
**Water quality — Guidelines for selective
immunoassays for the determination of
plant treatment and pesticide agents**

*Qualité de l'eau — Lignes directrices relatives aux dosages
immunologiques sélectifs pour la détermination des agents de traitement
des plantes et des pesticides*



Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 15089 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical, biochemical methods*.

Annexes A and B of this International Standard are for information only.

Water quality — Guidelines for selective immunoassays for the determination of plant treatment and pesticide agents

1 Scope

This International Standard specifies a guide for the selective quantitative analysis by immunoassays of environmental chemicals such as pesticides (including insecticides) or their metabolites in drinking, ground and surface water.

The application range of the procedure for the analysis of pesticides in drinking water applies to mass concentrations $\geq 0,05 \mu\text{g/l}$. Therefore, the determination limit should be in this case $\leq 0,05 \mu\text{g/l}$.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes*.

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques*.

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples*.

ISO/TR 13530:1997, *Water quality — Guide to analytical quality control for water analysis*.

3 Terms and definitions

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

3.1

affinity

strength of binding of antibody to analyte

NOTE The strength is defined by the equilibrium constant (K) of the reaction $\text{Ab} + \text{H} = \text{AbH}$, where Ab = antibody combining site and H = hapten; K is given by the mass action equation $K = c_{\text{AbH}}/(c_{\text{Ab}} \times c_{\text{H}})$.

3.2

analyte

substance to be determined

3.3

antibodies

serum proteins produced in vertebrates in response to immunization and which selectively bind the antigen or hapten, respectively

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NOTE 1 Monoclonal antibodies (mAb) are uniform populations of antibodies which are produced from a single cell clone of hybridoma cells.

NOTE 2 Polyclonal antibodies (pAb) are a mixed population of antibodies which are produced by several clones of plasma cells.

NOTE 3 Recombinant antibodies (rAb) are produced using recombinant techniques established in gene technology.

3.4 antibody conjugate

antibody covalently linked to a label such as an enzyme or a fluorochrome

3.5 antigen

substance that stimulates the production of antibodies and reacts with them

3.6 antiserum

immune serum obtained from the blood of immunized vertebrates after removal of cellular components and coagulation factors

NOTE It usually contains a number of different antibodies which can exhibit different affinities to the antigen/hapten.

3.7 coating conjugate

macromolecule bound to the hapten (also known as a hapten-carrier conjugate), which is immobilized to a solid phase

NOTE It is used to bind those antibody binding sites which are not occupied by the analyte.

3.8 competitive immunoassay

test which detects the proportion of antibody binding sites which have been occupied by the sample analyte

NOTE This is achieved by adding a tracer which binds to the unoccupied antibody binding sites and produces the measuring signal after a further reaction.

3.9 cross-reactivity

extent, to which an antibody or an antiserum reacts with a substance which structurally differs from the analyte

NOTE 1 The cross-reactivity of an antibody or an antiserum with this substance is determined by comparing the calibration curves. The reference curve obtained with the analyte is used as reference quantity (= 100 % cross-reactivity). The cross-reactivity is usually determined at the IC_{50} . The selectivity of an antibody or an antiserum, respectively, is inversely related to the cross-reactivities. An antibody or an antiserum, respectively, can display different affinities to different substances. With a given substance, the cross-reactivity of an antiserum can also vary within the measuring range. Usually the structure of the immunogen essentially determines the selectivity (the so-called specificity) of an antiserum. If cross-reactivities are due to a mixed population of antibodies as often occurs in an antiserum, the undesired antibodies may be removed by cross-absorption.

NOTE 2 All compounds (present in relevant concentrations) that exhibit cross-reactivity will create false positive results.

3.10 enzyme immunoassay EIA

immunochemical analysis which detects the occupancy of antibody binding sites by the analyte with the aid of a tracer (an enzyme-labelled hapten or an enzyme-labelled antibody) and consequently can be used to detect the analyte concentration in the medium

NOTE The detection procedure is based on the measurement of the enzyme activity of the tracer by means of substrate conversion.

3.11**enzyme substrate**

substance which is converted by the enzyme into a product that can be detected by a measuring device

3.12**excess standard**

analyte concentration which, once exceeded, produces no further decrease in the signal measured in the immunoassay

3.13**fluorescence immunoassay**

immunochemical detection procedure which is performed either as an immunoassay with fluorescent substrates or products, respectively, or as an immunoassay with fluorescence-labelled tracers or antibodies, respectively

3.14**hapten**

substance which, because of its small molecular size, does not evoke the production of antibodies unless it is covalently bound to an immunogen

NOTE Pesticides are examples of haptens.

