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Foodstuffs — Determination of okadaic acid in mussels — HPLC method with solid phase extraction clean-up, derivatization and fluorimetric detection

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National foreword

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Foodstuffs - Determination of okadaic acid in mussels - HPLC method with solid phase extraction clean-up, derivatization and fluorimetric detection

Produits alimentaires - Dosage de l'acide okadaïque dans les moules - Méthode par CLHP avec purification par extraction sur phase solide, dérivation et détection fluorimétrique

Lebensmittel - Bestimmung von Okadasäure in Muscheln - HPLC-Verfahren mit Reinigung durch Festphasenextraktion, Derivatisierung und fluorimetrischer Bestimmung

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Foreword

This document (EN 14524:2004) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2005, and conflicting national standards shall be withdrawn at the latest by February 2005.

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WARNING — The use of this standard can involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This document specifies a method for the quantitative determination of the content of okadaic acid in mussels and mussel products. The content of okadaic acid is determined as free extractable acid of mussel hepatopancreas. Okadaic acid, a fat-soluble toxin from dinophysis algae, is a main component of dinophysis toxins.

The method has been validated in an interlaboratory study according to ISO general principles on assessing accuracy of measurement methods and results. The limit of determination of this method (signal/noise = 10) is 100 µg/kg for okadaic acid in mussel hepatopancreas. The method has been validated for okadaic acid in cooked mussels at levels of 441 µg/kg to 1 467 µg/kg.

Laboratory experiences have shown that this method can also be used to determine other dinophysis toxins, e.g. dinophysis toxins 1, 2 and 3 (DTX-1, DTX-2 and DTX-3), see [1], [2], [3], [4], [5] and [6]

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.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987).*

3 Principle

Mussel hepatopancreas is separated from cooked mussels and homogenized. The toxins are extracted using methanol, derivatized with 9-anthryldiazomethane and the extract is cleaned up using a solid phase extraction (SPE) cartridge with silica gel. Chromatographic separation is performed on a HPLC-gradient system, followed by fluorescence measurement of the 9-anthryldiazomethyl ester of the toxin at 412 nm with excitation at 365 nm. Determination of okadaic acid is performed using external standards.

4 Reagents and materials

4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water according to grade 1 of EN ISO 3696.

4.2 Sodium sulfate, anhydrous

4.3 Nitrogen gas, volume fraction $\varphi \geq 99{,}999$ %

4.4 Acetone

4.5 Acetonitrile

4.6 Chloroform, stabilised with 2-methyl-2-butene

4.7 Ethyl acetate

4.8 Dichloromethane, stabilised with 2-methyl-2-butene

4.9 Methanol

4.10 Methanol solution, $\varphi = 80$ %

4.11 n-hexane

4.12 9-anthryldiazomethane (ADAM)

Weigh solid ADAM in discrete portions (e.g. 1 mg), store at ≤ -18 °C and dissolve in ethyl acetate just before use.

4.13 Derivatization solution, mass concentration $\rho = 1{,}5$ g/l in ethyl acetate (4.7)

4.14 Solvent mixture of n-hexane and dichloromethane

Mix 1 part per volume of n-hexane (4.11) with 1 part per volume of dichloromethane (4.8).

4.15 Solvent mixture of dichloromethane and acetone

Mix 9 parts per volume of dichloromethane (4.8) with 1 part per volume of acetone (4.4).

4.16 Solvent mixture of acetonitrile and dichloromethane

Mix 5 parts per volume of acetonitrile (4.5) with 1 part per volume of dichloromethane (4.8).

4.17 Mixture of methanol and ethyl acetate

Mix 1 part per volume of methanol (4.9) with 1 part per volume of ethyl acetate (4.7).

4.18 HPLC mobile phase solvent A

Mix 800 ml of methanol (4.9) with 200 ml of ethyl acetate (4.7).

4.19 HPLC mobile phase solvent B

Mix 700 ml of acetonitrile (4.5) with 300 ml of water.

4.20 Okadaic acid

WARNING — Okadaic acid is toxic. Gloves and safety glasses shall be worn at all times, and all standard and sample preparation stages shall be carried out in a fume cupboard.

4.21 Okadaic acid stock solution in methanol

Dilute okadaic acid with methanol (4.9) to a concentration of 1,0 µg/ml. This solution can be stored at -18 °C for at least 6 months.

4.22 Okadaic acid methylanthrylester

NOTE For the availability of this substance, contact your National Standardization Institute. It can for example be obtained from SIGMA-ALDRICH [1)].

