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EPA **Project Summary**

Development and Evaluation of a Quantitative Enzyme Linked Immunosorbent Assay (ELISA) for Polychlorinated Biphenyls

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A 96-well, microplate-based enzyme linked immunosorbent assay (ELISA) for the quantitative determination of PCBs (as Aroclors) in soil has been developed and evaluated. The method detection limits are 8.95 and 10.5 μg/Kg for Aroclors 1248 and 1242, respectively. The ELISA was characterized for potential cross-reactivity with 37 structurally related chlorinated benzenes, anisoles, and phenols which might potentially be present as co-pollutants in environmental samples. Cross-reactivity was found to be negligible.

Samples were extracted using a methanol shake extraction procedure derived from various field screening methods, including the EPA FASP (Field Analytic Screening Program) gas chromatography (GC) method for PCBs. Extraction efficiency was found to be greater than 90%, as determined by extraction of carbon-14 radiolabeled tetrachloro-biphenyl.

Three sets of real-world samples, obtained from Superfund sites and the EPA National Enforcement and Investigation Center (NEIC) were analyzed using the quantitative PCB plate ELISA. In addition, all samples were analyzed by outside confirmatory laboratories using standard EPA GC methods. Subsets of the samples were also extracted using supercritical fluid extraction and methanolic Soxhlet extraction. The results for the methanol shake extracts appeared to be statistically biased away from the confirmatory results. The ELISA results for the SFE and Soxhlet extracts, however, overlapped the GC results, within the error limits of the respective methods. The latter results demonstrated that the quantitative PCB plate ELISA can function in a highly precise and accurate manner as a detection and quantitation device when coupled to an efficient extraction procedure.

Introduction

This report details the development and evaluation of a 96-well microplate-based enzyme linked immunosorbent assay (ELISA) for the quantitative determination of polychlorinated biphenyls (PCBs) in soil. Procedures carried out during the developmental stage are described, along with accompanying performance characteristic data. In addition, the analysis of three sets of real-world soil samples, representing a wide variety of matrix challenges, is reported.

After the initial detection of polychlorinated biphenyls (PCBs) in the environment in 1966, mounting evidence led the U.S. Environmental Protection Agency (EPA) to classify them as suspected human carcinogens, due in part to their low rate of degradation, their tendency to bioaccumulate, and their carcinogenic nature. In 1976, the U.S. Congress banned PCB manufacture, processing, distribution and use, except for a handful of specific and limited uses. As a consequence, analytical method development resulted in the codification of a number of standard methods for PCBs. One such method, Method 8080, described in the EPA Office of Solid Waste and Emergency Response Manual SW-846, is representative of the majority of PCB analytical methods in that the method relies on rigorous overnight

Soxhlet extraction, followed by gas chromatography (GC) for quantitation of PCBs.

Several factors have generated increasing interest in immunochemically-based analytical methods, such as ELISA, for PCBs. In addition to the spiralling costs associated with regulatory compliance, current toxicological research has re-awakened the controversy surrounding the actual carcinogenicity and toxicity of PCBs. In an effort to expand the array of tools available for PCB research, immunochemically-based methods are being looked at to provide data under appropriate circumstances.

As a result of the Superfund Amendments and Reauthorization Act of 1986 (SARA), site monitoring and assessment will represent a large and possibly rising financial burden directly related to the collection, transport, and analysis of PCB containing samples. ELISA based methods have been shown to offer the potential for data outputs which are complimentary and in some cases comparable to established methods at a significantly lower cost on a per analysis basis. ELISA methods also show a distinct advantage with regard to timeliness of data generation.

Several ELISA-based methods for PCBs have become commercially available over the past several years. In general, these immunoassays are formatted to be used only for determining whether a given sample contains PCBs at a concentration above or below a set threshold value, although one currently marketed assay is intended for quantitative use. As such, these immunoassay kits are designed for rapid result generation, low cost, and ease of use by relatively untrained personnel. The benefits of such design criteria have become quite evident to EPA, which, under the Superfund Innovative Technology Evaluation (SITE) Program, officially mandated the use of low cost, rugged, field-portable methods to ease the burden of using expensive, time-consuming, GC or GC/MS methods for the characterization of contaminated areas.

