Immunoassay Screening Method for Polychlorinated Biphenyls (PCBs) in Insulating Oil (PCB sensor)

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Low level quantities of PCBs were mixed into insulating oil used in electrical machinery, mainly transformers, as recognized by the Japanese government in 2003, however production and use of PCBs has been prohibited since 1973. The necessity of testing approximately several million transformers for PCB contamination has stimulated urgent interest in development of measurement techniques. We report the performance of our developed rapid and simple immunoassay system for the detection of polychlorinated biphenyls (PCBs) in transformer oil.

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Introduction

Because polychlorinated biphenyls (PCBs) are nonflammable and possess insulating properties, they have been used in a variety of fields, from insulating oil for transformers and capacitors to heat transfer media. However, it has been revealed that PCBs are highly toxic due to several incidents including the Kanemi oil poisoning syndrome, caused by PCB-contaminated edible oil in 1968. Thus, the manufacture and import of PCBs were prohibited by the Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances (Chemical Substance Control Law) enacted in 1973. PCBs already in use were then stored away safely as waste materials. After the enactment of the law, PCB waste treatment was not sufficiently conducted for a long time. Under circumstances in which progress of environmental pollution due to the lost PCB waste became a serious concern, the Law Concerning Special Measures for Promoting Appropriate Treatment for Polychlorinated Biphenyl Waste (PCB Special Measures Law) came into force in June 2001 in order to address the PCB waste issue. The law stipulates completion of the detoxification treatment of stored PCBs by 2016.

PCBs are chemically stable and have low degradability. They also possess long distance mobility, and are highly accumulative in organisms and highly toxic to them. For this reason, PCBs have been designated as a persistent organic pollutant (POP) together with dioxins and nine chlorine-based agricultural chemicals that have similar properties to PCBs, such as DDT and chlordane. Furthermore, in order to prevent pollution from spreading globally, an international convention concerning usage regulations and disposal of POPs (the Stockholm Convention: POPs Convention) was adopted in May 2001. Japan ratified the POPs Convention in August 2002, promoting the PCB waste measures in a framework of international regulations, as well as their own regulations.

On the other hand, it was reported in 2003 that there was a possibility that insulating oil contained in certain heavy electric apparatuses currently in use may be contaminated with trace amounts of PCBs (low concentration PCB pollutants)[1]. It was estimated that the number of such heavy electric apparatuses reached several million[2]. In order to properly treat these apparatuses, there was a pressing need to clarify the PCB concentration of the insulating oil in the apparatuses and determine the presence of pollution. In other words, it became necessary to establish a fast and inexpensive measuring method to determine the presence of PCB pollution in an enormous number of oil samples. This need was not expected to arise when the initial PCB treatment plan was established under the PCB Special Measures Law enacted in 2001. In this report, I will describe conventional PCB analysis techniques using equipment and a new screening method for PCBs

using an immunoassay that our company has newly developed in order to meet the aforementioned requirements of society.

Method for Analyzing PCBs in Insulating Oil by Using Equipment

As shown in **Fig. 1**, PCB is a generic term for chemical substances in which chlorine has been substituted on a biphenyl backbone. As **Table 1** indicates, 10 types of homologue and 209 types of isomer (classified by sites of substitution and the number of substitutions) exist in theory. All these homologues and isomers are subject to the analysis. In general, gas chromatography (GC) is used for measuring PCBs. Isomers are separated as much as possible, and the peaks of the many isomers, shown in **Fig. 2**, are assayed and then totaled.

