
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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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).

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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.

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^{[1][3]}.

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 horemic 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-Q¹⁾ 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, KH_2PO_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 µm of pore size of a glass fibre filter and then air-dried overnight for extraction²⁾.

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 KH_2PO_4 , 8,0 g of NaCl and 0,2 g of KCl in 1 000 ml of water (6.1).

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 NaN_3 , passed through a 0,45 μm 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 NaN_3 and 55 g/l of DMSO, passed through a 0,45 μm 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 μm 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 kit²⁾) contains 0,12 $\mu\text{g/ml}$ IgG of anti-2,3,7,8-TCDD antibody and 0,06 $\mu\text{g/ml}$ of secondary antibody in sample buffer (6.11). Anti-3,3',4,4',5-PeCB antibody solution (P126 kit³⁾) contains 0,10 $\mu\text{g/ml}$ IgG of anti-3,3',4,4',5-PeCB antibody and 0,05 $\mu\text{g/ml}$ 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^[5] and ISO 17858^[6].

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.

7.3 Flow cell, stuffed with polymer beads coated with the antigen-mimic BSA conjugate (C.2.7) in an acrylic column.

There are two kinds of flow cell, a disassembly type and assembly types. A disassembly type flow cell is supplied pre-filled with polymer beads coated with the antigen-mimic BSA conjugate (C.2.7) and the entire flow cell is removed and replaced after several measurements. For assembly types, the flow cell remains in service much longer and the beads are automatically filled for each measurement.

8 Sampling

8.1 General

8.1.1 General

This document describes specific requirements for the sampling and samples with respect to the determination of dioxins or dioxin-like compounds in water samples. These compounds are hydrophobic and are present in water and wastewater as adsorbed on solid particles as well as the surface of wares used. Thus, collection, extraction and clean-up procedures suitable for these compounds are requested. For general information about sampling and samples, ISO 5667-1, ISO 5667-3, ISO 5667-6 and ISO 5667-10 shall be considered.

8.1.2 Bottles and material for sampling

Use clean glass bottles preferably with PTFE-lined (or acrylic-lined) caps. To avoid photodegradation of compounds of interest, preferably use amber glass bottles. If transparent glass bottles are used, wrap the bottles in aluminium foil or store them in a dark container.

8.1.3 Bottles and material pre-cleaning

After the routine cleaning procedure, additionally clean bottles and bottles and caps as follows: rinse the clean bottles and the caps three times with a minimum amount of acetone. Let the residual acetone evaporate (e.g. drying oven). Close the bottles immediately after drying. Rinse all glassware, spatulas etc. getting in contact with the sample three times with a minimum amount of acetone. Let the residual acetone evaporate.

8.1.4 Sampling

Use a 10 l bucket made from stainless steel for collection of water samples including drinking waters and river waters. A 3 l or more water sample is added into a 3,8 l of amber bottle and adjusted to pH 7 to pH 8 with Na_2CO_3 . For river waters, surface water is collected at a central area of a stream by the use of a 10 l of stainless steel bucket. A 3 l or more river water is added into a 3,8 l amber bottle, adjusted to pH 7 to pH 8 with Na_2CO_3 , and stored in a cold and dark room at 4 °C to 8 °C.

8.2 Preparation of sample

8.2.1 Extraction

For measurement of a pg/l LOQ level of dioxins and dioxin-like compounds in water samples, a 3 l or more water sample is submitted to extraction with suitable solvents such as dichloromethane and toluene, LLE, SPE or flocculation^[4] and Soxhlet extraction.

For the purpose of efficient collection of extremely low concentrations of dioxins and dioxin-like compounds in a large volume of water samples, a flocculant (6.9) should be used. A 300 mg of flocculant in 3 ml of a hexane-rinsed water is added to the water sample in a 3,8 l amber bottle (8.1.3) adjusted pH 7 to pH 8, stirred gently, allowed to stand for more than 50 min, agitated for a few seconds and then allowed to stand for more than 10 min. Thereafter, the water sample is filtered through 0,45 µm of pore size of a glass fibre filter set to Buchner funnel to collect flocs. Then, the flocs on the filter are air-dried

overnight in a draft. On the other hand, the amber bottle is rinsed three times with 5 ml of toluene. Then, both dried filter and rinsed toluene dehydrated with 30 g of Na_2SO_4 are extracted for instance in a high speed and high pressure extractor (SPE), which is an automatically operated device. The dried sample is added into a 40 ml volume sample vessel and then heated at 120 °C under 10^5 Pa for 60 s. A 120 ml of toluene is flashed through the sample vessel for 60 s and then N_2 gas is flashed. Stand it for 300 s and extract it for 240 s. This extraction cycle shall be repeated for three times.

Instead of SPE, Soxhlet extraction with 300 ml of toluene is carried out for both dried filter and rinsed toluene for more than 16 h according to ISO 18073^[5] or ISO 17858^[6].

8.2.2 Clean-up

The extracted samples are submitted to clean-up process by chromatography using a multilayer silica gel column and an active carbon or alumina column. An automate sample clean-up device is shown in [Annex B](#).

After extraction in flocculation and SPE or a Soxhlet extractor, a crude sample extract is cleaned up to remove interfering substances according to ISO 18073^[5] and ISO 17858^[6]. An automate sample clean-up device can be used to eliminate interferences of matrices instead of SPE and a Soxhlet. It consists of a multilayer silica gel column for purification, and an alumina column for concentration and solvent substitution. Both silica gel and alumina columns are each heated at 60 °C for 10 min and 90 °C for 10 min, respectively, for efficiency improvement.^[7] For more details, see [Annex B](#).

8.2.3 Sample solutions

The clean-up sample solutions are each diluted with DMSO for measuring under optimal conditions in a flow immunosensor. It shall be repeated for diluted sample solutions to measure at the range in which the response is proportional to the concentration, when measured values are out of the range of a calibration curve as shown in [Figure 1](#) (see [10.2](#)).

9 Procedure

9.1 General

All measurement procedures are carried out in an air-conditioned room at (24 ± 2) °C, since antibody antigen reaction and related antibody binding are dependent on temperature. All samples, reagents and a flow cell packed in a vacuum are stored in a refrigerator at 4 °C to 8 °C. Measurement conditions of standard chemicals and samples are $n = 3$ and one time.

9.2 Sample set up

A flow immunosensor based on the kinetic exclusion assay is used for measurement of an analyte in samples by the use of a monoclonal antibody specific to an analyte in a heterogeneous system. The measuring solutions set up five vials of a 10 ml volume at least for three times measurements. Each vial contains the measuring solutions as shown in [Table 1](#).

Table 1 — Example of an amount of each measuring solution in vials in the flow immunosensor model DXS-610⁴⁾

Measuring solution	Contents in				
	vial 1, b_0 (blank)	vial 2, port 1 (correction)	vial 3, port 2 (sample 1)	vial 4, port 3 (sample 2)	vial 5, port 4 (sample 3)
DMSO (6.7.4)	300 µl	No addition	60 µl	60 µl	60 µl
Correcting solution (6.13)	No addition	150 µl	No addition	No addition	No addition
Sample solution (8.2.3)	No addition	No addition	90 µl (sample 1)	90 µl (sample 2)	90 µl (sample 3)
Sample buffer (6.11)	4 200 µl	2 100 µl	2 100 µl	2 100 µl	2 100 µl
Antibody solution (6.16)	1 500 µl	750 µl	750 µl	750 µl	750 µl

The rate of 90 µl of a sample solution and 60 µl of DMSO shall be changed arbitrarily within a total of 150 µl for measuring among quantitative ranges. Since a total amount of measuring solution varies depending on a flow immunosensor, the instruction of apparatus maker should be followed. The correcting solution is used to correct the measured value of samples to correct influences due to temperature change, variety of reagent lot, etc. Therefore, the sample solution shall be diluted for measurement at the range between 20 % and 85 % of the maximum response to be shown in Figure 1 (10.2).

