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Nanotechnologies — Assessment of peroxidase-like activity of metal and metal oxide nanoparticles

Nanotechnologies — Evaluation de l'activité de type peroxidase des nanoparticules métalliques et d'oxydes métalliques

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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

Enzymes are the biological catalysts that control biochemical reactions. The enzyme peroxidase is a metalloenzyme with many isoforms. It catalyses the oxidation of various organic substrates by hydrogen peroxide, which is used extensively in biochemistry applications. Metal and metal oxide nanoparticles have a wide range of applications in biomedicine, environment protection, and some other fields, such as magnetic separation, detection, anti-bacterial, degradation of contaminants, medical imaging and tumour therapy. In recent years, an intrinsic peroxidase-like activity was observed in some metal and metal oxide nanoparticles, which means that these metal and metal oxide nanoparticles can catalyse the oxidation of substrates of natural peroxidase by hydrogen peroxide under mild reaction conditions in comparable efficiency and kinetics. Iron oxide (Fe_3O_4) nanoparticles are one representative material, and cobalt oxide (Co_3O_4) nanoparticles, copper oxide (CuO) nanoparticles, manganese oxide (MnO_2) nanoparticles, vanadium oxide (V_2O_5) nanoparticles, gold (Au) nanoparticles and platinum (Pt) nanoparticles have been reported to have the peroxidase-like activity as well. These findings extend enzyme mimics from organic compounds to inorganic nanomaterials.

Certain metal and metal oxide nanoparticles can catalyse the transfer of electrons from H_2O_2 to colorimetric indicator under physiological condition. This phenomenon is like the colorimetric reaction mediated by peroxidase and thus is called as peroxidase-like catalysis. Such catalytic property can be used to produce colorimetric, chemiluminescent or electrochemical signals which have great potential applications in biosensors, electrochemical sensors and immunoassays. The nanoparticles with peroxidase-like activity may have anti-tumour, antibacterial or antioxidant functions in biological system. In addition, the nanoparticles with such activity can have potential impacts on health, safety and the environment. Therefore, it is important to assess the peroxidase-like activity of a nanoparticle in practical applications.

The peroxidase-like activity of nanoparticles strongly depends on multiple factors including the composition, size, surface chemistry and crystal structure of the nanoparticles, as well as the measurement conditions. Therefore, it is important to establish a standard method for assessing the peroxidase-like activity of metal and metal oxide nanoparticles.

This document provides a specification for the assessment of peroxidase-like activity of metal and metal oxide nanoparticles. This protocol is useful to enterprises, research laboratories or institutions and metrological organizations that are working on nanomaterials used in biomedical applications and environment protection.

Nanotechnologies — Assessment of peroxidase-like activity of metal and metal oxide nanoparticles

1 Scope

This document specifies a method for assessing the peroxidase-like activity of metal and metal oxide nanoparticles by spectrophotometry. This document can serve as a reference for the measurements of peroxidase-like activities in other types of nanoparticles.

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 18153:2003, *In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values for catalytic concentration of enzymes assigned calibrators and control materials*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms, definitions and abbreviated terms

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following 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 Terms and definitions

3.1.1
nanoparticle
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

[SOURCE: ISO/TS 80004-2:2015, 4.4, modified — Note 1 to entry has been removed.]

3.1.2
catalytic activity
property of a component corresponding to the catalysed substance rate of conversion of a specified chemical reaction, in a specified measurement system

Note 1 to entry: In this document, the “component” is one kind of metal or metal oxide nanoparticles.

Note 2 to entry: In this document, the catalytic activity is the peroxidase-like activity of metal and metal oxide nanoparticles.

Note 3 to entry: The coherent derived SI unit is “katal” (kat), equal to “mole per second” ($mol \cdot s^{-1}$).

[SOURCE: ISO 18153:2003, 3.2, modified — Notes 1, 2 and 3 to entry have been added.]

3.1.3
specific catalytic activity
catalytic activity per unit mass of metal or metal oxide in nanoparticles

Note 1 to entry: Specific catalytic activity is expressed as $kat \cdot kg^{-1}$.

