Anal Bioanal Chem (2002) 373:685–690
DOI 10.1007/s00216-002-1311-1

SPECIAL ISSUE PAPER

An-Ping Deng · Vladimír Kolář · Robert Ulrich
Milan Fránek

Direct competitive ELISA for the determination of polychlorinated biphenyls in soil samples

Received: 14 November 2001 / Revised: 1 March 2002 / Accepted: 28 March 2002 / Published online: 29 May 2002

Abstract A direct competitive ELISA for determination of polychlorinated biphenyls (PCB) in soil samples was described. The standard calibration curve based on Delor 103 (Aroclor 1242) was constructed in the dynamic range of 10–1000 μg L^{-1} and a detection limit of 5.0–12.9 μg L^{-1} (or 0.5–1.29 μg g^{-1} soil) was achieved. When spiked soil samples were extracted with methanol recoveries were 90.6–106.3%. The effect of methanol and DMSO on assay signal and sensitivity was established. Eight PCB-contaminated soil samples were analyzed by ELISA and gas chromatography (GC). The ELISA results from Soxhlet extraction were in a good agreement with those of GC (correlation coefficient 0.9866; n=8). Except for one soil sample the results from ELISA with methanol extraction were not significantly different from those from GC.

Keywords Sheep antibody · PCB · Extraction · Gas chromatography · Correlation

Introduction

The polychlorinated biphenyls (PCB) are a class of 209 discrete congeners with 1–10 chlorine atoms attached to a biphenyl nucleus. PCB have characteristics, e.g. chemical inertness, heat resistance, non-flammability, low vapor pressure, and dielectric properties, that promoted their use in electrical equipment such as transformers or condensers. PCB have been recognized as ubiquitous environmental pollutants, because of their low rate of environmental degradation and tendency to bioaccumulate; there is also evidence of their carcinogenicity [1, 2, 3, 4, 5]. The high lipophilicity and stability of PCB has resulted in their widespread distribution in the global ecosystem and PCB occur in nearly all environmental matrixes (surface water, soil, rain, snow, etc.). Although manufacture of PCB was discontinued in the 1970s, available results indicate that from the beginning of the 1980s PCB levels have tended to decline only very slowly, so that an end of PCB exposure can hardly be expected in the near future [6].

Current methods for measurement of PCB are either non-specific or utilize complex laboratory-based instrumental techniques [7]. The former methods measure general properties of PCB, e.g. total chlorine content, whereas the latter are more time-consuming and expensive, typically requiring sample preparation, chromatographic separation, and detection. More rapid, simpler, and cost-effective methods are, therefore, urgently required for the detection of PCB in environmental, industrial, and food samples.

Immunochemical techniques such as radioimmunoassay (RIA) [3, 4], enzyme-linked immunosorbent assay (ELISA) [1, 5, 8], and use of immunosensors (IS) [9, 10, 11] have shown potential for providing simple, rapid, and economical methods for the analysis of PCB. Several PCB test kits are commercially available for semi-quantitative analysis of PCB in different matrixes, and considerable method-development activity has been focused on the quantitative determination of PCB in soil [1, 2, 8, 12, 13, 14].

In our previous studies [3, 15] PCB derivatives for the preparation of immunogen and hapten-peroxidase conjugates were synthesized. Polyclonal antibodies raised from 24 animals were evaluated with industrial PCB mixtures Delor 103, 104, 105, and 106 (the corresponding PCB analogs are the mixtures Aroclor 1242, 1248, 1254 and 1260). The cross-reactivity to Delor 103, 104, 105, and 106 for the sheep antibody used in this study was 100, 79.6, 31.7, and 13.5%, respectively. No cross-reaction with 19 other structurally related compounds was observed and only 5% cross-reactivity was found for dichlorodiphenyledichloroethylene (DDE) [15]. Other sheep antibodies with selectivity directed at Delor 106 proved to be appropriate immuno-reagents for quantitation of those PCB congeners that are most abundant in this industrial formulation [3].