9.3 Measuring procedure

The measurement in the flow immunosensor is processed as described as follows (an example of a flow immunosensor is shown in Annex C).

- Prior to use, sample solutions, sample buffer (6.11), correcting solution (6.13), antibody solutions (e.g. D48 kit and P126 kit) (6.16), measuring buffer (6.12), regeneration solution (6.14) and cleaning solution (6.15) are taken out of a refrigerator and brought to room temperature.
- A bottle for waste buffer, a bottle for effluent solutions, a bottle of measuring buffer, a bottle of regeneration solution, a bottle of rinsing water and a bottle of cleaning solution are set up to applied positions of the flow immunosensor.
- Sample solutions, sample buffer, correcting solution, antibody solutions and DMSO are used for preparation of the corresponding measuring solutions in five vials as described in Table 1.
- Then, five vials containing each of the corresponding measuring solutions are set up to applied positions of the instrument.
- Input information of measuring solutions and measuring conditions in the programme operated as normally used.

For using a disassembly type flow cell, when a new flow cell is used, a flow cell package is taken out of a refrigerator, brought to room temperature, taken off the seal and then set up to an applied position of the instrument. Then, the flow cell is flipped to remove air bubbles. The assembly type flow cell which can re-stuff beads (C.2.8) automatically is available.

- For aging of the flow cell, 0,2 ml of the measuring solution in vial 1 for blank is sent to the flow cell at a rate of 0,75 ml/min. This step is repeated twice.
- For measuring blank (B_0), 0,2 ml of the measuring solution in vial 1 for blank, 0,36 ml of measuring buffer for rinsing, 0,36 ml of regeneration solution and then 0,36 ml of measuring buffer are in turn sent to the flow cell at a rate of 0,75 ml/min. When the assembly type flow cell is used, rinsing the

4) Model DXS-610 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.

flow cell with regeneration solution is not necessary. The stuffed beads in the flow cell are thrown away after each measurement and new beads are filled automatically. The 1,18 ml of measuring buffer instead of 0,36 ml of measuring buffer is in turn sent to the flow cell. During these steps, the fluorescence detector records the fluorescence intensity change with time for 3 min (C.2.3). This process is repeated twice. When two recorded peaks are similar, second peak is accepted for blank (B_0).

- h) For measuring reference, flowing 0,2 ml of the measuring solution in vial 2 for reference, 0,36 ml of measuring buffer for rinsing, 0,36 ml of regeneration solution and then 0,36 ml of measuring buffer for regeneration to the flow cell, and recording the fluorescence intensity change with time for 3 min.
- i) For measuring sample 1, flowing 0,2 ml of the measuring solution in vial 3 for sample 1, 0,36 ml of measuring buffer for rinsing, 0,36 ml of regeneration solution and then 0,36 ml of measuring buffer to the flow cell, and recording the fluorescence intensity change with time for 3 min.
- j) For measuring samples 2 and 3, flowing 0,2 ml of each of the measuring solutions in vial 4 and vial 5, followed by the same steps as described above.
- k) Data processing is given in [Clause 10](#).
- l) After use of the instrument, 50 ml of cleaning solution is sent by a syringe pump to the flow cell, all pipelines and five vials which are filled up with cleaning position and keep for sterilization until the next use. Prior to the next use, the cleaning solution is replaced by measuring buffer.
- m) All vials used are disposed because of avoiding contaminations with used chemicals.

10 Data processing

10.1 Measurement data

Measurement of absorbance for a flow cell with a fluorescence detector at a defined wavelength (for instance at 650 nm of excitation and at 665 nm of emission) depends on a fluorescent-red label. The result of measurement of sample concentrations essentially depends on the precision of the calibration. Therefore, usually sixfold measurements of each of standard analytes at least are carried out to obtain a CV of less than 20 %. The means are used for further calculations in accordance with ISO 15089^[8] and JIS K 0461^[9]. In the flow immunosensor, vial 1 for blank (B_0 analyte free), vial 2 for a reference, and vial 3, vial 4 and vial 5 for each of three samples are in turn measured continuously.

10.2 Calibration curve

A calibration curve showing the dose-response is prepared for each of standard analytes in the range of 0,001 µg/l to 100 µg/l by the use of a program for example, DeltaGraph5.4v^[5]. The raw absorbance is displayed on the left axis and dose/concentration is on the horizontal axis as shown in [Figure 1](#). The raw absorbance data (B , binding of a specific analyte) are often converted to the percentage of the maximum signal, designated as B_0 . The percentage is expressed as B_{percent} as shown in [Formula \(1\)](#).

$$B_{\text{percent}} = B/B_0 \quad (1)$$

where

B is the binding of a specific analyte;

B_0 is the binding in the absence of a specific analyte (blank).

5) DeltaGraph5.4v^[5] is the trade name of a product supplied by Nihon Poladigital 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.

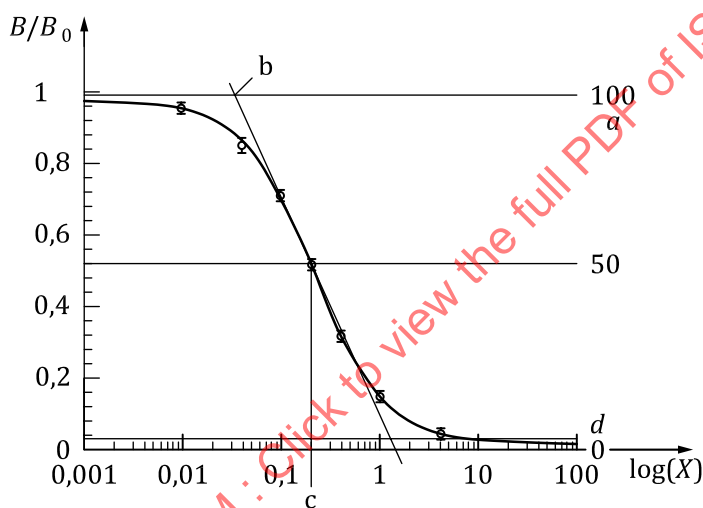
[Formula \(2\)](#) utilizes the entire response range but does not impose linearity on the flow immunosensor system. Instead, the four-parameter log fit generates additional variables representing the minimum response, a , the maximum response, d , inflection point in the central part of the curve, c , and slope at the inflection point, b , by iterative means of sixfold measurement values of standard analytes at least. The four-parameter curve fit which generates a sigmoidal response curve is commonly used for immunoassay data generation. [Figure 1](#) shows the sigmoidal response curve based on the four-parameter log fit.

$$y = \frac{a-d}{1+(x/c)^b} \quad (2)$$

where

y is the measured value;

x is the concentration of an analyte.



Key

X concentration of analyte, $\mu\text{g/l}$

Y B/B_0

Figure 1 — Sigmoidal response curve based on the four-parameter log fit

Examples of calibration curves of analytes 2,3,7,8-TCDD and 3,3', 4,4', 5-PeCB fortified in a drinking water are described in [Annex D](#).

