SIV-1

Quantitative Immunoassay for Polychlorinated Biphenyl Compounds in Electrical Insulating Oils

In Soo Kim
Life Science R&D, Research Park, LG Chemical Ltd.
104-1, Moonji-Dong, Yusong-Gu, Taejon 305-380, Korea

Abstract
The development and performance of a competitive indirect immunoassay for the quantitative measurement of polychlorinated biphenyl compounds in insulating oils is presented. Reagent preparation and the assay characterisation, optimisation and validation steps are described. The dynamic range of the assay for Aroclors 1254 and 1260 in methanol was 50-800 μg ml^{-1} with 50% signal inhibition values of 217 and 212 μg ml^{-1} respectively. Impending legislation in the UK is likely to decree that oils containing >50 μg ml^{-1} PCB be considered contaminated. Assay sensitivity increased with the degree of PCB chlorination. The assay of structurally related compounds of environmental concern yielded cross-reactivity values of under 0.6%. The immunoassay proved reliable for the analysis of transformer oils containing >70 μg ml^{-1} PCB, but over-estimated PCB levels in oils containing <20 μg ml^{-1} of the analyte with the oils requiring pre-treatment using either solid-phase extraction techniques or washing with KOH-ethanol/sulphuric acid to remove matrix interferents. The analytical performance of the assay was compared against a commercially available semi-quantitative immunoassay kit for PCBs in soil and water.

Introduction
There is increasing global concern regarding the utilisation and discharge of polychlorinated biphenyl (PCB) compounds into the environment. PCBs are a class of organo-halide that are routinely used as additives in various oil-based preparations and are important in the manufacture of products as diverse as plastics to pesticides. The ubiquitous nature of these materials coupled to their toxicity and recalcitrant nature has led to significant interest in the development of simple, rapid and low-cost methods for their analysis in a wide range of environmental matrices.

Structurally, a PCB is a chlorinated biphenolic compound with the general formula $C_{12}H_{(10-n)}Cl_n$. PCBs generally occur as mixtures, where n can vary from 1-10. The 10 sites available for possible chlorine substitution result in 209 possible PCB compounds or congeners [1]. There is now considerable concern regarding the presence of PCB congeners in insulating oils used within large-scale electrical supply systems. The chemical inertness, heat resistance, non-flammability, low vapour pressure and dielectric properties of PCBs led to their widespread usage as insulator oil additives from the 1930's until the 1970s when concerns regarding their carcinogenicity led to this practice being banned in the US and elsewhere [2]. Despite this fact, PCB-contaminated oils are still commonly encountered partly because some electrical units have never been refilled with PCB-free insulating oils, whilst those that have may still be contaminated due to inadequate plant and loading-line cleaning procedures. Some PCB contamination is believed to occur due to the re-use of incompletely reconditioned oil. Since attempts to identify a particular plant as contaminated have been unsuccessful, the only recourse of action has been to chemically analyse oils.

The current UK action plan [3] dictates that organisations with electrical equipment containing more than 5 litres of oil, contaminated with more than 50 μg ml^{-1} PCB will need to reduce these levels to comply with imminent new regulations. Indeed, the most recent EC Directive on the disposal of PCBs (96/59/EC) defines a range of chlorinated diphenolic and terphenolic compounds as PCBs. Consequently, this legal definition considers any preparation containing more than 50 μg ml^{-1} of PCB or PCB equivalent to be treated as a pure PCB preparation.

Current PCB measurement methods are either non-specific or utilise complex laboratory-based instrumental techniques [4]. The former methods measure general properties of the PCB analyte such as total chlorine content whilst the latter methods are generally more time consuming and expensive, typically requiring sample preparation, chromatographic separation and detection. Whilst the specific approaches are reproducible and of high-sensitivity, the increasing incidence of PCB compounds in the environment has created the need for more rapid, simpler and low-cost analytical procedures. Ideally, these techniques should have the durability and flexibility to be automated and applied either in the laboratory for routine analysis or in the field where assay speed and simplicity is desirable for the immediate implementation of appropriate remediation procedures.

Immunochemical techniques offer a simple, low-cost means of routinely and specifically measuring compounds in decentralised locations and have consequently been used for PCB analysis [5-13]. These methods have primarily been developed for the analysis of PCBs in predominantly polar matrices such as soils, water and milk. Indeed, two commercially available ELISA test kits for PCBs based on a competitive-format magnetic bead assay are also widely available (RaPID assay® and Envirogard® from SDI Europe Ltd., Alton, UK). However,

both of these systems have also been designed to work in essentially polar environments and are only semi-quantitative, yielding only a concentration range as opposed to a discreet numerical value. None of the above approaches has considered the analysis of PCBs in oils. Consequently, this paper describes the development of a rapid extraction and ELISA-based analysis method for the quantitative analysis of PCBs in electrical insulating oils. A key objective of the work has been to develop a simple protocol, amenable to both field-based and automated laboratory-based high-throughput analysis. The optimised procedure was compared against a commercial PCB ELISA kit.