4.23 Solution of okadaic acid methylanthrylester in methanol

Dilute okadaic acid methylanthrylester with methanol (4.9) to a concentration of 0,1 µg/ml. This corresponds to 1 600 µg/kg mussel hepatopancreas (in samples). Store the solution in a brown bottle. At ≤ -18 °C the solution is stable for at least 6 months.

4.24 Mussels free of okadaic acid and related compounds

5 Apparatus

5.1 General

Usual laboratory apparatus, and in particular:

5.2 Instrument for separation of hepatopancreas, e.g. scalpel

5.3 Blender or homogenizer

5.4 Centrifuge, capable of a centrifugal force up to 3 000 *g,* with suitable tubes, e.g. 20 ml

5.5 Pear shaped flask, e.g. 10 ml and 25 ml capacity

5.6 Glass sample tubes with stoppers, e.g. 10 ml capacity

5.7 One-mark volumetric flask, 20 ml capacity

5.8 Analytical balance, accurate to the nearest 0,1 mg

5.9 Solid phase extraction (SPE) cartridges, (3 ml) filled with 650 mg silica gel with solvent reservoir (approx. 10 ml), commercially available

5.10 Suitable pipettes of various volumina

1) This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN. Equivalent products may be used if they can be shown to lead to the same results.

5.11 Apparatus for solvent removal using nitrogen gas

5.12 HPLC apparatus, comprising the following

5.12.1 HPLC pump, for gradient elution at e.g. 0,8 ml/min constant flow rate

5.12.2 Injection system, capable to deliver an injection volume of e.g. 20 µl

5.12.3 Analytical reverse-phase separating column which ensures a baseline resolution of the peaks of the derivatives from all other peaks, for example:

— RP-C_{18}

— a length of 250 mm;

— an inner diameter of 4 mm;

— a stationary phase with particle size of 5 µm.

Columns of other dimensions may also be used.

5.12.4 Fluorescence detector, fitted with a flow cell and set at 365 nm (excitation) and 412 nm (emission).

Specific properties of the detector shall be regarded.

5.12.5 Data system

5.13 Rotary evaporator, with evaporation flask and water bath

5.14 Laboratory shaker for tubes

5.15 Ultrasonic bath

5.16 Glass funnel, with paper filter, 90 mm diameter

6 Sampling

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Samples shall be deep frozen until use or shall be extracted immediately.

NOTE Storage of mussel samples in frozen state at –18 °C has no negative influence on the result of this method.

7 Procedure

7.1 Preparation of test sample

Transfer fresh mussels into boiling water and cook for 10 min. Separate 20 g to 30 g of hepatopancreas material from approximately 1 kg of mussels (including the shells). The hepatopancreas is the green or brown part of the mussel. If only smaller sample amounts of hepatopancreas material are available, the whole material has to be prepared. Homogenize the hepatopancreas material with the homogenizer or blender (5.3) and start immediately with the preparation or otherwise store the homogenate at –18 °C.

7.2 Extraction procedure

Transfer 1,00 g ± 0,02 g of homogenate to a centrifuge tube (5.4) and stir with 9 ml of the methanol solution (4.10) using the homogenizer (5.3). Centrifuge the mixture at approximately 3 000 *g* for 5 min. Transfer the supernatant to a one-mark volumetric flask (5.7).

For a second extraction, add 9 ml of the methanol solution (4.10), stir and centrifuge the mixture and transfer the resulting supernatant to the volumetric flask (5.7). Fill the flask to the calibration mark with the methanol solution (4.10).

7.3 Removal of fat

Pipette 5,0 ml of the sample extract as prepared in 7.2 into a centrifuge tube (5.4) and mix with 4 ml of n-hexane (4.11). Centrifuge the mixture at approximately 3 000 *g* for 5 min. Remove and discard the hexane layer at the top.

Add another 4 ml of n-hexane (4.11), mix and centrifuge as described. Remove and discard the hexane layer at the top. Repeat this procedure a third time.

Add 2 ml of water and 4 ml of chloroform (4.6) to the remaining water layer. Stir the mixture and centrifuge at 3 000 *g* for 5 min. Remove the chloroform layer at the bottom and transfer it to a conical flask.