3.2 Abbreviated terms

ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt)
DMSO	dimethyl sulfoxide
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
IONPs	iron oxide nanoparticles
NPs	nanoparticles
OPD	o-phenylenediamine
TMB	3,3',5,5'-tetramethylbenzidine

4 Principle

The HRP reaction can be expressed by Formula (1):

$$\text{Substrate}_{\text{reduced}} + H_2O_2 \xrightarrow{\text{Horseradish Peroxidase}} \text{Substrate}_{\text{ox}} + H_2O \quad (1)$$

To evaluate the activity of HRP, chromogenic substrates are often employed, such as TMB, OPD, ABTS, among these, TMB can be the most widely used one.

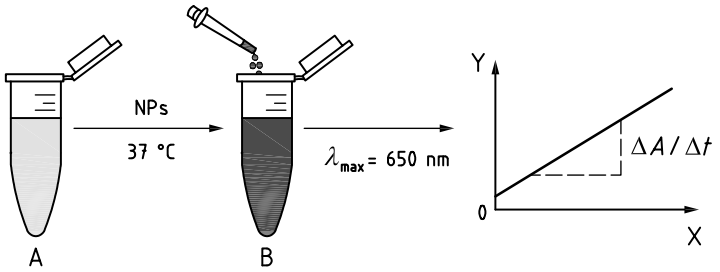
Some metal and metal oxide NPs can also catalyse the substrates of horseradish peroxidase in the presence of H_2O_2, which is referred as peroxidase-like activity in this specification. For the substrate TMB, the chemical reaction can be expressed by Formula (2):

$$\text{TMB} + H_2O_2 \xrightarrow{\text{metal and metal oxide NPs}} \text{TMB}_{\text{ox}} + H_2O \quad (2)$$

The reaction of Formula (2) generates a blue colour oxidized product that has a characteristic absorption peak at a wavelength of 650 nm. The absorbance is measured as a function of time at (37 ± 1) °C (see Figure 1). The measurement time can be determined based on the linear range of the progressing curve of the peroxidase reaction. During the initial phase of the reaction within the first few percent progression towards total completion, there is a linear phase of the reaction. It is recommended to record the absorbance within the linear phase of the reaction. To determine the enzymatic activity, it is sufficient to calculate from the change in absorbance per unit of time during this linear phase.

Usually, metal or metal oxide NPs show peroxidase-like activity under acidic conditions and the activity is weak or even vanishes under neutral or base conditions. The relevance of determining the peroxidase-like activity under acidic environment is considered as the low pH exists ubiquitously in biological systems including lysosomes, tumours, wounds, stomach and in environmental systems including polluted water. At acidic conditions, the dissolution of ions is possible and therefore inclusion of an additional control is needed (see 7.5).

The solubility of TMB is significantly decreased in solutions of pH ≥ 5,5. This document is applicable for suspensions with a pH value in the range of 3,5 to 5,5.



Key

X	time, in s	A	TMB + H_2O_2
Y	absorbance	B	TMB_{ox} + H_2O

Figure 1 — Schematic of peroxidase-like activity measurement for metal or metal oxide NPs

The number of peroxidase-like activity units of metal or metal oxide NPs is calculated according to the Lambert-Beer law:

The initial change rate of absorbance (min^{-1}) is obtained from the slope of the early, linear phase, of the experiment, as shown in Figure 1. After deducting the reagent blank rate, the number of peroxidase-like activity units of metal or metal oxide NPs is calculated according to Formula (3).

$$b_{\text{nano}} = \frac{V}{\varepsilon \times l} \times \frac{\Delta A}{\Delta t} \tag{3}$$

where

b_{nano} is the number of enzyme activity units of metal or metal oxide NPs, in kat;

V is the total volume of the reaction solution, in l;

ε is the molar attenuation coefficient of the TMB derivative, which is 39 000 $mol^{-1} \cdot l \cdot cm^{-1}$;

l is the optical path length of the cuvette, in cm;

$\Delta A/\Delta t$ is the initial change rate of absorbance of the reaction solution after correcting with a reagent blank rate, in s^{-1}.

The specific catalytic activity of NPs, a_{nano}, is calculated by dividing b_{nano} by the mass of the tested NPs.

The peroxidase-like activity of metal or metal oxide NPs is calculated according to Formula (4).

$$a_{\text{nano}} = \frac{b_{\text{nano}}}{m_{\text{M}}} \tag{4}$$

where

a_{nano} is the peroxidase-like activity of metal or metal oxide NPs, in $kat \cdot mg^{-1}$;

m_{M} is the mass of metal or metal oxide NPs, in mg.

NOTE See Annex A for the example from measurement and calculation of the mass.

