ELISA method and Eurofins kit. To assess the significance, results were compared with those obtained through the HRGC/HRMS method using a T-test. This analysis was conducted using SPSS version 26 software (IBM, USA).

RESULTS AND DISCUSSION

ANTIBODY TITRATION

The ELISA plates were coated with hapten-BSA with the optimal concentration of 0.1 µg/mL according to Sugawara's study (Sugawara et al. 1998). 100 µL of each of three commercially available anti-dioxin antibodies (ABIN934378 from Antibodies online, German, CABT-L4232 from Creative Diagnostics, USA and YII-YM010-EX from Cosmobio, Japan) (dilution 1:500 with their reconstitution buffer) were added to the plates at a 1:500 dilution in their respective reconstitution buffers to determine their hapten-binding ability (Đặng et al. 2022). The results revealed that the CABT-L4232 antibody exhibited the highest binding ability to hapten BSA coated plate, with the highest OD_{450} value. Therefore, CABT-L4232 was selected in this study.

Antibody concentration is crucial in an ELISA reaction because it affects antigen detection ability and relates to the excess of antibodies (Chen et al. 2013; Sugawara et al. 2002, 1998). Studies by Sugawara utilized antibodies with dilutions ranging from 1:5000 to 1:3500 (Sugawara et al. 2002, 1998). Hence, antibodies were initiated at 1:1000 to optimize antibody concentrations (Figure 1).

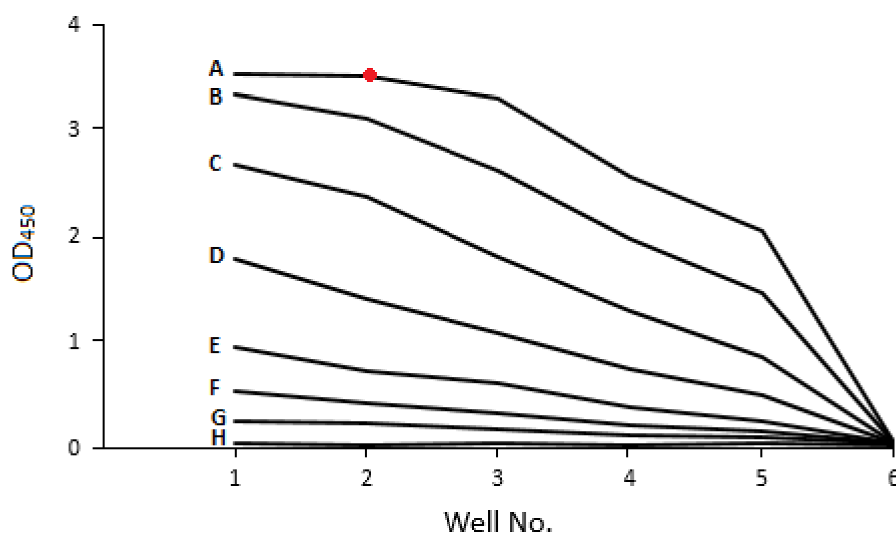

FIGURE 1. Titration of anti-dioxin antibody and conjugated antibody. Anti-dioxin antibody dilutions: 1-1:1000, 2-1:2000, 3:1:4000, 4-1:8000, 5-1:16000 and 6-no anti-dioxin antibody. Conjugated antibody dilutions: A-1:1000, B-1:2000, C-1:4000, D-1:8000, E-1:16000, F-1:32000, G-1:64000, H-no conjugate antibodies. The red point presented at which the OD_{450} is the highest and both anti-dioxin antibody and conjugated antibody saturated with hapten coated on the plate. Therefore, 1:2000 and 1:1000 were chosen for the dilutions of anti-dioxin antibody and conjugated antibody, respectively