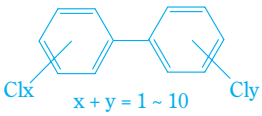

Fig. 1 The chemical formula of PCBs

Table 1 Name of homologue and number of isomer of PCBs

Number of chlorine	Name of homologue	Number of isomer
1	Mono chlorinated	3
2	Di chlorinated	12
3	Tri chlorinated	24
4	Tetra chlorinated	36
5	Penta chlorinated	42
6	Hexa chlorinated	36
7	Hepta chlorinated	24
8	Octa chlorinated	12
9	Nona chlorinated	3
10	Deca chlorinated	1
Total		209

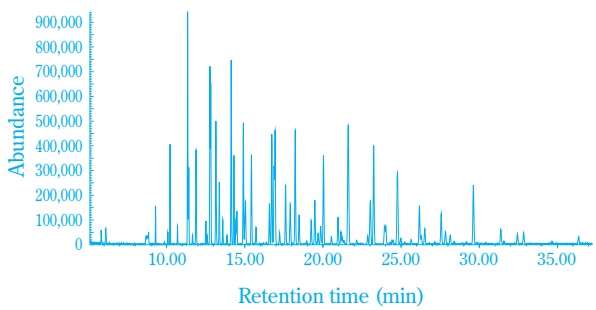

Fig. 2 Total ion chromatogram of PCBs (Measurement by GC-LRMS)

Thus, the analysis requires many procedures. Although various types of detectors for connection to a GC are available, and can be chosen according to the required accuracy, sensitivity and budget, electron capture detectors (ECD) which have high selectivity toward chemicals containing halogens have been most widely used. Mineral oils, whose primary components are aliphatic hydrocarbons, are mainly used for insulating oil. However, because it is difficult to separate and selectively detect PCBs only by using the physical properties of measuring apparatus, pretreatment the sample is required. **Fig. 3** depicts an example of the pretreatment flow of the official method for detoxified insulating oil. It is necessary to conduct complicated pretreatment that requires advanced skills for the following reasons: Aliphatic hydrocarbons are highly lipophilic, like PCBs, and mineral oils contain chemicals other than their primary components, albeit in trace amounts, which adversely affect the measurement.

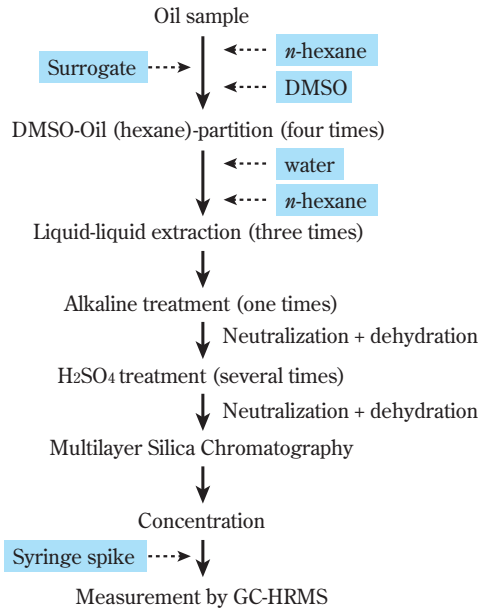

Fig. 3 PCBs analytical procedure (Official method for decomposed insulating oil)

Currently, there is no official method to measure PCBs in insulating oil before detoxification to determine the presence of low concentration PCB pollution in heavy electric apparatuses. However, official measuring methods and quasi-official measuring methods have been established for waste oil such as treated oil and wash oil derived from detoxification, as well as environmental samples such as water, sediments, air

and organisms. Over the last few years, various simplified analytical methods have been developed by improving such official methods[3]. It is considered a pre-requisite that the apparatus used for simplified analysis can detect PCBs at least up to 0.5mg/kg –which is the standard concentration in oil after detoxification– or lower when determining the presence of PCB pollution. Therefore, our company has been developing a GPC/GC-ECD method (lower detection limit: 0.1mg/kg) which uses gel permeation chromatography (GPC) for pretreatment, that can effectively separate PCBs from insulating oil, and a GC-LRMS method (lower detection limit: 0.05mg/kg) that takes advantage of the high selectivity of a low resolution mass spectrometer (LRMS)[4), 5)]. Although the procedures for simplified instrumental analyses are less complicated than the GC-HRMS method (lower detection limit: 0.05mg/kg) that uses a high resolution mass spectrometer (HRMS), which enables ultra high sensitive detection (this method has been adopted as the official method for detoxified insulating oil), all of these techniques require at least few days for pretreatment and the measurement itself, and their costs are relatively high. It is therefore impossible in realistic terms to measure very large numbers of samples that may contain PCBs on a daily basis. For this reason, we have developed a new analytical method, focusing on bioassays, which are fast and highly cost effective, although they are slightly less accurate in terms of measurement than instrumental analyses.