10.3 Calculation of measured concentration

Binding of a certain dose, designated as B and the maximum signal (blank) designated as B_0 , the percent bound is expressed as B/B_0 . Measurement concentration of a sample is obtained by extrapolation of percent bound (B/B_0) to calibration curve defined by [Formula \(3\)](#), where the measurement sample value is calculated by [Formula \(4\)](#).

$$C_s = X \times n \times \frac{V_E}{V'_E} \times \frac{v}{V} \quad (3)$$

where

C_s is the (reference) measurement concentration in micrograms per litre, $\mu\text{g/l}$;

X is the measurement sample value in micrograms per litre, $\mu\text{g/l}$;

n is the dilution rate;

v is the amount of liquid sample for measurement in millilitres, ml;

V_E is the extract amount in millilitres, ml;

V'_E is the aliquot of the extract in millilitres, ml;

V is the sample collection quantity in litres, l.

$$X = X_s \times \frac{C_R}{X_{RS}} \quad (4)$$

where

X_s is the measured value of the environmental sample solution in micrograms per litre, $\mu\text{g/l}$;

X_{RS} is the actual value of the calibration solution in micrograms per litre, $\mu\text{g/l}$;

C_R is the calibration solution concentration in micrograms per litre, $\mu\text{g/l}$.

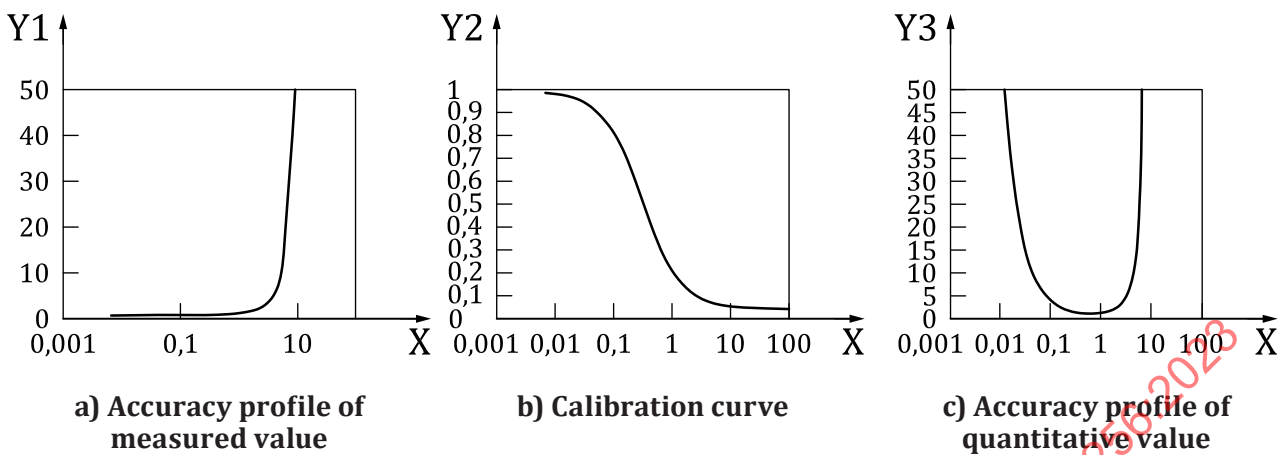
11 Validation

11.1 General

Calibration curves can be characterized by their range of measurement, LOD, LOQ and the potential region of non-quantifiable detections lying between those two parameters. Validation usually consists of replicating the calculations performed by the software by an alternative means such as a calculator or spreadsheet. Confirmatory analyses can be used for method of validation as described in [11.2](#).

11.2 Accuracy profile

Accuracy profiles are prepared according to ISO 11843-5^[10] and JIS K 0461^[9]. In precision profiles, CV calculated from measured values at least more than six times are displayed on the left axis and dose/concentration is on the horizontal axis. [Figure 2](#) a) is an accuracy profile of measured values, [Figure 2](#) b) is a calibration curve and [Figure 2](#) c) is an accuracy profile of quantitative values^{[9][11][12]}.



- Key**
- X concentration of analyte, µg/l
 - Y1 percentage of the coefficient of variation of the measured value
 - Y2 B/B_0
 - Y3 percentage of the coefficient of variation of the quantitative value

Figure 2 — Examples of accuracy profiles

As shown in [Figure 3](#), the percentage of the coefficient of variation of the measured values (r_m) is converted to the percentage of the coefficient of variation of the quantitative values (r_c) through the process of converting SD of measured values to SD of quantitative values by the use of differential coefficient (dY/dX) of calibration curve [see [Formula \(5\)](#)]:

$$r_c = \frac{r_m Y}{\left(\frac{dY}{dX}\right) X} \tag{5}$$

where

- r_c is the percentage of the coefficient of variation of the quantitative value;
- r_m is the percentage of the coefficient of variation of the measured value;
- X is the quantitative value;
- Y is the measured value.

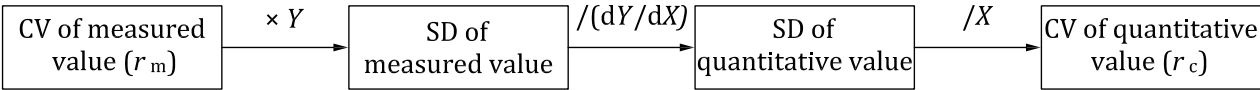
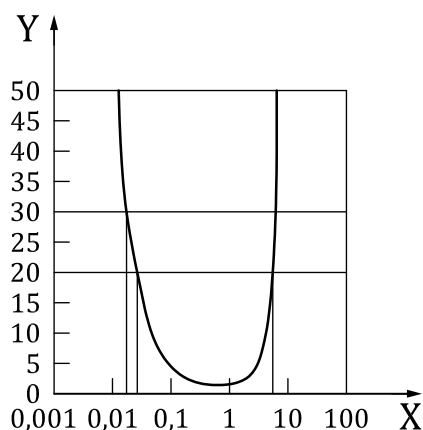


Figure 3 — Conversion of CV of measured values to CV of quantitative values

11.3 Range of quantitation

A lower LOQ is the minimum concentration of an analyte in a sample and an upper limit is the maximum concentration. Both limits correspond to 20 % or less of CV of quantitative values as shown in [Figure 4](#).

**Key**

X concentration, $\mu\text{g/l}$

Y percentage of the coefficient of variation of the quantitative value

NOTE If a linear calibration line can be forced through the origin, a lower and an upper limit, L , of quantitation are 10 times of the standard deviation, s , of measured values. When this is defined as a conversion value, the lower limit is: $L = 10s/dY/dX$, which is adapted for $(s/dY/dX)/L = 1/10$. In this case, the left is a lower limit and the right is an upper limit of quantitation concentration and correspond to 20 % or less of CV of quantitative values.

Figure 4 — Illustration of a range of quantitation and a limit of detection

Examples of calculation of LOD and LOQ for 2,3,7,8-TCDD and 3,3',4,4',5-PeCB fortified in drinking water are described in [Annex D](#).

11.4 Limit of detection

A lower limit of detection LOD is determined according to ISO 11843-5^[10] and JIS K 0461^[9]. The lower limit of detection is the minimum concentration of an analyte in a sample. The limit corresponds to 30 % or less of CV of quantitative values as shown in [Figure 4](#).

NOTE If a linear calibration line can be forced through the origin, a lower limit of detection, L , is 3,3 times of the standard deviation, s , of measured values. This is defined as a concentration converted from a calibration curve. The lower limit of detection is: $L = 3,3s/dY/dX$ which is adapted for $(s/dY/dX)/L = 1/3,3$. In this case, the left side is a lower limit of detection concentration and corresponds to 30 % or less of CV of quantitative values.

11.5 Evaluation of data

Selection of a LOD or a LOQ will impact the eventual data evaluation. If these parameters are established too low, some samples that are true negatives are likely to be scored as detections. On the other hand, if these parameters are too high, some samples actually containing residues are likely to be non-detections. These situations can also be described, respectively, as generating false positive and false-negative results. Minimization of these errors can be achieved by prudent adjustment of the sensitivity of the measurement to match the results of the detection or by the use of the automated flow immunosensor.