The ELISA assay's absorbance reached its highest value (3.5) at a dilution of 1:1000 for conjugate antibody (row A) and dilutions of 1:1000 and 1:2000 for anti-dioxin antibody (wells 1 and 2). Thus, the anti-dioxin antibody at 1:2000 was saturated with the amount of hapten coated on the plate, and the conjugate antibody at 1:1000 was saturated with the anti-dioxin antibody retained on the plate. The OD_{450} signals in wells 6 (without anti-dioxin antibody) were very low (0.4-0.6), showing that the interaction between the conjugate antibody and the hapten is negligible. Similarly, the signals of the H-row wells (without conjugated antibody) were also very low (0.4), indicating a low background signal of the reaction, and the generated signals resulted from the conjugate enzyme-substrate reaction. Therefore, the dilutions of 1:2000 for the anti-dioxin antibody and 1:1000 for the conjugated antibody were selected for further study. In this study, the commercial antibody exhibited a weak affinity for hapten, which is the reason why the antibody's concentration is higher compared to that of previous studies (Sugawara et al. 2002, 1998).

OPTIMIZATION OF DIOXIN RECONSTITUTION BUFFER'S COMPOSITION

Optimization of organic solvents

Because dioxin is highly hydrophobic and only soluble in non-polar organic solvents, it is important to investigate an organic solvent for preparing the analyte solution that is both dioxin-soluble and compatible with antibodies. Most studies by Sugawara and Shan utilized DMSO as the primary solvent for dissolving dioxin (Shan et al. 2001; Sugawara et al. 1998). In another study by Sugawara, MeOH was added to the buffer (Sugawara et al. 2002). The effects of DMSO and MeOH were evaluated using an organic solvent-to-water ratio of 1:1.

When MeOH was added to the analyte solution, the OD_{450} value increased compared to that of the solution containing only DMSO and water (the OD_{450} of the blank was 1.233 ± 0.042 in the DMSO/H_2O buffer and 1.615 ± 0.058 in the DMSO/MeOH/H_2O buffer). The relative signal reduction (B/B_0 value) of the TCDD sample in DMSO/MeOH/H_2O buffer was also higher than that of DMSO/H_2O buffer (B/B_0 value of samples 2.5, 12.5 and 25 pg/well were 0.96, 0.91 and 0.89 in DMSO/

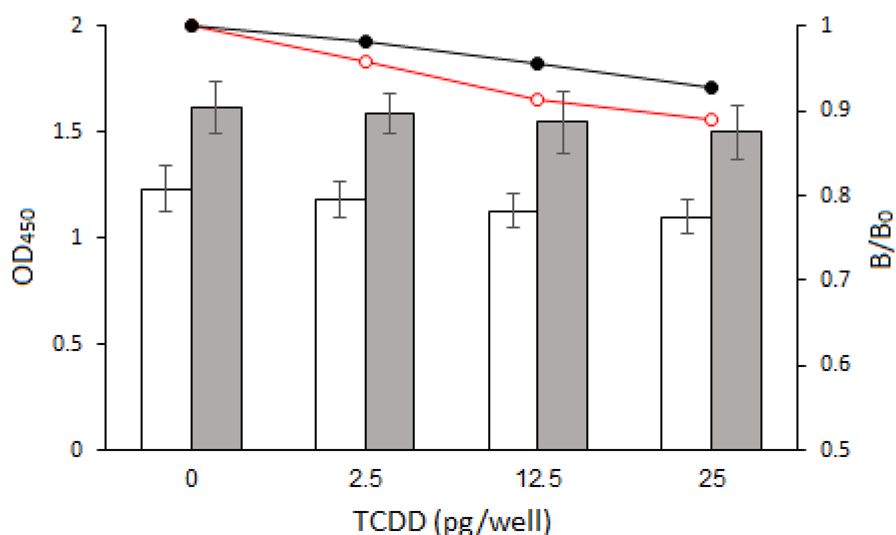

FIGURE 2. OD_{450} values of the dioxin standard reconstituted in DMSO:H_2O ratio of 1:1 (column □) and DMSO:MeOH:H_2O ratio of 1:1:2 (column ■); B/B_0 ratio of the standard mixed in DMSO:H_2O ratio of 1:1 (○) and DMSO:MeOH:H_2O ratio of 1:1:2 (●). In this case, the TCDD's B/B_0 ratio in DMSO:H_2O ratio of 1:1 (red line) is lower than in DMSO:MeOH:H_2O ratio of 1:1:2. So, DMSO:H_2O ratio of 1:1 was chosen

