
**Water quality — Biochemical and
physiological measurements on fish —
Part 2:
Determination of
ethoxyresorufin-O-deethylase (EROD)**

iTeh STANDARD PREVIEW
*Qualité de l'eau — Mesurages biochimiques et physiologiques sur
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Partie 2: Dosage de l'éthoxyrésorufine-O-dééthylase (EROD)*

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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 2.

The main task of technical committees is to prepare International Standards. 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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
- an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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

ISO/TS 23893-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 23893 consists of the following parts, under the general title *Water quality — Biochemical and physiological measurements on fish*:

- *Part 1: Sampling of fish, handling and preservation of samples*
- *Part 2: Determination of ethoxyresorufin-O-deethylase (EROD) [Technical Specification]*

Introduction

The measurement of pollution biomarkers in fish, such as the measurement of biotransformation enzyme activities, is likely to provide information about exposure levels, bioavailability and the early biological effects of substances present in aquatic ecosystems. The measurement of the EROD enzyme activity allows the diagnosis of the exposure of fish to inducers of the P450 1A cytochrome, such as certain polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and dioxins. A large amount of research work bears witness to the extent of the studies conducted (see Bibliography).

An induction of EROD activity reflects the presence of inducers such as those mentioned above. On the other hand, the absence of induction does not necessarily reflect the absence of exposure of the fish to organic contaminants, account being taken of the inhibition phenomena of the EROD induction of possible modification of the bioavailability of the inducers or of low exposure concentrations.

The application of a standardised reference method is strongly advised within a monitoring programme. The intercalibration exercises on the measurement of the EROD enzyme activity undertaken since 1991 have revealed multiple sources of errors, which are very easy to avoid (dilution of resorufin, determination of proteins, calculation of the enzyme activity, etc.) once laboratories have become familiar with the analysis of enzyme activities and the possible error factors.

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Water quality — Biochemical and physiological measurements on fish —

Part 2: Determination of ethoxyresorufin-O-deethylase (EROD)

1 Scope

This part of ISO 23893 specifies a method for measuring the ethoxyresorufin-O-deethylase (EROD) enzyme activity on a post-mitochondrial fraction of fish liver homogenate (subcellular fraction in which the EROD activity is located) employing a cell or microplate fluorimetric method.

It applies to fish that are sampled in their natural environment (fresh water or salt water) or exposed to substances or effluents in a laboratory.

This method is applicable for EROD values greater than or equal to 1 pmol/(min·mg) of proteins. A higher sensitivity may be achieved by using a cell (test tube) procedure.

2 Normative references

[ISO/TS 23893-2:2007](https://standards.iteh.ai/catalog/standards/sist/730eff44-462e-45a2-b0ba-789ef894152c/iso-ts-23893-2-2007)

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The following referenced documents are indispensable for the application 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 23893-1, *Water quality — Biochemical and physiological measurements on fish — Part 1: Sampling of fish, handling and preservation of samples*

3 Principle

Samples of fish are collected and dissected as described in ISO 23893-1 to obtain pieces of liver. Homogenates of fish liver are prepared by crushing (homogenisation) and the supernatant S9 fraction recovered by centrifugation. The EROD enzyme activity in the S9 fractions is determined by measurement of the increase in fluorescence due to the transformation of the 7-ethoxyresorufin into resorufin. The fluorescence is reported in quantities of resorufin by means of a calibration range (external calibration of resorufin or of rhodamine B). The EROD activity is related to the quantity of proteins in the S9 fraction.

4 Test environment

All of the tests and handling operations with the S9 fraction shall be carried out at a temperature close to 4 °C (e.g. handling in crushed ice), except the enzyme reaction which shall be performed at 20 °C ± 2 °C.

5 Reagents

Unless otherwise specified, use only reagents of recognised analytical grade.

5.1 Ultra pure water, having a conductivity below 1 µS/cm.

5.2 Potassium chloride, 150 mmol/l solution.

Dissolve 11,2 g of KCl (relative molecular mass 74,6) in 1 l of water (5.1). This solution is stable for 6 months at a temperature of 4 °C ± 3 °C.

5.3 Phosphate buffer, 100 mmol/l; pH 7,8 ± 0,1.

The ionic composition and pH can affect the EROD activity, and optimal conditions may vary between species. The following buffer is expected to provide measurable and comparable values of EROD activity for most species of fish.

Prepare the following two solutions, A and B:

- Solution A: dissolve 17,4 g of K₂HPO₄ (relative molecular mass 174,2 as the anhydrate) in 1 l of water (5.1);
- Solution B: dissolve 13,6 g of KH₂PO₄ (relative molecular mass 136,1 as the anhydrate) in 1 l of water (5.1).

Adjust solution A to pH 7,8 ± 0,1 with solution B.

This solution is stable for 6 months at a temperature of 4 °C ± 3 °C.