As for information, the result of interlaboratory trial of the measurement of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB in water samples by using the flow immunosensor is described in [Annex F](#).

11.6 Quality control

The fortified sample water with 2,3,7,8-TCDD and 3,3',4,4',5-PeCB are each submitted to recovery tests in HRGC/HRMS and in the flow immunosensor after preparations as described in [8.2.1](#) through [8.2.2](#). Ensure that the recovery rate is more than 80 %. [Annex E](#) shows the results of recovery tests for the fortified river water samples with 2,3,7,8-TCDD and 3,3',4,4',5-PeCB.

[Annex F](#) shows the results of the interlaboratory trial of measurement of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB for drinking water, river water and wastewater.

12 Test report

The test report shall contain at least the following information:

- a) the test method used, together with a reference to this document, i.e. ISO 23256:2023;
- b) specificity of antibodies used for a flow immunosensor;
- c) identity of the sample;
- d) sample pre-treatment, if required;
- e) calibration curve of the standard solutions as well as the zero standards according to [Clause 10](#);
- f) expression of the results according to [Clause 10](#);
- g) statement on the validity criteria according to [Clause 11](#);
- h) any deviations from this method;
- i) any unusual features observed;
- j) the date of the test;
- k) report of all circumstances that can have affected the results.

Annex A (informative)

Specificity of mouse monoclonal antibodies used for the flow immunosensor

Table A.1 shows the cross-reactivities of anti-2,3,7,8-TCDD antibody and anti-3,3',4,4',5-PeCB antibody towards certain congeners of each of PCDDs and PCBs measured in the flow immunosensor, model DXS-610 (see Annex C).

Table A.1 — Cross-reactivity of anti-2,3,7,8-TCDD antibody and anti-3,3',4,4',5-PeCB antibody towards certain congeners of each of PCDDs and PCBs

Dioxin and dioxin-like compound	Congener	Symbol	TEF ^[16]	C ₁₅₀ %	
				Anti-2,3,7,8-TCDD antibody	Anti-3,3',4,4',5-PeCB antibody
PCDDs	2,3,7,8-TCDD	D48	1	100	<0,2
	1,2,3,7,8-PeCDD	D54	1	7	<0,2
	1,2,3,4,7,8-HxCDD	D66	0,1	<1	<0,2
	1,2,3,6,7,8-HxCDD	D67	0,1	<1	<0,2
	1,2,3,7,8,9-HxCDD	D70	0,1	<1	<0,2
	1,2,3,4,6,7,8-HpCDD	D73	0,01	<1	<0,2
	OCDD	D75	0,000 3	<1	<0,2
PCDFs	2,3,7,8-TCDF	F83	0,1	10	<0,2
	1,2,3,7,8-PeCDF	F94	0,03	<1	<0,2
	2,3,4,7,8-PeCDF	F114	0,3	4	<0,2
	1,2,3,4,7,8-HxCDF	F118	0,1	<1	<0,2
	1,2,3,6,7,8-HxCDF	F121	0,1	<1	<0,2
	1,2,3,7,8,9-HxCDF	F124	0,1	<1	<0,2
	2,3,4,6,7,8-HxCDF	F130	0,1	<1	<0,2
	1,2,3,4,6,7,8-HpCDF	F131	0,01	<1	<0,2
	1,2,3,4,7,8,9-HpCDF	F134	0,01	<1	<0,2
	OCDF	F135	0,000 3	<1	<0,2
DL-PCBs	3,4,4',5-TCB	#81	0,000 3	<1	9
	3,3',4,4'-TCB	#77	0,000 1	1	40
	3,3',4,4',5-PeCB	#126	0,1	<1	100
	3,3',4,4',5,5'-HxCB	#169	0,03	<1	70
	2',3,4,4',5-PeCB	#123	0,000 03	<1	<0,2
	2,3',4,4',5-PeCB	#118	0,000 03	<1	<0,2
	2,3,3',4,4'-PeCB	#105	0,000 03	<1	<0,2
	2,3,4,4',5-PeCB	#114	0,000 03	<1	<0,2
	2,3',4,4',5,5'-HxCB	#167	0,000 03	<1	<0,2
	2,3,3',4,4',5-HxCB	#156	0,000 03	<1	1
	2,3,3',4,4',5'-HxCB	#157	0,000 03	<1	<0,2
	2,3,3',4,4',5,5'-HpCB	#189	0,000 03	<1	<0,2

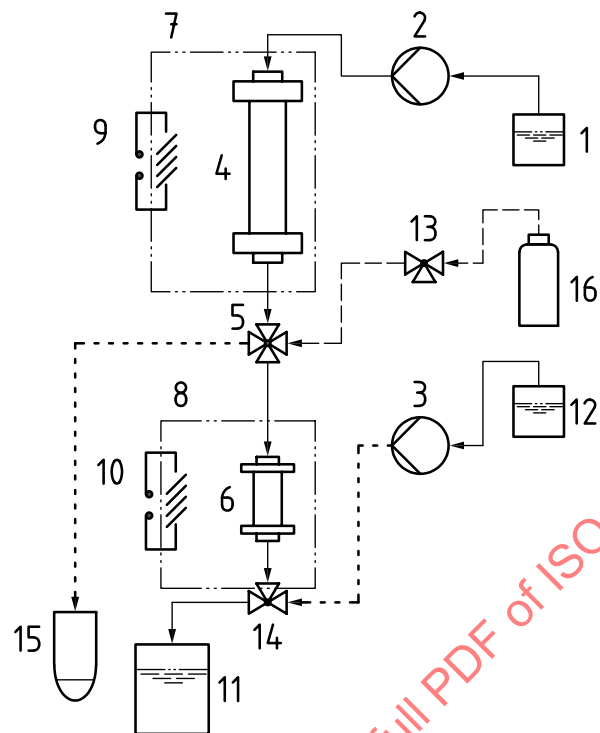
Annex B (informative)

Example of the automate sample clean-up and concentration device

B.1 General

An automated sample clean-up and concentration device (model SPD-600⁶⁾) consisting of a multi-layer silica gel column (14 mm diameter and 300 mm length) containing 0,1 g of glass wool, 2,3 g of silica gel, 10,4 g of 440 g/kg H₂SO₄ silica gel, 0,2 g of silica gel, 3,6 g of 100 g/kg AgNO₃ silica gel and 1,5 g of silica gel for purification, and an alumina column (6 mm diameter and 50 mm length) containing 0,1 g of glass wool, 1,0 g of alumina and 0,1 g of glass wool for concentration and solvent substitution can be used. [Figure B.1](#) shows a diagram of the clean-up and concentration device model SPD-600. A crude extract in up to maximum of 5 ml hexane is applied onto the silica gel column which is heated at 60 °C for 10 min. During heating, 85 ml of hexane is pumped into the column at the rate of 2,5 ml/min and then, hexane is removed from the alumina column by N₂ gas purging. Thereafter, for concentration and solvent substitution, the alumina column is heated at 90 °C for 10 min. During heating, the alumina column is eluted with 1,9 ml of DMSO by back flushing to give approximately 0,4 ml to 0,6 ml of DMSO eluent which is a sample solution used for preparation of measuring solutions^[13].

6) Model SPD-600 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.

