VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF DOMOIC ACID IN SHELLFISH AND FINFISH BY RP-HPLC USING UV DETECTION

INDEX

I REPORT ON THE FIRST PHASE OF THE VALIDATION STUDY OF THE DOMOIC ACID DETERMINATION BY HPLC-UV (METHANOLIC EXTRACTION) 3
1. INTRODUCTION 3
2. PARTICIPANTS 3
3. MATERIALS 4
4. METHODS 5
OUTCOME OF THE STUDY (First phase) 6
1. ABILITY OF THE LABORATORIES TO PRODUCE ACCEPTABLE CALIBRATION CURVES. ... 6
2. DETECTION LIMIT 7
3. ABILITY OF THE LABORATORIES TO QUANTIFY DOMOIC ACID IN SOLUTION 8
4. RECOVERY 10
PRELIMINARY CONCLUSIONS OF THE STUDY 11
5. REPEATIBILITY AND REPRODUCIBILITY 12
II REPORT ON THE SECOND PHASE OF THE VALIDATION STUDY OF THE DOMOIC ACID DETERMINATION BY HPLC-UV (METHANOLIC EXTRACTION) 15
1. INTRODUCTION 15
2. PARTICIPANTS 15
3. MATERIALS 16
List of materials: 16
Homogeneity studies: 17
Stability studies: 17
4. METHODS 18
OUTCOME OF THE STUDY (Second phase) 19
5. BLANK MATERIALS 21
6. PRECISION: REPEATABILITY AND REPRODUCIBILITY 22
7. CONCLUSIONS 23

I REPORT ON THE FIRST PHASE OF THE VALIDATION STUDY OF THE DOMOIC ACID DETERMINATION BY HPLC-UV (METHANOLIC EXTRACTION)

1. INTRODUCTION

The purpose of the first phase of the validation study of the Domoic acid determination by HPLC- UV (methanol extraction) was to evaluate:

- The ability of the laboratories to produce acceptable calibration curves.
- Detection limit.
- The ability of the laboratories to quantify DA in solution.
- Recovery efficiencies from CRM from the NRC (MUS-1B).

2. PARTICIPANTS

AUSTRIA

Egon Hellwig

Austrian Agency for Health and Food Safety Food Control and Research Vienna

BELGIUM

Chrysostome Nsengiyumva

Institut d 'Hygiene et d ' Épidemiologie Section Denrées Alimentaries

DENMARK

Kevin Jorgensen/Lene Bai Jensen

Danish Veterinary and Food administration Institute of Food Research and Nutrition

FINLAND

Karin Blom berg

Finnish Customs Laboratory

FRANCE

Sophie Krys, Elisabeth Gleizes

Agence Fran9aise de Sécurité Sanitaire des Aliments

GERMANY

Reinhard Tiebach

Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinarmedizin

GREECE

Ignatia Kaniou

Center of Veterinary Institutions of Thessaloniki

IRELAND

Terry McMahon/Philipp Hess

Marine Institute Fisheries Research Centre

ITALY

Anna Milandri, Alfiero Ceredi

Centro Ricerche Marine

THE NETHERLANDS

H.P.Van Egmond, H.J. Van Den Top

National Institute of Public Health and the Environment (RIVM)

NORWAY

Lai Nguyen, Tore Aune

Department of Pharmacology, Microbiology and Food Hygiene Norwegian College of Veterinary Medicine

PORTUGAL

Paulo Vale

Instituto de Investigacion das Pescas e do Mar

UNITED KINGDOM

Elizabeth A. Smith

FRS Marine Laboratory

SPAIN

F Arévalo, C. Salgado, J. Correa

Centro de Control del Medio Marino (CCMM).

Pedro A. Burdaspal, Teresa M ª Legarda

Centro Nacional de Alimentación (CNA)

Coordination:

Aurea Míguez

EU-CRL on Marine Biotoxins

3. MATERIALS

Each laboratory was supplied with two ampoules (A and B) containing approx. 0.5 ml of DA solutions in acetonitrile: water (1: 9), of unknown concentration.
Solution A was prepared by taking 250 µL of DACS- l D and diluting up to 25 ml, yielding a solution of a nominal concentration of 0.88 µg /ml
Solution B was prepared by taking 1 mi of DACS-l D and diluting up to 25 ml yielding a solution of a nominal concentration of 3.51 µg /ml.
The solutions were ampoule D (2-ml amber ampoules) under nitrogen in approx. 0.5 ml portions. After preparation, the ampoules were stored in a refrigerator at 2-8 ºC.
Homogeneity was checked by analyzing five randomly selected ampoules for each solution. The coefficients of variation were 0.91 % for solution A and 0.19 % for solution B and therefore homogeneity was considered acceptable.
Samples were sent by courier on 18.02.02 and were received in good condition by the participants between 19 and 22 of February, with the exception of the Marine Institute (Ireland) that received one of the ampoules broken. Another set of ampoules was sent to the Marine Institute a few days after.
For the calibration curves, each laboratory was requested to use DACS-l D produced by the Institute for Marine Biosciences (IMB), National Research Council (Canada).