In this study the sheep antiserum raised against 4,4′-dichlorobiphenyl-thyroglobulin immunogen [15] has been

A.-P. Deng · V. Kolář · R. Ulrich · M. Fránek (✉)
Veterinary Research Institute,
Hudcova 70, 621 32, Brno, Czech Republic
e-mail: franek@vri.cz

applied to the determination of PCB (Delors) in soil samples, by use of the direct ELISA format. Delor 103 was used as calibrator for quantification. The effect of different amounts of organic solvents on the assay signal (here absorbance) and sensitivity was investigated. The extraction efficiency of the simpler extraction method (shaking with methanol) was evaluated. Real soil samples contaminated with PCB were extracted by shaking with methanol and in a Soxhlet apparatus. The results from ELISA were compared with those obtained from gas chromatography.

Experimental

Reagents, solutions, and apparatus

The production of sheep polyclonal antiserum and the preparation of PCB-peroxidase conjugate have been described elsewhere [15]. PCB standard (Delor 103) was obtained from the Slovak Institute of Metrology (Slovakia). Horseradish peroxidase (HRP), dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), and dimethyl sulfoxide (DMSO) were purchased from Sigma (USA); tetramethylbenzidine (TMB) and bovine serum albumin (BSA) was from Serva (Germany); 30% hydrogen peroxide (H_2O_2) was from Merck (Switzerland); and methanol was from Panta (Czech Republic). Na_2CO_3, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, NaCl, and all other chemicals were of analytical grade.

Na_2CO_3–$NaHCO_3$ solution, 50 mmol L^{-1}, pH 9.6, was used for coating microtitre plates; 10 mmol L^{-1} phosphate–buffered saline (PBS) containing 145 mmol L^{-1} NaCl, pH 7.2, was used for diluting peroxidase-PCB conjugate; and 10 mmol L^{-1} PBST (containing 0.1% Tween-20) was used for washing microtitre plates. Methanol (1%), DMSO (2%), and BSA (0.75%) in pure water was used for preparation of Delor 103 standard solution (assay solution without methanol was used for diluting soil extracts). The substrate buffer was 0.1 mol L^{-1} sodium acetate; pH was adjusted to 5.5 by addition of 1.0 mol L^{-1} citric acid. The substrate (TMB/H_2O_2 solution) was prepared by mixing TMB stock solution in DMSO (1%, w/v, 100 µL), H_2O_2 (5%, w/v, 10 µL), substrate buffer (500 µL), and pure water (10 mL). The stop solution was 2.0 mol L^{-1} H_2SO_4.

Microtitre plates (type P) were from GAMA (Ceske Budejovice, Czech Republic). The ELISA plate reader (MF) with software (Genesis 2.0) for absorbance measurement and data analysis was from Labsystems (Helsinki, Finland). The microtitre plate washer (812 SW1) was from SLT (Vienna, Austria) and the microtitre plate shaker (MTS 4) from IKA (Germany). Milli-Q equipment for water purification was from Millipore (USA).

Preparation of Delor 103 standard solution

Delor 103 stock solution was prepared by diluting Delor 103 with hexane to a final concentration of 1000 µg mL^{-1} and stored at –20 °C until used. Solvent exchange is required in PCB standard preparation, because hexane is incompatible with the immuno-reaction in the assay. Delor 103 stock solution in hexane (0.5 mL) was placed in a glass tube, the hexane was evaporated with a stream of nitrogen, and DMSO (0.5 mL) was added to dissolve the Delor 103 in the tube. Delor 103 standard solutions with concentrations of 10, 50, 100, 250, 500, 2000 µg L^{-1} were prepared by diluting PCB stock solution in DMSO with assay solution.

Preparation of soil samples

PCB-free soil samples were collected from the top layer of maize field and stored at –20 °C. Before spiking with Delor 103 solution they were dried in air, ground, and blended. To prepare soil samples spiked with PCB at concentrations of 4, 8, 16, 32, and 64 µg g^{-1}, different volumes of Delor 103 stock solution in hexane were added to five glass beakers containing 50 g soil. Pure hexane was added so that the total volume of solution in the five beakers was the same. When the soil was thoroughly wetted it was kept at room temperature for several days with a filter paper on the top of the beaker to exclude dust. After complete drying in air the spiked soils were homogenized with a glass rod and kept at –20 °C until use.

Eight real soil samples suspected of being contaminated with PCB were obtained from Enviro Technology Today (Lehnice, Czech Republic). After removal of stones, they were dried in air, ground, blended, and kept at –20 °C until use.