**Key**

- 1 solvent reservoir (hexane)
 - 2 solvent delivery pump (hexane)
 - 3 solvent delivery pump (toluene or DMSO)
 - 4 multilayer silica gel column
 - 5 2-way valve
 - 6 alumina column
 - 7 constant temperature chamber for multilayer silica gel column
 - 8 constant temperature chamber for alumina column
 - 9 heater for constant temperature chamber 5
 - 10 heater for constant temperature chamber 8
 - 11 waste bottle
 - 12 solvent reservoir (toluene or DMSO)
 - 13 3-way solenoid valve for nitrogen flow
 - 14 3-way solenoid valve for toluene or DMSO flow
 - 15 sample collection vial
 - 16 nitrogen cylinder
- hexane
 - - - - - N₂ gas
 DMSO

Figure B.1 — Example of a diagram of the clean-up and concentration model SPD-600

Annex C
(informative)

Example of the flow immunosensor

C.1 General

The flow immunosensor (model DXS-610) based on the kinetic exclusion assay theory uses mouse monoclonal antibodies specific to each of antigens/analytes in a heterogeneous assay system for rapid detection of analytes in a number of samples. The flow immunosensor mainly consists of vials for specific antibody bound to a secondary antibody with a fluorescent label in measuring buffer, an assembly-type flow cell containing beads coated with an antigen-mimic BSA conjugate to hold a free specific antibody bound to a secondary antibody with a fluorescent label, a syringe pump to transport each of measuring solutions into vials, a measuring buffer, a regeneration solution and then a measuring buffer to a flow cell. During the process, a fluorescence detector records a fluorescence intensity change with time in a flow cell for approximately 3 min to give a sensorgram. The system of a flow immunosensor is operated automatically according to a set-up program. A free specific antibody bound to a secondary antibody with a fluorescent label is held on beads coated with an antigen-mimic BSA conjugate in the flow cell and then detected by the fluorescence detector. ^{[1][2][3]} Figure C.1 shows an example of the flow immunosensor model DXS-610.

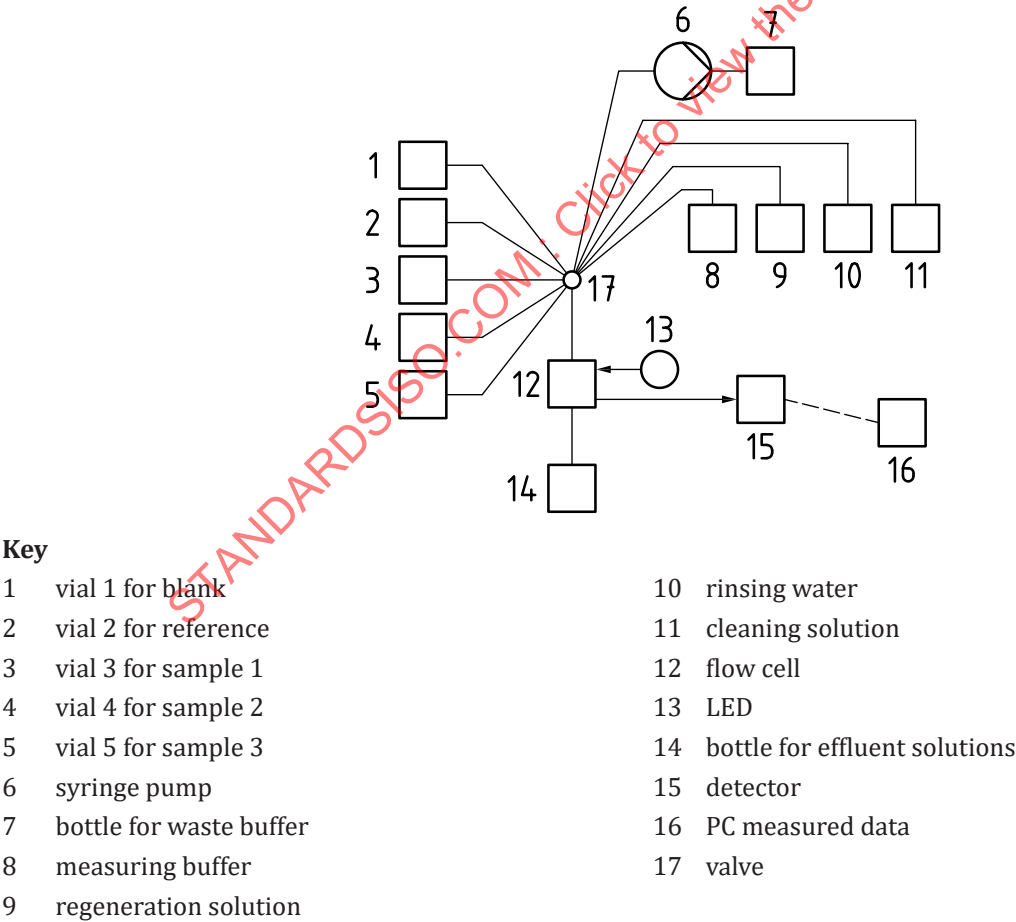
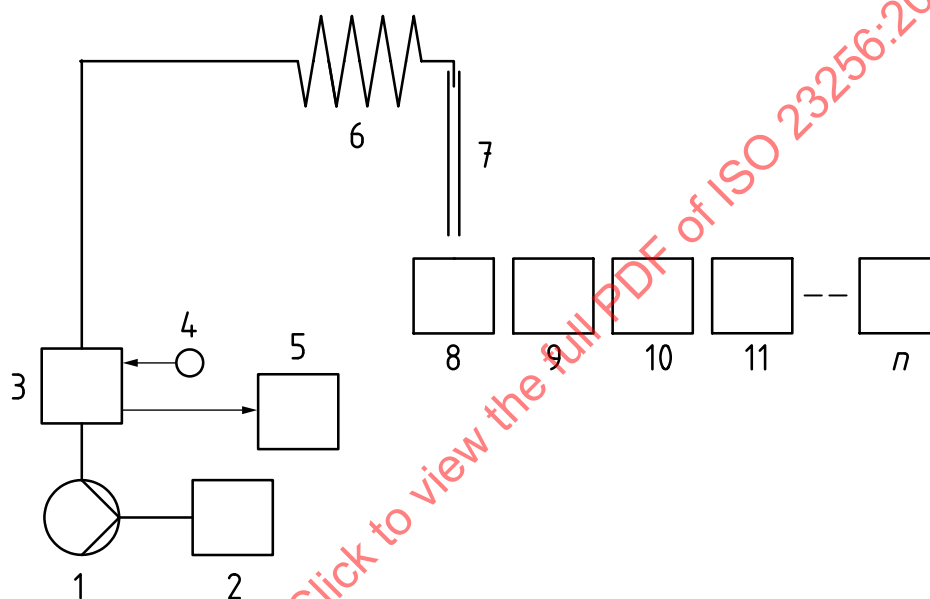


Figure C.1 — Simplified the flow immunosensor model DXS-610 diagram

The flow immunosensor model KinExA 4000⁷⁾ based on the kinetic exclusion assay (3.1.11) theory uses mouse monoclonal antibodies specific to each of antigens/analytes in a heterogeneous assay system for rapid detection of analytes in a number of samples. A disassembly type flow cell contains beads coated with an antigen-mimic BSA conjugate to which fluids of standards, samples, a secondary antibody with a fluorescent label are drawn through the flow cells by a syringe pump and fresh beads (replaced for each measurement), standards, samples, a secondary antibody with a fluorescent label are arrayed in glass test tubes on the KinExA 4000s integrated autosampler. During the process, a fluorescence detector records a fluorescence intensity change with time in a flow cell for approximately 3 min to give a sensorgram. The system of a flow immunosensor is operated automatically according to a set-up program. A free specific antibody bound to a secondary antibody with a fluorescent label is held on beads coated with an antigen-mimic BSA conjugate in the flow cell and then detected by the fluorescence detector.^{[1][2][3][14]} Figure C.2 shows a simplified flow diagram of the flow immunosensor model KinExA 4000.