Materials and Methods

Reagents

General chemical and biological reagents were of analytical grade and were purchased from Sigma-Aldrich or Merck (both Poole, UK). Deionised-Reverse osmosis water was purified using an Elgastat system (Elga, High Wycombe, UK). PBST solutions were prepared from 10 mM buffer salts, (pH 7.4), 0.15 M NaCl and 0.05% v/v Tween 20. The carbonate buffer used was 0.2 M at pH 9.6. Aroclors 1242, 1248, 1254, 1260 and 1262 (Ultra Scientific, North Kingstown, RI, USA) were supplied as 100 $\mu g\ ml^{-1}$ solutions in methanol and diluted in methanol as required. For cross-reactivity studies, the structurally similar non-PCB compounds pentachlorophenol (PCP), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (TCPAA) and 2-(2,4,5-trichlorophenoxy)-propionic acid (TCPPA) from Sigma-Aldrich were used. The National Grid Company (Leatherhead, UK) donated an unused PCB-free transformer oil and 5 used oils containing PCBs. The used oils had the following GC determined PCB concentrations in $\mu g\ ml^{-1}$: A, 4; B, 35; C, 51; D, 10; E, 6. Oil clean-up was facilitated using C18 solid phase extraction (SPE) columns containing 500 mg resin in 3 ml capacity columns, from IST Ltd. (Hengoed, UK).

Anti-PCB monoclonal antibody (MAb, Research Diagnostics Inc. Flanders, NJ, USA) was diluted 10-fold to 500 $\mu g\ ml^{-1}$ protein, aliquoted and stored at –20 °C with no significant loss in activity over 1 year. Goat anti-mouse antibody-horseradish peroxidase (GAM-HRP) conjugate (Sigma-Aldrich) was similarly aliquoted and stored at –20 °C and diluted in 10 % v/v foetal bovine serum (FBS, Sigma-Aldrich) with PBST as required and was stable for 1 month at 4 °C. Coating antigen was synthesised by conjugating bovine serum albumin (BSA) to TCPPA using 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) and adsorbed onto microtitre well walls. Residual adsorption sites were blocked using SuperBlock PBS buffer (Pierce, Chester, UK). TCPPA and BSA solutions were prepared in 0.1 M 2-[N-morpholino] ethane sulphonic acid (MES) buffer, pH 4.5, containing 50% v/v n,n-dimethylformamide (DMF). Conjugate protein concentrations were determined using the Coomassie Plus protein assay (Modified Bradford method, Pierce). O-phenylenediamine (OPD) substrate was purchased as 5 mg tablets from Sigma-Aldrich and dissolved in 0.15 M citrate buffer, pH 5 (citric acid 7.3 g, Na_2HPO_4-$2H_2O$ 11.86 g). 1-Step TURBO TMB was purchased from Pierce and used as received. Hydrogen peroxide stock solution (30% w/w) was from Sigma-Aldrich.

Instrumentation

Two types of microtitre plate were evaluated: Polysorb® 96-well (A/S Nunc, Roskilde, DK) and ImmunoWare® 8-well EIA Strip Plates (Pierce). During incubation, the plates were mechanically shaken with an iEMS incubator/Shaker HT (LabSystems, Finland) before being washed using an 8 channel manual washer (Nunc). The extent of colour development in the plates was determined using a Titertek Multiscan MCC Microwell plate reader (LabSystems). Coating antigen conjugate was recovered using a Sephadex G-25 size exclusion column (PD-10, Pharmacia, Sweden).

Preparation and immobilisation of coating antigen

Coating antigen was prepared by reacting EDC with the carboxyl group on the PCB analogue TCPPA. The amine-reactive O-acylisourea intermediate was then reacted with amine groups on BSA to form a stable carrier-protein-analogue conjugate. First, 1 ml of 5mg ml^{-1} TCPPA and 400 µl of 10 mg ml^{-1} BSA in MES buffer, pH 4.5 were stirred with 200 µl of freshly prepared 10 mg ml^{-1} EDC in water for 2 hours at 25°C. The Sephadex G-25 column was pre-washed with 0.2 M carbonate buffer, pH 9.6 and loaded with the reaction mixture. Conjugated material was eluted using 0.2 M carbonate buffer and collected as 0.5 ml fractions. The protein concentration of each fraction was determined using the Coomassie protein assay according to the manufacturers instructions using BSA as the calibration standard. Fractions 4-8 contained the greatest amount of protein and were pooled; the combined protein concentration was 1.3 mg ml^{-1}. The conjugate was diluted to 1 mg ml^{-1} in 0.2 M carbonate buffer and stored at 4°C until required. The TCPPA:BSA conjugation ratio was determined photometrically to be approximately 20:1.

Coating antigen stock solution was appropriately diluted in carbonate buffer and 150 • l volumes dispensed into microtitre plate wells. The wells were sealed and incubated at 25°C for 4 h to allow adsorption of coating antigen onto the well walls, washed ×3 with PBST and excess fluid removed by inversion and rapping on an

absorbent towel. Wells were blocked with SuperBlock PBS buffer for 30 min at 25°C, then washed and dried as before. The plates were sealed and stored at 4°C until required.

ELISA procedure

A 90 µl volume of appropriately diluted anti-PCB MAb in PBST and 10 µl of sample or standard in methanol, was added to each TCPPA-coated well. The plates were then incubated at 37 °C (primary incubation) for a given time, then washed and rap-dried as before. Next, 100 µl volumes of 1:4000 GAM-HRP conjugate in FBS-PBST were added to each well before incubation at 37°C (secondary incubation) for a given time, washing and rap-drying.