Immunoassay Screening Method for PCBs in Insulating Oil

1. Immunoassay

Bioassaying is a testing method that uses actual organisms, biochips such as receptors and antibodies, and genes. Its characteristic is that its procedures are generally simpler than those of instrumental analyses. The purpose (i.e. definition) of an assay is not to measure the quantity of specific chemical substances, but to determine the pass or failure of target chemicals to specific standards, or to determine their classification. The greatest feature of an assay is that it is used for screening for specific chemicals. Although bioassays have been used in clinical tests for a long time, they have recently begun gathering attention in the field of environmental analyses. For example, in 2005, four types of techniques were approved as official methods, including the Ah-receptor method, which is a simplified method for measuring dioxins developed by Sumitomo Chemical. In Europe, screening[6)] has been adopted as a detection method for dioxins contained in food. In the U.S.A. also, the Environmental Protection Agency (EPA) is currently testing a method using immunoassay for screening a variety of chemicals[7)]. In addition, over several hundred measuring kits for exogenous endocrine disrupting chemicals and agricultural chemicals are available in the market, although they were not used as official methods for the immunoassay that we adopted for our new development. Furthermore, for PCB measuring methods, several types of immunoassay have been reported[8)]. Standardization of these assays as the Japanese Industrial Standard's (JIS) general rules is currently being discussed.

Immunoassay is referred to as an immunochemical measurement method which uses an antigen-antibody reaction to measure antigen or antibody protein content. The antigen-antibody reaction occurs between the antigen and the antibody created in the organism's body against the antigen. It is an immune system through which antibodies recognize the antigen as an alien substance, and attempt to protect the organism from the alien substance (antigen) originating outside the organism's body, thereby causing detoxification or a biological reaction such as excretion. Through an immunoassay, the degree of such a reaction is measured, and the result of this measurement is then used to measure the quantity of the substance. Realistically, antibodies created by immunizing a laboratory animal (such as a mouse) are used, with the substance being measured, or one similar to this, used as an antigen. As **Fig. 4** shows, the antibody is a glycoprotein molecule that has a Y-shaped model structure. It recognizes the antigen in the top half near the tips of the V-shaped part, which are called variable domains. As the combi-

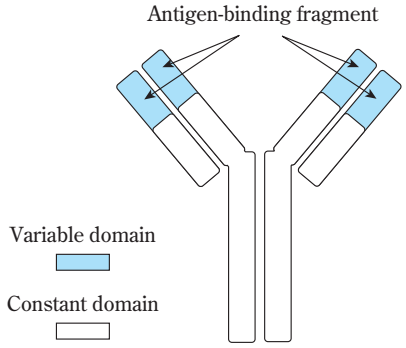

Fig. 4 Structure of antibody

nation of various amino acid sequences present in the variable domains changes, various types of antibodies that can bind to fight against a specific antigen can be created. Furthermore, antibodies can be classified into two types: polyclonal antibodies in which various types of antibody molecule are present, and monoclonal antibodies obtained from clones derived from a single antibody-forming cell. For immunoassays applied to chemicals having far lower molecular weights than proteins, such as PCBs, monoclonal antibodies that have high specificity are generally used.

The initial development of the immunoassay measurement principle goes back to the 1930s. Although it has a long history, the first time an immunoassay was put to practical use was towards the end of the 1950s, when the radio immunoassay, in which the antigen was labeled using a radioactive substance, was developed[9]. Since then, a variety of techniques have been developed using various methods such as a method of separating the protein being measured from those which are not, a labeling method for the antigen or antibody being measured, and a detection method for the labeled substances[10]. Today, non-radioactive immunoassays have become more popular due to exposure and disposal issues, and a batch type enzyme-linked immunosorbent assay (ELISA), which uses an enzymatic reaction as a labeled substance, is most widely used[9), 10)]. We adopted the KinExA system, which can measure chemicals with higher sensitivity than the ELISA through a flow-style measuring method that uses monoclonal antibodies. As we will not discuss details of the KinExA system in this report, please refer to other documents for more details[11)–13)].