Key

- | | | | |
|---|-----------------------|----|--------------------------------|
| 1 | syringe pump | 7 | autosampler tip |
| 2 | sample waste | 8 | coated beads |
| 3 | flow cell | 9 | bead waste |
| 4 | LED | 10 | buffer |
| 5 | fluorescence detector | 11 | samples, label up to 270 total |
| 6 | flexible line | | |

Figure C.2 — Simplified flow diagram of the immunosensor model KinExA 4000

C.2 Apparatus and materials

C.2.1 Flow cell

A flow cell contains beads coated with an antigen-mimic BSA conjugate to hold a free specific antibody bound to a secondary antibody with a fluorescent label. A syringe pump transports each measuring solution in vials, a measuring buffer, a regeneration solution and then a measuring buffer to a flow cell. During the process, a fluorescence detector records a fluorescence intensity change with time in a flow cell for approximately 3 min to give a sensorgram^{[1][2][3]}.

7) KinExA 4000 of Sapidyn Instruments Inc 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.

The disassembly type flow cell is 2 mm in diameter and 12 mm in length (37,7 μl of volume) and is supplied containing fine-grained polymer beads with a 100 μm of diameter. The assembly type flow cell is 1,6 mm in diameter and 30 mm in length. A variety of bead types and sizes can be used and are packed into a column approximately 4 mm long, approximately 8 μl of volume. The beads are coated for instance with 6-(2,4,5-trichlorophenoxy) hexanoic BSA conjugate for holding a free specific antibody bound to a secondary antibody with a fluorescent-red label Cyanine 5 or Alexa[®] Fluor 647⁸⁾. The assembly type flow cell is packaged and kept in a refrigerator until use.

During flowing a measuring solution, measuring buffer, regeneration solution and then measuring buffer to a flow cell, a free specific antibody bound to the secondary antibody with a fluorescent-red label held on beads coated with the antigen-mimic BSA conjugate is detected by the use of a fluorescence detector at 650 nm of excitation and at 665 nm of emission.

C.2.2 Syringe pump system

A syringe pump system is able to supply each measuring solution, measuring buffer and regeneration solution at a constant flow rate of 0,75 ml/min with less pulsation to a flow cell in a flow immunosensor device and to feed the solutions at a constant speed to the measurement device.

C.2.3 Fluorescence detector

The fluorescence detector capable of detecting the fluorescence intensity of Cyanine 5 or Alexa[®] Fluor 647 label at 650 nm of excitation and at 665 nm emission accurately. It records the fluorescence intensity change with time. For using the flow immunosensor model DXS-610, the fluorescence intensity during flowing a measuring solution, measuring buffer, regeneration solution and then measuring buffer for approximately 3 min. The detector capable of tracking the fluorescence intensity change is preferable with the lapse of time. The change of the fluorescence is recorded to give a sensorgram as shown in [Figure C.3](#). The range of the two lines indicates difference between vial 1 (B_0) and vial 2 in the intensity of fluorescence-red dependent on the amount of antigen/analyte-free antibody bound to antigen-mimic BSA conjugate on beads. Concentrations of an analyte are calculated according to the set up program FIS-Win⁹⁾ from measured difference values between vial 1 (B_0) and vial 2.

8) Cyanine 5 and Alexa Fluor 647 are examples of suitable products available commercially. These examples are given only as information for the convenience of users of this document and do not constitute an endorsement by ISO of these products.

9) DioFlock is the trade name of a product supplied by Miura 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.

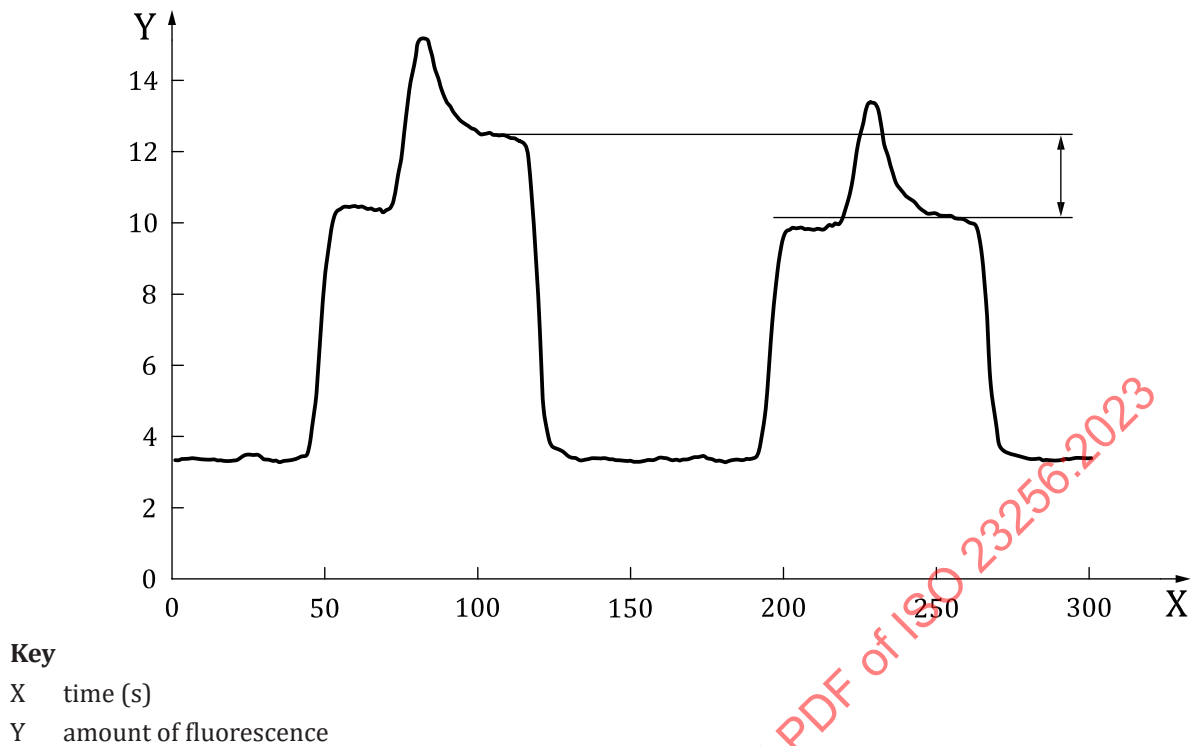
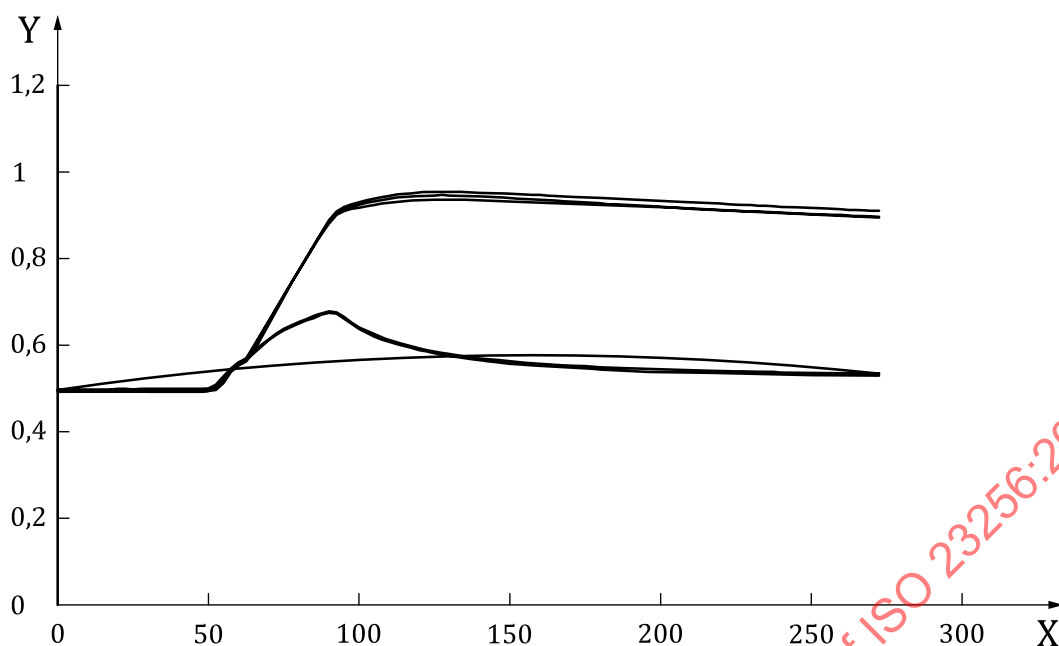