3.15**heterogeneous immunoassay**

test which requires a separation of solid phase-bound and unbound tracers in order to detect the occupancy of antibody binding sites by the analyte and thus the analyte concentration in the sample

3.16**immunoassay**

quantitative assay which is based on the selective analyte/antibody binding and uses tracers for the detection of the free or occupied antibody binding sites, respectively

3.17**immunogen**

substance which triggers an immune response after injection into a vertebrate

3.18**inhibition concentration****IC**

analyte concentration which reduces the measuring signal of the zero standard (= 100 %), in the case of IC₅₀ to 50 % of the zero standard

3.19**luminescence immunoassay****LIA**

immunochemical assay which is either performed as an immunoassay detecting luminescent substrate or product, respectively, or luminescence-labelled tracer

3.20**solid phase immunoassay**

heterogeneous immunoassay which uses (depending on the test type) antibodies or coating conjugates, immobilized to a solid phase

NOTE Usually, both test types are known as ELISA (enzyme-linked immunosorbent assay) when enzyme-labelled tracers are used.

3.21**tracer**

labelled hapten (or antigen) or labelled antibody which, in the case of competitive immunoassays, is used to detect the percentage of antibody binding sites not being occupied by the analyte

3.22

zero standard

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

4 Interferences

Interferences are caused by improper sampling, for instance due to the choice of equipment or materials which adsorb or liberate the substances to be analysed. Assay conditions, for instance pH or sample components such as metal ions, humic acids, salinity and solvents influencing the test components (for instance matrix effects), can interfere with the analysis. The influence of matrix effects may be assessed by spiking samples with known amounts of the analyte.

5 Principle

Immunoassays are methods which use antibodies produced against defined analytes or analyte groups as biochemical sensors for the quantification of analyte concentrations. These assays are particularly useful as screening assays. All immunoassays for the detection of haptens are based on the principle of the competitive immunoassay. Assays for pesticides have been reported (see references [1] to [4] in the bibliography). A typical procedure, as an example of an EIA, is described below.

The solid phase variant requires either immobilized antibodies and dissolved hapten tracers (variant a, direct immunoassay) or immobilized coating conjugate and dissolved antibody tracers (variant b, indirect immunoassay) in constant ratios. The antibodies are applied in limiting amounts. Therefore components which are not bound to the coated solid phase can be removed by washing prior to the final detection. This also includes most of the interfering matrix effects. The more tracer molecules are bound, the lower is the analyte concentration in the sample.

The immobilization can be achieved by passive adsorption or by covalent binding to functional groups of the solid phase.

The calibration is performed with solutions of known analyte concentrations.

6 Reagents

6.1 General

Use reagents and water of high purity (for instance "for residue analysis"). Information on type and origin of antibodies or antisera, respectively, as well as the cross-reactivities shall be stated in the report. Information on storage and stability of the used reagents shall be requested from the supplier. The selectivity of the antibodies or antiserum, respectively, shall guarantee that sample concentrations deduced from a calibration curve do not deviate by more than $\pm 10\%$ from the actual analyte concentrations in these samples. If the specificities of the antibodies or antiserum, respectively, are low, the interfering analyte shall be removed from the samples by suitable chemical-physical procedures. Cross-reacting antibodies of an antiserum can be removed by cross-absorption.

6.2 Buffered washing solution, used for washing for instance phosphate buffered saline (PBS; pH 7,6) with a phosphate concentration of 80 mmol/l (prepared with NaH_2PO_4 and Na_2HPO_4) and a sodium chloride concentration of 85 g/l.

6.3 Acid, for instance sulfuric acid, $c(\text{H}_2\text{SO}_4) = 1 \text{ mol/l}$, for adjusting the pH and stopping the enzyme reaction.

6.4 Base, for instance sodium hydroxide, $c(\text{NaOH}) = 1 \text{ mol/l}$, for pH adjustment and for stopping the enzyme reaction.

7 Apparatus

7.1 Solid phase, consisting of plastics, glass or magnetic particles for use in heterogeneous immunoassays, for instance microwell plates, test tubes, beads, magnetic particles, or membranes.

7.2 Multipipettes, for instance variable pipettes 10 μl to 500 μl , fixed volume pipettes 10 μl , 100 μl , 200 μl , multichannel pipettes for instance 300 μl , and dispensers.