HRP activity was determined using either 1-Step TURBO TMB or OPD. The former substrate was supplied ready for use, whilst the latter was prepared immediately before required by adding 15 µl of 30 % v/v H_2O_2 to 15 ml of 1mg ml^{-1} OPD in 0.15 M citrate buffer. In both cases, 100 µl of substrate was dispensed to each well. The plates were incubated for 30 min or until the blank reached an optical density (OD) of 1.0 after which the reaction was stopped by adding 100 µl of 1M H_2SO_4. The plate was read at 492/630nm. All tests were performed a minimum of 3 times.

Data evaluation

The logit-log model, the most widely used procedure for immunoassay data evaluation [14], was used to analyse the data. The model represents a continuous sigmoidal function with a single inflection point, described by the equation:

$$y = (a-d)/(1+(x/c)^b) + d$$

where a represents the maximum current at zero analyte (upper asymptote), b the slope of the curve at mid-point (50% signal reduction), c the analyte concentration at mid-point and d the residual current at infinite dose (lower asymptote; background current + non-specific binding). These constant values were calculated using the curve fit (math) function of Sigma plot for Windows (Jandel Scientific). The practical quantitative range of the ELISA was defined as the linear portion of the sigmoidal curve, which was also calculated using Sigma plot. The assay limit of detection (LOD) was defined as the analyte concentration corresponding to $[OD_{zero\ analyte} - (2 \times SD_{zero\ analyte})]$. Accuracy is a measure of the systematic error in the assay and was reported as % bias, calculated as 100% × [(measured value – true value)/true value)]. Cross-reactivity was calculated as 100% × (c-value Aroclor 1254 standard/ c-value cross-reacting sample). Extraction efficiencies were calculated as 100% × (measured value/expected value).

Transformer oil preparation

Direct dilution: Oil samples were diluted 10-fold in 2-propanol, 50-fold in methanol and assayed.

Liquid-liquid extraction: Oil samples (100 µl) were added to 1 ml of extraction solvent, either methanol, acetonitrile or dimethyl sulphoxide (DMSO) and vigorously agitated using a bench top vortex mixer for 1 min. After phase separation, 100 µl of the upper phase was diluted 500-fold in methanol and immediately assayed.

KOH-ethanol/sulphuric acid extraction: Oil samples (100 µl) were vortexed with 1 ml of 1 M KOH in ethanol for 5 min. Next, 1 ml of n-hexane was added and the mixture vortexed for a further minute. After phase separation, the upper hexane phase was transferred to a fresh glass tube, 1 ml of concentrated sulphuric acid added and the mixture vortexed for 1 min. After phase separation, the hexane phase was removed and evaporated under nitrogen. The residual material was re-dissolved in 1 ml of 2-propanol, then diluted 500-, 1000- and 2000-fold in methanol prior to assay.

Solid phase extraction: A C18 SPE column was pre-wetted with 2 ml of 2-propanol followed by 1 ml of 10% v/v transformer oil in methanol. Retained materials were eluted with 10 ml 2-propanol then 10 ml n-hexane. Each fraction was collected separately, mixed well and 100 µl fractions evaporated under nitrogen, re-dissolved and diluted 500- or 1000-fold in methanol and assayed.

Commercial PCB assay kit

The optimised assay was tested in parallel with a semi-quantitative PCB kit, tube format (RaPID assay, SDI Europe Ltd.). The kit uses an indirect competitive assay format with anti-PCB antibody covalently immobilised to paramagnetic particles and used according to the manufacturers instructions. The 5 PCB-containing transformer oil samples and a 100 µg ml^{-1} Aroclor 1260 standard were prepared by the KOH-ethanol/sulphuric acid method and diluted 500- and 10 000-fold (500- and 40 000-fold for the Aroclor 1260 standard) in methanol. Extracts (200 µl) were incubated with 250 µl PCB-enzyme conjugate at room temperature for 15 min. prior to bead recovery using a magnetic tray (SDI). The tubes were washed twice with wash buffer, filled with 500 µl of substrate/chromogen and incubated for 20 min. The reaction was halted using 500µl 2 M sulphuric acid and the absorbance measured at 450 nm.

Results and Discussion

ELISA optimisation

Optimisation of coating antigen and primary antibody concentration
Optimisation was achieved using the checkerboard titration method in which serial dilutions of both coating antigen and primary antibody were incubated together. Since no sample was present, a 100µl volume of primary antibody was used. Primary and secondary incubation times of 1 h and OPD substrate (incubation temp. 25°C) were used in all tests. The highest OD readings were recorded at a coating antigen and primary antibody concentration of 10 µg ml^{-1} and 1.25 µg ml^{-1} respectively. These concentrations were correspondingly employed in subsequent assays unless otherwise stated.

Choice of microtitre plate
Both the Polysorb and Immunoware plates were coated with 10 µg ml^{-1} TCPPA-BSA and incubated with 5, 2.5, 1.25, and 0.625 µg ml^{-1} primary antibody according to the assay procedure used for antibody optimisation. The Immunoware plate was found to give a ~40% higher response than the Polysorb at the same primary antibody dilution, indicating greater protein binding capacity and was used in subsequent experiments.