5 Physicochemical characterization of metal or metal oxide NPs

Prior to the assessment of peroxidase-like activity of metal or metal oxide NPs, the size (distribution), shape, composition, surface chemistry and crystal structure of the nanoparticles should be

characterized according to Reference [1], as the peroxidase-like activity strongly depends on these factors.

6 Apparatus and reagents

6.1 Apparatus and appliances

6.1.1 Spectrophotometer.

A calibrated standard spectrophotometer covering visible wavelength range shall be used.[2] The spectrophotometer shall be turned on 1 h prior to the measurement to allow the baseline to stabilize.

6.1.2 Thermometer, with an accuracy equal to or under ±1,0 °C.

6.1.3 pH meter, with a resolution equal to or under 0,01 and an accuracy of ±0,002.

6.1.4 Thermostatic water bath.

The thermostatic water bath of the spectrophotometer is used to control the tank of cuvette at a constant temperature of (37 ± 1) °C.

6.1.5 Electronic balance, with a precision of 0,01 mg and a repeatability (calibration weight) of ≤0,015 mg (5 g).

6.1.6 Adjustable pipette, of 200 µl (uncertainty, u < 0,3 %) and 1 000 µl (u < 0,3 %).

6.1.7 Volumetric flask, of volume (10 ± 0,04) ml and (100 ± 0,2) ml.

6.1.8 Cuvette, with an optical path length of (10 ± 0,05) mm.

6.2 Reagents

All essential reagents for the assay are listed in Table 1.

Table 1 — Reagents

Classification	Chemical name	Name
Reagents	Anhydrous sodium acetate (AR) [a]	sodium acetate
	Anhydrous acetic acid (AR) [a]	glacial acetic acid
	3,3',5,5'-Tetramethylbenzidine (AR) [a]	TMB
	30 % hydrogen peroxide (AR) [a]	30 % H_2O_2
	Horseradish peroxidase (≥4,167 × 10^{-6} kat/mg solid)	HRP
Solvent	Double distilled water, grade 2, in accordance with Reference [3]	double distilled water
	Dimethyl sulfoxide (AR)	DMSO

[a] AR represents the analytical-reagent grade.

[b] GBW08616, GBW(E)130070 and GBW(E)130071 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Table 1 *(continued)*

Classification	Chemical name	Name
Standard substance	Standard solution of metal elements, such as standard solution of iron elements (e.g. GBW08616)[b]	NA
	Potassium biphthalate (e.g. GBW(E)130070)[b]	NA
	Mixed phosphate (e.g. GBW(E)130071)[b]	NA

[a] AR represents the analytical-reagent grade.

[b] GBW08616, GBW(E)130070 and GBW(E)130071 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

7 Solution preparation

7.1 General requirements

The mass given for each component in solution refers to 100 % content. If the content of the chemical substance is less than 100 % [e.g. y (%)], the factor (F_{content} = 100/y) should be used to calculate the mass of a chemical substance equivalent to the given mass.

The uncertainty should be within ±0,5 % when weighing with an electronic balance.

7.2 TMB solution

Prepare the TMB solution of 4,16 mmol·l^{-1} in DMSO. The TMB should be fully dissolved. The TMB solution is recommended to store in separate packages. Repeated freezing-thawing should be avoided. The solution must not be used if it is observed to be discoloured or if the absorption spectrum has changed. The absorption spectrum of TMB can be found in Reference [4].

7.3 Buffer solution

Prepare the sodium acetate solution of 0,2 mol·l^{-1} and the glacial acetic acid solution of 0,2 mol·l^{-1} with double distilled water.

Mix above two solutions in a certain proportion to prepare the buffer solution with a pH value ranging 3,5 to 5,5 (see Clause 4).

7.4 Nanoparticle dispersion solution

Prepare the stock dispersion solution of metal or metal oxide NPs according to Reference [5]. The stock solution may be diluted to ensure the absorption values fitting within the instrumental detection limit.

7.5 Additional control solution

Metal ions may be released from the surface of nanoparticles in acidic medium and may contribute to the catalytic activity. To subtract the possible contribution, an additional control solution can be prepared (see Reference [6]).

Prepare the additional control solution by mixing the nanoparticle dispersion solution with the buffer solution at (37 ± 1) °C for the same amount of time as the measurement time length. The nanoparticles are then removed from the solution by using ultrafiltration, the ultrafiltration pore size should be smaller than the size of the NPs, or, to remove magnetic nanoparticles from the solution, a magnet can be used. The resulting solution is referred to as the additional control solution.