While the benefits of using commercial immunoassay kits are evident, the data which they generate is complimentary but not comparable with current GC-based methods for PCBs. Consequently, there exists a large gulf between these analytical methods.

In the broadest of terms, the report describes work on an immunoassay aimed at bridging this gap between the GC based instrumental methods and the commercial immunoassay kits. Such an immunoassay procedure will provide data which are quantitative and comparable to the GC-based methods for many applications, such as site characterization, mapping concentration isopliths, and monitoring remedial activities, while providing high throughput analytical procedures which are, to a great degree, as inexpensive, rapid, and simple as the kit immunoassays.

Conclusions

Assay Performance

The quantitative PCB plate ELISA was characterized over the course of assay development and subsequent analysis of three sets of real-world samples obtained from EPA SITE demonstrations and regulatory activities. Based on the Aroclors which contaminated these real-world samples the bulk of data are focused on Aroclors 1242 and 1248. Initial characterization data show that this assay could be used for Aroclors 1254 and 1260 equally well with similar performance characteristics. The remainder of the discussion centers around Aroclors 1242 and 1248.

The assay described in the current study is intended for the analysis of PCB contamination in solid matrices such as soil, sediment, clays and paper pulp, and thus the samples required extraction prior to analysis. The methanol-based shake extraction procedure employed during the present study was chosen for its simplicity, and was based on extraction procedures common to a number of field methods. Preliminary extraction studies, with a wide variety of matrices, using a radiolabeled tetrachloro-biphenyl suggested that the extraction procedure would optimally provide an average extraction efficiency of 92% with a relative standard deviation (RSD) of variation of ±4%.

Extraction of commercially available “PCBs in soil” standard reference materials (SRMs), followed by quantitation with the plate ELISA provided an indirect measure of extraction efficiency. ELISA results for Aroclor 1248 SRMs suggest extraction efficiencies greater than 90%, while for Aroclor 1242, employing 5 PCB levels, efficiencies ranging from 53% to 91% were observed. In all cases for the 1242 SRMs, the reported value is within the EPA defined advisory range as specified by SW-846 Method 8080/81.

The assay had detection limits of 1.31 ng/mL for Aroclor 1248 with a σ of 0.9 ng/mL and a detection limit of 1.6 ng/mL for Aroclor 1242 with a σ value of 0.61 ng/mL. The detection limit in soil (based on a 5 gram sample) is 9.0 ng/g, σ = 6.0 ng/g for Aroclor 1248 and 10.5 ng/g, σ = 4.1 ng/g for Aroclor 1242 after correcting for the dilution factor imposed by adding soil extracts to assay solution. The assay had a quantitation range of about 8 ng/mL to 200 ng/mL in assay solution, corresponding to soil concentrations of about 50 ng/g to 1330 ng/g, or 0.05 mg/Kg to 1.33 mg/Kg. Samples extracts containing greater than about 1.3 µg/mL PCBs require appropriate dilution to bring the PCB concentration into the working range of the assay.

The assay provided the long-term reproducibility required for use as a quantitative tool. Based on repeated measures of Aroclor 1248 soil SRMs over a 6 month period, determinations were carried out with RSD’s for all SRMs of less than 10%. Repeated measures of Aroclor 1242 soil SRMs over a 3 month period provided similar performance. Dependant on PCB level, RSD’s ranged from 30% to 5%.

The quantitative PCB plate ELISA was found to be highly selective for PCBs; it exhibited very little cross-reactivity with a large number of compounds which might potentially co-contaminate environmental samples and interfere with accurate measurement of PCB concentrations. The assay exhibited selectivity for PCBs which will allow the plate ELISA to be used in the presence of a wide range of commonly occurring chlorinated anisoles, benzenes and phenol co-pollutants. The 37 compounds which were studied in the preliminary development stage cross-react no more than about 3% relative to Aroclor 1248.

