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**Water quality — Detection of selected congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls — Method using a flow immunosensor technique**

*Qualité de l'eau — Détection d'une sélection de congénères de dibenzo-*p*-dioxines polychlorées et de biphényles polychlorés — Méthode utilisant la technique d'immunodétection en flux*

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Published in Switzerland

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## Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## Introduction

Persistent organic pollutants (POPs) including dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (DL-PCBs) in water and wastewater are analysed by instrumental methods such as gas chromatography/mass spectrometry (GC-MS) and high-resolution gas chromatography/ high-resolution mass spectrometry (HRGC/HRMS). These methods are accurate and precise, but labour-intensive, time-consuming, and costly. Alternatively, immunoassays are used for monitoring of POPs. These methods have similar sensitivity and selectivity, but are more cost-effective and able to manage large loads of sample well. The use of immunoassays is suitable for timely detection of selected congeners of PCDDs and PCBs in water and wastewater in advance of subsequent confirmatory methods.

Recently, automated immunoassay methods including a flow immunosensor have been developed for detection of POPs in environmental samples. These methods reduced manual operations such as pipetting, time-consumption and coefficient of variation. Therefore, practical use of these methods can play an important role in timely, continuous cost-saving monitoring of water and wastewater in both developed and developing countries. This method can be applicable to timely continuous monitoring of selected congeners of dibenzo-*p*-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) in water and wastewater to prioritize those for subsequent confirmatory determination.

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# Water quality — Detection of selected congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls — Method using a flow immunosensor technique

**WARNING** — The PCDDs, PCDFs and PCBs are among the most hazardous chemicals. Therefore, all work with PCDDs, PCDFs and PCBs require the utmost care; the national safety measures which correspond to those for hazardous substances shall be strictly adhered to. The responsibility of the user is to establish appropriate safety and health practices.

**IMPORTANT** — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

## 1 Scope

This document specifies methods and principles for detection of selected congeners of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) in water and wastewater using a flow immunosensor. The flow immunosensor utilizes antibodies specific to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) and 3,3',4,4',5-pentachlorobiphenyl (3,3',4,4',5-PeCB), which have the highest toxic equivalent factor (TEF) value among the congeners of each of PCDDs and PCBs. The method is applicable to timely monitoring of selected congeners of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB in water and wastewater to prioritize those for subsequent confirmatory determination.

This document specifies practical methods and procedures for sampling, extraction, clean-up, measurement in a flow immunosensor, data processing and validation of measurement results. The combined use of automated instruments for extraction, clean-up, and flow immunosensing can reduce time-consumption and labour-intensity, while providing reproducible precise data. This method can provide the lower limit of quantification (LOQ) for 2,3,7,8-TCDD and 3,3',4,4',5-PeCB of 28 pg/l and 152 pg/l, respectively at 20 % or less of coefficient variation (CV) depending on sampling, extraction, clean-up and measurement conditions.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

ISO 5667-3, *Water quality — Sampling — Part 3: Preservation and handling of water samples*

ISO 5667-6, *Water quality — Sampling — Part 6: Guidance on sampling of rivers and streams*

ISO 5667-10, *Water quality — Sampling — Part 10: Guidance on sampling of waste water*

## 3 Terms, definitions, and abbreviated terms

### 3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

### 3.1.1

#### **analyte**

selected congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls which bind to specific monoclonal *antibodies* (3.1.2)

Note 1 to entry: [Annex A](#) gives information of the specificity of mouse monoclonal antibodies used for the flow immunosensor.

### 3.1.2

#### **antibody**

class of serum proteins that are induced by exposing to an immunogen and will bind specifically to *antigen/analyte* (3.1.3)/(3.1.1) forming an antibody-antigen complex

Note 1 to entry: An antibody with a fluorescent label is used as a secondary antibody for binding to an antibody specific to antigen/analyte.

### 3.1.3

#### **antigen**

molecule which selectively binds to an *antibody* (3.1.2)

Note 1 to entry: Antigens are analytes in the case of selected congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls.

### 3.1.4

#### **conjugate**

large molecule covalently coupled with a small molecule

Note 1 to entry: An antigen-mimic BSA conjugate is used for coating polymer beads.