H_2O buffer, and 0.98, 0.96 and 0.93 in DMSO/MeOH/H_2O buffer, respectively). These data demonstrate that MeOH increases the affinity between the antibody and hapten but reduces the affinity of the antibody for dioxin. Compared with DMSO, MeOH has a higher polarity, which decreases the solubility of dioxins in the buffer. Therefore, the suitable solvent for the ELISA analysis is a DMSO:H_2O ratio of 1:1. Most studies for detecting dioxin in soil used the same solvents (Shan et al. 2001; Sugawara et al. 1998), except for Sugawara's study, which added methanol to the solvent to analyze dioxin in human milk (Sugawara et al. 2002). Since milk has a high fat content, methanol helps to dissolve fat and dioxin in the reconstitution buffer more effectively.

Optimizaion of DMSO concentration

DMSO is an organosulfur solvent commonly used in ELISA assays at a concentration of 50% to dissolve dioxin (Shan et al. 2001; Sugawara et al. 1998). To assess the impact of DMSO concentration on the solubility of dioxins and the affinity between antibodies and dioxin, a dioxin standard was prepared using various concentrations of DMSO-containing solvents and analyzed by ELISA (Figure 3) following the indirect competitive ELISA procedure.

The OD_{450} signals of the control samples gradually increased with increasing the DMSO concentrations (gained 1.232 ± 0.053 at DMSO-free solution) and reached a maximum in the 100% DMSO solution (2,204 ± 0.078). At 50% DMSO concentration, the antibody's affinity for dioxin was at its highest, as indicated by the lowest B/B_0 value of TCDD samples (B/B_0 values for TCDD samples at 2.5, 12.5 and 25 pg/well were 0.89, 0.84, and 0.78, respectively). As a result, 50% DMSO (DMSO:H_2O ratio of 1:1) was selected for preparing dioxin solutions. This finding aligns with the results of Sugawara and Shan's studies (Shan et al. 2001; Sugawara et al. 1998).

Optimization of surfactant concentration

Dioxin is lipophilic and insoluble in water, therefore, surfactants have been used to enhance dioxin solubility (Shan et al. 2001; Sugawara et al. 1998). In this study, the ability to solubilize dioxin of reconstitution buffer containing Triton X-100 at various concentrations was investigated (Figure 4).

As Triton X-100 was added to the buffer at increasing concentrations, the OD_{450} signal gradually decreased, ranging from 2.854 ± 0.085 at zero concentration to 2.153 ± 0.079 at 0.1% Triton X-100-addition. The assay's