For the recovery experiments, each laboratory was requested to use MUS 1 B produced by the Institute for Marine Biosciences (IMB), National Research Council (Canada).

4. METHODS

As the purpose of this study is to validate a method, all the participants were asked to follow strictly protocol that had been previously discussed and agreed by the participants and the coordinator. Only the type and dimensions of the HPLC column and some chromatographic conditions (flow rate, injection volume, and percentage of acetonitrile) were left to the choice of the participants (see chromatographic conditions in Table I).

TABLE I

Lab.	Column	Dimensions	Mobil phase	Vol. Inj.	Ret. time	Flow ml/min	Temp.ºC	Separation DA/epiDA
1	Supelcosil LC-PAH	250x4 ,6mm,5µm	10%Acet/Water0, 1% TFA	20	23.2	1	30	No
2	Bondapak WaCl 8RP	300x3,9mm, 10	13%Acet/Water 8, 5% H3P04	20	10.2	1	Room temp	Yes
3	Vydac 201TP54 RPC18	250x4,6mm,5µm	l 1%Acet/Water0, 1% TFA	20	7.5	1	50	Yes
4	Vydac 20 ITP54 RPC 18	250x4,6mm,5µm	l 1%Acet/Water0, 1% TFA	20	9.6	1	Room temp	Yes
5	Vydac 201TP54 RPC 18	l 50x4,6mm,5µm	10%Acet/Water0, 1% TFA	20	8.8	1	40	Yes
6	Nucleosil I 00-5Cl 8	250x4mm,5µm	Gradient Acet/Water, formic acid	10	10.2	0.7	35	No
7	Symmetry WatersRPCl 8	250x4 ,6mm,5µm	10%Acet/Water0 , 1% TFA	20	14.2	1	40	Yes
8	Vydac 201TP 104 RPCl 8	250x4 ,6mm,5µm	10%Acet/Water0, 1% TFA	20	9.5	1	40	Yes
9	Phenomenex Jupiter C 18	250x4,6mm,5µm	10%Acet/Water0, 02% TFA	20	11.7	1	40	Yes
10	Phenomenex Luna C 18	150x3mm,5µm	10%Acet/Water0 , 1% acet.acid	20	7.4	0.5	45	No
11	LiChrospeher 1OORPC1 8	250x4,6mm	10%Acet/Water0, 1% TFA	20	16.6	1	40	Yes
12	Nucleosil 100-5C 18	125x3mm,5µm	10%Acet/Water0, 1% Form	5	7.4	0.45	40	Yes
13	Vydac RPCl 8	250x4,6mm,5µm	10%Acet/Water0, 1% TFA	10	6	1.5	40	Yes
14	LiChrospeher 1OORPC18	125x4mm,5µm	10%Acet/Water0 , 1% acet.acid	20	10	0.6	Room temp	No
15	Spherisorb Cl 8/0DS-2	l 50x4,6mm ,5µm	10%Acet/Water0, 1% TFA	20	16.5	1	30	yes

Participants were asked to carry out the calibration curve, the analysis of samples A and B, and the determination of the recovery in the same day.

OUTCOME OF THE STUDY (First phase)

1. ABILITY OF THE LABORATORIES TO PRODUCE ACCEPTABLE CALIBRATION CURVES.

Participants were asked to prepare:

1) A calibration curve of at least four points within the mass range of e.g. 0.2 µg/mL to 25 µg/mL by accurately diluting the standard solution DACS-1 D with acetonitrile:water (1:9). At least one of the points of the calibration series should be≤lµg/mL.

2) Linearity plots by using the following formula:

$$\% \, Yi / Xi = \frac{\frac{Yi}{Xi}}{\sum_{i=1}^{N} \left(\frac{Yi}{Xi} \right) / N} \bullet 100$$

Xi: mass calibration point (i = 1 ...N)
Yi: peak area calibration point (i = 1 ...N)
N: number of calibration points

Participants were asked to determine whether each individual % Yi / Xi fell between 100 ± 10%, and to eliminate each calibration point which did not fulfil this requirement. In case that more than one calibration point was eliminated, the calibration series should not be considered linear. Table II shows the maximum and minimum values of % Yi / Xi found by each participant

TABLE II

Lab.	r	% Y/X max	% Y/X min
1	0.9994	104.7	95.2
2	0.99998	106.0	92.2
3	0.9999	100.6	98.7
4	0.99995	102.5	96.2
5	0.9993	105.6	98.0
6	0.9991	110.0	86.0
7	0.999945	100.8	97.5
8	0.9997	107.3	90.5
9	0.99994	106.5	92.5
10	0.9999	101.2	98.3
11	1	101.1	97.5
12	0.9991	110.0	94.0
13	0.99669	107.1	90.3
14	0.99993	106.5	97.4
15	0.99999	101.3	97.9

All the participants produced acceptable calibration curves and only one from the 15 participants (Lab. 6) failed in meeting the acceptance criterion for the linearity plot.