5.4 Glycerol (20 %) in phosphate buffer solution.

Add a mass fraction of 20 % of glycerol (C₃H₈O₃, relative molecular mass 92,1) to the phosphate buffer (5.3). The final concentrations are then 20 % glycerol and 100 mmol/l phosphate.

5.5 Resorufin (108 mg/l) stock solution.

Dissolve in the dark, shaking for 2 h, about 10,8 mg of resorufin (sodium salt C₁₂H₆NNaO₃; relative molecular mass 235,2) in 100 ml of dimethylsulfoxide (DMSO). Read the optical density at 572 nm on the spectrophotometer. Calculate the exact resorufin concentration, *c*, in millimoles per litre, using Equation (1):

$$c = \frac{D}{\epsilon l} \quad (1)$$

where

D is the optical density (corresponding to the absorbance wavenumber in cm⁻¹);

ε is the molar extinction coefficient {for resorufin, values of *ε* = 73,2 (mmol/l)⁻¹ cm⁻¹ at 572 nm (Reference [17]) and 54,0 ± 1,1 (mmol/l)⁻¹ cm⁻¹ (Reference [36]) have been reported};

l is the optical pathlength, in centimetres.

Prepare this solution at the time of determination and store aliquots of this solution frozen at -20 °C and sheltered from the light. These aliquots can be stored for 6 months.

NOTE Resorufin is very unstable under daylight conditions.

5.6 Resorufin working solution.

Dilute the stock solution (5.5) with DMSO to obtain approximately 10 ml of 11,5 µmol/l working solution. Prepare this solution at the time of determination.

5.7 Rhodamine B standard solution.

Dissolve 25 mg of rhodamine B ($C_{28}H_{30}N_2O_3$; relative molecular mass 442,55) in 250 ml of ethylene glycol monomethyl ether. Dilute this solution with ethylene glycol monomethyl ether, so as to obtain a standard 0,1 $\mu\text{mol/l}$ solution to be stored in aliquots. This solution remains stable for 6 months in the dark and at a temperature of $4\text{ }^\circ\text{C} \pm 3\text{ }^\circ\text{C}$.

5.8 Nicotinamide adenine dinucleotide phosphate (NADPH).

For the microplate method, dissolve 19,2 mg of β -NADPH ($C_{21}H_{26}N_7Na_4O_{17}P_3$; relative molecular mass 833,35) in 2 ml of water (5.1) to obtain a concentration of 10 mmol/l.

Prepare this solution at the time of determination and keep it sheltered from light in an ice bath.

NOTE For the cell (test tube) method, 41,7 mg of NADPH are dissolved in 1 ml of water (5.1) to obtain a concentration of 50 mmol/l.

5.9 7-Ethoxyresorufin stock solution

Prepare a stock solution of concentrated 7-ethoxyresorufin ($C_{12}H_6NaO_3$; relative molecular mass 235,17), e.g. 5 mg/ml, in DMSO. Store this solution in the dark at room temperature for a maximum of 1 year.

5.10 7-Ethoxyresorufin (46 $\mu\text{mol/l}$) working solution

Measure the exact concentration of the stock solution (5.9) by spectrophotometry at 482 nm using Equation (1). At 482 nm, the molar absorption coefficient is $2,25 \times 10^4\text{ (mol/l)}^{-1}\text{ cm}^{-1}$.

Dilute the stock solution (5.9) with DMSO in order to obtain a 46 $\mu\text{mol/l}$ working solution for the microplate method. Prepare this solution at the time of determination.

NOTE A 400 $\mu\text{mol/l}$ working solution is used for the cell (test tube) method.
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5.11 β -Naphthoflavone dissolved in peanut oil

For the injection of a dose of β -naphthoflavone ($C_{19}H_{12}O_2$, relative molecular mass 272,3) into the fish at a mass per body mass fraction of 50 mg/kg (injection of 10 μl of solution into the oil per gram of fish), prepare a solution of β -naphthoflavone in peanut oil at a concentration of 5 mg/ml. This solution is shaken and brought up to a temperature of $45\text{ }^\circ\text{C} \pm 5\text{ }^\circ\text{C}$ using a water bath in order to improve the homogeneity.

NOTE A dose even 10-fold lower than 50 mg/kg can be sufficient for EROD induction. It is possible, therefore, that the exact dose to be used is of little importance.

6 Apparatus

Usual laboratory and dissection equipment and in particular the following.

6.1 Cryogenic tubes.**6.2 Liquid nitrogen container or freezer, set at a temperature below $-70\text{ }^\circ\text{C}$.****6.3 Cell or microplate spectrofluorimeter, for 96-well microplates.**

NOTE The use of white opaque microplates allows a significant reduction of the fluorimetric background noise.

6.4 Centrifuge.**6.5 Homogeniser, of Potter Elvehjem or equivalent type.**

6.6 pH meter.