Again add 4 ml of chloroform, stir and centrifuge as described above. Remove the chloroform layer and add it to the chloroform of the first extraction. Dry the chloroform by adding approximately 1 g of sodium sulfate (4.2). Wait for 30 min and filter the chloroform using a glass funnel with paper filter (5.16). Collect the filtrate into a pear shaped flask (5.5). Wash the conical flask and the filter with additional 6 ml of chloroform. Remove the chloroform with a rotary evaporator (5.13) at approximately 40 °C under 250 hPa to dryness. Dissolve the residue in 10,0 ml of methanol (4.9).

This solution can be stored in a refrigerator for at least 24 h at 4 ^{o}C to 8 ^{o}C.

7.4 Derivatization with 9-anthryldiazomethane (ADAM)

Pipette 0,50 ml of the methanolic solution prepared in 7.3 into a sample tube (5.6). Remove the solvent using a gentle stream of nitrogen (4.3). Dissolve the residue in 50 µl of derivatization solution (4.13). Treat the mixture in an ultrasonic bath (5.15) for 2 min or with a shaker (5.14) for 1 min. Add 150 µl of methanol (4.9) and mix again with ultrasonic bath or shaker. The derivatization reaction is finished after 60 min at room temperature in the dark. Remove the solvent using a gentle stream of nitrogen (4.3).

NOTE Okadaic acid methylanthryl ester is decomposed by light and forms a substance which shows a peak with a retention time only a few minutes later than that of okadaic acid methylanthryl ester, if chromatographic conditions described in this document are used. Therefore, it is recommended to work under reduced light.

7.5 Clean up

For all steps of this procedure dropping speed shall not exceed 1 drop per second. Precondition the SPE cartridge (5.9) using 10 ml of n-hexane/dichloromethane (4.14). Dissolve the sample in 1 ml of n-hexane/dichloromethane and transfer the sample onto the preconditioned SPE cartridge. Wash the sample tube with 1 ml of n-hexane/dichloromethane and transfer the washing solution on the cartridge. Wash the cartridge with 2 ml of n-hexane/dichloromethane (4.14) and 8 ml of dichloromethane/acetone (4.15). Elute the toxin with 6 ml of acetonitrile/dichloromethane (4.16) and collect in a suitable (e.g. 10 ml) pear shaped flask (5.5). Remove the solvent with the rotary evaporator (5.13) at 40 °C under 250 hPa until about 100 µl is left and finally dry the sample using nitrogen.

Dissolve the residue in 200 µl of methanol/ethyl acetate (4.17) and transfer the solution to a HPLC sampling vial.

NOTE It is important, that the various washing and elution solutions run exactly to the surface of the silica gel. The cartridge should not be allowed to run dry.

7.6 Preparation of the standard solutions

Prepare a toxin free mussel hepatopancreas extract from DSP uncontaminated mussels (4.24) in accordance with 7.1, 7.2 and 7.3. Prepare standard solutions by adding suitable volumina of okadaic stock solutions (4.21) to 0,5 ml of the toxin free mussel hepatopancreas extract in accordance with Table 1. Derivatize the standard solutions in accordance with the procedure described for test samples in 7.4. Purify the derivatized standard solutions in accordance with 7.5.

Table 1 — Preparation of standard solutions in toxin-free hepatopancreas extract

Standard solution number	**Toxin free sample extract** (ml)	**Okadaic stock solution (4.21)** (µl)	**Corresponding content of okadaic acid in mussel hepatopancreas** (µg/kg)
1	0,5	5	400
2	0,5	10	800
3	0,5	15	1 200
4	0,5	20	1 600
5	0,5	25	2 000

Inject 20 µl of each standard solution prepared in Table 1 into the HPLC apparatus.

7.7 HPLC operating conditions

When a column meeting the specifications in 5.12.3 and the HPLC mobile phases specified in 4.18 and 4.19 were used, the following settings were found to be appropriate for the analysis of okadaic acid.

— total flow rate HPLC mobile phase: 0,8 ml/min;

— time programme according to Table 2;

— fluorescence detection: excitation wavelength of 365 nm and emission wavelength of 412 nm;

— injection volume: 20 µl.

Table 2 — Time programme for HPLC mobile phase

Time (min)	**Part of mobile phase A (4.18) of total flow** (%)	**Part of mobile phase B (4.19) of total flow in** (%)
0,1	20	80
50	20	80
51	90 [a]	10 [a]
81	90 [a]	10 [a]
82	20	80
110	STOP	
[a] This mixture is used after each analysis to wash out strongly retarding substances.		

Depending on the HPLC apparatus and chromatography column, the retention time of okadaic acid varies between 20 min and 30 min. A suitable concentration of okadaic acid methylanthrylester should be used for optimization of the chromatography, e.g. 0,1 µg/ml, corresponding to 1 600 µg/kg hepatopancreas.