Validation of ELISA Performance with Real-world Samples

Validation of ELISA using comparative results obtained by standard instrumental methods is based on several important assumptions. Statistically, this procedure can become confounded by sampling errors, sample preparation differences, and inter-lab variation, even before variability is introduced by true inter-method differences. Comparison of the quantitative PCB plate ELISA soil sample results with results generated using standard methods such as SW-846 Method 8080/81 is made difficult by the fact that performance data for the standard methods is typically limited to either solution phase measurement data or a limited number of soil matrices. This lack of availability of extensive soil analysis performance data for standard methods points out the difficulty of comparing results across soil samples; each soil matrix may present new challenges a particular method cannot meet. Extraction procedures which work well for sandy soils may provide irregular performance characteristics when applied to oily clay samples or sediments.

ELISA Analysis of Kansas City Samples

ELISA analysis of Aroclor 1248 contaminated clay samples obtained as sample splits from the Kansas City, MO Indian Creek Superfund site provided data which are interesting considering the above discussion. Using a paired t-test as the basis for decision, it was found that the PCB levels as reported by the CLP laboratory were not equivalent to the PCB levels as determined by the quantitative PCB plate ELISA. The average relative percent difference was found to be 46%.

One problem with such an approach is the implicit assumption that both the ELISA and CLP reported values represent the true mean for each respective method. The large error bars for the methods suggest that this is not likely to be the case. One undesirable alternative would be running enough replicates of each sample to ensure the validity of this assumption. This degree of rigor is possible with immunoassay, however, given the time and expense of the CLP analyses, this is not practical.

Another problem is the distinction (or lack thereof) between preparative and determinative steps in the data generation. The ELISA and CLP methods employed very different extraction procedures. It is conceivable that most of the measured difference between methods is due to sample preparation alone.

Four alternative hypotheses can explain the data. The first is: the quantitative PCB plate ELISA is not accurate but the CLP method is. The second is: some interferant or interferants in the samples themselves causes assay performance degradation. The third hypothesis is: the quantitative PCB plate ELISA and CLP method are both accurate, but extraction performance varied greatly between methods. Finally, the fourth hypothesis is: there is a large quantity of some non-PCB cross-reacting species present in the samples which elevates the apparent concentration of PCBs as measured by the quantitative PCB plate ELISA.

The first hypothesis can be ruled out. Data generated for the commercial soil SRMs as well as quantitation of spiked solutions shows that the ELISA can accurately measure PCB concentrations. The second hypothesis can be ruled out as well. Data collected during parallelism studies with the Kansas City samples show that there are no significant non-specific matrix contributions for the ELISA results.

The third hypothesis cannot be readily discounted. Based on earlier work (Spittler, 1986), it might be suspected that extraction procedures employing methanol would work better than hexane/acetone extractions as called for in the CLP method. Hexane/acetone extraction as specified in the CLP method may be optimal for a mixture containing all the chlorinated analytes covered by the method, but not for the specific, more focused use of PCB extraction exclusively. Of course, the simple approach taken in a methanol-based shake extraction used for the ELISA might offset the gain realized from substitution of methanol. Results for the SFE extracts of the Kansas City samples illustrate these extraction issues. Using the identical ELISA procedure, it was found that the SFE extracts gave results which converged toward the CLP results. The SFE-ELISA results were equivalent to the CLP results by a paired t-test ($t = 0.8729$, $p = 0.39$), whereas the ELISA results for the same samples extracted by the methanol shake procedure were not ($t = 2.118$, $p = 0.046$).

The fourth hypothesis cannot be ruled out easily either. If there are cross-reacting compounds, they are not commonly occuring chlorobenzenes, phenols, or anisoles. One possibility is the presence of PCB metabolites, such as polychlorinated biphenylols, which would not be detected by standard methods, but which may nevertheless be measured by ELISA.