6.7 Spectrophotometer.

7 Sampling and preparation of samples

7.1 Sampling of fish

Sampling should be carried out in the natural environment by fishing or in a laboratory on fish exposed to substances or effluents as specified in ISO 23893-1.

Sample at least 10 fish of the same species and sex and of uniform size from each group to be examined for EROD activity.

Do not take samples during the spawning season because the habitat of the fish and their physiological activities may be modified by sexual activity.

Taking into account the factors likely to influence EROD activity, the following conditions shall be determined and recorded in the test report:

- a) water temperature;
- b) a general description of the health condition of each fish (sex, length, body mass, liver mass, gonad mass, presence of external and internal injuries) — this is usually reported in connection with sampling as described in ISO 23893-1.

Depending on the objectives of the study, ensure that the control fish (from the reference location or laboratory group) are taken from an environment of satisfactory ecological quality. Handle the control fish and their samples in the same manner as those from the examined or experimentally treated groups, except for exposure to the substance(s) of concern.

7.2 Killing of fish and dissection

After fishing or on completion of exposure, the fish are killed and the livers dissected one by one on removal from the water as described in ISO 23893-1. Sample a piece of less than 1 g from the same part of the liver from each fish as soon as possible after killing it. Be careful not to contaminate the liver with bile during dissection.

7.3 Storage

If the determination cannot take place on the day of sampling, the samples of liver shall be frozen immediately to below -70°C , for instance by using liquid nitrogen or dry ice. The samples can thereafter be stored for 3 months in liquid nitrogen or at a temperature below -70°C (6.2).

If measurements of EROD are to be made on the day of sampling, then the preparatory step (8.1) shall be started within 1 h and the liver samples shall be stored at below 4°C .

8 Procedure

8.1 Preparation of the fractions

Prior to conducting the determination, wash the liver sample in potassium chloride solution (5.2) and then homogenise it in 4 ml of phosphate buffer (5.3) by crushing in a homogeniser (6.5). If smaller (or bigger) liver samples are used, then the volume of the buffer should be reduced (or increased) to keep the 1 g:4 ml tissue:buffer ratio constant. Perform this operation at a temperature of about 4°C by maintaining the

phosphate buffer at that temperature. Crushing shall occur within 0,5 min and 2 min, and the crushing conditions and time shall be identical for each sample. Adequate crushing results from five to 10 strokes for most liver samples.

Centrifuge the homogenates at 10 000g (about 90 000 m/s²), at a temperature of 4 °C ± 3 °C for 10 min to 20 min. Recover the supernatant liquid (S9 fraction) and store it at a temperature of 4 °C ± 3 °C. Perform the enzyme determination within 1 h of supernatant recovery.

NOTE It is possible to freeze the liver homogenates rapidly in cryogenic tubes (6.1) to below -70 °C, using for example liquid nitrogen, under the conditions given in 7.3 provided that the liver samples (7.3) have not previously been frozen. In the case of prior freezing, the liver samples shall be homogenised beforehand as indicated above, but using a glycerol in phosphate buffer (5.4).

8.2 Determination of protein

For each S9 fraction, carry out a protein determination according to Reference [22] or Reference [2], for which methods commercial test kits are readily available. The concentration of protein standard [bovine serum albumin (BSA)] shall be checked using a spectrophotometer if in-house standards are used.

8.3 Determination of the EROD activity

8.3.1 Calibration

8.3.1.1 General

Carry out a calibration taking a resorufin working solution (5.6) or rhodamine B standard solution (5.7) as a basis. The fluorescence of rhodamine B is 1,2 times higher than that of resorufin at equivalent molar concentrations.

8.3.1.2 Calibration with resorufin

The linearity of the calibration curve shall be determined; samples should be measured in the linear part of the curve. Highly active samples have to be diluted to achieve this. The linear part of the curve is dependent on the apparatus used and its settings.

Establish the range by dilution of the resorufin working solution (5.6) with DMSO at the following dilution factors: 1; 5; 10; 50; 100.

Prepare the reaction mixture according to 8.3.2.1, replacing 7-ethoxyresorufin by resorufin and the S9 fraction by a protein solution (BSA or protein extract of the fish species under study). A concentration of protein which is as similar as practically possible to that of the S9 fractions being analysed should be used.

Measure the fluorescence according to 8.3.2.2. The measured fluorescence is proportional to the resorufin concentration. Ensure that only the linear part of the standard curve is used for quantification.

8.3.1.3 Calibration with rhodamine B

Introduce the rhodamine B standard solution (5.7) into the cell or into 3 wells of the microplate depending on the spectrofluorimeter (6.3) being used.

NOTE Taking into account the rhodamine B/resorufin intercalibration (under reaction mixture conditions), the rhodamine B solution is used as such as a standard. It is not necessary to add any reagent.

Measure the fluorescence according to 8.3.2.2. This fluorescence, divided by 1,2, is equal to the fluorescence of a resorufin solution having a concentration of 0,1 µmol/l.