8 Measurement procedure

8.1 Measurement condition

Specific measurement conditions are shown in Table 2.

Table 2 — Measurement conditions

Parameter	Index
Temperature	(37 ± 1) °C
Detection wavelength	(650 ± 1) nm
Optical path	(10,00 ± 0,05) mm
Incubation time	(60 ± 10) s
Measurement time	≥300 s (record the absorbance within the linear phase of the reaction)
Measurement point	≥11 (interval, $I \leq 30$ s)

8.2 Measurement procedure

8.2.1 Control (37 ± 1) °C in the cuvette with the thermostatic water bath.

8.2.2 Place the cuvettes containing double distilled water in the spectrophotometer for baseline calibration and recording the reference.

8.2.3 Prepare the reagents and testing samples (see Clause 6).

8.2.4 The buffer solution, dispersion solution of metal or metal oxide NPs, additional control solution, TMB solution and 30 % H_2O_2 are equilibrated to 37 °C in the thermostatic water bath.

8.2.5 Add the above solutions to the reaction beaker following the procedure in Table 3.

Table 3 — Measurement procedure

Volume	Measurement procedure
2,00 ml	Buffer solution added to reaction beaker and incubated to 37 °C.
100 µl	Dispersion solution of metal or metal oxide NPs, or additional control solution, added to reaction beaker and mixed with vortex mixer completely.
100 µl	TMB solution added to the reaction beaker, mixed with vortex mixer completely, and incubated for 60 s. At the end of incubation, the temperature of the solution in the reaction beaker should reach 37 °C.
200 µl	30% H_2O_2 added to reaction beaker, mixed with vortex mixer completely. Transfer appropriate amount of reaction solution to the cuvette and measure the changes of absorbance (650 nm) within the specific time.

8.2.6 Specific measurement process is shown in Figure 2.

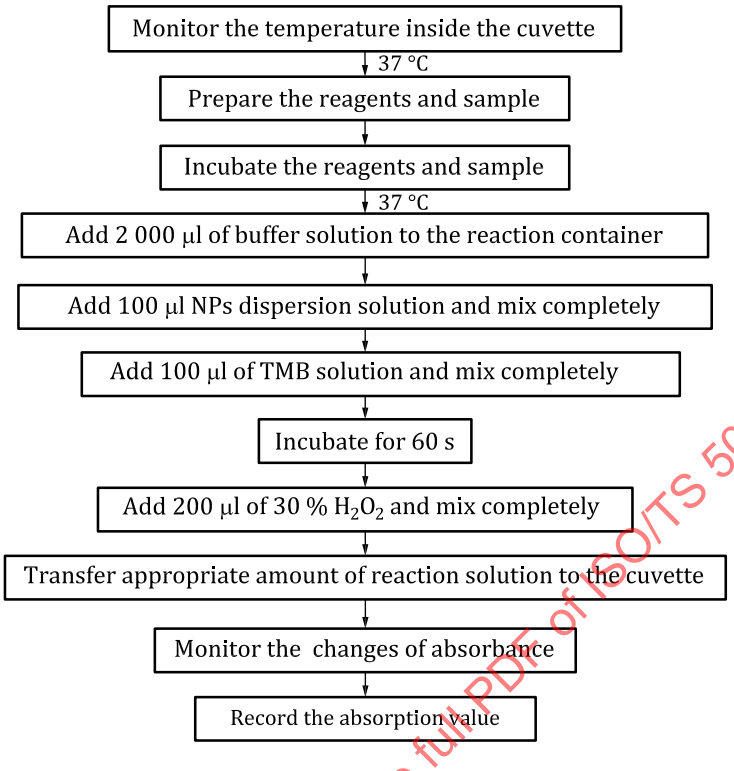


Figure 2 — Measurement flowchart

8.3 Measurement of reagent blank absorption

In the measurement flowchart (see Figure 2), the 30 % H_2O_2 or the dispersion solution of metal or metal oxide NPs is replaced by double distilled water and the absorption value is measured following the procedure in 8.2.

It is recommended to use the mean value of three measurements to substitute into the calculation.

8.4 Positive control measurement

The dispersion solution of metal or metal oxide NPs is replaced by HRP, and the absorption value is measured following the procedure in 8.2.