Comparison of OPD and TMB substrates
A 90 µl volume of 1 µg ml^{-1} MAb in PBST and 10 µl of serially diluted Aroclor in methanol (800, 600, 400, 200 100, 50 and 0 µg ml^{-1}) were added to TCPPA-coated Immunoware plates and the immunoassay procedure followed. Bound label activity was determined using either OPD or TMB. OPD was found to be ~3-fold more sensitive than TMB (Figure 1) and hence was used in subsequent experiments, although, unlike TMB, it is carcinogenic and less convenient to prepare.

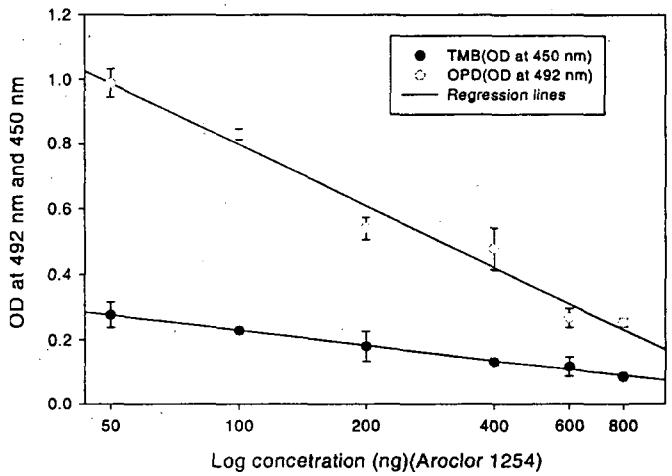

Figure 1. Comparison of assay performance using OPD and TMB as enzymatic substrates. Antigen coating, 10 µg ml^{-1}; primary antibody, 1.25 µg ml^{-1}; GAM-HRP, 1:4000; OPD or TurboTMB, 1mg ml^{-1}. Primary/secondary incubations, 1 h; substrate incubation, 15 min. Error bars = SD, (n=4).

Optimisation of incubation times
Primary antibody (100 µl of 1.25 µg ml^{-1}) was incubated in TCPPA-coated plates for 30, 60, 90, 120, 150, and 180 min., with subsequent assay steps being performed as before (secondary incubation for 1 h). Equilibrium binding was achieved at a primary incubation time of 150 min. This relatively long incubation time highlights the problem of solid-phase (heterogeneous) immunoassays compared to solution-based (homogeneous) assays, the limiting factor being the time taken for antibody to diffuse from the bulk solution and achieve equilibrium binding with the solid phase support. The commercially available homogeneous RaPID Assay and Envirosys assay (SDI) test kits have incubation times of ca. 30 min. By using an elevated incubation temperature of 37 °C and a mechanical shaker/incubator, the primary incubation binding equilibrium was achieved in 30 min. Under the same conditions, the secondary incubation time achieved equilibrium by 50 min., although an incubation time of 30 min (75% of equilibrium OD value) was used in subsequent studies for reasons of assay speed. The heterogeneous PCB assays reported in the literature have incubation times of hours [15,16].

ELISA characterisation

Dose response of selected Aroclors

The performance of the fully optimised PCB ELISA assay was determined for each of the standard Aroclors (n=8). Aroclor concentrations of 50 000, 10 000, 5000, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 μg ml^{-1} were tested, with a methanol blank. Results are shown in Table 1. The c-values indicate that the assay is most sensitive towards Aroclor 1262, and showed reduced sensitivity with decreasing degree of chlorination. The supplier states that the primary antibody has a specificity of 112%, 100%, 67% and 40% to Aroclor 1260, 1254, 1248 and 1242 respectively. Thus, it would appear that the antibody reacts more favourably with the more highly chlorinated (higher numbered) PCBs. However, Aroclors 1254 and 1260 were selected for further experimentation due to their relative abundance in insulating oils. The practical quantitative range of the ELISA for these two compounds was 30-1000 μg ml^{-1} for both Aroclor types, with the c-values positioned centrally on the calibration curve. The linear calibration profiles and regression data for these Aroclors is shown in Table 2.

The assay LODs for Aroclors 1254 and 1260 were determined for eight separate assays (n=4) performed during a 7-month period. The data is summarised in Table 2. Mean LODs of 24.94 ± 7.08 and 38.21 ± 18.67 μg ml^{-1} were recorded for Aroclors 1254 and 1260 respectively. No significant decrease in assay sensitivity was observed over the 7 month period, indicating a high degree of assay repeatability.

Aroclor	a (OD)	b (OD ml ng^{-1})	c (ng ml^{-1})	d (OD)
1242	1.816	0.932	803	0.025
1248	1.851	0.938	332	0.099
1254	1.933	1.044	217	0.109
1260	1.986	1.077	212	0.149
1262	1.987	0.983	120	0.125

Table 1. Logit-log data for the 5 Aroclor standards assayed using the optimised ELISA.