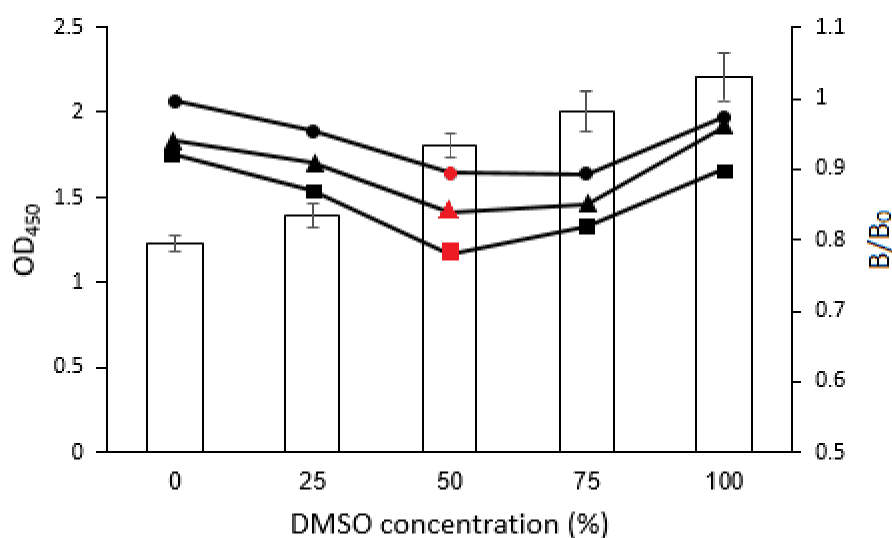

FIGURE 3. OD_{450} value of control sample (column □) and B/B_0 ratio of the standard at 2.5 pg/well (●), 12.5 pg/well (▲) and 25 pg/well (■) in DMSO concentrations. The DMSO concentration of 50% was chosen because of the lowest TCDD's B/B_0 ratio given

ability to detect dioxin using the antibody reached its maximum with a solution containing 0.05% Triton X-100 (B/B_0 of TCDD samples 2.5, 12.5, and 25 pg/well were 0.92, 0.89, and 0.86, respectively). Therefore, a Triton X-100 concentration of 0.05% is suitable for solubilizing the dioxin in the ELISA assay. This result differs from a previous study by Okuyama, which identified the optimal Triton X-100 concentration at 0.01% (Okuyama et al. 2004). However, it's important to note that in this study, TCDD concentrations used (2.5 - 25 pg/well) were higher than those in Okuyama's study (1 - 12.5 pg TMDD/well) when evaluating the effect of Triton X-100 in the reconstitution buffer. Additionally, TCDD is less soluble than TMDD in polar solvents, necessitating a higher concentration of Triton X-100 in the reconstitution buffer for better TCDD dissolution in the DMSO/H_2O solution.

OPTIMIZATION OF INCUBATION TIME

Antigen-antibody binding is a non-covalent and reversible interaction (Soltis & Hasz 1982). The performance of the ELISA assay depends on the incubation time of the antibody-antigen complex, which is in turn influenced by the affinity between the two components. Therefore, it is essential to investigate this factor to determine the optimal incubation time. Furthermore, ELISA signals depend on the amount of substrate that reacts with the enzyme to produce a yellow color. In an acidic solution, the yellow color is less stable and can change to a colorless form. Hence, it is important to consider both substrate incubation time and the time of measurement after incubation to achieve a high and stable signal. Table 1 displays the OD_{450} values of the control samples and the B/B_0 ratios of the TCDD samples in the assays evaluating the effect of incubation time.

When adjusting the incubation time of antibodies and dioxin, the OD_{450} signals of the blank samples remain stable (in the range of 2.0 to 2.1), but there were variations in the B/B_0 ratios of the TCDD samples. These B/B_0 ratios gradually decreased and reached their lowest values, stabilizing after 60 min of incubation (0.94, 0.91, and 0.86 for 2.5, 12.5, and 25 pg TCDD/well, respectively). Sugawara et al. (1998) also determined 60 min as the optimal time for antibody-dioxin incubation. In another study, Van Emon and Chuang (2013) selected a 30-minute incubation time for antibody-PCBs. The main factor affecting incubation time is the affinity of antibodies for antigens, with antibodies having higher affinity requiring less incubation time and vice versa.

During the substrate incubation step, the OD_{450} signals gradually increased from 0.252 ± 0.012 (at 0 min) to 2.307 ± 0.057 after 20 min. The B/B_0 ratios of TCDD samples gradually decreased and reached their lowest values when the substrate incubation time was extended to 10 min (0.95, 0.88 and 0.82 for 2.5, 12.5 and 25 pg