Figure C.3 — Example of sensorgrams using the DXS-610

For using the flow immunosensor model KinExa 4000, the change of fluorescence intensity during flowing a measuring solution is recorded continuously. The signal of steady fluorescence intensity is used for calculation. [Figure C.4](#) describes signal traces from the lowest standard at 28 pg/ml of 2,3,7,8-TCDD and the highest standard at 1 971 pg/ml of 2,3,7,8-TCDD by using the flow immunosensor model KinExA 4000.

**Key**

X time (s)

Y amount of fluorescence

NOTE Triplicate repeats of each are shown.

Figure C.4 — Signal traces using the KinExA 4000 from the lowest 2,3,7,8-TCDD standard and the highest 2,3,7,8-TCDD standard

C.2.4 Data system

Capable of collecting, recording and storing measurement data according to a set-up program, for example, FIS-Win in a computer. The KinExA Pro software supplied with the KinExA 4000 instrument performs this function.

C.2.5 Specific monoclonal antibody

Specific monoclonal antibodies (IgGs) are selected for measurement of analytes in a flow immunosensor. Mouse 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. 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 monitoring of PCDDs and PCBs in the flow immunosensor. The inhibition concentration 50 value of each of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB towards anti-2,3,7,8-TCDD antibody and anti-3,3',4,4',5-PeCB antibody is 0,16 µg/l and 0,34 µg/l, respectively. The sensitivity and specificity in a flow immunosensor largely depend on affinity and cross-reactivity of monoclonal antibodies used for the corresponding antigens/analytes.

C.2.6 Secondary antibody

Goat anti-mouse IgG antibody [pepsin-cleaved fragment (Fab')₂ of H and L chains] specifically binds to the constant region of specific mouse monoclonal IgG antibodies. The anti-mouse IgG antibody[(Fab')₂ of H and L chains] is labelled with a fluorescent label such as Cyanine 5 or Alexa[®] Fluor 647 by the coupling method using N-hydroxysuccinimide. The fluorescent-red labelled anti-mouse IgG antibody is used for binding to specific monoclonal antibodies described in [C.2.5](#) as a secondary antibody for flow immunosensing.

C.2.7 Antigen-mimic BSA conjugate

Add 15 mg/ml of BSA (3.2) in PBS (6.10) into 550 ml of DMSO (6.7.4) and then the mixture is combined with a 450 µl of 10 mmol/l of 6-(2,4,5-trichlorophenoxy) hexanoic acid 2,5-dioxopyrrolidin-1yl ester in DMSO to prepare a 5 mg/ml of 6-(2,4,5-trichlorophenoxy) hexanoic-BSA conjugate (antigen-mimic BSA conjugate) in PBS containing 5 % DMSO. This solution is used for coating of polymer beads with antigen-mimic BSA conjugate.

C.2.8 Beads

For instance, polymer beads (100 µm diameter) composed of polystyrene and polymethacrylic acid are suspended in PBS containing 5 % DMSO and then mixed with 5 mg/ml of 6-(2,4,5-trichlorophenoxy) hexanoic-BSA conjugate in PBS containing 5 % DMSO. The beads coated with the antigen-mimic BSA conjugate (C.2.7) are blocked with 1 % of a blocking reagent such as Block Ace^{®10)} in water containing 5 % DMSO and the solution is then replaced with PBS containing 5 % DMSO for preparation of beads coated with the antigen-mimic BSA conjugate.

10) Block Ace[®] is an example of a suitable product available commercially. This example is given only as information for the convenience of users of this document and do not constitute an endorsement by ISO of these products.

Annex D (informative)

Recoveries, calibration curves, ranges of quantitation of the standard analytes 2,3,7,8-TCDD and 3,3',4,4',5-PeCB in drinking water

D.1 Recoveries of the standard analytes 2,3,7,8-TCDD and 3,3',4,4',5-PeCB in drinking water

D.1.1 Sampling and spiking

Collect 3 l of drinking water in a 3,8 l amber bottle and adjusted to pH 7 to pH 8 with Na₂CO₃. Add 1 ml of acetone solution of a mixture of 0,6 ng/ml of 2,3,7,8-TCDD and 3,0 ng/ml of 3,3',4,4',5-PeCB or a mixture of 6 ng/ml of 2,3,7,8-TCDD and 30 ng/ml of 3,3',4,4',5-PeCB into the drinking water sample.

D.1.2 Flocculation and extraction

The fortified water sample was treated with a 0,3 g of a flocculant¹¹⁾ for dioxins in a wastewater. The flocs formed were collected by filtration with 0,5 µm of pore size of glass fibre filter and then air-dried overnight in a draft. The dried flocs on the filter and toluene rinsed an amber bottle were subjected to extraction in the high speed and high pressure extractor model E-916¹²⁾ and a Soxhlet extractor.

D.1.3 Clean-up and concentration

The crude extract was subjected to the automated sample clean-up and concentration device model SPD-600 (see [Annex B](#)) to give a purified sample in DMSO solution.

D.1.4 Measurement in the flow immunosensor and in HRGC/HRMS

The purified sample in DMSO was submitted to measurement in the flow immunosensor model DXS-610 (see [Annex C](#)) and the purified sample in toluene was analysed in a HRGC/HRMS (JMS-700D or JMS-800D) in accordance with ISO 18073^[5] and ISO 17858^[6] for recovery tests. The results are shown in [Tables D.1](#) and [D.2](#). The recovery of 2,3,7,8-TCDD was 94 % and 97 % for Soxhlet extraction, and 89 % and 95 % for high speed and high pressure extraction, respectively, in HRGC/HRMS and the flow immunosensor. Similarly, the recovery of 3,3',4,4',5-PeCB was 103 % and 91 % for Soxhlet extraction, and 99 % and 97 % for high speed and high pressure extraction, respectively in HRGC/HRMS and the flow immunosensor.

11) DioFlock is the trade name of a product supplied by Miura 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.

12) Model E-916 is the trade name of a product supplied by BUCHI Labortechnik AG. 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.