As previously described, for the measurement of PCBs in an enormous number of insulating oil samples, it is most suitable to use the concept of screening in order to detect the pollutants. In this report, we will introduce a screening method for PCBs in insulating oil using the immunoassay (PCB sensor), which is most suitable for treating a large number of samples. Furthermore, this method has been designed to be able to screen PCBs in insulating oil before detoxification at 0.5mg/kg.

2. Measurement Principle of PCB Sensor

This method uses the antigen-antibody reaction that occurs between the anti-PCB antibodies and the PCBs (antigen) to detect the PCBs. **Fig. 5** depicts the detection principle. Firstly, antibodies and PCBs are mixed together to reach a state of binding equilibrium. As the antibody concentration is fixed, the rate of binding between the antibodies and PCBs depends on the PCB concentration. Next, the mixed solution is transferred to a detection cell coated with a thin membrane containing a PCB analog (pseudo-antigen). When the mixed solution passes through the detection cell, antibodies that are not bound to PCBs react with the pseudo-antigen. They are then captured on the membrane. If the solution contains a large amount of PCBs, the antibodies react with the PCBs contained in the solution, and do not react with the pseudo-antigen on the membrane as much. Therefore, antibodies bound to PCBs pass through the membrane, thus reducing the amount of antibodies captured on the membrane. As the antibodies accumulated on the membrane have already been labeled with a gold colloid (purple-red), the PCB concentration can be calculated by measuring the light absorbency of the membrane using an absorptiometer. More specifically, if the PCB concentration is low, the amount of antibodies captured on the membrane increases. This makes the membrane red, resulting in the membrane having a higher light absorbency. Conversely, if the PCB concentration is high, the membrane does not become colored at all, resulting in a lower light absorbency.

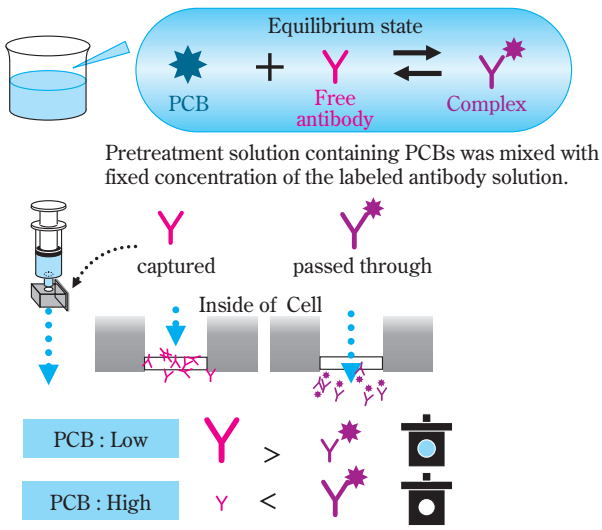

Fig. 5 Principle of measurement for immunoassay

3. Pretreatment Method

In general, when conducting an immunoassay, it is essential to eliminate foreign elements that may adversely affect the antigen-antibody reaction. Howev-

er, in order to validate its simplicity and rapidity as a screening method, it is crucial to simplify the procedures for extracting PCBs from insulating oil samples. In the course of the new development, we have established an extremely simple pretreatment method (and made it in the form of a kit), thus enabling a pretreatment which is both simple and rapid[14)–18)]. **Fig. 6** shows the simplified immunoassay pretreatment procedure. First, an oil sample is added to a multi-layer column containing a fuming sulfuric acid-impregnated silica gel, and dissolvent refining treatment is then conducted on assay-inhibiting substances. Next, the PCBs are eluted from the multi-layer column using hexane. After adding a fixed amount of dimethylsulfoxide (DMSO) to the eluate, the *n*-hexane is concentrated and then eliminated using a rotary evaporator in order to replace the solvent with DMSO. At this point, most of the insulating oil layer that has not been broken down is separated from the DMSO layer in the multi-layer column, and only the DMSO layer used for the immunoassay is extracted to complete the pretreatment. The length of time required for these pretreatment procedures is only about 15 minutes for a single sample. Since many samples can be simultaneously treated in the column processing stage, it is possible to treat more than 60 samples per day.