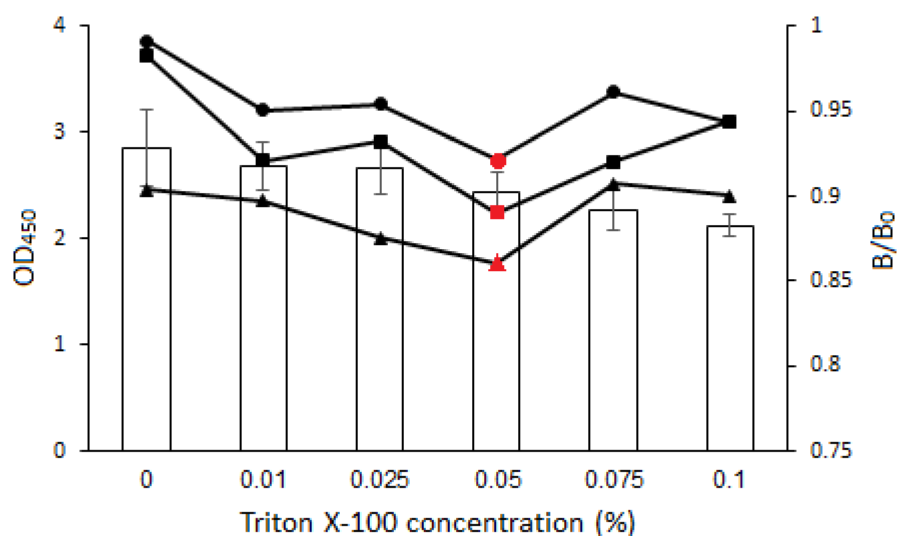

FIGURE 4. OD_{450} values of control samples (column □) and B/B_0 ratio of the standard at 2.5 pg/well (●), 12.5 pg/well (▲) and 25 pg/well (■) with various concentrations of Triton X-100. The TCDD sample's B/B_0 ratio in the reconstitution buffer contain 0.05% Triton X-100 is the lowest when compared with that at the same TCDD concentration in other Triton X-100 concentration, so 0.05% Triton X-100 was added in reconstitution buffer

TCDD/well, respectively). These ratios remained steady with longer incubation time. Therefore, achieving a high OD_{450} signal is not necessarily indicative of effective dioxin detection. Additionally, excessively high OD_{450} values can lead to instrumentation errors (Crowther 2009). Consequently, the optimal substrate incubation time was determined to be 10 min.

Some dioxin detection kits recommend a substrate incubation time of 20 min. This recommendation may be influenced by the specific substrate solution used in the kit, such as TMB T0440, which contributes to a faster substrate enzyme reaction rate compared to other TMB solutions (Labiano et al. 2015). After adding the stop solution, the B/B_0 ratios of the TCDD samples remained constant. However, the OD_{450} signals gradually decreased from 1.657 ± 0.063 to 1.581 ± 0.048 after 20 min. Notably, this change was not significant within the first 5 min. Therefore, ELISA absorbance should be measured within 5 min after adding the stop solution.

IC_{50} VALUE AND LIMIT OF DETECTION (LOD)

The IC_{50} represents the antigen concentration that inhibits 50% of the antibody binding to the immobilized antigen (Rajan & Sidhu 2012). This value serves as an indicator of the ELISA method's ability to detect dioxin. A lower IC_{50} value indicates a higher affinity between the antibody and dioxin, signifying that the ELISA method can detect dioxin at lower concentrations. The limit of detection (LOD) is the lowest concentration of dioxin that can be reliably detected, with the OD_{450} value of the sample containing dioxin being significantly lower than that of the blank. In this study, standards were prepared in the reconstitution buffer and ELISA assays with increasing dioxin concentrations under optimized conditions were conducted to determine the IC_{50} value. (Figure 5). The LOD was calculated according to the formula provided by Clinical and Laboratory Standards Institute with 90% confidence interval.