7.3 Magnetic rack, consisting of a rack with a magnetic base to be used during the washing step for immunoassays with antibodies bound to ferromagnetic particles.

8 Sampling and sample preparation

8.1 Sampling

Perform sampling in accordance with ISO 5667-1, ISO 5667-2 and ISO 5667-3. The immunoassay is generally performed with raw water samples. Sample pretreatment should be considered, if necessary. It is possible to concentrate samples by suitable chemical-physical procedures.

8.2 Coating of solid phases

Coated solid phases can be obtained commercially. Otherwise they should be prepared for enzyme immunoassays as follows:

- a) by incubating diluted solutions of the antibody or antiserum, respectively, in a suitable buffer solution, for instance 50 mmol/l carbonate buffer (pH 9,6), prepared with Na_2CO_3 and NaHCO_3 . This is sometimes referred to as direct ELISA;
- b) coating the solid phases with a hapten-carrier conjugate before starting the assay. This is sometimes referred to as indirect ELISA.

Usually proteins are applied as carriers. After coating and a subsequent washing step, blocking of the unspecific binding sites can be necessary for some assays. Furthermore, it is necessary to prepare a standard calibration series of the respective pesticide, for instance in distilled water.

8.3 Enzyme tracer

The hapten-enzyme tracers (direct ELISA) or antibody-enzyme tracers (indirect ELISA) used for the enzyme immunoassay are either prepared shortly before use in a suitable dilution in buffer solution or they are provided by the supplier in a stabilized form ready for use. The enzyme substrate is prepared shortly before use by dissolving one or more components in a buffer solution or it is obtained in a stabilized form from the supplier.

8.4 Solutions

Prepare washing solutions for rinsing the solid phase (see 9.1.2) as well as suitable acids or bases for terminating the enzyme reaction (see 9.1.3) prior to the measurements.

8.5 Sample preparation

Prior to the assay, all water samples, reagents, solutions, equipment and coated solid phase are brought to a defined temperature. The enzyme reaction is carried out at constant temperature ($\pm 0,5$ $^{\circ}\text{C}$) in the range between 20 $^{\circ}\text{C}$ and 37 $^{\circ}\text{C}$. If the pH of the water samples is not in the range between 7,0 and 7,5 or another value is chosen as suitable for the assay, a pH adjustment (for instance with NaOH or H_2SO_4) should be carried out.

The resulting dilution of the samples has to be taken into account in the calculation.

9 Procedure

IMPORTANT — It is necessary to include specialists or special facilities for investigations in accordance with this International Standard.

If this procedure is applied, it should be checked in each individual case (depending on the task) whether and as far it is necessary to define additional marginal conditions.

9.1 Direct enzyme immunoassay (with enzyme-hapten tracers, variant a)

9.1.1 Binding reaction

Combine the standard or the water sample, respectively, together with the tracer (see 8.3) in suitable dilutions with the coated solid phase. Incubate in the dark until the immunoreaction is finished, usually within 30 min to 60 min.

9.1.2 Washing step

Remove the portions of standard, sample and tracer, which have not been bound during the binding reaction, by rinsing several times with a buffered rinsing solution (for instance PBS, see 6.2).

9.1.3 Enzyme reaction

In a further step the hapten-bound enzyme tracer converts an added substrate.

Choose the incubation time and the substrate concentration so that with the given tracer concentration the substrate is not completely converted. Terminate the enzyme reaction either for the end-point determination by adding acid (6.3) or base (6.4) after the incubation time or measure continuously.

9.1.4 Determination of the converted substrate

The converted substrate is determined by an appropriate instrumental method. Guidance on the choice of appropriate instrumentation is usually given with the commercial IA kits.

9.1.5 Example of a direct immunoassay

NOTE A direct enzyme immunoassay may be carried out as in the flowchart and pipetting scheme given in annex A.

9.1.5.1 Binding reaction

Pipette individually 200 μl of standard or sample, respectively, into the appropriate number of precoated wells of the microwell plates. Prepare a suitable working dilution (if necessary in two consecutive steps) of a peroxidase tracer.

First, add 10 μl of the tracer to 10 ml of buffered washing solution (6.2) in a dilution of 1:1 000 by volume. Pipette 50 μl of peroxidase-tracer dilution into each cavity. Mix the standard solution or water sample, respectively, and tracer on a vertical shaker for 30 s or by careful circular movements of the plate.