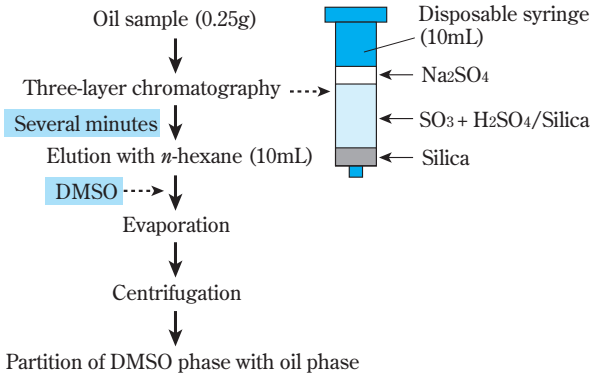

Fig. 6 Extraction and clean-up procedure for immunoassay

This method does not require any complex procedures. It also uses only general-purpose instruments, thus requiring no advanced skills. Moreover, it can be conducted in a limited space and it has excellent portability for the following reasons: All the instruments used for the method are of a portable size and weight, the method does not require a large number of samples, and it only requires small columns and flasks. In addition, a feature is that it only uses a minimal amount of organic solvents (*n*-hexane: approximately 10mL, DMSO: 0.25mL). In addition, the fuming sulfuric acid-impregnated silica gel is sealed in a glass tube as a kit, thus significantly improving the safety and analytical accuracy over methods that use liquid fuming sulfuric acid.

4. Assay Procedures

Fig. 7 shows a simplified measurement procedure. As with the pretreatment, we have developed a method that can be used easily and rapidly as a screening method through the use of a kit. Firstly, the DMSO extract (on which the aforementioned pretreatment has been performed) and the antibody solution are mixed. Then a binding equilibrium reaction is allowed to occur between the PCBs and antibody at room temperature for one hour. Next, this mixed solution is placed in a syringe and transferred to a detection cell having the antibody-capturing membrane by using a syringe pump at a constant flow rate (8.5mL/min). After washing the membrane with saline (biochemical buffer solution), the membrane is dried in order to remove moisture, which can affect light transmittance. Lastly, the light transmittance of the membrane is measured using a dedicated portable absorptiometer. Although the entire procedure requires approximately two hours, the actual man-hours required for the procedure, excluding the binding equilibrium reaction and membrane drying process, is only about 10 minutes. Furthermore, since many samples can be treated simultaneously, it is possible to measure more than 80 samples per day.

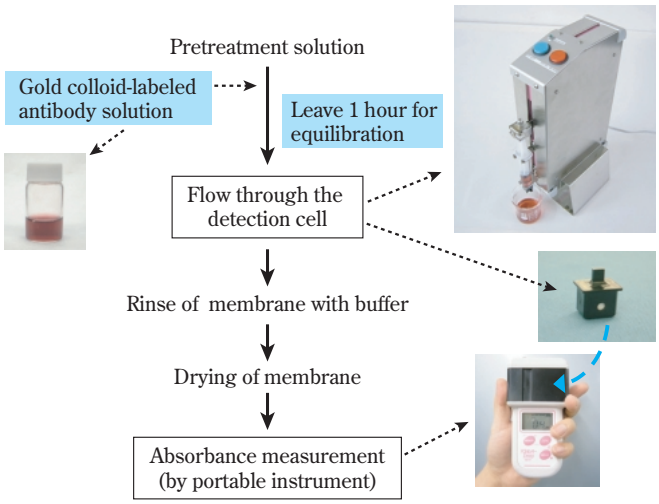

Fig. 7 Immunoassay procedure

As with the pretreatment, no advanced skills are required for this method. All the instruments used have high portability, and it is possible to perform the procedure in a limited space. In particular, as the portable absorptiometer is a hand-held type, it requires no complicated settings, thus providing excellent simplicity, which is an important attribute for the rapid analysis of multiple samples.