2. DETECTION LIMIT

Detection limit was calculated as the concentration corresponding to a signal-to-noise ratio of 3, expressed in µg /ml. The results obtained by the participants are showed in Table III.

TABLE III

Lab.	**Vol. Inj. (µl)**	**Detection limit (ug/ml)**
1	20	0.03
2	20	0.01
3	20	0.01
4	20	0.009
5	20	0.023
6	10	0.1
7	20	0.02
8	20	0.1
9	20	0.0048
10	20	0.02
11	20	0.05
12	5	0.048
13	10	0.1
14	20	0.142
15	20	0.0079

Detection limit ranged from 0.0048 µg/mL to 0.142 µg/mL.

From the results, detection limit seems good enough for all the participants to detect and quantify DA concentrations below 1µg/ml and therefore, below the European regulatory limits of 20 µg/g (Directive 91/492/CEE) and 4.6 µg/g (EU Decision 2002/226/CEE).

3. ABILITY OF THE LABORATORIES TO QUANTIFY DOMOIC ACID IN SOLUTION

Statistical analysis and evaluation of the results:

As the main objective of this study was to evaluate the ability of the participants to quantify DA, the results were considered as if they were originated from a proficiency test. The statistical procedure selected for evaluation of the results was that recommended in the IUPAC/ISO/AOAC International Harmonized Protocol for Proficiency Testing of Chemical Analytical Laboratories, that uses the value of z score. This statistical treatment considers that the analytical results are normally distributed. **The statistics of a normal distribution mean that about 95% of data points will lie between a z-score of -2 and + 2. Performance is considered satisfactory if a participant's z- score lies within this range.**

Participant's z-scores were calculated as follows:

$$Z = (x - X) / SD$$

Where:

x = the participant's reported result

X = the assigned value

SD = standard deviation

The assigned value was calculated as the mean of the all data submitted. For the two solutions used in the study, the calculated assigned value was very close to the nominal value.

Table IV shows the results for solutions A and B and z score for all the participants and the mean value, SD and RSD % (relative standard deviation).

TABLE IV

Lab.	Solution A	Z score	Solution B	Z score
1	0.90	-0.11	3.50	0.19
2	0.91	0.16	3.33	-0.88
3	0.88	-0.08	3.6	0.95
4	0.88	-0.08	3.29	-1.16
5	0.82	-0.56	3.4	-0.41
6	1.12	1.84	3.45	-0.07
7	0.94	0.4	3.57	0.75
8	1.07	1.44	3.53	0.48
9	0.89	o	3.49	0.2
10	0.85	-0.32	3.32	-0.95
11	0.90	0.08	3.43	0.20
12	0.91	0.16	3.7	1.63
13	0.82	-0.56	3.44	-0.14
14	0.78	-0.88	3.34	-0.82
15	0.96	0.56	3.74	1.9
Mean	0.909		3.475	
SD	0.09		0.135	
RSD %	**9.9%**		**3.9%**	

All the laboratories met the z-score≤|2| (criterion).

Reproducibility expressed as RSD % ranged from 3.9% to 9.9%.

4. RECOVERY

For recovery experiments, each participant was requested to use MUS-lB.
Participants were asked to homogenate and accurately weigh two replicates of ca 4 g of the mussel homogenate (MUS-l B) and to carry out for each one of the replicates , the extraction and determination following the protocol.

Participants were asked to determine DA in each one of the two replicates with and without SAX cleanup step and to report both results . In the case that DA and epi-DA peaks were not resolved , participants were asked to report the total DA peak area found in the box corresponding to the sum of DA and epi-DA peak areas.

Data on recoveries for each replicate (Rl and R2) calculated on the basis of DA peak area and on the basis of the sum of DA and epi DA (SUM), without and with clean-up step and shown in Table V.