There are certain applications for which data provided by the quantitative ELISA could prove very useful. The reported data for the Kansas City samples demonstrate the use of ELISA as a bridge between GC methods and semi-quantitative immunoassay test kits. The majority of the samples had concentrations well below 5 mg/Kg. At this level, relative percent differences of 100% may correspond to as little as 0.033 mg/Kg (the detection limit of the CLP method). For example, to easily obtain a quantitative PCB result of 0.1 mg/Kg, $\pm$ 0.1 may have great value when the option is either GC analysis or a semi-quantitative "less than 5 mg/Kg" result obtained through use of a commercial, semi-quantitative ELISA. The quantitative PCB plate ELISA allows for the measurement of PCB concentration in a way which provides more information than the semi-quantitative ELISAs currently available commercially, while using an assay procedure of similar complexity.

ELISA Analysis of Allied Paper/ Portage Creek/Kalamazoo River Samples

ELISA analysis of Kalamazoo samples obtained from the Allied Paper/Portage Creek/Kalamazoo River Superfund site in Michigan provides further amplification upon the points discussed above. The data can be thought of as consisting essentially of two subsets, the low level samples (PCB concentrations below approximately 30 mg/Kg) and the high level samples (PCB concentrations greater than 30 mg/Kg). For the low level samples, the bulk of the ELISA and SW-846 Method 8081 results overlap one another within the 95% confidence intervals of the respective methods. For the high level samples, ELISA and Method 8081 results overlapped in a similar manner after more vigorous methanolic Soxhlet extraction prior to ELISA analysis. ELISA analysis of extracts obtained using a 20 min. shake in methanol resulted in measured values of PCB up to a factor of 6.5 lower than ELISA results for methanolic Soxhlet extracts. Again, parallelism studies and spike recovery data demonstrated that the quantitative PCB plate ELISA, as applied to the Allied Paper/Portage Creek/Kalamazoo River samples, showed high accuracy and no assay degradation due to matrix artifacts. Thus, the potential utility of the quantitative PCB plate ELISA as a determinative method for PCBs in sediment, soil and paper waste was demonstrated by the results.

The results for the high concentration samples illustrate the need for differentiating the sample preparation from the determinative step itself. The ELISA results for the simple "20 minute methanolic shake" extracts and the ELISA results for the methanolic Soxhlet extracts are very different. Clearly, only the extraction efficiency plays a significant role in altering the ELISA results.

The fact that the ELISA results for the methanolic Soxhlet extracts are convergent with the CLP data (the ELISA results appear to be slightly lower than the CLP results with a calculated mean RPD of -17%) gives rise to the hypothesis that the quantitative PCB plate ELISA, as the determinative step, provided comparable data to the GC, for these environmental samples, provided that appropriate extraction procedures were used.

ELISA Analysis of NEIC Samples

The results from ELISA analysis of samples obtained from the EPA Enforcement and Investigation Center (NEIC) illustrate a number of points. Analysis of several serial dilutions of the sample extracts demonstrated that the assay was not subject to performance degradation due to matrix artifacts. The ELISA results were generally higher than the corresponding GC results, with an average RPD of 37%.

The results for this data set raise the issue of calibration, an issue which is universal to any determinative method for Aroclors. The samples were characterized by NEIC as being mixtures of Aroclors 1242/1254/1260. The apparent bias high on the part of the ELISA may be due wholly to selection of the calibration mixture. In addition, the PCB levels reported by NEIC most likely have a built in bias, due to analyst judgement calls on assigning peak patterns to the various Aroclors.

Taken as a complete method, 20-min. methanolic shake extraction followed by ELISA determination of PCBs appears to have bias away from standard GC based methods, at least statistically speaking. The quantative PCB plate ELISA data for the three sets of real-world samples contain more information than results which may be generated employing commercially available semi-quantitative ELISA-based methods. Thus, the quantitative PCB plate ELISA fulfills the goal of providing an easily performed method bridging the performance gap between GC methods and semi-quantitative ELISA.

The real-world data indicate that extraction procedures play a major role in the ELISA results. Statistically speaking, in these studies, it is improper to compare the accuracy of the ELISA determinative step with the GC determinative step, because the extraction procedures confound matters.