Wrap in aluminium foil and incubate at constant temperature, for instance 20 °C, for 1 h.

9.1.5.2 Washing step

After incubating, the plate should be washed three times with a microplate washer (or manually) using 300 μl of buffered washing solution per well. Remove the remaining buffered washing solution after the last run by tapping the plate on tissue (cotton).

9.1.5.3 Enzyme reaction

After preparing the enzyme substrate, start the stop watch and pipette in rows 200 μl of substrate in each well in a 10-s or 15-s measure.

After a reaction time of 10 min, add 100 μl acid (6.3) per cavity in the same order and the same measure. If there is no intensive colour of the zero control solution 10 min after substrate incubation, the termination of the reaction should be delayed. After adding acid (6.3) to each cavity, a colour change from blue to yellow is observed. Proper mixing is obtained by gently swirling the plate.

9.1.5.4 Absorbance measurement

Measure the absorbances with a vertical photometer for microwell plates at a defined wavelength (for instance 450 nm) which depends on the product of the enzyme reaction. The wells with zero standards should result in absorbance values between 1,0 and 1,5.

The result of the determination of sample concentrations essentially depends on the precision of the calibration (see clause 10). Therefore, usually threefold measurements are carried out to obtain coefficients of variation (CV) of approximately 10 %. The means are used for further calculations. Sample concentrations lying beyond the measuring range (the range of the calibration curve) cannot be taken into account.

9.2 Measurement of the zero standard

Run with each measuring series a zero standard (method blank) to check for eventual problems with the equipment and the reagents.

Prepare and analyse water (6.1) in the same way as a real sample for the performance of the measurement of the zero control.

If a zero standard lies above the detection limit, the problem shall be located by systematic investigations in order to remove the contamination source. The results obtained from the batch of samples containing this zero standard should be discarded.

9.3 Calibration

Calibrate each immunoassay for its analyte with individual standard solutions. Their concentration should be checked regularly in accordance with the respective ISO standards. The concentration shall not deviate more than 3% from the given concentration.

Calibration solutions may, for instance, contain the following mass concentrations: 0 $\mu\text{g/l}$; 0,03 $\mu\text{g/l}$; 0,10 $\mu\text{g/l}$; 0,30 $\mu\text{g/l}$; 1,0 $\mu\text{g/l}$; 10 $\mu\text{g/l}$; 1 000 $\mu\text{g/l}$ [excess standard, for use in equation (1)]. At least five solutions with mass concentrations between the zero and the excess standard are required for quantification.

The reference function obtained for the analyte can only be applied for the mass concentration range covered.

10 Validity criteria

For validity criteria, see ISO/TR 13530.

The following validity criteria can only be considered useful if the immunoassay method is in routine use and is under statistical control. In order to ensure that the latter is the case, a full performance validation exercise shall be carried out for each analyte prior to the first application of the method in a laboratory. This exercise will establish the parameters upon which the routine Quality Assurance and Control can be based using a control charting approach.

The desired coefficient of variation (CV) of the replicate determinations of a sample depends on the purpose of the measurement and on the matrix. For quantification, the CV should not exceed 10 %.

At least one blank spiked with the analyte at the concentration equivalent to the level of interest should be analysed with each batch of samples. The result for this spiked blank should be used as a point on the control chart.

11 Calculation

11.1 Replicates

In order to obtain coefficients of variation between 5 % and 10 %, replicate determinations should be carried out. The desired CV determines the number of replications. Often 3 replicate analyses are sufficient for this purpose.

11.2 Calculation of the B/B_0 -values

A normalization of the data should be carried out for several purposes, for instance for the direct comparison of different reference curves.

The data from the spectrometric, fluorometric or luminometric determination are converted to B/B_0 -values using equation (1). These values, expressed as a percentage, indicate the occupancy of antibody binding sites by the enzyme tracer or the coating conjugate.

$$B/B_0 = \frac{(A - A_0)}{(A_0 - A_e)} \times 100 \quad (1)$$

where:

- B is the binding of the tracer in the presence of the sample;
- B_0 is the binding of the tracer in the absence of the sample;
- A is the absorbance of the sample or the calibration standard (without dimension);
- A_0 is the absorbance of the zero standard (without dimension);
- A_e is the absorbance of the excess (without dimension).