TABLE 1. Effect of antibody-dioxin incubation time, substrate incubation time, and measurement time

Step	Time (min)	OD_{450} of the blank	B/B_0 ratio of TCDD samples		
			2.5 pg/well	12.5 pg/well	25 pg/well
Antibody - dioxin incubation	30	2.121 ± 0.056	0.97	0.96	0.98
	60	2.124 ± 0.063	**0.94**	**0.91**	**0.86**
	90	2.087 ± 0.082	0.99	0.93	0.88
	120	2.043 ± 0.079	0.97	0.90	0.87
Substrate incubation	0	0.252 ± 0.012	0.98	0.99	0.98
	5	0.954 ± 0.045	0.99	0.95	0.94
	10	1.657 ± 0.064	**0.95**	**0.88**	**0.82**
	15	2.157 ± 0.074	0.95	0.88	0.82
	20	2.307 ± 0.057	0.95	0.87	0.83
Measurement	0	1.657 ± 0.063	0.95	0.88	0.82
	5	**1.656 ± 0.062**	0.94	0.88	0.82
	10	1.643 ± 0.058	0.95	0.88	0.82
	15	1.618 ± 0.053	0.95	0.88	0.82
	20	1.581 ± 0.048	0.95	0.88	0.82

In this table, the time for antibody-dioxin incubation and substrate incubation (60 minutes and 10 minutes, respectively) was chosen based on the lowest TCDD sample's B/B_0 ratio (the bold number). Meanwhile, the OD_{450} value was stable during the first 5 minutes, so 5-minute was chosen for the time to measure the OD_{450}

The IC_{50} and the LOD values for the competitive ELISA assay were found to be 8500 pg/well and 2.02 pg/well, respectively. These results are notably higher than those reported in previous studies on ELISA method for dioxin detection. For instance, some studies conducted by Sugawara showed that their ELISA assays could detect dioxin at 0.5 pg/well with an IC_{50} of 12 pg/well (Sugawara et al. 1998). Another study by Shan also reported a LOD of 4 ppt (equivalent to 0.2 pg/well) and the IC_{50} value of 36 ± 6.0 ppt (equivalent to 1.8 ± 0.3 pg/well) when employing 2,3,7-trichloro 8-methyldbenzo-*p*-dioxin (TMDD) as the surrogate standard (Shan et al. 2001). The primary reason for this difference is the affinity of the antibodies to dioxin. Since dioxin itself is insoluble in the intracellular water environment and lacks the ability to generate an immune response, antibodies were generated in mouse cells with BSA-conjugated dioxin as an immunogen, rather than dioxin itself. The absence of the conjugated BSA component may explain the weak affinity between the antibody and dioxin in the sample. When compared to the Dioxin (TCDD) ELISA kit, both the IC_{50} and LOD parameters of the competitive ELISA assay in this study were higher than those of the kit (Table 2).

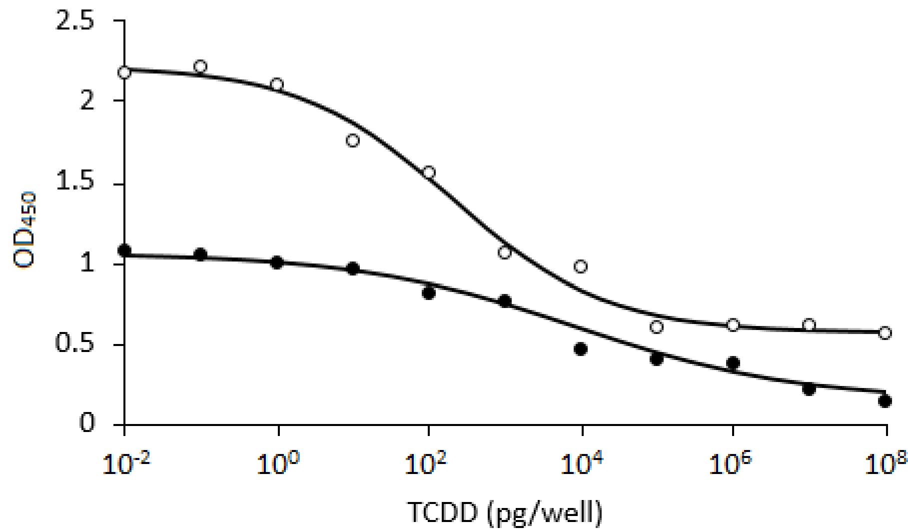

FIGURE 5. ELISA inhibition curve for Dioxin (TCDD) in ELISA kit (Eurofins) (○) and in the optimized competitive ELISA method (●). Each point represents the average for triplicate measurements. The details were described in Table 2