7.8 Calibration graph

Prepare a calibration graph at the beginning of the analysis. Plot the peak response against the corresponding content of okadaic acid in mussel hepatopancreas in µg/kg. Ensure that the linearity check is carried out (see [7]).

7.9 Identification

Identify okadaic acid by comparing the retention time of the sample with that of the standard substance. Sometimes it can be necessary to identify the okadaic acid peak by injection of a sample spiked with standard solution. Typical chromatograms are given in Annex A.

7.10 Determination

Carry out the determination by external standards. Integrate the peak area or determine the peak height, and compare the results with the calibration graph. If the okadaic acid methylanthrylester response is outside the calibration graph, adjust the amount of sample injected by concentrating or diluting the sample test solution.

8 Precision

8.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are given in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given in Annex B.

8.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible interval will exceed the repeatability limit *r* in not more than 5 % of the cases.

The values are:

$\bar{x}$ = 441 µg/kg r = 110 µg/kg (uncontaminated mussel material with 450 µg/kg okadaic acid added)

$\bar{x}$ = 819 µg/kg r = 226 µg/kg (contaminated mussel material)

$\bar{x}$ = 1 467 µg/kg r = 365 µg/kg (uncontaminated mussel material with 1 500 µg/kg okadaic acid added)

8.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.

The values are:

$\bar{x}$ = 441 µg/kg R = 151 µg/kg (uncontaminated mussel material with 450 µg/kg okadaic acid added)

$\bar{x}$ = 819 µg/kg R = 278 µg/kg (contaminated mussel material)

$\bar{x}$ = 1 467 µg/kg R = 492 µg/kg (uncontaminated mussel material with 1 500 µg/kg okadaic acid added)

9 Test report

The test report shall contain the following data:

— all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

— reference to this document;

— date and type of sampling procedure (if known);

— date of receipt;

— date of test;

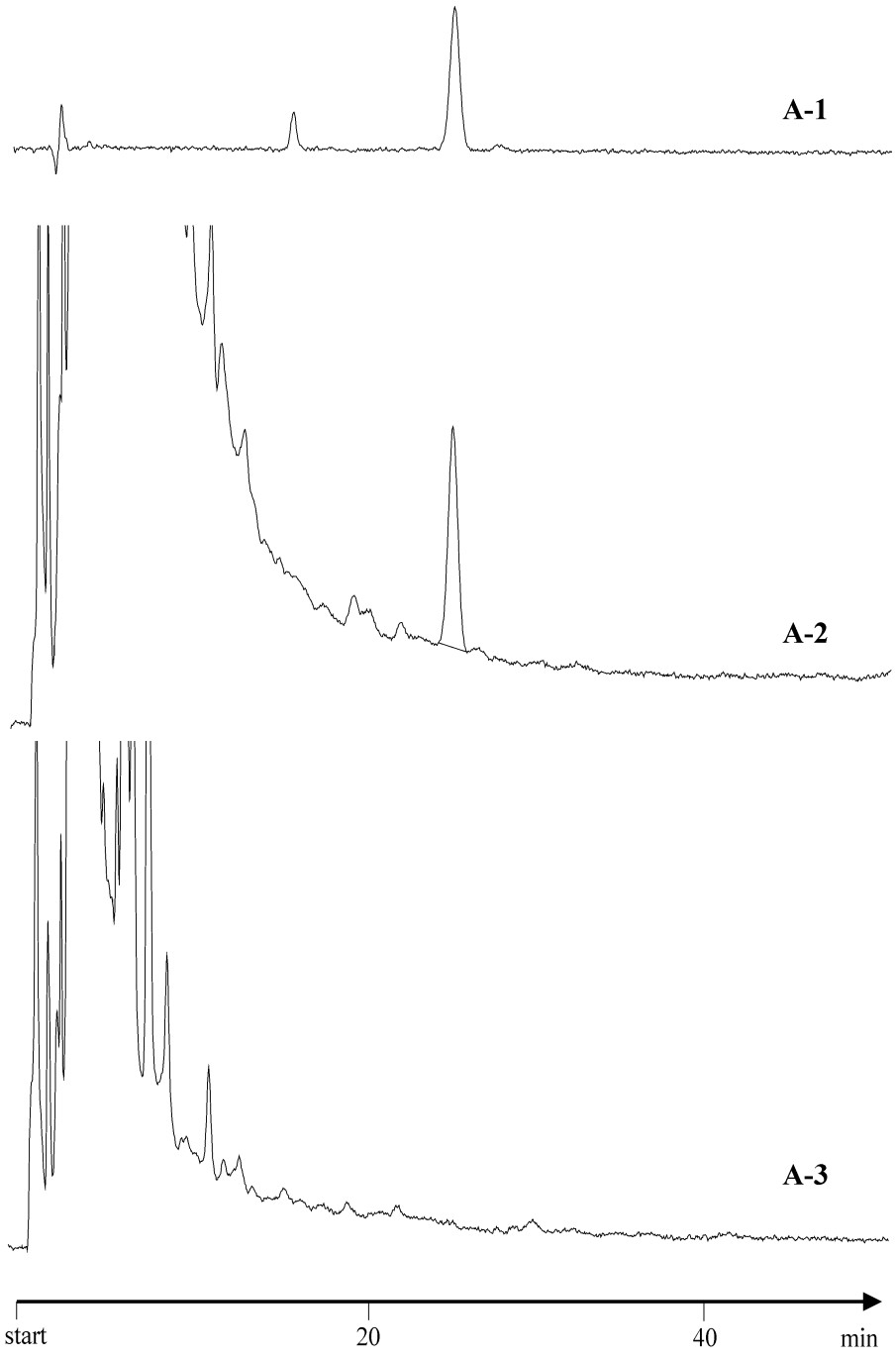
— test results and the units in which they have been expressed;