Extraction of soil samples

Methanol extraction and Soxhlet extraction were employed in this study. In principle, extraction by shaking with methanol was performed as previously described for atrazine in soil [16]. Briefly, 5 g soil was placed in glass flasks with stoppers and 5 mL pure methanol was added. The flasks were sealed, shaken vigorously for 1 min, and left to settle at room temperature for 30 min. Approximately 2 mL of the extract (upper phase) was transferred to another flask and centrifuged at 2500 rpm for 10 min. The supernatant was diluted 100 times with assay solution and used for standard ELISA procedures.

Soxhlet extraction was performed by extracting 5 g soil with a 1:1 mixture of petroleum ether and acetone in a Soxhlet extractor for 4 h. After purification on a column containing 14 g activated Florisil and evaporation the extract was dissolved in 1 mL isooctane for PCB analysis by gas chromatography. Substitution of isooctane by DMSO was required when the extract was used for ELISA.

ELISA procedure

Sheep polyclonal antibody (200 µL) diluted with coating buffer (1:40,000) was pipetted into the wells of microtitre plates and incubated overnight at room temperature. Unbound compounds were removed by washing three times with PBST. Delor 103 standards (100 µL) in the concentration range 10–2000 µg L^{-1} or soil extracts diluted 1:100 with assay solution were added to the wells in triplicate. PCB-peroxidase conjugate diluted 1:400,000 with PBS (50 µL) was also added to the same wells. The plate was shaken on a microtitre plate shaker for 1 min followed by incubation for 1 h at room temperature. The plates were washed three times as before, and 200 µL enzyme substrate (H_2O_2+TMB solution) was then added. After the plates had been shaken for 15 min, the enzymatic reaction was stopped by addition of 50 µL 2 mol L^{-1} H_2SO_4. Absorbance was measured with an ELISA reader at 450 nm and the concentrations of PCB in the soil samples were calculated from a standard curve generated during the same run. The standard curve was plotted in the form of absorbance against log C or $\%B/B_0$ against log C (where B and B_0 were the values of absorbance measured at standard concentrations and at zero concentration, respectively).

Gas chromatography

Gas chromatography (GC) was performed with a Varian Star 3600 CX GC (Varian, USA) with ^{63}Ni electron-capture detector (ECD) and a dual column system (007-2 and 007-1701 columns, 60 m× 0.25 mm×0.25 µm film, Quadrex Corporation, USA). Samples were injected by means of an 8200 CX autosampler. Hydrogen was used as carrier gas at a flow of 3.0 mL min^{-1}. The oven temperature was 100 °C (for 2 min)→150 °C (at 20 ° min^{-1})→250 °C (at 5 ° min^{-1}) and held for 15 min. On-column injection was performed at an initial injector temperature of 100 °C; the injector temperature was then programmed to 225 °C at 250 °C min^{-1}. The detector temperature was 300 °C and the detector make-up gas was

nitrogen at a flow of 30.0 mL min^{-1}. For chromatogram evaluation the Delor 103 peaks selected were those with the retention times 14.8, 15.7, 15.8, 18.3, and 19.4 min.

Results and discussion

Stability and sensitivity of the ELISA

The standard calibration curve for Delor 103 was constructed in the concentration range 10–2000 μg L^{-1}. The same ELISA procedure was repeated 10 times over 6 weeks. As shown in Fig. 1, the stability of the established ELISA procedure was good – the relative standard deviation for each calibration point on 10 curves was within 4.5 to 13.9%. The IC_{50} values (concentrations of Delor resulting in 50% binding inhibition in optimized assays) for 10 curves were in the range 140 to 220 μg L^{-1}. The detection limits, defined as the detectable concentration equivalent to twice the standard deviation of zero binding, were within 5.0 to 12.9 μg L^{-1}, which is low enough for quantification of PCB in soil samples. PCB levels of 50 μg g^{-1} or more constitutes soil contamination [7, 17]. Because 5 g soil was extracted with 5 mL organic solvent in this ELISA and the soil extract was diluted 100 times for ELISA, the detection limit achieved by use of this ELISA for soil analysis will be 0.5–1.29 μg g^{-1} soil, which is approximately 40–100 times lower than the value of 50 μg g^{-1}. The advantage of analyzing soil samples by means of a highly sensitive ELISA is that interference from other components in the matrix can be greatly reduced, or eliminated, simply by appropriate dilution of the soil extract with the assay buffer.