Table D.1 — Recovery of 2,3,7,8-TCDD in drinking water

Extraction	Amount added (2,3,7,8-TCDD pg/l) (A)	HRGC/HRMS (2,3,7,8-TCDD pg/l) (B)	Flow immunosensor (2,3,7,8-TCDD pg/l) (C)	Recovery (B/A)	Recovery (C/A)
Soxhlet extraction	207	193	201	94 %	97 %
High speed and high pressure extraction	207	183	196	89 %	95 %

Table D.2 — Recovery of 3,3',4,4',5-PeCB in a drinking water

Extraction	Amount added (3,3',4,4',5-PeCB pg/l) (A)	HRGC/HRMS (3,3',4,4',5-PeCB pg /l) (B)	Flow immunosensor (3,3',4,4',5-PeCB pg/l) (C)	Recovery (B/A)	Recovery (C/A)
Soxhlet extraction	1 040	1 067	946	103 %	91 %
High speed and high pressure extraction	1 040	1 033	1 003	99 %	97 %

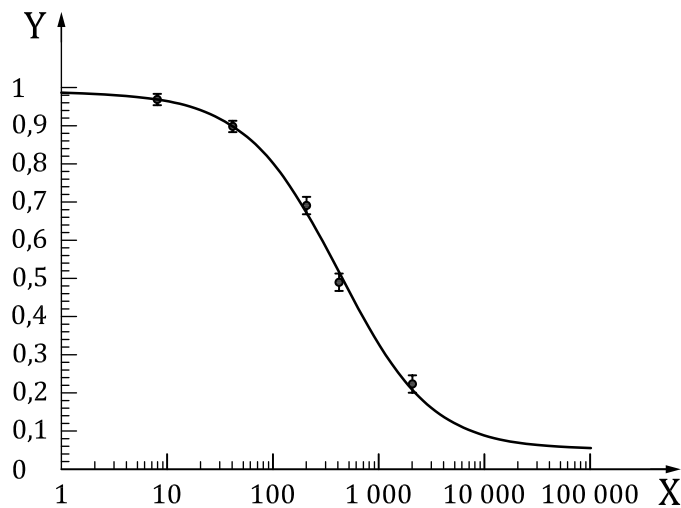
D.2 Calibration curves, LOD and LOQ of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB in drinking water

D.2.1 Measurement procedure

One millilitre of acetone solution containing each of five concentrations of the standard analytes 2,3,7,8-TCDD corresponding to 8 pg/l, 40 pg/l, 200 pg/l, 400 pg/l and 2 000 pg/l, and 3,3',4,4',5-PeCB corresponding to 36 pg/l, 180 pg/l, 900 pg/l, 1 800 pg/l and 9 000pg/l was added into a 3 l of the drinking water sample adjusted to pH 7 to pH 8 with Na₂CO₃. The fortified water sample was treated with 0,3 g of flocculant for dioxins in wastewater, extracted in the high speed and high pressure extractor model E-916, purified in the automated sample clean-up and concentration device model SPD-600 and then measured in the flow immunosensor model DXS-610.

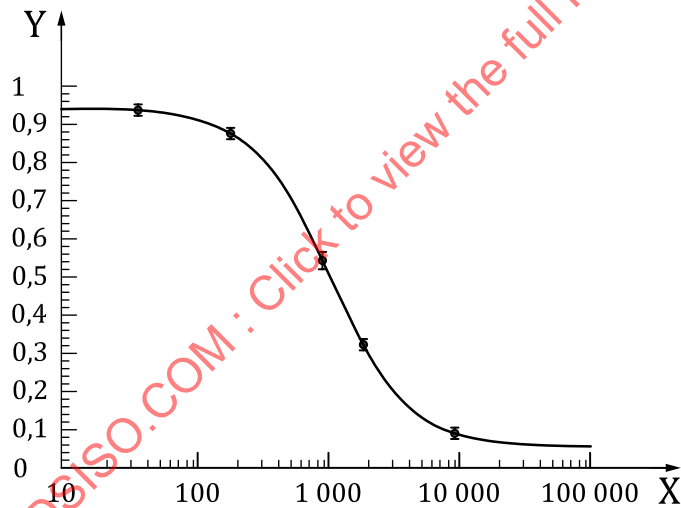
D.2.2 Calibration curve

The each of five concentrations of the standard analytes 2,3,7,8-TCDD and 3,3',4,4',5-PeCB were measured six times. The calibration curves of 2,3,7,8-TCDD and 3,3',4,4',5-PeCB were each prepared as specified in [10.1](#) through [10.2](#). The results are shown in [Figures D.1](#) and [D.2](#).



Key
X 2,3,7,8-TCDD, pg/l
Y B/B_0 of quantitative value

Figure D.1 — Calibration curve of the reference analyte 2,3,7,8-TCDD in a drinking water



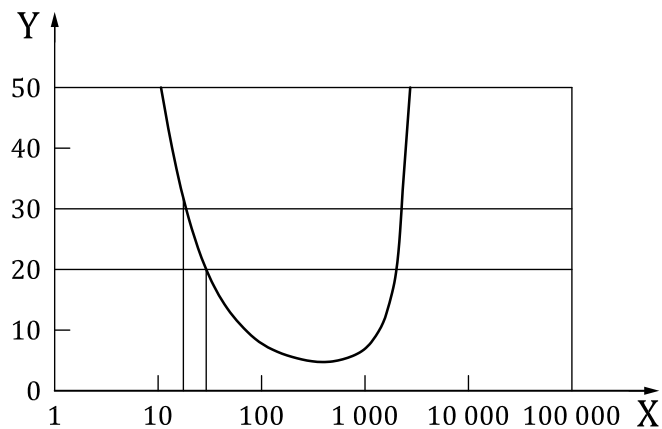
Key
X 3,3',4,4',5-PeCB, pg/l
Y B/B_0 of quantitative value

Figure D.2 — Calibration curve of the reference analyte 3,3',4,4',5-PeCB in a drinking water

D.2.3 Calculation of LOD and LOQ

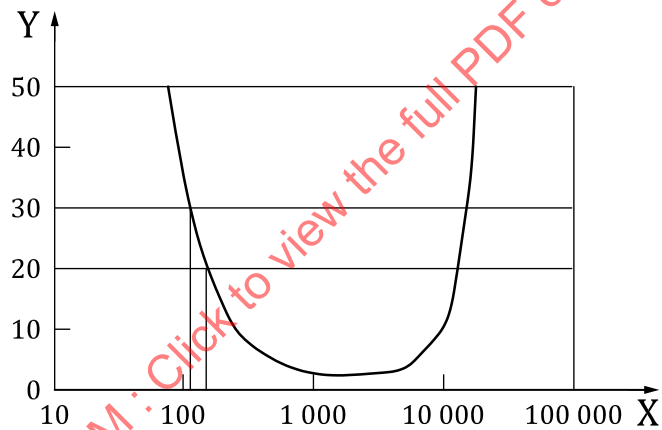
The calculation of LOD and LOQ was carried out as specified in [Clause 11](#). The results are shown in [Figures D.3](#) and [D.4](#).

In measurement of the analyte 2,3,7,8-TCDD in a drinking water in the flow immunosensor model DXS-610 using the monoclonal antibody specific to 2,3,7,8-TCDD, LOD and LOQ were 18 pg/l and 28 pg/l, respectively. Similarly, in measurement of the analyte 3,3',4,4',5-PeCB in the flow immunosensor model DXS-610 using the monoclonal antibody specific to 3,3',4,4',5-PeCB, the LOD and LOQ were 112 pg/l and 152 pg/l, respectively.



Key
X 2,3,7,8-TCDD, pg/l
Y percentage of the coefficient of variation of the quantitative value

Figure D.3 — Illustration of LOD and range of quantitation of 2,3,7,8-TCDD in a drinking water



Key
X 3,3',4,4',5-PeCB, pg/l
Y percentage of the coefficient of variation of the quantitative value

Figure D.4 — Illustration of LOD and range of quantitation of 3,3',4,4',5-PeCB in a drinking water