— any particular points observed in the course of the test;

— any operations not specified in the method or regarded as optional, which might have effected the results.

Annex A
(informative)

Typical chromatogram



Key

A-1 Typical chromatogram of okadaic acid methylanthrylester standard

A-2 Typical chromatogram of okadaic acid methylanthrylester in mussel hepatopancreas (819 µg/kg)

A-3 Typical chromatogram of okadaic acid free mussel hepatopancreas

Figure A1 — Typical chromatogram

Operating conditions for Figure A.1:

Injection volume: 20 µl

Column: Nucleosil 100-5 C_{18}, length of 250 mm, inner diameter of 4 mm, stationary phase particle size of 5 µm

Flow rate: 0,8 ml/min

Mobile phase: methanol/ethyl acetate and acetonitrile/water

Derivatization: with 9-anthryldiazomethane

Detection: fluorescence (λ_{ex} = 365 nm, λ_{em} = 412 nm)

Annex B
(informative)

Precision data

The following data were obtained in an interlaboratory study organized by the Federal laboratory for fish and fish products, Cuxhaven, Germany in accordance with ISO 5725-2, -4 and –6. The method used and described in this document is based on the work of the German paragraph 35 LMBG group "mussel toxins" at the Federal German Institute of Consumer's Health and Veterinary Medicine ("BgVV"). The method was tested by 9 laboratories on homogenized mussel samples [8].

Table B.1 — Precision data

Sample	A [a]	B [b]	C [c]	D [d]
Year of interlaboratory test	2000	2000	2000	2001
Number of laboratories	9	9	9	9
Number of laboratories retained after eliminating outliers	9	9	9	9
Number of outliers (laboratories)	0	0	-	0
Number of accepted results	45	45	-	52
Mean value $\bar{x}$, µg/kg	819	1 467	0	440,7
Repeatability standard deviation s_r, µg/kg	80	129	0	38,8
Repeatability relative standard deviation RSD_r, %	9,76	8,79	0	8,89
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	226	365	0	109,7
Reproducibility standard deviation s_R, µg/kg	98	174	0	53,3
Reproducibility relative standard deviation RSD_R, %	11,96	11,86	0	12,23
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	278	492	0	150,9
Recovery, %	102 [e]	101 [e]	-	97 [e]

a okadaic acid contaminated mussel material from Wadden sea area, harvested October 1995; stored at –20 °C at VUA Cuxhaven since Oct. 1995. Determination in 1995: 880 µg okadaic acid per kg material; result of homogeneity study (Dec. 1999): 800 µg/kg

b uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999; addition of 1 500 µg/kg okadaic acid per kg material; result of homogeneity study (Dec. 1999): 1 450 µg/kg

c uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999

d uncontaminated mussel material from Wadden sea area, harvested July 1999, stored at –20 °C at VUA Cuxhaven since July 1999; addition of 450 µg/kg okadaic acid per kilogram of material; result of homogeneity study (Dec. 2000): 453 µg/kg

e based on homogeneity study result

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