### 3.1.5

#### **cross-reactivity**

ability of an *antibody* (3.1.2) to bind to certain molecules which structurally related to an *antigen* (3.1.3)

### 3.1.6

#### **flocculation**

physical process of contact and adhesions wherein the aggregates form larger-size clusters called flocs being excluded from suspension for water treatment

### 3.1.7

#### **flow immunosensor**

immunosensor based on a *kinetic exclusion assay* (3.1.11) using a monoclonal *antibody* (3.1.2) in a heterogeneous assay system for rapid detection of an *analyte* (3.1.1) in a number of samples

Note 1 to entry: The system of a flow immunosensor is operated automatically according to a program.

### 3.1.8

#### **IgG**

#### **immunoglobulin-G**

antibody molecule, consisting of two identical heavy(H)-chains and two identical light(L)-chains held together with disulfide bonds

Note 1 to entry: Both H- and L-chains consist of the variable and constant regions. An antigen binding site is presented in the variable region.



### 3.1.9 immunoassay

immunochemical detection procedure based on specific *antibody-antigen* (3.1.2)-(3.1.3) binding theory often using a tracer for the detection of a free or bound antibody

Note 1 to entry: In a heterogeneous immunoassay, one of immunoreagents is immobilized on a solid support and requires a washing step to separate the bound and free immunoreagents.

### 3.1.10 inhibition concentration

IC

$C_{i50}$

analyte concentration which reduces the measuring signal of the *zero standard* (3.1.14), in the case of 50 % inhibition of the zero standard that expresses 50 values

### 3.1.11 kinetic exclusion assay

binding between *antibody* (3.1.2) and *antigen* (3.1.3) that reaches equilibrium in solution, and then the three species such as the specific antibody, the antigen and the antibody-antigen complex that are present

Note 1 to entry: A kinetic exclusion assay measures the concentration of the free antibody without perturbing the equilibrium. The format of the assay is to measure a free specific antibody with a label.

### 3.1.12 monoclonal antibody

*antibody* (3.1.2) population, possessing identical selectivity and affinity produced by a single antibody-producing cell line

Note 1 to entry: Monoclonal antibodies specific to selected congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated biphenyls are prepared for measurement of these analytes.

### 3.1.13 procedure blank

solution prepared in the laboratory, using reagent water or other blank matrix

### 3.1.14 zero standard

analyte-free standard (blank) which is used for calibration

## 3.2 Abbreviated terms

BSA	bovine serum albumin
CV	coefficient of variation
DMSO	dimethyl sulfoxide
GC-MS	gas chromatography/mass spectrometry
HpCB	heptachlorobiphenyl
HpCDD	heptachlorodibenzo- <i>p</i> -dioxin
HpCDF	heptachlorodibenzofuran
HRGC	high-resolution gas chromatography
HRMS	high-resolution mass spectrometry

HxCB	hexachlorobiphenyl
HxCDD	hexachlorodibenzo- <i>p</i> -dioxin
HxCDF	hexachlorodibenzofuran
IgG	immunoglobulin G
LED	light emitting diode
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
OCDD	octachlorodibenzo- <i>p</i> -dioxin
OCDF	octachlorodibenzofuran
PBS	phosphate-buffered saline
PCDD	polychlorinated dibenzo- <i>p</i> -dioxin
PCDF	polychlorinated dibenzofuran
PCB	polychlorinated biphenyl
PeCB	pentachlorobiphenyl
PeCDD	pentachlorodibenzo- <i>p</i> -dioxin
PeCDF	pentachlorodibenzofuran
POP	persistent organic pollutant
PTFE	polytetrafluoroethylene
SD	standard deviation
SPE	solid phase extraction
TCB	tetrachlorobiphenyl
TCDD	tetrachlorodibenzo- <i>p</i> -dioxin
TCDF	tetrachlorodibenzofuran
TEF	toxic equivalent factor

## 4 Principle

### 4.1 Flow immunosensor

A flow immunosensor based on kinetic exclusion assay principles consists of a flow cell containing a capture reagent immobilized on a solid phase matrix such as azlactone, sepharose, polymethyl methacrylate, polystyrene, or the like, along with an antibody specific for the analyte and a fluorescent label used for detection. In many cases, the fluorescent label is attached to a second anti-species antibody that recognizes and binds to the analyte specific antibody. In practice, the capture reagent used on the solid phase is frequently either the analyte itself, an analogue of the analyte or a protein

conjugate of either the analyte or an analogue. To perform the assay, a suitable sample (which can be extracted and/or concentrated from a large environmental sample) is mixed with the specific antibody and the secondary antibody (C.2.6) with a fluorescent label, and then incubated for a period of time to allow binding to occur. After incubation, the antibody mixture is flowed over the solid phase matrix and free (not bound to analyte) antibody bound to the secondary antibody with a fluorescent label from the solution binds to the solid phase capture reagent. A fluorescent signal is measured from the captured antibody. The largest fluorescent signal occurs when there is zero analyte present and the presence of analyte results in reduced signal levels<sup>[1][3]</sup>.