8.5 Additional control measurement

The dispersion solution of metal or metal oxide NPs is replaced by additional control solution and the absorption value is measured following the procedure in 8.2.

9 Data analysis

The procedure for determining the peroxidase-like activity of the metal or metal oxide NPs is as follows:

a) Measured the time dependence of the absorbance at 650 nm for metal and metal oxide NPs (A_{MOi}) and two blank solutions (A_{B1i} and A_{B2i}) with time (t_i),

where

i is the time measurement point and i = 0, 1, 2,…;

A_{MOi} is the absorbance at 650 nm at t_i for metal or metal oxide NPs;

A_{B1i} is the absorbance at 650 nm at t_i for blank solution with 30 % H_2O_2 replaced by double distilled water;

A_{B2i} is the absorbance at 650 nm at t_i for blank solution with the dispersion solution of metal or metal oxide NPs replaced by double distilled water;

t_i is the time taken to measure the absorbance.

b) Calculate the relative absorbance at 650 nm for the metal or metal oxide NPs (A_{Mi}) at time (t_i) using Formula (5),

$$A_{Mi} = A_{MOi} - A_{B1i} - A_{B2i} \tag{5}$$

where

i is the time measurement point and i = 0, 1, 2...;

A_{Mi} is the relative absorbance at 650 nm for metal or metal oxide NPs by subtracting the absorbance of the blank solutions from the absorbance of the metal or metal oxide NPs at time (t_i).

c) Calculate the initial rate of absorbance change ($\Delta A/\Delta t$) according to Formula (6),

$$\frac{\Delta A}{\Delta t} = \frac{\left(A_{Mj} - A_{Mi}\right)}{\left(t_j - t_i\right)} \tag{6}$$

where

t_j and t_i are the time taken to measure the absorbance, j, i= 0, 1, 2, ... and $j > i$;

A_{Mj} and A_{Mi} are the relative absorbance at 650 nm for the metal or metal oxide NPs at time t_j and t_i, respectively.

d) Calculate the number of enzyme activity units of metal or metal oxide NPs (b_{nano}) according to Formula (3) in Clause 4.

e) Measure the mass of the metal or metal oxide NPs (m_{M}).

f) Calculate the peroxidase-like activity of metal or metal oxide NPs (a_{nano}) according to Formula (4) in Clause 4.

10 Measurement uncertainties

The uncertainties in the calculation of the peroxidase-like activity of metal or metal oxide NPs may derive from[7]:

a) a broadening effect originating from agglomeration of metal or metal oxide NPs in the sample;

b) optical absorption by impurities including metal or metal oxide NPs in the sample;

c) the systematic and statistical uncertainties associated with deducing from Formula (2) to Formula (6);

d) traces of water in the solvent that cause spurious absorbance.

11 Test report

The test report should fully describe the test procedures used, to allow the experiment to be reproduced.

The test report shall include the following:

a) the sample;

b) test results;

c) test procedures;

d) complete identification of the nanoparticles tested (composition, size distribution, purity, etc.);

e) procedures for test sample preparation and storage condition of the test sample;

f) identification of the used reagent (trade-name, manufacturer's code, catalogue or formulation number, batch number or date of manufacture, sensitivity, etc.);

g) the International Standard used (including its year of publication);

h) any deviations from the procedure;

i) any unusual features observed;

j) the date of the test.

NOTE For more information on the physicochemical parameters, see Reference [1].

Annex A
(informative)

Measurement for the mass from the tested metal and metal oxide nanoparticles

A.1 Solid or powder samples

To calculate the mass of tested nanoparticles in 100 µl dispersion solution, weigh 10 mg of the solid or powder 3 times and calculate the average mass value. Add the powder in 10 ml of H_2O to prepare an original dispersion solution and calculate the average concentration (µg/µl). The mass of tested nanoparticles in 100 µl dispersion solution is calculated by multiplying the average concentration by 100 µl.

A.2 Solution samples

To estimate the mass of tested nanoparticles in 100 µl dispersion solution, freeze dry or vacuum dry 100 ml of sample solution to obtain the solid or powder samples. Weigh the powder three times and calculate the average mass value. The mass of tested nanoparticles in the solution sample is calculated by dividing the average mass of the powder sample by 1 000.