TABLE V

Lab.	R1/DA	R2/DA	R1/SUM	R2/SUM	R1/DA Clean up	R2/DA Clean up	R1/SUM Clean up	R2/SUM Clean up
1			100	104.1			92.2	95.9
2	95.5	93.1	93.l	90.7	91.4	93.4	90.3	91.8
3	100	100	99.5	99.2	90.3	97.2	89.5	96.4
4	95.l	96.8	96.2	97.9	93.4	89.2	92.2	88.2
5	111.1	108.3	113.3	110.3	95.3	93.3	96.9	89.7
6			90.2	93.l			78.6	80.4
7	101.1	100.7	102.7	98.9	78.6	76.2	78.4	75
8	96.4	95.2	97.4	95.8	70.6	87.6	70.20	85.9
9			97.7	101.1			84.6	97.6
10			90.1	88.7			90.2	89.8
1 1			97.2	96.8			85.7	85
12	104.4	101.9	104.6	101.8	89.4	90.6	89	90.3
13	95.7	89	95.l	88.l	87.2	86.9	86.8	86.5
14	102.7	105.5	100.2	103.3	94.7	93.3	93	91.8
15	83.9	86.5	82.6	84.6	84.7	80.8	83.5	80.8
Mean %	98.6	97.7	**97.3**	97	87.6	88.9	**86.7**	**88.3**
Min. %	83.9	86.5	**82.6**	84.6	70.6	76.2	**70.2**	75
Max. %	111.1	108.3	**113.3**	110.3	95.3	97.2	**96.9**	96.4

Recovery with Clean-up step is approx. 10% less than recovery without clean-u p step.

When the protocol was applied without clean-up step, 13 from the 15 laboratories obtained recoveries between 90 and 110% for the replicate 1 and 11 from the 15 for the replicate 2. The lowest value was 82.6 % and the highest was 113.3%.
When the protocol was applied with clean-up step, 12 from the 15 laboratories obtained recoveries between 80 and 100% for the replicate 1 and 14 from the 15 for the replicate 2. The lowest value was 70.2% and the highest was 96.9%.

Recovery is poorer when clean-up step is included in the procedure.

PRELIMINARY CONCLUSIONS OF THE STUDY

1) The 15 participants produced acceptable calibration curves and only one failed in meeting the acceptance criterion for the linearity plot.

2) Sensitivity achieved by all the participants is good enough to detect and quantify DA concentrations below 1µg/ml and therefore, below the regulatory limits of 20 µg/g (Directive 91/492/CEE) and 4.6 µg /g (EU Decision 2002/226/CEE).

3) Z-score values were satisfactory for all the participants for solution A (mean value 0.91µg/ml) and solution B (mean value 3.48 µg/mL). Reproducibility expressed as RSD% was 9.9% for the determination of the DA concentration of solution A and 3.9% for the determination of the DA concentration of solution B.

4) When the protocol was applied without clean-up step, 13 from the 15 laboratories obtained recoveries between 90 and 110% for the replicate 1 and 11 from the 15 for the replicate 2. The lowest value was 82.6% and the highest was 113.3%. When the protocol was applied with clean-up step, 12 from the 15 laboratories obtained recoveries between 80 and 100% for the replicate 1 and 14 from the 15 for the replicate 2. The lowest value was 70.2 % and the highest was 96.9%. Recovery is poorer when clean-up step is included in the procedure.

The results of this first phase of the validation study are satisfactory and encourage to go ahead with the second phase of the study. All the laboratories that participated in the first phase of the study seem to be in the conditions of facing the second phase. Those laboratories that obtained recoveries lower than 90% and higher than 110% when the protocol was applied without clean-up step will be encouraged to investigate the causes and to improve the performance.

ANNEX

Although this was not the objective of the first phase of the validation study, the following statistical treatment was done as a first approach to check the precision (repeatability and reproducibility) of the method for the analysis of shellfish samples. The calculations were done considering the two replicates of certified reference materials as two blind samples. The results were considered as if they were originated from a proficiency test.

5. REPEATABILITY AND REPRODUCIBILITY

Data on repeatabi lity (RSDr %) and reproducibility (RSDR %) were calcu lated on the basis of the sum of DA and epi DA (SUM), without and with clean-up step (Table VI).

TABLE VI

Lab.	R1/SUM	R2/SUM	R1/SUM Clean up	R2/SUM Clean up
1	39	40.6	35.9	37.4
3	36.3	35.4	35.2	35.8
3	38.8	38.7	34.9	37.6
4	37.5	38.2	36	34.4
5	44.2	43	37.8	35
6	35.2	36.3	30.6	31.3
7	40.1	38.6	30.6	29.3
8	38	37.4	27.4	33.5
9	38.l	39.4	33	38. l
10	35.l	34.6	35.2	35
11	37.9	37.7	33.4	33.2
12	40.8	39.7	34.7	35.2
13	37.1	34.4	33.9	33.7
14	39.l	40.3	36.3	35.8
15	32.2	33	32.6	31.5
Mean	**37.9**		**34.2**	
RSDr %	**2.2**		**5.0**	
RSDR %	**7.2**		**7.5**	

The reproducibility is not significantly different between the procedure with clean up (RSDR% 7.5) and the procedure without clean up (RSDR% 7.2) step. However the repeatability of the procedure with clean-up step (RSDr % 5.0) was worse than that of the procedure without clean-up step (RSDr % 2.2).

Table VII shows the results for R l/SUM and R2/SUM, z score and CV (repeatability intralaboratory) for al i the participants, as well as the RSDr % and the RSDR %.