## 4.2 Specific antibody

Specific antibodies (IgG) are selected for measurement of analytes in a flow immunosensor. Monoclonal anti-2,3,7,8-TCDD antibody and anti-3,3',4,4',5-PeCB antibody show the highest affinity to 2,3,7,8-TCDD and 3,3',4,4',5-PeCB, respectively as shown in [Annex A](#). Each of these congeners is known to be present in the environment and has the highest TEF value among the congeners of each of PCDDs and PCBs. Thus, both antibodies are used as the representatives for detection of selected congeners of PCDDs and PCBs in water and wastewater using a flow immunosensor. When antibodies specific for other congeners or other related compounds (e.g. PCDFs) are available, the application of the immunosensor described in [4.1](#) are readily expanded for detection of those related compounds or congeners.

## 5 Interferences

PCDDs, PCBs and other dioxin-like compounds are hydrophobic and largely present in water and wastewater as adsorbed on solid particles. When these compounds are spiked into water samples in containers, these compounds are quickly adsorbed on the surface of containers. The use of either inappropriate sampling devices or a sampling flask, or both, can influence the test result because of the possible adsorption of these compounds leading to false-negative results. On the other hand, these compounds can be released into the sample from sampling flasks, especially when wares used are contaminated with these compounds, and false-positive results can be generated. If filtered samples are tested in order to remove the sample solid particles, the dioxins and dioxin-like compounds which are adsorbed on particles cannot be detected.

Measurement conditions, for instance pH or sample components such as hormic acids, salinity and solvents influencing the detection which so-called matrix effects can interfere with antibody binding. The interference of matrix effects shall be assessed by spiking samples or reference samples with known amounts of the analyte. In any case, extraction and clean-up are necessary to avoid interference. In addition, air bubbles in measuring solutions and in a flow cell interfere not only antibody binding but also fluorescence detection. Therefore, care shall be taken when preparing measuring solutions, particularly by stirring gently at a room temperature.

Monoclonal antibodies specific to antigen/analyte are used for a flow immunosensor as shown in [Annex A](#). These antibodies cross-react against structurally related compounds and also bind to non-specific compounds to show matrix effects. The extracted samples are each submitted to clean-up process in an automate sample clean-up and concentration device as shown in [Annex B](#), in which a multiphase silica gel column works to eliminate polynuclear aromatic hydrocarbons and sulfur compounds. In addition, the clean-up samples are also submitted to HRGC/HRMS analysis to confirm the removal of matrices.

## 6 Reagents

Use reagents with a high purity for instance the grade “for residue analysis”. Information on type and origin of antibodies as well as the cross-reactivity are stated in [Annex A](#). Information on storage and stability of the used reagents shall be requested according to the information of the supplier. If sensitivity in a flow immunosensor is lower than expected, analyte-interfering substances such as matrices and air bubbles shall be removed from samples and measuring solutions by suitable procedures.

**6.1 Water**, complying with grade 3 as defined in ISO 3696.

For instance, a Milli-Q<sup>1)</sup> water is recommended for the preparation of a buffer and a regeneration solution.

**6.2 Sodium chloride**, NaCl, >995 g/kg purity.

**6.3 Potassium chloride**, KCl, >990 g/kg purity.

**6.4 Potassium dihydrogen phosphate**,  $\text{KH}_2\text{PO}_4$ , >995 g/kg purity.

**6.5 Sodium hydroxide**, NaOH >970 g/kg purity.

**6.6 Disodium hydrogen phosphate dodecahydrate**,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , >980 g/kg purity.

### 6.7 Organic solvents

Solvents used for spiking, extraction, clean-up and measurement in the following:

**6.7.1 Propanone** (acetone), >995 ml/l purity.

**6.7.2 Toluene**, >990 ml/l purity.

**6.7.3 Hexane**, >960 ml/l purity.

**6.7.4 Dimethyl sulfoxide** (DMSO), >995 ml/l purity.

**6.7.5 Ethanol**, >995 ml/l purity.

### 6.8 Standard solution.

The standard acetone solutions of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB are each diluted with acetone for spiking into a 3 l or more water sample and for confirmation of spiking concentration by HRGC/HRMS analysis. For preparation of sample solutions, a part of each acetone solution of both standard chemicals is diluted with DMSO.