Annex B
(informative)

Example of calculation and uncertainty evaluation from the peroxidase-like activity of iron oxide nanoparticles

B.1 Measurement problem

The peroxidase-like activity of IONPs a_{nano} is determined by dividing the number of enzyme activity units b_{nano} contained in the tested IONPs by the mass of IONPs m_{IONPs}. As specified in this document, b_{nano} is obtained by measuring the generation of blue oxidized product per unit time catalysed by IONPs in the presence of H_2O_2 at 37 °C under an acidic condition of pH = 3,6 and is calculated according to Beer-Lambert law.

B.2 Mathematical model

From Formulae (3) and (4), the measurand a_{nano} can be expressed as

$$a_{nano} = \frac{V}{\varepsilon \times l \times m_{IONPs}} \times \frac{\Delta A}{\Delta t} \tag{B.1}$$

where

a_{nano} is the peroxidase-like activity of IONPs, in kat·mg^{-1};

V is the total volume of reaction solution, in l;

ε is the molar attenuation coefficient of TMB derivative (39 000 mol^{-1}·L·cm^{-1});

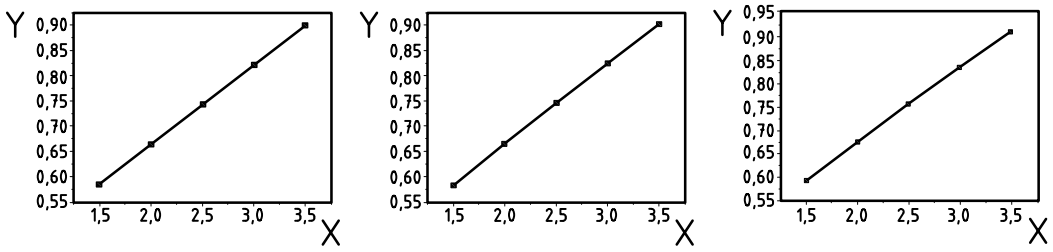
l is the optical path of cuvette, in cm;

m_{IONPs} is the mass of IONPs, in mg;

$\Delta A / \Delta t$ is the initial change rate of absorbance of the reaction solution after correcting with reagent blank rate, in s^{-1}.

B.3 Example of calculation for the peroxidase-like activity of IONPs

As an example, the spectral changes of TMB oxidation products with time catalysed by IONPs was shown in Figure B.1, independent repeated three times under the same measurement conditions.



Key

X time, in min

Y absorbance (650 nm)

Figure B.1 — Spectral changes of TMB oxidation products with time (n = 3)

Consider that three independent sets of repeated measurement of $\Delta A / \Delta t$ are obtained via the same procedure (see Clause 8.2), resulting in the data given in Table B.1. The corresponding values of a_{nano} are calculated according to Formula (B.1). The arithmetic mean of a_{nano} and the standard deviation calculated from Formulae (B.2) and (B.3) are also given in Table B.1.

$$\bar{a}_{nano} = \frac{1}{n}\sum_{i=1}^{n} a_{\text{nanoi}} \tag{B.2}$$

$$s_{(a_{\text{nanoi}})} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(a_{\text{nanoi}} - \bar{a}_{\text{nano}})^2} \tag{B.3}$$

Table B.1 — Values of the input quantities V, ε, l, m_{Fe} and $\Delta A / \Delta t$ obtained from three sets of repeated measurements and the calculated a_{nano}

Input quantities	V, l	0,002 4		
	ε, $\text{mol}^{-1}\cdot\text{l}\cdot\text{cm}^{-1}$	39 000		
	l, cm	1 000		
	m_{IONPs}, mg	$m_{\text{Fe}} = C_{\text{Fe}} \times V_{\text{Fe}} = 0{,}066 \times 0{,}1 = 6{,}6 \times 10^{-3}$		
	$\Delta A / \Delta t$, s^{-1}	1	2	3
		0,002 62	0,002 65	0,002 65
a_{nano}, $\text{kat}\cdot\text{mg}^{-1}$		$2{,}44 \times 10^{-8}$	$2{,}47 \times 10^{-8}$	$2{,}47 \times 10^{-8}$
Arithmetic mean, $\text{kat}\cdot\text{mg}^{-1}$		$2{,}46 \times 10^{-8}$		
Standard deviation, $\text{kat}\cdot\text{mg}^{-1}$		$1{,}73 \times 10^{-10}$		

B.4 Uncertainty evaluation for the peroxidase-like activity of IONPs

B.4.1 Type A evaluation of standard uncertainty

Using the measurement procedure of 8.2, the peroxidase-like activity of IONPs are measured four measurements, in each of three groups on each of three days, and the calculated a_{nano} were shown in Table B.2.