Effect of solvent concentration on the assay

Non-polar (or low polarity) organic solvents are usually required for extraction of hydrophobic environmental pollutants such as PCB in matrixes. The extracts can be directly applied to ELISA analysis if the organic solvents used are water-miscible, because ELISA is usually performed in the aqueous phase. If not, evaporation of the solvent and re-constitution in a suitable assay-compatible solvent are needed before the ELISA analysis [18]. In this study the assay solution used for PCB analysis was a mixture of methanol, DMSO and BSA. Methanol is a water-miscible solvent used for extraction of PCB from soil samples, thus the assay solution for the preparation of Delor 103 standard solutions should contain the same amount of methanol as those in diluted soil extracts. The assay solution was supplemented with DMSO and BSA, because DMSO (a water-miscible solvent) has proven to be an effective solubilizer of hydrophobic PCB [3] and BSA is a common protein used to reduce non-specific adsorption of the analyte on to the wells of the microtitre plate. Because two organic solvents (methanol and DMSO) are used in the assay, it is necessary to investigate the effect of different concentrations of these solvents on the assay.

Delor 103 standards in the concentration range 10–2000 ng mL^{-1} were prepared with assay solution containing 5, 10, 20, and 30% methanol or 2, 5, 10, and 20% DMSO. The calibration solutions were subjected to the ELISA procedure and the curves obtained were plotted in the form of absorbance against logC. As shown in Fig. 2a,b, the absorbance at the same calibration point decreased with increasing concentrations of methanol or DMSO. Consequently the standard curves are gradually shifted downwards. The IC_{50} values calculated from the standard curves were not significantly changed, however, because the IC_{50} values for 5, 10, 20, or 30% methanol were 218, 157, 167, or 208 ng mL^{-1} and the IC_{50} values at 2, 5, 10, or 20% DMSO were 196, 166, 141, or 152 ng mL^{-1}, respectively. It is obvious that the assay sensitivities still remained in a reasonable range, even though the signal decreased when the concentrations of methanol (or DMSO) were increased from 5 to 30% (or from 2 to 20%); this indicates this ELISA is insensitive to organic solvents, even at high concentrations. Because soil extracts were diluted 1:100 for ELISA analysis in this study, in other words the concentration of methanol in the diluted soil extract was only 1%, the composition of the assay solution for the preparation of Delor 103 standard solutions was selected as 1% methanol, 2% DMSO, and 0.75% BSA.

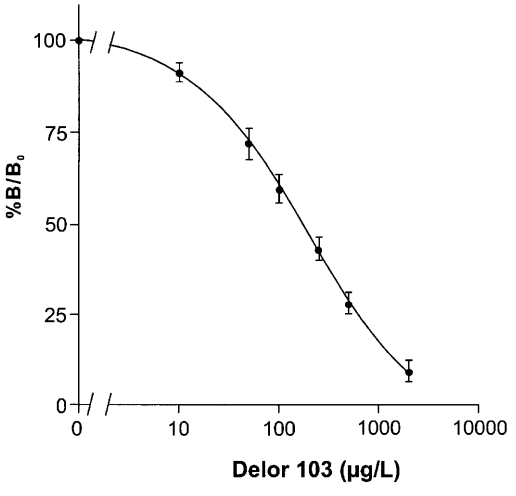

Fig. 1 Standard curve for Delor 103. The bars show the standard deviations for 10 replicate assays. Dilutions: sheep antiserum 1:40,000, PCB-HRP (1:400,000)

Evaluation of extraction by shaking with methanol

Before the analysis of real soil samples, the precision and accuracy of the simpler extraction method (extraction with methanol by shaking) were evaluated. PCB-free and Delor 103-spiked soil samples were extracted by shaking with methanol and the extracts were subjected to standard ELISA procedures. Extraction of the soil samples and ELISA analysis were repeated six times within 2 weeks. As shown in Table 1, for a PCB-free sample the measured value was 0.9 μg PCB g^{-1} soil, which is just in the range

