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Kakovost vode - Biokemijske in fiziološke meritve v ribah - 2. del: Določevanje etoksiresorufin-O-deetilaze (EROD)

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

Qualité de l'eau - Mesurages biochimiques et physiologiques sur poisson - Partie 2: Dosage de l'éthoxyrésorufine-O-dééthylase (EROD)

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Water quality — Biochemical and physiological measurements on fish —

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

Qualité de l'eau — Mesurages biochimiques et physiologiques sur poisson —

Partie 2: Dosage de l'éthoxyrésorufine-O-dééthylase (EROD)

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Foreword

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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:

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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order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn.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again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.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.

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

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 K_2HPO_4 (relative molecular mass 174,2 as the anhydrate) in 1 l of water (5.1);

— Solution B: dissolve 13,6 g of KH_2PO_4 (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 ($C_3H_8O_3$; 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 $C_{12}H_6NNaO_3$; 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):

$$c = \frac{D}{\varepsilon l} \tag{1}$$

where

D is the optical density (corresponding to the absorbance wavenumber in cm^{-1});

ε is the molar extinction coefficient {for resorufin, values of $\varepsilon = 73{,}2$ $(mmol/l)^{-1}$ 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 µmol/l solution to be stored in aliquots. This solution remains stable for 6 months in the dark and at a temperature of 4 °C ± 3 °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 µmol/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 $2{,}25 \times 10^4$ $(mol/l)^{-1}$ cm^{-1}.

Dilute the stock solution (5.9) with DMSO in order to obtain a 46 µmol/l working solution for the microplate method. Prepare this solution at the time of determination.

NOTE A 400 µmol/l working solution is used for the cell (test tube) method.

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 °C ± 5 °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 °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.