Series	1	2	3	4	5	6	7	8	Mean LOD ± SD
Aroclor 1254[a]	19.55	16.52	22.70	16.72	33.23	33.05	26.39	31.37	24.94 ± 7.07
Aroclor 1260[b]	18.41	37.96	5.31	49.03	45.84	35.08	62.98	51.09	38.21 ± 18.68

[a]: $y = 2.425 - 0.793x$; $r^2 = 0.998$.
[b]: $y = 1.956 - 0.610x$; $r^2 = 0.984$.

Table 2. Optimised ELISA detection limits in ng ml^{-1} for Aroclor 1254 and 1260. Eight separate assays were performed over a 7 month time period. Each calibration curve was constructed from an 11 point plot (n=4) and the resultant linear equations displayed as footnotes.

In order to calculate inter-assay precision, the assay was performed on three separate days. In each assay, the samples were analysed in triplicate to allow calculation of intra-assay precision. The data for 600 μg ml^{-1} Aroclor 1254 is reported here. Intra-assay precision varied from 1.4%-6.3% relative SD (RSD), whilst inter-assay precision varied from 2.1-6.3% RSD with a mean value of 3.8%. The overall average measured concentration was 637.9 μg ml^{-1}, yielding a bias of + 6.3%. Shah *et al.* [17] recommends that the total RSD of an assay should not exceed 15% (20% at the lower limit of the assay range). Using these definitions, the optimised ELISA can therefore be considered both accurate and precise for the analysis of Aroclor 1254 standard.

Cross reactivity

The following stock solutions: 2,4-D, 5 mg ml^{-1}; PCP, 10 mg ml^{-1}; TCPAA, 2.5 mg ml^{-1}; TCPPA, 2.5 mg ml^{-1}; were serially diluted in methanol to final concentrations of 4.9, 78.1, 2.4 and 2.4 μg ml^{-1} respectively and assayed using the optimised ELISA procedure (n=4). The resultant Logit-log c values were 572 000, 1 294 000, 52 853 and 78 359 μg ml^{-1} for 2,4-D, PCP, TCPAA and TCPPA respectively. Thus, all of these potentially cross-reacting compounds exhibited less than 0.4% cross-reactivity relative to Aroclor 1254. As expected, TCPAA and TCPPA had the highest cross-reactivities due to their close structural similarity to the TCPPA-BSA coating antigen, although the extent of binding was sufficiently low to permit application of the assay in most situations. These findings suggest there is a significant structural difference between free TCPPA and

conjugated TCPPA and that the PCB-antibody binding reaction is affected by factors other than PCB proximal structure. The very low cross-reactivity exhibited by the highly chlorinated PCP suggests that PCB-primary antibody binding is influenced by both the proximal and distal structure of the antigen. 2,4-D, chosen for it's structural similarity in the proximal region of the PCBs, had a cross-reactivity of under 0.06%.

Organic solvent tolerance of ELISA
PCBs, like many environmental contaminants, are relatively hydrophobic and as such are best extracted from the sample matrix using organic solvents. Whilst for assay purposes it is possible to evaporate the solvent and re-constitute in the aqueous phase, it is preferable for reasons of assay simplicity, to perform the analysis directly within the organic solvent extract. This factor introduces a paradox since antibodies, by their nature, will favour the aqueous phase. However, it is becoming widely appreciated that antibodies, like enzymes, can retain biological activity in organic solvents. Since the purpose of this study was to develop a simple immunoassay to measure PCBs in insulating oils, the performance of the assay in the presence of a number of organic solvents was assessed. Since the assay requires a wash step prior to the measurement process, it is only the primary incubation that is performed in the organic phase.

Ten microlitre volumes of acetonitrile, propanol, ethanol, methanol and PBST were pipetted (n=4) into individual antigen-coated microtitre wells containing 90 µl of primary antibody and the optimised assay procedure performed. Taking the OD_{492} response of the PBST control to be 100%, the corresponding responses for acetonitrile (least polar), propanol, ethanol and methanol (most polar) were 86%, 75%, 49% and 75% respectively. It is apparent that the presence of organic solvent decreases antibody-PCB binding and that the extent of antibody binding is not solely dependent upon solvent polarity.

The latter observation does not concur with other studies [18,19] that show a correlation between decreasing antibody-antigen binding affinity and increasing solvent hydrophobicity. However, Giraudi and Baggiani [20], using an testosterone-anti-testosterone test system, found that binding affinity was more influenced by solvent molecular mass rather than solvent polarity, suggesting that binding inhibition is related to the ability of the solvent to displace water from around the antigen. It has been demonstrated that some antibodies, either free or immobilised, are able to retain a residual binding activity in 90% v/v methanol and ethanol and in methanolic solutions containing 50% v/v acetone, diethyl ether or benzene [21]. Since the binding of hydrophobic antigens to antibody binding sites may be *via* hydrophobic interactions, lowering the polarity of the surrounding water miscible solvent will result in the reduced effectiveness of these binding mechanisms. Interestingly, despite reductions in signal intensity, assay sensitivity can actually be improved in the presence of some polar organic solvents [22].