5. Assay Performance

(1) Cross Reactivity toward PCB Compositions

Insulating oil products manufactured in Japan that have been contaminated with PCBs contain one or more of the four types of PCB mixture (Kanechlor (KC)-300, 400, 500, 600)[1]. **Fig. 8** shows the PCB congener patterns in four PCB mixtures measured by GC-LRMS. Since all these PCB mixtures contain only trace amounts of mono-, di-, nona- and deca-chloro PCBs, the figure only shows six types of congener (tri- to octa-chloro PCBs). While KC-300's main component is trichloro PCBs, KC-600 is mainly composed of hexa-chloro PCBs. This means that the congener pattern differs depending on the mixture. Therefore, in order to measure each PCB mixture with the same accuracy, it is important to choose antibodies that are least affected by the congener patterns.

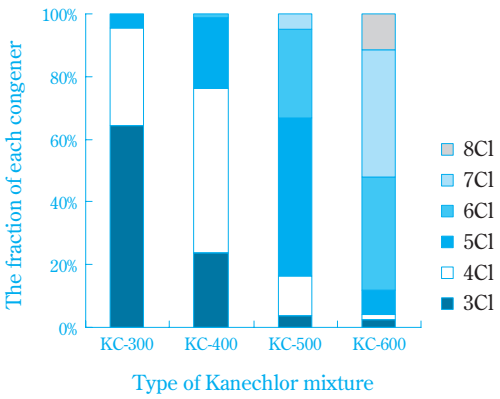

Fig. 8 PCB congener patterns in each Kanechlor

Fig. 9 shows calibration curves obtained after the pretreatment and measurement of several insulating oils containing different types of Kanechlor using the PCB sensor method. These curves almost overlap each other. It can be considered that this screening method is highly applicable for the following reasons: The effect of the differences between each type of Kanechlor (i.e. PCB congener patterns) on the measurement results is minimal for the antibody chosen, and the dependence on the compositional ratio of the Kanechlors contained in the insulating oil is low.

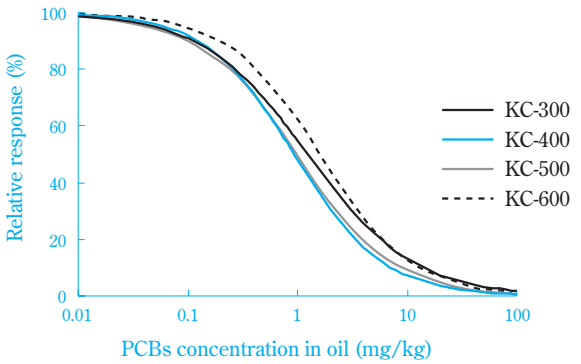

Fig. 9 Calibration curve in each Kanechlor

(2) Measurement Accuracy

In accordance with the general rules of a competing immunoassay[19] used for water samples, the limits of detection and determination of the entire measurement procedure, including pretreatment have, been confirmed. As a result, it has been verified that the lower detection limit is 0.2mg/kg, the lower limit of determination is 0.5mg/kg and the upper limit of determination is 3mg/kg. Furthermore, **Fig. 10** shows the results of comparison between the measured values obtained through the immunoassay and those obtained through GC-HRMS, which is considered to be the most accurate instrumental analytical method. The correlation between the immunoassay and the GC-HRMS method is expressed in the form of a linear line with an