11.3 Curve fitting

Equation (2) is frequently used for obtaining the calibration function (see reference [6]). However, several other options are available. Figure 1 is given as an example.

$$y = d + \left[\frac{a - d}{1 + \left(\frac{\rho}{\rho_{IC50}} \right)} \right] \times b \quad (2)$$

where:

- y is the measured value, for instance absorption;
- d is the lower asymptote of the curve;
- a is the upper asymptote of the curve;
- ρ is the analyte mass concentration, for instance in micrograms per litre;
- ρ_{IC50} is the analyte mass concentration, for instance in micrograms per litre, at IC_{50} ;
- b is the slope of the curve at the IC_{50} .

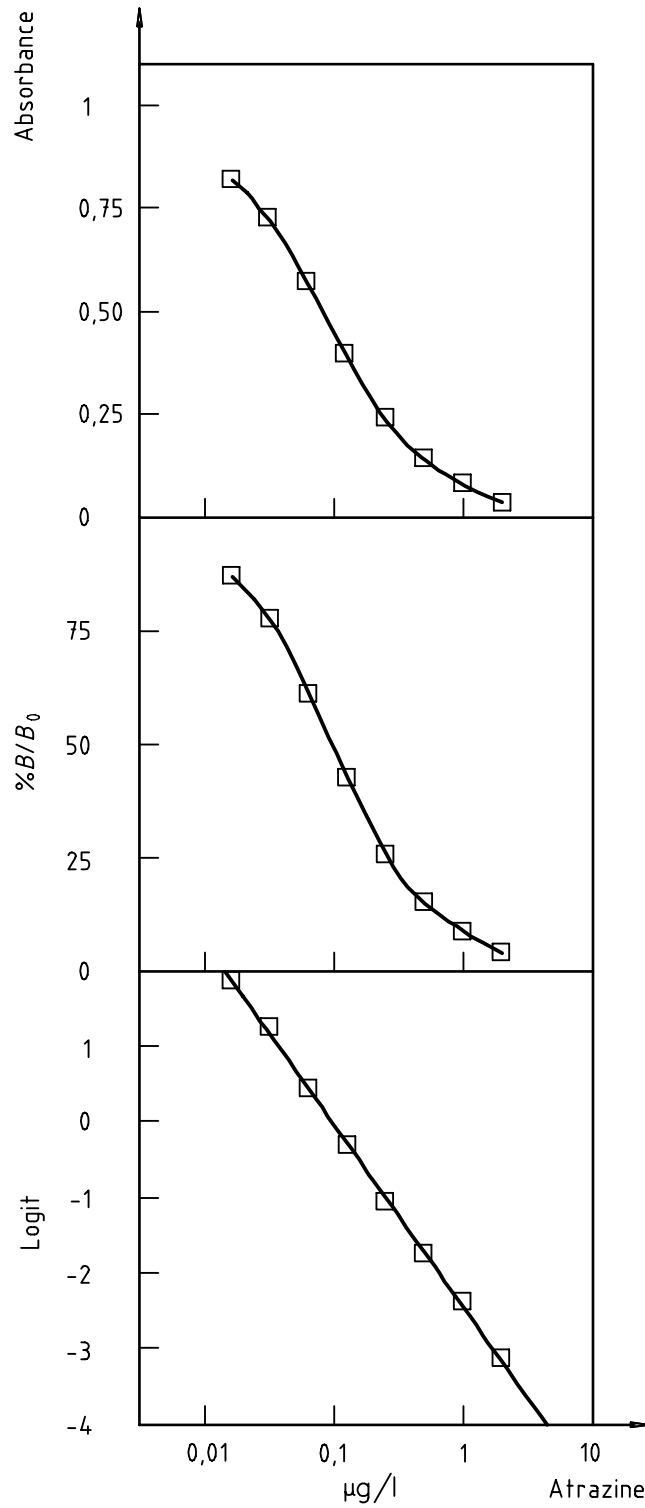


Figure 1 — Calibration curve for atrazine obtained with a competitive EIA based on monoclonal antibodies (K4E7^[5])

Computer programmes are available for the calculation.

For details of the calculation of mass concentrations and their limits of confidence see [6].

The analyte mass concentrations are given as values rounded to three significant figures.

EXAMPLE Atrazine 0,572 µg/l; 1,27 µg/l

12 Precision

As an example of the type of performance that can be obtained using this immunoassay is provided in annex B, i.e. an interlaboratory trial ^[7] was conducted using EIA for the determination of atrazine in different water samples.