The effect of solvent on the physiochemical properties of the antigen may also contribute to the overall binding process. For example, it has been found that decreased antibody-antigen binding affinity is apparent in solvents that are most suitable for antigen dissolution. The TCPPA-BSA coating antigen complex is soluble in pure aqueous solution by virtue of the hydrophilic BSA carrier protein. It is probable that the TCPPA molecule retains its non-polar nature in the conjugated form, thus, this non-polarity partly explains the relatively high antibody-PCB binding observed in the presence of propanol and acetonitrile. Although the most favourable binding characteristics were apparent in acetonitrile and methanol, the latter solvent was selected for further study due to its widespread usage in environmental sample extractions [23]. The behaviour of antibodies in organic solvents has been reviewed by Setford *et al.* [24]

Detection of PCBs in Transformer Oil

Evaluation of extraction methods
The performance of the assay for quantitatively determining PCBs in insulating oils was investigated. In order to minimise the matrix effect, a number of alternative sample preparation strategies were examined. Since a primary aim of the work was to develop an automatable assay, a simple sample preparation procedure was required. Tests were performed on unused PCB-free transformer oil either used as supplied or spiked with 100 or 200 • g ml^{-1} of Aroclor 1254 or 1260. Calibration curves were prepared using the same standards in methanol All tests were repeated 4 times.

Direct dilution
This was the simplest and hence the most easily automatable sample preparation method. The blank and spiked (1254, 100 µg ml^{-1}) transformer oils were immiscible in methanol and thus required pre-dilution in 2-propanol co-solvent before methanol dilution. An ELISA PCB concentration of 10.13 ± 0.16 µg ml^{-1} was determined in the spiked oil, equating to a recovery of approximately 10% of the expected value.

Liquid-liquid extraction
Extraction efficiencies of 19.6, 21.7 and 19.8% were obtained for methanol, acetonitrile and DMSO respectively for the 500-fold diluted PCB spiked (1254, 100 μg ml^{-1}) transformer oil. Assay repeatability varied from 1.4-3.0% RSD for the 3 solvents used. Increased extraction time, as well as solvent type had no significant effect on PCB recovery.

KOH-ethanol/sulphuric acid extraction
This approach, previously used for animal fats digestion [26], was examined as a means of reducing interferent co-extraction by degrading the long chain aliphatic interferents present in the transformer oil matrix. The final diluted methanol extracts were assayed by the ELISA method. The spiked transformer oil had a PCB level of 200 μg ml^{-1} Aroclor 1254 equating to PCB concentrations of 200, 100 and 50 μg ml^{-1} for the 500, 1000 and 2000-fold diluted samples respectively, assuming a 100% recovery of analyte. The actual recorded values were 179 ± 14, 97 ± 8, and 37 ± 2 μg ml^{-1} respectively, equating to extraction efficiencies of 89.5, 97.0, and 74.0 %. The 1:2000 diluted sample, with an expected Aroclor 1254 concentration of 50 μg ml^{-1}, was at the lowest limit of the assay range and may account for the decreased extraction efficiency observed. The higher extraction efficiencies recorded for the 1:500 and 1:1000 diluted samples indicates that the KOH-ethanol/sulphuric acid extraction method, with appropriate extract dilution, may represent a satisfactory means of minimising matrix effects.

Solid phase extraction
The ELISA results for unused oil spiked with 100 μg ml^{-1} Aroclor 1260 and extracted using a C18 SPE column are shown in Table 3. At the 500-fold dilution, the 2-propanol phase contained 59.5 % of the expected PCB concentration whilst the subsequently eluted hexane phase contained 28 % of the expected PCB concentration giving a combined Aroclor 1260 recovery of 87.5%. At the 1000-fold dilution, the 2-propanol and hexane phases contained 66 % and 38% of the expected PCB concentration respectively, equating to an overall recovery of 104 %. The SD values suggest that there was no significant difference in Aroclor 1260 recovery at each dilution indicating that SPE column treatment may be an effective way of sample pre-treatment.

Dilution	Eluate	Measured (ng ml^{-1})	SD (ng ml^{-1})	Expected (ng ml^{-1})	Efficiency (%)
1:500	2-propanol	119	27	200	59.5
	hexane	56	5.6	200	28.0
1:1000	2-propanol	66	6.6	100	66.0
	hexane	38	1.9	100	38.0

Calibration curve-fit correlation (n=8): $r^2 = 0.988$, $y = 1.591 - 0.481\ x$

Table 3 ELISA based measurement of unused transformer oil spiked with 100 μg ml^{-1} Aroclor 1260 standard and extracted using a C18 SPE column.

Since matrix effects are a prime cause of problems in PCB analysis in complex samples [27], numerous sample preparation methods have been reported. Most methods employ a combination of liquid-liquid extraction followed by florisil, silica gel, aluminium oxide or benzenesulphonic acid column adsorption chromatography. Shu *et al.* [28] report a method in which over 98 % of the components of an oil matrix were removed by DMSO liquid-liquid extraction, with further removal of 80 % of the remaining oil components by HPLC. Although recoveries in excess of 90% were recorded, the method requires a time-consuming and instrumentally complex chromatographic step. In this study, similar PCB recoveries were achieved using rapid, simple automatable methods based on KOH/ethanol treatment or SPE column methods. The high sample dilutions used in these latter approaches should act to minimise matrix effects in the subsequent sensitive ELISA step.