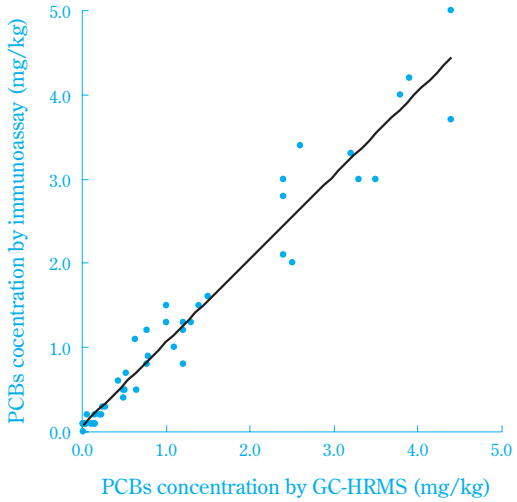

Fig. 10 Correlation between immunoassay and GC-HRMS in used oil (50 samples)

incline of 0.99 and a y-intercept of 0.069. It also shows an excellent coefficient of determination (r^2) of 0.96. As a result, it can be concluded that the immunoassay has sufficient accuracy as a screening method for determining PCB concentration in insulating oil at the lower limit of 0.5mg/kg.

6. Screening Test

(1) Overview of Screening Test[20), 21)]

Fig. 11 shows an overview of the screening test. A screening test is a quick method for assaying the quantity of a target substance contained in a sample to verify whether or not it exceeds a regulatory concentration. For assaying, the screening concentration (cut-off concentration) is determined as the regulatory concentration of the substance being measured. A value that exceeds the cut-off concentration is considered to be positive, and one that does not is considered to be negative. In general, in order to reduce the risk of misjudgment (i.e. a positive sample being determined as negative), the cut-off concentration is often set lower than the regulatory concentration. In other words, conducting screening with a cut-off concentration that has been multiplied by a safety factor increases the probability of detecting a sample that may contain the target substance at a concentration which exceeds the regulatory concentration. Uses of screening in blood tests for health examinations and in examinations for mad cow disease (BSE) are well known examples. It is effective to use screening as a simplified measurement method to detect positive samples having a low possibility of existence from an enormous number of samples, as it is practically impossible to conduct precise assays on such a large number of samples due to the time and cost involved. The concept of screening was also suitable for the assaying we conducted on this occasion to detect trace amounts of PCB contamination in insulating oils. Screening tests are often combined with more precise analytical methods in order to further confirm the results of the screening once the sample has been determined as positive (higher than the cut-off concentration). At this stage, because it is mandatory to report concentration when managing PCB-contaminated insulating oils whose concentration exceeds the regulatory level, it is ideal to screen the target substance by immunoassay first, then assay those samples determined as positive using a previously mentioned conventional instrumental analytical method.

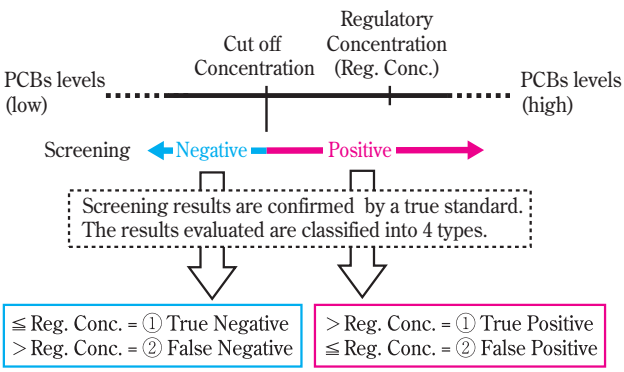

Fig. 11 Definition for screening criteria

(2) Examples of Screening Tests

We have evaluated the performance of screening using the PCB sensor method by conducting the screening on 274 used oil samples. For this evaluation, the PCB concentrations measured by the GPC/GC-ECD method[4)] are considered as the true values, and are then compared to the measurement values obtained from the immunoassay screening. **Fig. 12** shows comparisons of the PCB concentration distributions between the immunoassay screening and GPC/GC-ECD methods. In both methods, samples containing PCBs at less than 0.2mg/kg account for more than 50% of the total, showing a similar distribution in which samples having a higher concentration account for a lower proportion of the samples. In general, by taking values measured by a more precise method as true values, the performance of the screening can be evaluated by the probability of the occurrence of false-negatives, in which samples determined as being negative by the screening turn out to be positive according to the true values due to exceeding the