The data generated during development, evaluation, and application of the ELISA strongly suggest that the quantitative PCB ELISA can function in an accurate and highly precise manner when considered as a “detection and quantitation device” separate from the sample preparation itself.

Recommendations

The analyses conducted in the course of the current study strongly suggest that the PCB ELISA has great potential for use as a determinative step in PCB analysis, in particular, when coupled with an appropriate sample preparation procedure. To ascertain the performance of ELISA as a “detector system,” it will be important to remove the statistical ambiguity resulting when the data being generated by two detectors (GC/electron capture detector and ELISA) are actually the result of measurements on two distinct soil extracts. The two soil extracts are very likely different in their PCB concentrations, and thus, even in the best case scenario of 100% accuracy in the measurement step, the results will of course differ.

To remove the contribution of errors in soil extraction, it is suggested that further experiments be carried out in which samples are extracted and portions of the extracts are quantitated by both GC and ELISA. Any extract cleanup procedures should be carried out before splitting the extract. Alternatively, a study design could be structured such that analyses could be carried out on extracts which had been cleaned up as well as extracts which had not been subjected to additional clean-up steps, thereby allowing for checks on the possible effects of the cleanup procedure.

Further work could be carried out to allow unambiguous comparison between the PCB ELISA and GC/ECD as quantitation techniques. One possible scenario would entail re-extraction of the Kansas City and/or Allied Paper samples, followed by analysis using ELISA and GC (in-house and/or CLP laboratory) of splits derived from these extracts.

The results of this study suggest that ELISA has the capability of providing useful data for certain applications. The indirect inhibition format was used because, generally, it is one of the more sensitive formats which can be chosen from the myriad of ELISA formats. In addition, this format prevents exposure of the enzyme to potential interferants present in the original sample. The assay can be configured to allow even greater ease of use. For example, the equilibration times may be reduced allowing a one day assay without a substantial change in performance.

The plate ELISA format can be easily automated using any number of the readily available robotic plate ELISA instruments. This would permit screening of large numbers of samples, and in addition, it could allow for the carrying out of extensive parallelism studies on a routine basis. Extensive quality assurance of the ELISA data output could thus be ensured.

A note of caution is raised with respect to simple or abbreviated extraction procedures. Typically, most of the commercially available semi-quantitative ELISAs for soil screening rely upon “quick shake” extraction procedures, enabling extreme streamlining of the entire ELISA based procedure. The experiences noted in the current study reflect the possible dangers in assuming that these extractions always perform adequately.

The quantitative PCB plate ELISA can be used to measure PCBs with high accuracy and precision when coupled with appropriate sample preparation procedures. Further utilization should include coupling the quantitative PCB plate ELISA with efficient and potentially fieldable extraction methods, such as supercritical fluid extraction. Additionally, the quantitative PCB plate ELISA could be coupled with rapid Soxtec™ type extraction procedures, potentially in mobile laboratories, enabling rapid, relatively non labor-intensive measurement of PCBs. This would be an analytical scheme of high utility, and acceptance, as use of Soxtec type extraction procedures for PCBs already has precedence in such methods as EPA SW-846 Method 3541, Automated Soxhlet Extraction.

Interest in the application of the quantitative PCB plate ELISA has been generated within a number diverse groups, such as the EPA Great Lakes National Program Office and the Fish and Wildlife Service, National Fisheries Contaminant Research Center. In order to facilitate the application of the quantitative PCB plate ELISA, the ELISA procedure, coupled to a suitable extraction procedure should be subject to peer verification through such avenues as the Association of Official Analytical Chemists (AOAC) Peer-Verified Methods program.

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Mention of trade names or commercial products does not constitute endorsement of recommendation for use.

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The complete report, entitled "Development and Evaluation of a Quantitative Enzyme Linked Immunosorbent Assay (ELISA) for Polychlorinated Biphenyls," (Order No. PB95-100038/AS; Cost: $19.50; subject to change) will be available only from:

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