Optimised ELISA versus a commercial assay kit
A comparison of the ELISA results obtained for the optimised quantitative ELISA system and commercial RaPID assay system for the 5 used transformer oil samples (A-E) and the standard sample is shown in Table 4. The oil samples were prepared by KOH-ethanol/sulphuric acid extraction and the PCB levels determined simultaneously by both assay methods. The calibration curves for the two assay methods are given in Table 4. A stronger linear correlation was observed for the optimised assay ($r^2 = 0.988$) compared to the commercial test kit ($r^2 = 0.945$).

The standard Aroclor sample, oil C and oil B with PCB concentrations of 200, 102 and 70 μg ml^{-1} respectively, yielded efficiencies of 84.5, 87.3 and 88.6% respectively using the optimised ELISA method and thus were in good agreement with the expected PCB values (Table 4). However, for oils A, D and E which had significantly

Sample	Measured value (ng ml^{-1})		RSD (%)		Expected value* (ng ml^{-1})		Efficiency (%)	
	ELISA	RaPID	ELISA	RaPID	ELISA	RaPID	ELISA	RaPID
A 1260	169	2.2	2.5	10.7	200	2.5	84.5	88.0
A	24	0.45	4.7	5.5	8	0.4	436.3	112.5
B	62	0.82	1.6	12.8	70	3.5	88.6	23.4
C	89	4.53	8.9	6.0	102	5.1	87.3	88.8
D	26	0.50	2.8	11.9	20	1.0	130.0	50.0
E	25	0.378	4.7	6.6	12	0.6	208.3	63.0

ELISA calibration curve (n=4): $r^2 = 0.988$, $y = 1.870 - 0.593\ x$
RaPID Assay calibration curve (n=2): $r^2 = 0.945$, $y = 0.121 - 0.097\ x$
*Value expected for 100% extraction efficiency. Based on GC data

Table 4. Optimised quantitative ELISA system versus a commercial PCB test kit for the analysis of 5 PCB-contaminated transformer oils and a 100 μg ml^{-1} Aroclor 1260 standard after extraction using the KOH-ethanol/sulphuric acid method.

lower PCB values (8-20 μg ml^{-1}), the ELISA assay significantly overestimated the PCB concentration by 130-436%. It should be noted that these latter oils had PCB levels at the lowest limit of the optimised ELISA analytical range. Whilst further refinement is necessary to improve the assay performance at this lower limit, it should be noted that impending regulation in the UK will identify samples with PCB concentrations in excess of 50 μg ml^{-1} to be classified as contaminated. By overestimating the PCB levels in contaminated samples will minimise the number of genuinely contaminated oils being graded as 'PCB-free'. Ultimately, to eradicate this problem, the assay LOD must be improved. This could be achieved by employing more effective clean-up procedures to allow the analysis of less dilute oil samples, or by reducing the quantity of primary antibody used in the assay. Alternatively, an increase in sample volume may improve assay sensitivity. For example, the sample volume in the RaPID assay accounts for 21 % of the total assay reaction volume, compared with only 10% in the optimised ELISA.

The RaPID assay data did not display the concentration-response trends observed with the optimised ELISA. The Aroclor 1260 standard and oils A and C yielded efficiency values of 88.0 – 112.5%, whilst oils B, D and E, with efficiency values of 23.4-63.0% under-estimated the PCB levels expected in the oils. It is interesting to note that the ratio of the efficiency of the optimised ELISA over the RaPID assay varies from 2.60-3.87 for all of the oils tested excluding oil C. The cause of this general over-estimation by the optimised ELISA test (and underestimation by the commercial kit) has to be elucidated.

The RaPID assay system had a total assay time of 35 min compared to 75 min for the optimised ELISA. However, the latter system operates within a microtitre plate format and hence is more amenable to automation than the magnetic bead assay. Whilst the assay time of the current system is acceptable for most applications, it could be reduced by directly conjugating the enzyme label to the primary anti-PCB antibody thereby obviating the secondary incubation step. A preliminary evaluation indicates that this assay format has a similar analytical performance to the current ELISA with an assay time of <50 min. This assay format would be simpler-to-use and hence more attractive, but the influence of the solvent and matrix on enzyme label activity requires further attention.

Conclusions

The fully optimised assay had a dynamic range of 50-800 ng ml^{-1} with 50% signal inhibition values of 217 and 212 μg ml^{-1} for the Aroclors 1254 and 1260 (high relative abundance in insulating oils) in methanol. The assay proved more sensitive to the more highly chlorinated PCB standards tested. The acceptable limit of PCBs in electrical plant insulating oils has yet to be defined in the UK, but it would appear that impending legislation will select a value around 50 μg ml^{-1}, which is close to the detection limit of the developed assay. An improvement in assay LOD is therefore desirable. This could be achieved using an anti-PCB antibody of higher PCB-binding affinity, reduced amounts of primary antibody or by improving the sample preparation method to remove greater quantities of interferents, allowing lower sample dilutions to be employed.