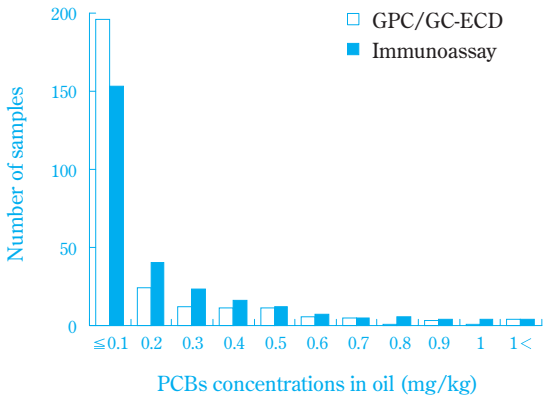

Fig. 12 Comparison of sample distributions between immunoassay screening and GPC/GC-ECD (274 samples)

regulatory concentration, and false-positives, in which samples determined as positive by the screening turn out to be negative according to the true values. In our evaluation, we have set the regulatory concentration at 0.5mg/kg and calculated the probability of the occurrence of false-negatives and false-positives for various cut-off concentrations. **Table 2** shows the evaluation results. In the GPC/GC-ECD method, the number of samples that exceeded the regulatory concentration (true positives) was 20 and the number of samples that were lower than the regulatory concentration (true negatives) was 254.

Table 2 False positive and false negative at various cut off concentrations (Regulatory concentration : 0.5mg/kg)

Number (rate)	0.5 mg/kg	0.4 mg/kg	0.3 mg/kg	0.2 mg/kg
False positive	12 (9.1%)	23 (15%)	39 (24%)	61 (40%)
False negative	1 (5%)	1 (5%)	0 (0%)	0 (0%)

If the cut-off concentration is multiplied by the safety factor and set to below 0.3mg/kg, false-negatives do not occur at all, thus providing perfect accuracy. If the cut-off concentration is set to 0.3mg/kg, 39 samples turn out to be false-positives (false-positive rate: 24%, percentage of false-positives relative to all the samples: 14%). However, considering the cost and time required for more precise method conducted on large number of samples, it is definitely worth using a screening method. On the other hand, if the cut-off concentration is set to 0.4 – 0.5mg/kg, one sample turns out to be a false-negative (false-negative rate: 5%, percentage of false-negatives relative to all the samples: 0.4%). However, if the cut-off concentration is set to 0.4mg/kg, the number of false-positives decreases to 23 samples (false-positive rate: 15%, percentage of false-positives relative to all the samples: 8%), thus improving the cost effectiveness. From these results of our evaluation using 274 samples, it has been confirmed that the method shows excellent performance in many ways, including cost effectiveness when the cut-off concentration is set to 0.3 – 0.5mg/kg. In addition, the false-negative rate and false-positive rate can be greatly affected not only by the accuracy of the measurement method, but also by the concentration distributions of the samples, in particular, by the percentage of samples near the regulatory concentration. Therefore, when applying the screening method, if the population subject to the measurement can be obtained prior to the screening, more efficient screening can be conducted by setting a cut-off concentration which takes economic benefits into account.

Furthermore, using standard deviation and the Gaussian function of the measurement method when analyzing a sample having an arbitrary concentration "n" times enables us to statistically estimate the false-positive rate and false-negative rate of the arbitrary population at each cut-off concentration[22].

Conclusion

The PCB sensor method that uses the concept of screening is an extremely effective, rapid and inexpensive simplified method that has never existed before for the determination of the presence of pollution by trace amounts of PCBs in a large volume of samples. We hope that the scheme, in which more precise instrumental analysis is separately conducted on the samples determined as positive by the screening, will become further recognized and widely adopted in the world.

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