13 Test report

The test report shall contain the following details:

- a) reference to this International Standard, i.e. ISO 15089:1999.
- b) identification of the sample;
- c) sample pretreatment, if required;
- d) calibration curve of the standard solutions as well as the zero standards in accordance with 9.3;
- e) statement of the results in accordance with 11.3;
- f) statement on the validity criteria in accordance with clause 10;
- g) all deviations from this procedure and statement of all circumstances which may have influenced the result;
- h) cross-reactivities of the applied antibodies.

Annex A (informative)

Flowchart and pipetting scheme

The flowchart and pipetting scheme of the immunoassay described in this International Standard are given in Figure A.1 and Table A.1.

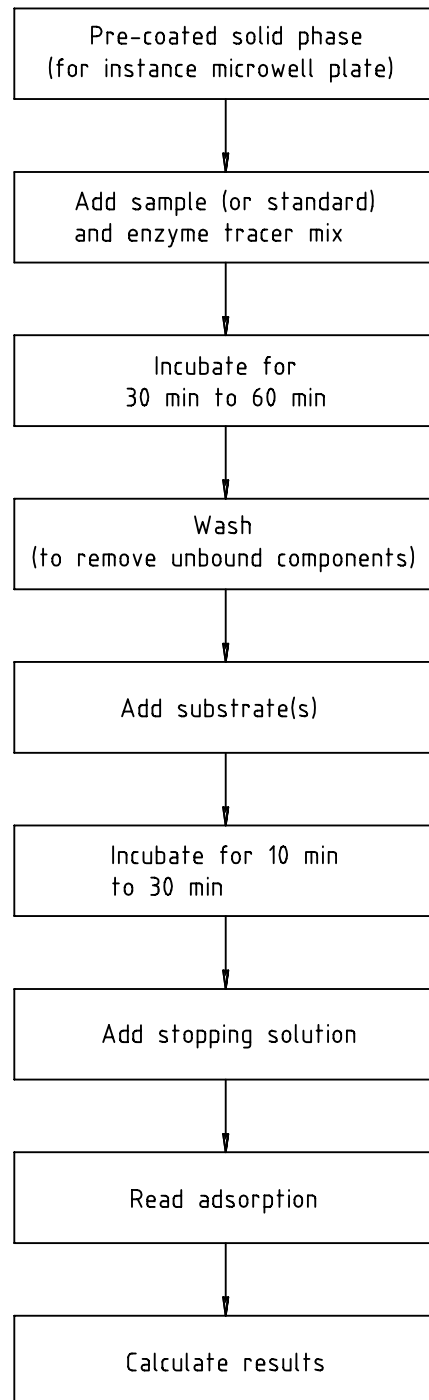


Figure A.1 — Flowchart of the immunoassay

Table A.1 — Pipetting scheme of the immunoassay

Step	Item	Volume	Comment
1	standard or sample	200 µl	per pre-coated well
2	tracer	50 µl	mix for 30 s and incubate for 1 h
3	—	—	
4	washing solution	300 µl (3 ×)	use microplate washer
5	substrate	200 µl	incubate for 10 min to 30 min
6	stopping solution (for instance H ₂ SO ₄)	100 µl	
7	—	—	read absorption

Annex B (informative)

Precision data from an interlaboratory trial

Table B.1 presents precision data obtained from an interlaboratory trial conducted using enzyme immunoassay to determine the concentration of atrazine when spiked in two of three different types of water samples.

Table B.1 — Precision data from an interlaboratory trial for the determination of atrazine using EIA

Sample	l	n	o %	x_{nom} $\mu\text{g/l}$	x $\mu\text{g/l}$	Recovery %	σ_R $\mu\text{g/l}$	CV_R %	σ_r $\mu\text{g/l}$	CV_r %
1	15	56	11,1	—	0,003	—	0,0016	52,8	0,006	18,9
2	15	57	9,5	0,130	0,125	95,8	0,0153	12,3	0,0080	6,4
3	14	54	8,5	0,115	0,116	100,5	0,0234	20,2	0,0109	9,4
l	number of laboratories (after elimination of outliers)									
n	number of measured values (after elimination of outliers)									
o	outliers									
x_{nom}	nominal true value									
x	total mean									
σ_R	reproducibility standard deviation									
CV_R	reproducibility coefficient of variation									
σ_r	repeatability standard deviation									
CV_r	repeatability coefficient of variation									
Sample 1: Drinking water										
Sample 2: Ground water from a deep well, spiked at 0,130 $\mu\text{g/l}$										
Sample 3: Spring water from tunnel, spiked at 0,115 $\mu\text{g/l}$										

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