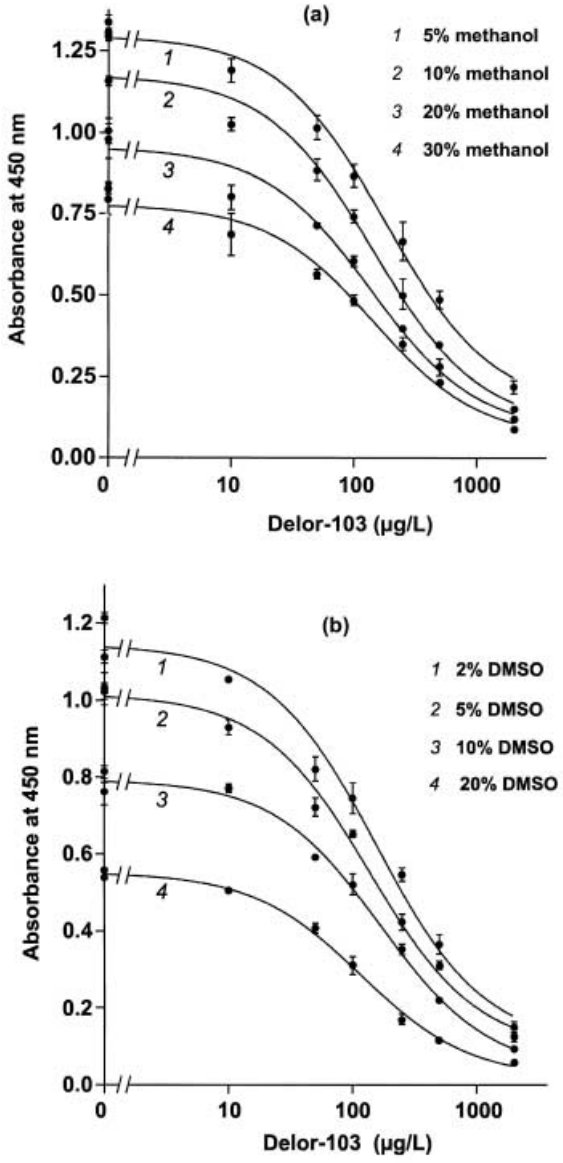

Fig. 2 The effect of methanol (**a**) and DMSO (**b**) on assay signal and sensitivity

of the detection limit of ELISA for soil analysis, indicating that interference by the matrix was negligible when the extract was diluted 100 times with the assay solution. For the soil samples spiked with Delor 103 concentrations of 4–64 µg g^{-1} soil the measured concentrations were 5.1–58.9 µg PCB g^{-1} soil. For samples spiked with the highest levels, 8–64 µg g^{-1} soil, inter-assay relative standard deviations (RSD) were 13.2–15.1%, whereas for the lowest spike level, 4 µg PCB g $soil^{-1}$, the RSD was higher – 25.4%. The accuracy of the extraction method was expressed as the recovery [recovery (%)={(conc. measured–conc. in PCB-free soil)/conc. spiked}×100%]. It was observed that recoveries of added PCB from spiked soils by shaking with methanol were in the range 90.6–106.3%, in accordance with the results of Lawruk et al. [1] and Johnson and Van Emon [8] who obtained recoveries of approximately 90%, irrespective of sample type and total Aroclor level.

Analysis of real soil samples

Extracts obtained by shaking with methanol were analyzed by ELISA only, whereas Soxhlet extracts were analyzed by ELISA and GC. As shown in Table 2, it is apparent that the soil samples were heavily contaminated with PCB, because the PCB concentrations measured by ELISA and GC for most of the soil samples were much higher than 50 µg g^{-1}. For soil samples No. 4 and No. 6 the PCB content measured by GC and ELISA was even higher than 1000 µg g^{-1} soil. It should be noted that the PCB concentration in our randomly collected samples did not always correspond with the concentration range of the extraction procedure evaluated. This drawback was, on the other hand, compensated by obtaining data for high-level soil contamination.

The ELISA results both from the extract obtained by shaking with methanol and from the Soxhlet extract were similar in trend to those from GC although, as it seemed from the values in Table 2, ELISA provided rather semi-quantitative values. One exception is the No. 2 soil sample extracted by shaking with methanol, in which the measured concentration is 26.5 µg g^{-1} soil, approximately one-twenty-fifth that measured by GC (668.3 µg g^{-1} soil). A possible explanation is that the PCB were strongly adsorbed on charcoal particles in the soil matrix of the sample No. 2 and shaking with methanol did not completely desorb the hydrophobic analyte from the matrix.