TABLE VI

Lab.	R1/SUM	Z score	R2/SUM	Z score	Mean	CV (%)
1	39	0.36	40.6	1.04	39.8	2.8
2	36.3	-0.61	35.4	-0.89	35.9	1.8
3	38.8	0.29	38.7	0.33	38.8	0.2
4	37.5	-0.18	38.2	0.15	37.9	1.3
5	44.2	**2.24**	43	1.93	43.6	1.9
6	35.2	-1.01	36.3	-0.56	35.8	2.2
7	40.l	0.76	38.6	0.3	39.4	2.7
8	38	o	37.4	-0.15	37.7	1.1
9	38.1	0.04	39.4	0.59	38.8	2.4
10	35.l	- 1.05	34.6	-1.19	34.9	1
11	37.9	-0.04	37.7	-0.04	37.8	0.4
12	40.8	1.01	39.7	0.7	40.3	1.9
13	37.1	-0.32	34.4	-1.26	35.8	5.3
14	39.l	0.4	40.3	0.93	39.7	2.1
15	32.2	**-2.09**	33	-1.78	32.6	1.7
Mean	**37.9**					
RSDr %	**2.2**					
RSDR%	**7.2**					

The laboratories that obtained recoveries clearly lower than 90% and higher than 110% when the protocol was applied without clean-up, presented results with z score≥|2|.

Table VIII shows the results for R1/SUM and R2/SUM with clean up step, z score and CV (repeatability intralaboratory) a for all the participants , as well as the RSDr % and the RSDR %.

TABLE VIII

Lab.	R1/SUM Clean up	Z score	R2/SUM Clean up	Z score	Mean	CV (%)
1	35.9	0.78	37.4	1.18	36.7	2.9
2	35.2	0.52	35.8	0.48	35 .5	1.2
3	34.9	0.41	37.6	1.26	36.3	5.3
4	36	0.82	34.4	-0.04	35.2	3.2
5	37.8	1.49	35	0.2	36.4	5.4
6	30.6	-1.19	31.3	-1.19	31	1.6
7	30.6	-1.19	29.3	**-2.11**	30	3.3
8	27.4	**-2.38**	33.5	-0.41	30.5	14.1
9	33	-0.3	38.1	1.46	35.6	10.1
10	35.2	0.52	35	0.2	35.l	0.4
11	33.4	-0.15	33.2	-0.53	33.3	0.4
12	34.7	0.33	35.2	0.28	35	1
13	33.9	0.04	33.7	-0.33	33.8	0.4
14	36.3	0.93	35.8	0.53	36.1	1
15	32.6	-0.45	31.5	-1.22	32.1	2.4
Mean	**34.2**					
RSDr %	**5.0**					
RSDR %	**7.5**					

The laboratories that obtained recoveries clearly lower than 80% when the protocol was applied with clean up, presented results with z score≥|2|

II REPORT ON THE SECOND PHASE OF THE VALIDATION STUDY OF THE DOMOIC ACID DETERMINATION BY HPLC-UV (METHANOLIC EXTRACTION)

1. INTRODUCTION

The purpose of the second phase of the validation study of the domoic acid (DA) determination by HPLC-UV (methanol extraction) was to determine the accuracy and the precision of the method in shellfish and finfish by using an aqueous:methanol (50:50) extraction followed by HPLC-UV detection. Performance characteristics were determined for the method with and without a purification and preconcentration strong anion exchange (SAX), solid phase extraction cleanup step. The study was planned in two phases. The first phase was concluded with satisfactory results.
The specific objective of the second phase of the study is the determination of:

- Reproducibility and repeatability

2. PARTICIPANTS

AUSTRIA

Egon Hellwig

Austrian Agency for Health and Food Safety Food Control and Research Vienna

BELGIUM

Chrysostome Nsengiyumva

Institut d 'Hygiene et d ' Épidemiologie Section Denrées Alimentaries

DENMARK

Kevin Jorgensen/Lene Bai Jensen

Danish Veterinary and Food administration Institute of Food Research and Nutrition

FRANCE

Sophie Krys, Claire Fabre

Agence Franc;aise de Sécurité Sanitaire des Aliments

GERMANY

Reinhard Tiebach

Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinarmedizin

GREECE

Ignatia Kaniou

Center of Veterinary Institutions of Thessaloniki

IRELAND

Biotoxin-Chemistry National Reference Laboratory Dublin.