The analysis of diluted insulating oils indicated that direct oil analysis was an unreliable method of sample preparation due to the presence of matrix interferents. Two sample preparation methods were found to be suitable for interferent removal - solid sorbents and washing with KOH-ethanol then sulphuric acid. The latter

method, combined with the optimised ELISA process, proved reliable for the analysis of transformer oils containing >70 μg ml^{-1} PCB, but over-estimated PCB levels in oils containing <20 μg ml^{-1} of the analyte. The assay was compared to a commercially available semi-quantitative PCB magnetic bead format ELISA. Sample clean-up was by the KOH-ethanol/sulphuric acid method. Both methods yielded reliable data for the quantification of an Aroclor 1260 standard in methanol, but for 4 of the 5 oils tested, the ELISA method determined the PCB levels to be 2.6-3.9 times higher than the commercial test kit. The major benefit of the optimised ELISA over the commercial kit was simplicity of automation, allowing high sample throughputs. Preliminary data shows that reduced assay times are achievable by conjugating the assay label directly to the primary antibody.

The authors wish to thank the National Grid Company, Leatherhead, UK, for the supply of insulating oil samples and Dr J.A. Bolbot for his many helpful discussions.

References

1. Ballschmiter, K. and Zell, M.F.Z. *Fresnius J. Anal. Chem.* 302 (1980) 20.
2. Rouse, T.O. Mineral Insulating Oil in Transformer Oil. *IEEE Electrical Insulation Magazine.* 14 (1998) 6-16
3. UK Action Plan for the Phasing Out and Destruction of Polychlorinated Biphenyls and Dangerous PCB Substitutes. March 1997 – Dept. Environment (PCBs).
4. Alford-Stevens, A.L. *Env. Sci. Tech.* 20 (1986) 1194.
5. Luster, M.I., Albro, P.W., Clark, G., Chea, K., Chaudhary, S.K., Lawson, L.D., Corbett, J.T. and McKinney, J.D. *Toxicol. Appl. Pharmacol.* 50 (1979) 147.
6. Newsome, W.H. and Shields, J.B. *Intern. J. Environ. Anal. Chem.* 10 (1981) 295.
7. Sisak, M., Franek, M., and Hruska, K. *Anal. Chim. Acta.* 311 (1995) 415.
8. Charles, P.T., Conrad, D.W., Jacobs, M.S., Bart, J.C. and Kusterbeck, A.W. *Bioconjugate Chem.* 6 (1995) 691.
9. Roberts, M.A. and Durst, R.A. *Anal.Chem.* 67 (1995) 482.
10. Zhao, C.Q., Anis, N.A., Rogers, K.R., Kline Jr., R.H., Wright, J., Eldefrawi, A.T. and Eldefrawi, M.E. *J. Agric. Food Chem.* 43 (1995) 2308.
11. Johnson, J.C. and Van Emon, J.M. *Anal. Chem.* 68 (1996) 162.
12. Lawruk, T.S., Lachman, C.E., Jourdan, S.W., Fleeker, J.R., Hayes, M.C., Herzog, D.P. and Rubio, F.M. *Environ. Sci. Technol.* 30 (1996) 695.
13. Bender, S. and Sadik, O. *Environ. Sci. Technol.* 32 (1998) 788.
14. Rodbard, D. and Cooper, J.A. In vitro Procedures with Radioisotopes in Medicine; International Atomic Energy Agency: Vienna, 1970; pp 659-673.
15. Vo-Dinh, T., Pal, A., and Pal, T. *Anal. Chem.* 66 (1994) 1264.
16. Chiu. Y-W., Carlson, R.E., Marcus, K.L., and Karu, A. *Anal. Chem.* 67(1995) 3829.
17. Shah, V.P., Midah, K.K., Dighe, S., McGilveray, I.J., Skelly, J.P., Yacobi, A., Layloff, T., Viswanathan, C.T., Cook, C.E., McDowall, R.D., Pittman, K.A. and Spector, S. *Pharm. Res.* 9 (1992) 588.
18. Russell, A.J., Trudel, L.J., Skipper, P.L., Groopman, J.D., Tannenbaum, S.R., and Kilvanov, A.M. *Biochem. Biophys. Res. Com.* 158 (1989) 80.
19. Stocklein, W., Gebbert, A. and Schmid, R.D. *Anal. Lett.* 23(1990) 1465.
20. Giraudi, G. and Baggiani. *Biochm. et Biophys. Acta.* 1157 (1993) 211.
21. Matsuura, S., Hamano, Y., Kita, H., and Takagaki. *J. Biochem.* 114 (1993) 273.
22. Stocklein, W.F.M., Scheller, F.W. and Abuknesha, R. *Sens. Actuat. B* 24 (1995) 80.
23. Spittler, T.M. *Env. Samp. Hazardous Wastes.* 267 (1984) 37.
24. Setford, S.J., Kroger, S. and Turner, A.P.F. *Analusis*, 27 (1999) 600.
25. Setford, S.J. *Trends Anal. Chem.* In press
26. Tanabe, S., Kannan, N., Subramanian, A., Watanabe, S. and Tatsukawa, R. *Environ. Pollut.* 47 (1987) 147.
27. Li, K., Caron, T., Jocelyn-Pare, J.R., Lanoy, M. and Chiu, C. Proceedings of the Electric Power Research Institute. (1991) 48.1 - 48.3.
28. Shu, Y.Y., Dowdall, J.E., Chiu, C. and Lao, R.C. *Intern. J. Environ. Anal. Chem.* 60 (1995) 185.