When ELISA results were compared with those from GC, ELISA results for Soxhlet extracts were in close agreement with those of GC – the correlation coefficient was 0.9866 (n=8); this agreement is apparent from Fig. 3a,b. For extracts obtained by shaking with methanol the correlation coefficient for the ELISA and GC results was 0.9465 (n=7, i.e. excluding soil sample No. 2). The slope 0.7916 from the linear regression equation in Fig. 3a indicated that values for the Soxhlet extracts were underestimated whereas the slope 1.2041 in Fig. 3b showed that ELISA values for extracts obtained by shaking with methanol were overestimated compared with results from GC.

Table 1 Precision and accuracy for extraction of PCB from spiked soil samples by shaking with methanol

Conc. spiked (µg g^{-1} soil)	0	4	8	16	32	64
Average conc. measured (µg g^{-1} soil)±SD	0.9±0.8	5.1±1.3	9.4±1.4	16.6±2.5	29.9±4.2	58.9±7.8
Inter-assay CV (%) (n=6)	–	25.4	14.9	15.1	14.0	13.2
Recovery (%)	–	105.0	106.3	98.1	90.6	90.6

Table 2 PCB concentrations in real soil samples as measured by ELISA and GC

No. soil	ELISA (μg g^{-1} soil)		GC (μg g^{-1} soil)
	Methanol shake extract	Soxhlet extract	Soxhlet extract
1	2150	800	990
2	26.5	368	668
3	93.1	278	204
4	3190	2090	2540
5	291	111	116
6	1910	1530	1880
7	89.4	141	82.0
8	31.8	117	34.4

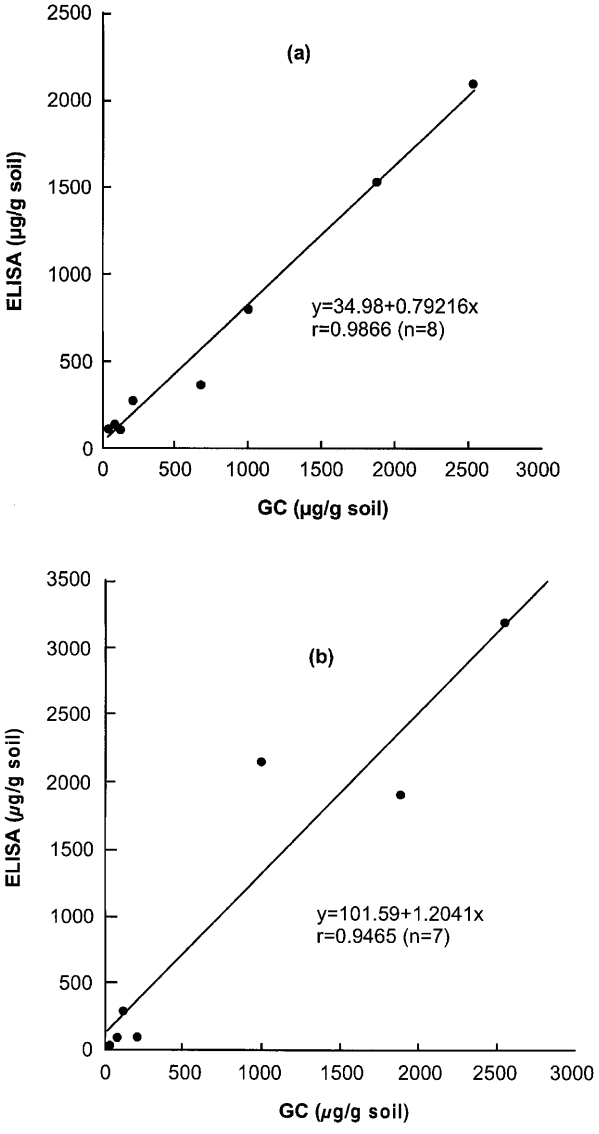

Fig. 3 Correlation of the results from determination of PCB residues in soil samples: (**a**) correlation of ELISA and GC results for Soxhlet extract; (**b**) correlation of ELISA and GC results for extract obtained by shaking with methanol

Because ELISA detection is based on specific recognition of the total amount of PCB in a sample, the selectivity of the antibody can affect quantification of this analyte in a real analysis. Thus different response to a standard PCB formulation and to PCB in real samples is indicated. This might be a partial cause of the discrepancies between ELISA and GC results. The sample extraction procedure plays a major role in the overall analytical result even the accuracy and precision of ELISA for analysis of spiked samples are high [2, 8, 13]. The data obtained by Johnson and Van Emon [8] demonstrated that ELISA was capable of providing quantitative results for PCB when Soxhlet or supercritical-fluid extraction was used. ELISA analysis of the extract obtained by shaking with methanol, on the other hand, gave results which were not always in a close agreement with the values obtained by use of conventional GC-based methodologies [8]. A sample matrix containing substances that might interfere with immunochemical analysis is, moreover, another factor with potential to affect the results. Samples with a high organic matter content resulted in reproducible overestimation when ELISA was performed after methanol extraction [2]. Matrix interferences and their influence on immunochemical detection are usually hardly predictable. It should be noted that a method such as GC could, in some circumstances, also be a source of errors contributing to differences between the independent techniques.