### 6.9 Flocculant.

A flocculant consisting of activated charcoal powder, poly aluminium chloride, silica gel and sodium carbonate is added into a water sample adjusted to pH 7 to pH 8 at a rate of 0,1 g/l. Then, flocs formed are collected by filtration with a 0,45  $\mu\text{m}$  of pore size of a glass fibre filter and then air-dried overnight for extraction<sup>[4]</sup>.

### 6.10 Phosphate-buffered saline.

Prepare 50 mmol/l of PBS at pH 7,4 by dissolving 2,9 g of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0,2 g of  $\text{KH}_2\text{PO}_4$ , 8,0 g of NaCl and 0,2 g of KCl in 1 000 ml of water (6.1).

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1) Milli-Q is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

**6.11 Sample buffer.**

Used for sample preparation, with PBS (6.10) containing 1 g/l of BSA and 0,2 g/l of  $\text{NaN}_3$ , passed through a 0,45  $\mu\text{m}$  of pore size of a polyvinylidene difluoride filter. The buffer prepared in a glass bottle is kept in a refrigerator.

**6.12 Measuring buffer.**

PBS containing 1 g/l of BSA, 0,2 g/l of  $\text{NaN}_3$  and 55 g/l of DMSO, passed through a 0,45  $\mu\text{m}$  polyvinylidene difluoride filter. The buffer in a glass bottle is kept in a refrigerator.

**6.13 Correcting solution.**

Prepare 0,6 ng/ml of 2,3,7,8-TCDD in DMSO and 1,6 ng/ml of 3,3',4,4',5-PeCB in DMSO by the dilution of each of the standard chemicals in DMSO. The solution prepared in a glass bottle is kept in a refrigerator.

An antigen-mimic, 3-[6-(2,4,5-trichlorophenoxy) hexanoylamino] propionic acid, is also used as a reference at a concentration of 9,5 ng/l in DMSO.

**6.14 Regeneration solution.**

Prepare 1 g/l of NaOH (6.4) and 55 g/l of DMSO in water passed through a 0,45  $\mu\text{m}$  polyvinylidene difluoride filter. The solution prepared in a glass bottle is kept in refrigerator.

**6.15 Cleaning solution,** prepare 20 ml/l ethanol (volume fraction) in a glass bottle.

**6.16 Antibody solution.**

Antibody solution is prepared to mix with each antibody which is bound specifically to 2,3,7,8-TCDD or 3,3',4,4',5-PeCB and the second with a fluorescent label as a secondary antibody (C.2.6) in sample buffer (6.11). A fluorescent label should have a high intensity of emission and absorption at a certain wavelength which can be detected by a photodetector of an immunosensor.

Anti-2,3,7,8-TCDD antibody solution (D48 kit<sup>2)</sup>) contains 0,12  $\mu\text{g}/\text{ml}$  IgG of anti-2,3,7,8-TCDD antibody and 0,06  $\mu\text{g}/\text{ml}$  of secondary antibody in sample buffer (6.11). Anti-3,3',4,4',5-PeCB antibody solution (P126 kit<sup>3)</sup>) contains 0,10  $\mu\text{g}/\text{ml}$  IgG/ml of anti-3,3',4,4',5-PeCB antibody and 0,05  $\mu\text{g}/\text{ml}$  of secondary antibody (C.2.6) in sample buffer. D48 kit and P126 kit are each used for the detection of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB, respectively. Both solutions are in glass bottles that are covered with an aluminium foil for protection against light and kept in a refrigerator.

**7 Apparatus and materials**

All water sample containers are rinsed with acetone, toluene and hexane in turn, and dried prior to use. Apparatus used for extraction, clean up and concentration are referred to in ISO 18073<sup>[5]</sup> and ISO 17858<sup>[6]</sup>.

**7.1 Flow immunosensor (4.1).**

**7.2 Vial,** add a mixed antibody solution (6.16) and either samples or correcting solution (6.13), and then mix gently.

2) D48 kit is the trade name a product supplied by Seeds Tec Co., Ltd. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3) P126 kit is the trade name of a product supplied by Seeds Tec Co., Ltd. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.