Marine Institute Fisheries Research Centre

ITALY

Anna Milandri, Alfiero Ceredi Centro Ricerche Marine

THE NETHERLANDS
H.P.Van Egmond, H.J. Van Den Top
National Institute of Public Health and the Environment (RIVM)
NORWAY
The Norwegian School of Veterinary Science
Institute of Pharmacology, Microbiology and Food Hygiene
PORTUGAL
Paulo Vale
Instituto de Investigacion das Pescas e do Mar
UNITED KINGDOM
Col in Megginson
FRS Marine Laboratory
SPAIN
F. Arévalo, C. Salgado, J. Correa
Centro de Control del Medio Marino (CCMM).
Teresa M ª Legarda
Centro Nacional de Alimentación (CNA)
Coordination:
EU-CRL on Marine Biotoxins

3. MATERIALS

Each laboratory was supplied with 14 bottles (labelled as 01/DA/03 to 14/DA/03) containing approx. 6 grams per sample. Samples were thawed and tissues homogenized with a blender (the day of the preparation). Subsamples from each homogenate were immediately taken, while still well mixed, and conditioned into sealed plastic bottles.

List of materials:

01/DA/03 and 02/DA/03: blind duplicate of spiked clam. This sample was carefully prepared by spiking 196.5 g of blank clam with 28.8525 g of a solution prepared with 21.2325 g of water and 10.8395 g of a certified solution of domoic acid CRM-DA-d (formerly DACS I D), to obtain a nominal value of 4.28 µg DA+EA/g (EA: epidomoic acid).(CRM-DA-d solution was obtained from the Certified Reference Materials Program, National Research Council of Canada, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada and had a certified concentration of 89.0 µg DA+EA/g).

03/DA/03 and 04/DA/03: blind duplicate of clam naturally contaminated.

05/DA/03 and 06/DA/03: blind duplicate of spiked mussel. This sample was carefully prepared by spiking 187.84 g of blank mussel with 102.6 g of a mussel tissue reference material for domoic acid, CRM-ASP-MUS-b (formerly known as MUS IB). The nominal

concentration was and 13.78 µg DA+EA/g.
(CRM-ASP-MUS-b was obtained from the Marine Analytical Chemistry Standards Program, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada and had a certified concentration of 39.0 µg DA+EA/g)

07/DA/03 and 08/DA/03: blind duplicate of blank mussel.

09/DA/03 and 11/DA/03: blind duplicate of gonad scallop naturally contaminated.

10/DA/03 and 12 /DA/03: blind duplicate of whole body scallop naturally contaminated.

13/DA/03 and 14/DA/03: blind duplicate of anchovy naturally contaminated.

Homogeneity studies:

Homogeneity was checked by analyzing 3 subsamples (4 g each) per material under repeatability conditions. For these purpose, 3 bottles of each material were randomly chosen, the same day of the material preparation , and subsequently extracted with 16 mL of methanol: water (50:50) (single-step dispersive extraction) and analyzed by HPLC with UV detection. The results obtained are the following:

Material	Day of preparation & analysis	Results* (µg/g)			C.V (%)
Spiked clam	02/06/03	4.25	4.12	4.13	1.7
Clam	28/05/03	17.7	17.7	17.6	0.23
Spiked mussel	02/06/03	13.4	13.1	12.9	1.7
Gonad scallop	30/05/03	2.44	2.42	2.41	0.63
Whole body scallop	30/05/03	9.34	9.46	9.40	0.64
Anchovy	29/05/03	83.9	83.0	83.2	0.55

**Results show the sum of domoic-acid + epidomoic acid*

Taking into account the results from these studies, it was considered that materials homogeneity was acceptable.

Stability studies:

After conditioning subsamples from each homogenate into sealed plastic bottles and taking 3 bottles of each material for analysis, samples were immediately frozen and kept in the frozen state until the stability studies were conducted.

Stability was checked by analyzing the samples in two different days (within a 12 day interval), that coincided with the beginning and the end of the established period for the study. The analyses were carried out by the same analyst. Each day 2 subsamples (n = 2) of each material were randomly chosen and thawed. Then 4 g samples were extracted with 16 mL of methanol:water (50:50) (single-step dispersive extraction) and analyzed by

HPLC with UV detection. The results obtained are showed in the following table:

Material	Results* (µg/g) (09.06.03)	Results* (µg/g) (20.06.03)	C.V (%)
Spiked clam	3.15	3.23	1.8
Clam	17.0	16.4	2.7
Spiked mussel	11.4	11.0	2.6
Gonad scallop	2.06	2.10	1.2
Whole body scallop	8.81	8.81	0.0
Anchovy	83.5	82.4	1.2

**Reported results are mean values of two replicates and show the sum of domoic acid + epidomoic acid*

Taking into account the results from these studies, it was considered that materials stability was acceptable. However, when comparing results from the first study and the latter, it was possible to detect a decrease in the concentration of DA, which is supposed to be due to the freezing operation. For this reason recovery (for the spiked samples) was not calculated.

4. METHODS

As the purpose of this study is to validate the method, all the participants were asked to strictly follow the protocol that it had been previously discussed and agreed by the participants and the coordinator. Only the type and dimensions of the HPLC column and some chromatographic conditions (flow rate, injection volume, and percentage of acetonitrile) were left to the choice of the participants.