Conclusions

A method for the determination of PCB in soil samples has been developed on the basis of a specific sheep polyclonal antibody for lower chlorinated PCB congeners. The method enables assessment of the total concentration of PCB by comparison with Delor 103 (Aroclor 1242) as calibration standard. The high stability of the assay was apparent from results from 10 replicate ELISA analyses. The detection limit of 5.0–12.9 μg L^{-1} (or 0.5–1.29 μg g^{-1} soil) achieved by this ELISA was low enough for quantification of PCB in soil samples. The recoveries of PCB extracted from spiked soil samples by shaking with methanol were 90.6%–106.3%, indicating that methanol was a suitable organic solvent for PCB extraction. Although increasing the concentrations of organic solvents resulted in a decrease in the assay signal, the sensitivity of the assay remained approximately the same in the calibration range used. Eight real soil samples were collected and analyzed by ELISA and the results were compared to those from GC analysis. Correlation of ELISA and GC results for Soxhlet extracts was good, although some of the values measured were different. This disparity might be ascribed to different sample composition, extraction method, or detection of the complex analyte. It seemed from the data obtained that the ELISA presented is a potential means of screening soil for PCB contamination, especially when the analysis of large-scale soil samples is required. When fast decisions are required, for example in field analysis at contaminated sites (on-site analysis), a

simple device such as the immunosensor would be superior to conventional analytical methods.

Acknowledgements This work was supported by the Czech Ministry of Agriculture-MZE-M03-99-01 program and by the Grant No. 525/97/0924 of the Czech Grant Agency. The authors thank D. Fojtova for her excellent technical assistance.

References

1. Lawruk TS, Lachman CE, Jourdam SW, Jourdan SW, Fleeker JR, Hayes MC, Herzog DP, Rubio FM (1996) Environ Sci Technol 30:695–700
2. Pullen S, Haiber G, Scholer HF, Hock B (1996) Int J Environ Anal Chem 65:127–138
3. Fránek M, Hruška K, Šišák M, Diblíková I (1992) J Agric Food Chem 40:1559–1565
4. Sisak M, Fránek M, Hruska K (1995) Anal Chim Acta 311: 415–422
5. Chiu YW, Carison RE, Marcus KL, Karu AE (1995) Anal Chem 67:3829–3839
6. Bucholski KA, Begerow J, Winneke G, Dunemann L (1996) J Chromatogr A 754:479–485
7. Kim IS, Setford SJ, Saini S (2000) Anal Chim Acta 422:167–177
8. Johnson JC, Van Emon JM (1996) Anal Chem 68:162–169
9. Zhao CQ, Anis NA, Rogers KR, Kline RH, Wright J, Eldefrawi AT, Eldefrawi ME (1995) J Agric Food Chem 43:2308–2315
10. Carlo MD, Lionti I, Taccini M, Cagnini A, Mascini M (1997) Anal Chim Acta 342:189–197
11. Laschi S, Fránek M, Mascini M (2000) Electroanalysis 12: 1293–1298
12. Harrison RO, Melnychuk N (1995) Int J Environ Anal Chem 59:179–185
13. Chuang JC, Miller LS, Davis DB, Peven CS, Johnson JC, Van Emon JM (1998) Anal Chim Acta 376:67–75
14. Johnson JC, Van Emon JM, Clarke AN, Wamsley BN (2001) Anal Chim Acta 428:191–199
15. Fránek M, Pouzar V, Kolar V (1997) Anal Chim Acta 347: 163–176
16. Deng AP, Fránek M, Kolar V (1999) Food Agric Immunol 11: 135–144
17. Lambert N, Fan TS, Pilette J-F (1997) Sci Total Environ 196: 57–61
18. Setford SJ (2000) Trends Anal Chem 19:330–339