TABLE 2. IC_{50} and LOD values of Dioxin (TCDD) ELISA kit (Eurofins) and the indirect competitive ELISA assay in this study

	OD_{450} max (A)	OD_{450} min (D)	Slope (B)	IC_{50} (pg/well) (C)	A/D	LOD (pg/well)	R^2
Dioxin ELISA kit	2.217	0.567	0.43	200	3.91	0.37	0.9893
Indirect competitive ELISA	1.052	0.145	0.3	**8500**	7.26	**2.02**	0.9943

The results show that the indirect competitive ELISA method gives IC_{50} and LOD value of 8500 pg/well and 2.02 pg/well, respectively, which are much higher than those of the ELISA kit

COMPARISON OF THE COMPETITIVE ELISAS AND THE HRGC/HRMS

The World Health Organization has compiled a list of the most toxic dioxin congeners and their Toxic Equivalency Factors (TEFs) when comparing their toxicity with that of 2,3,7,8-TCDD. On that basis, the TEQ of a sample was defined by multiplying the concentration of each congener by its TEF value (Van den Berg et al. 2006). The HRGC/HRMS method is capable of accurately quantifying the 17 most toxic congeners of dioxin; making it the standard for determining dioxin concentrations in soil (U.S. Environmental Protection Agency 1994). Immunoassay uses a specific antibody to detect dioxin and determine the TEQ value of the sample through the TCDD standard curve. An antibody can cross-react with other congeners in a dioxin sample by its affinity towards those congeners. Therefore, the closer the antibody cross-reactivity to the TEF value of the congener is, the more accurate the ELISA results will be (Harrison & Carlson 1997; Shan et al. 2001). In this study, 12 dioxin-contaminated soil samples were extracted for dioxin analysis by both the optimized competitive ELISA method and the Dioxin (TCDD) ELISA kit. The results were compared with those obtained using the HRGC/HRMS method (Figure 6). Most of the results analyzed by the kit were lower than those of the HRGC/HRMS method, while the results of the optimized competitive ELISA method were higher. The difference between the ELISAs and HRGC/HRMS results was statistically significant with $p < 0.05$ (Table 3) using the T-test analysis. Hence, both the Dioxin (TCDD) ELISA kit and the optimized competitive ELISA method showed the ability to detect dioxin. However, the correlations between the two ELISAs and the HRGC/HRMS are not equal, mainly due to the use of different antibodies in the two assays, which have different cross-reactivity to the dioxin congeners.

Measured TEQ value of TCDD of the optimized ELISA was positively correlated to that of HRGC/HRMS determination following the relation equation $y = 1.8481x + 91.756$ with $R^2 = 0.75$. Thus, the ELISA response of the dioxin samples was about 1.8 times that of the comparable dioxin content determined by HRGC/HRMS. Although this difference is significant, the correlation result suggests that the optimized ELISA is a potential method for preliminarily screening dioxin contamination prior to a HRGC/HRMS analysis.