Samples were sent the 3rd of June 2003 and reached the participants no later than 5th June. The analytical work had to be finalized before June 20th and results had to be sent before June 30th

Participants were asked to comply with the following requirements:

- For a given sample, extraction and DA determination should be carried out in the same day.
- The determination of DA in the sample had to be carried out with and without SAX cleanup step and both results had to be reported.
- Participants had to make duplicate injections of each extract. Peak area from each injection had to be reported and the DA concentration had to be calculated on the basis of the average areas. In the case that DA and EA were resolved by the chromatography column, DA area and the SUM of DA and EA peak areas had to be reported, and the DA concentration had to be calculated in two ways: on the basis of the DA peak area and on the basis of the sum of the areas of both peaks. If DA and EA peaks were not resolved, total peak area had to report in the box corresponding to the sum of DA and EA peaks.

- If all the samples were not going to be analyzed in the same <lay, participants had to take into account the following:

-For a given sample, extraction and DA determination should be carried out in the same day.

-A calibration curve should be made each day of analysis.

-Samples should be analyzed in a consecutive order, according to the labelling and following the scheme below:

a)-Study performed in two days:
Day 1: Samples 01 to sample 06; Day 2: sample 07 to sample 14.

b)-Study performed in three days:
Day 1: Sample 01 to sample 04; Day 2: sample 05 to sample08; Day 3: Sample 09 to sample 14.

OUTCOME OF THE STUDY (Second phase)

This study was conducted taking into account the *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (AOAC, 2002). All the results sent by the participants were checked to find not valid data before the statistical analysis. This not valid data are represented in table I and II as empty cells.
These guidelines establish the Cochran test, Single Grubbs test and Pair Grubbs test to find outliers.

Table I shows all valid raw data without SAX cleanup and Table II shows all valid raw data with SAX cleanup. Taking into account that participants reported their results on the basis of the average areas (injection 1 and 2), the average areas were used for the statistical analysis.

For statistical analysis only data obtained from total peak area (sum of DA and EA areas) was considered.

Table I. Raw valid data results (µg/g) without SAX procedure

Nº lab	Spiked clam		Clam		Spiked mussel		Gonad scallop		Whole body scallop		Anchovy	
1												
2	3.00	2.90	18.8	17.5	11.2*	12.7*	2.30	2.10	8.30	8.50	86.8	87.3
3	3.50	3.80	17.6	17.7	13.7	13.1	2.40	2.40	9.60	9.90	84.2	85.5
4	3.51	3.66	19.0	18.9	12.6	12.6	2.75	2.73	10.3	10.5	94.6	97.4
5	2.85	2.92	18.4	18.1	13.4	13.1	2.73	2.74	10.2	10.0		
6	3.58	3.58	18.0	17.9	12.5	12.8	2.27	2.24	10.0	10.6	89.l	89.4
7	3.23	3.07	15.1	14.2	10.7	11.0	2.58	2.40	7.86	7.75	77.3	77.8
8	3.80	3.60	19.2	19.5	13.4	13.1	4.00	3.70	12.1	11.7	96.0	96.7
9	4.03	3.50	19.7	20.5	13.4	13.4	3.49	3.67	10.7	10.8	107*	97.2*
10							2.16	2.35	9.00	8.93	80.2	81.4
11	3.25	2.24	15.2	14.4	10.7	10.2	2.37	2.21	8.84	8.60	76.8	79.0
12	4.14	3.77	17.9	18.4	13.2	13.0	3.66	3.75	10.4	10.5	85.1	82.2
13	3.70	4.20	16.7	16.2	10.4	10.3	2.80	2.50	8.60	8.90	80.9	79.9
14	2.63	2.76	17.2	17.0	11.9	11.9	2.13	2.17	8.99	9.04	83.3	82.2

**Outlier.*

Empty cells: not valid data

Table II. Raw valid data results (µg/g) with SAX procedure (µg/g)

Nº lab	Spiked clam		Clam		Spiked mussel		Gonad scallop		Whole body scallop		Anchovy	
1												
2	2.60	2.60	15.7	15.3	10.3	10.4	2.10	2.20	7.70	7.80	73.2	77.8
3	2.70	2.50	17.1	15.4	10.3	11.8	2.20	2.20	8.50	8.70	81.4	87.4
4	2.93	2.77	20.8	19.0	13.l	13.8	2.76	2.74	10.7	10.8	93.0	96.7
5	0.72*	0.72*	5.65	5.21	6.01	5.94	1.07*	0.95*	3.85	4.13		
6												
7	2.18	2.12	9.63	10.7	10.0	9.85	2.04	1.97	7.56	7.44	73.0	70 .6
8	3.10	2.90	16.9	16.4	11.2	11.3	2.60	2.20	10.2*	9.10*	81.2	83.5
9	2.30	2.01	15.8	16.6	11.5	11.5	2.14	2.18	9.00	9.24	85.9	82.2
10							1.88	1.98	6.96	6.98	65.2	67.2
11	2.77	1.99	13.6	13.2	9.19	8.59	1.98	2.04	7.46	7.48	59.0	61.0
12	2.91	2.53	14.9	15.6	10.9	11.0	2.47	8.24	8.48	10.5	70.0	75.6
13	1.70	1.30	11.8	11.8	8.50	7.90	1.70	1.50	7.50	7.10		
14	2.69	2.83	16.2	17.5	11.5	10.4	2.11	2.22	8.29	8.75		