Table B.2 — Calculation of results of a_{nano}

Date	Group	a_{nano} kat·mg^{-1}			
		$n = 1$	$n = 2$	$n = 3$	$n = 4$
Day 1	I	$2,48 \times 10^{-8}$	$2,44 \times 10^{-8}$	$2,07 \times 10^{-8}$	$2,23 \times 10^{-8}$
	II	$2,74 \times 10^{-8}$	$2,43 \times 10^{-8}$	$2,39 \times 10^{-8}$	$2,57 \times 10^{-8}$
	III	$2,71 \times 10^{-8}$	$2,35 \times 10^{-8}$	$2,44 \times 10^{-8}$	—
Day 2	IV	$2,15 \times 10^{-8}$	$2,29 \times 10^{-8}$	$2,12 \times 10^{-8}$	$2,31 \times 10^{-8}$
	V	$2,35 \times 10^{-8}$	$2,21 \times 10^{-8}$	$2,21 \times 10^{-8}$	$2,18 \times 10^{-8}$
	VI	$2,41 \times 10^{-8}$	$2,29 \times 10^{-8}$	$2,18 \times 10^{-8}$	$2,07 \times 10^{-8}$
Day 3	VII	$2,44 \times 10^{-8}$	$2,24 \times 10^{-8}$	$2,46 \times 10^{-8}$	$2,46 \times 10^{-8}$
	VIII	$2,28 \times 10^{-8}$	$2,57 \times 10^{-8}$	$2,22 \times 10^{-8}$	$2,36 \times 10^{-8}$
	IX	$2,48 \times 10^{-8}$	$2,17 \times 10^{-8}$	$2,44 \times 10^{-8}$	$2,40 \times 10^{-8}$
NOTE "—" in this table is outlier removing according to Grubbs criterion.					

Substitution of the values from Table B.2 into Formulae (B.2) and (B.3) then given $\bar{a}_{nano} = 2,35 \times 10^{-8}$ kat·mg^{-1} and $s_{(a_{nanoi})} = 1,64 \times 10^{-9}$ kat·mg^{-1}.

The type A standard uncertainty $u_{A(a_{nano})}$, that is the experimental standard deviation of the mean $s_{(\bar{a}_{nano})}$ is $2,78 \times 10^{-10}$ kat·mg^{-1}, calculated according to Formula (B.4).

$$u_{A(a_{nano})} = s_{(\bar{a}_{nano})} = \frac{s_{(a_{nanoi})}}{\sqrt{n}} = \frac{1,64\times10^{-9}}{\sqrt{35}} = 2,78\times10^{-10} \text{ kat·mg}^{-1} \quad \text{(B.4)}$$

B.4.2 Type B evaluation of standard uncertainty

B.4.2.1 General

B.4.2.1.1 Introduction

According to Formula B.1, it can be analysed that the components of type B uncertainty are contributed by V, ε, l, m_{IONPs} and $\Delta A/\Delta t$. Therefore, the type B relative standard uncertainty can be expressed as:

$$\frac{u_{B(a_{nano})}}{a_{nano}} = \sqrt{\left[\frac{u(V)}{V}\right]^2 + \left[\frac{u(\varepsilon)}{\varepsilon}\right]^2 + \left[\frac{u(l)}{l}\right]^2 + \left[\frac{u(m_{IONPs})}{m_{IONPs}}\right]^2 + \left[\frac{u\left(\frac{\Delta A}{\Delta t}\right)}{\frac{\Delta A}{\Delta t}}\right]^2} \quad \text{(B.5)}$$

B.4.2.2 Relative uncertainty of the total volume of reaction solution, $u(V)/V$

Since the reaction volume of IONPs, TMB solution and 30 % H_2O_2 was 100 µl, 100 µl and 200 µl, respectively, which is much smaller than that of Solution 1 (2 000 µl), the uncertainties generated by them are insignificant and can be ignored. Therefore, the source of $u(V)$ is the adjustable pipette (range 100 µl to 1 000 µl) and is provided by the pipette manufacturer. The relative uncertainty of V is given by

$$\frac{u(V_{1\ 000})}{V} = 0,002$$