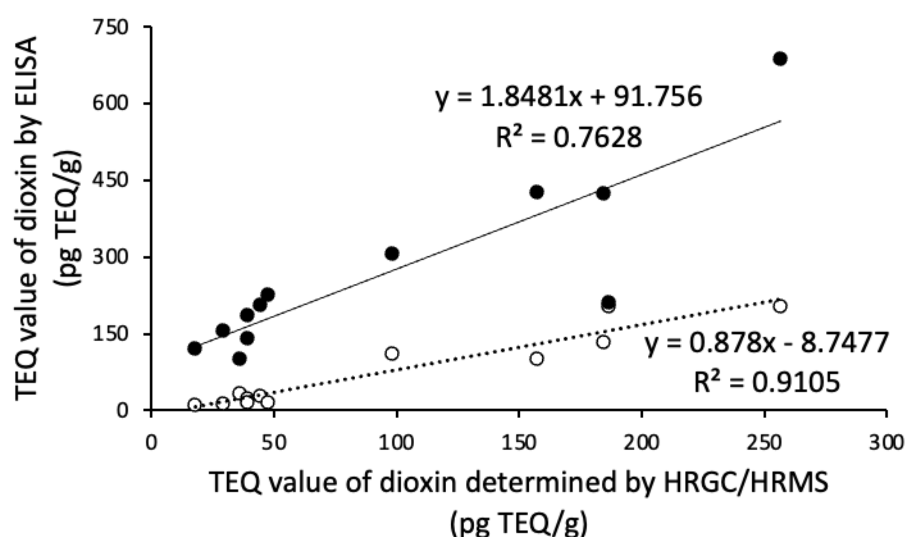

FIGURE 6. Correlation between the assay values (TCDD equivalent) obtained by the Dioxin (TCDD) ELISA kit (○), the competitive ELISA method in this study (●) and the TEQ value obtained by the HRGC/HRMS method. T-test analysis between the HRGC/HRMS and ELISA's results were described in Table 3

TABLE 3. T-test analysis between the HRGC/HRMS and ELISAs results

Pair	Paired differences					t	df	*p*
	Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
HRGC/HRMS vs Eurofins	20.27	24.23	6.99	4.88	35.67	2,899	11	**0.014**
HRGC/HRMS vs ELISA	171.96	107.42	31.01	240.21	103.71	5.546	11	**0.000**

When compared with the HRGC/HRMS, both ELISA method's TEQ values are significantly different withthose of the HRGC/HRMS ($p < 0.05$)

Conclusions

In this study, several ELISA assay conditions were optimized to achieve the best dioxin detection in the immunoassay using commercial antibodies. The optimal dilution for the anti-dioxin antibody and the conjugate antibody is 1:2000 and 1:1000, respectively. The suitable dioxin reconstitution buffer is DMSO/H_2O with a 1:1 ratio, supplemented with 0.05% Triton X-100. The optimal incubation time for the antibody and dioxin is 60 min, while the substrate incubation is 10 min. The sample should be measured within 5 min after adding the stop solution to obtain a high and stable signal. All of these parameters reduce the total time required for dioxin analysis to 4-5 h, significantly faster compared to that of other similar ELISA studies in Vietnam (which may take up to 20 h). In this competitive ELISA analysis, the IC_{50} and the LOD were determined to be 8500 pg/well and 2.02 pg/well, respectively. The results obtained with this method in analyzing dioxin-contaminated soil extracts outperformed those of HRGC/HRMS and Dioxin (TCDD) ELISA kit (Eurofins). However, a positive correlation between the obtained results suggests that ELISA measurements using commercial reagents offer a cost-effective and rapid approach to screening dioxin contamination, with some optimizations that consider the hydrophobic and lipophilic nature of dioxin.

Using commercial antibodies, which have been evaluated by the manufacturer for their dioxin binding ability, saves time and costs when screening samples. While HRGC/HRMS is expensive, complex to implement, time-consuming, and rare in Vietnam, developing a rapid dioxin detection kit using readily available commercial antibodies and an optimized ELISA method is essential due to its , low cost, speed, and effectiveness for on-site dioxin screening. Moreover, commercial dioxin detection ELISA kits have a low and narrow working range (2,5 – 50 ppt), which is not suitable for the high dioxin contamination hotspots in Vietnam (ranging from thousands to hundreds of thousands ppt). Therefore, optimizing competitive ELISA for the creation of rapid dioxin detection kits provides a significant and effective resource for dioxin screening that is tailored for the specific situation of dioxin contamination in Vietnam.

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