**Outlier*

Empty cells: not valid data

5. BLANK MATERIALS

This material was used to check the presence of false positive (a positive value on a blank material). Ali the results sent by the participants were checked to find not valid data.
Taking into account the results sent by the participants, if it is assumed that a value lower than limit of quantification (<LOQ) or lower than limit of detection (<LOD) is a negative result, there wouldn't be tendency of the method to produce false positives.

Table III. Valid data results without SAX procedure

Nº lab	Blank mussel (07/DA/03)	Blank mussel (08/DA/03)
1		
2	< LOD	< LOD
3	< LOQ	< LOQ
4	< LOD	< LOD
5	< LOD	< LOD
6		
7	< LOD	< LOD
8	< LOD	< LOD
9	< LOQ	< LOQ
10	< LOD	< LOD
11	<LOQ	<LOQ
12	<LOD	<LOD
13		
14	< LOD	< LOD

Cells in white reflect not valid results, mainly due to report results which areas were below the lower calibration standard.

Table IV. Valid data results with SAX procedure

Nº lab	Blank mussel (07/DA/03)	Blank mussel (08/DA/03)
1		
2	< LOD	< LOD
3	< LOQ	< LOQ
4		
5	< LOD	< LOD
6		
7	< LOD	< LOD
8	< LOD	< LOD
9	< LOD	< LOD
10	< LOD	< LOD
11	<LOQ	<LOQ
12	<LOD	<LOD
13	<LOD	< LOD
14	< LOD	< LOD

Cells in white reflect not valid results, mainly due to report results which areas were below the lower calibration standard.

6. PRECISION: REPEATABILITY AND REPRODUCIBILITY

These specific figures were calculated following the *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (AOAC, 2002). This protocol requires the calculation and reporting of mean, percent recovery (% Rec), HORRAT, repeatability (within-laboratory, S_r) and reproducibility (interlaboratory, S_R) standard deviations, and repeatability and reproducibility relative standard deviations (RSD_r, RSD_R, respectively).
Performance characteristics are showed in table V and VI.

Table V. Interlaboratory results for DA SUM (DA+EA) by HPLC-UV (methanolic extraction) without SAX procedure.

Material	Level (µg/g)	Nº labsª(b)	Mean (µg/g)	S_r	Repeatability RSD, %	S_R	Reproducibility RSD_R %	Reproducibility HORRAT
Spiked clam	4.28*	12(0)	3.38	0.28	8.3	0.51	15	1.1
Clam		12(0)	17.7	0.40	2.3	1.6	9.0	0.87
Spiked mussel	13.8*	11(1)	11.6	0.37	3.2	2.0	17	1.6
Gonad scallop		13(0)	2.72	0.12	4.3	0.62	23	1.7
Whole body scallop		13(0)	9.63	0.18	1.9	1.2	12	1.0
Anchovy		11(1)	85.1	1.1	1.3	6.6	7.7	0.94

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).
**Spiking levels*

Table VI. Interlaboratory results for DA SUM (DA+EA) by HPLC-UV (methanolic extraction) with SAX procedure.

Material	Level (µg/g)	Nº labsª(b)	Mean (µg/g)	S_r	Repeatability RSD, %	S_R	Reproducibility RSD_R %	Reproducibility HORRAT
Spiked clam	4.28*	10(1)	2.47	0.24	9.6	0.47	19	1.4
Clam		11(0)	14.3	0.70	4.9	4.0	28	2.6
Spiked mussel	13.8*	11(0)	10.2	0.45	4.4	2.0	19	1.7
Gonad scallop		11(1)	2.16	0.11	5.2	0.31	14	0.99
Whole body scallop		11(1)	7.85	0.17	2.2	1.7	21	1.8
Anchovy		9(0)	76.9	2.7	3.6	10	14	1.7

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).
**Spiking levels*

7. CONCLUSIONS

- The results of this second phase of the validation study are satisfactory when participants use the method without SAX procedure. On the other hand, when SAX procedure is used, results are in general poorer than without SAX in all the performance characteristics of the method.

- It was no possible to calculate the recovery in spiked samples because after the homogeneity and stability studies, it was observed a decrease in the sample concentration, probably due to freezing process. Nevertheless, this parameter was calculated in the phase 1 with reference material.