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Assessment of a new lateral flow immunochromatographic (LFIC) assay for the okadaic acid group of toxins using naturally contaminated bivalve shellfish from the Portuguese coast

* P. Vale, * S.S. Gomes, * J. Lameiras, * S.M. Rodrigues, * M.J. Botelho, ** M.V. Laycock,

* Instituto Nacional dos Recursos Biológicos – IPIMAR, Av. Brasília, s/n, 1449-006, Lisboa, Portugal

** Jellett Rapid Testing Ltd., 4654 Route 3, Chester Basin, Nova Scotia, Canada B0J 1K0

Abstract:
A new rapid assay for okadaic acid group of toxins based on lateral flow immunochromatographic (LFIC) test strips developed by Jellett Rapid Testing Ltd. was assessed with several naturally contaminated bivalves from the Portuguese coast. One prototype was evaluated using samples harvested during 2005, extracted with 80% methanol, followed by dilution with the running buffer of a methanolic extract after alkaline hydrolysis for esters. The second prototype was assessed using samples harvested during 2006, extracted with 100% methanol, and after alkaline hydrolysis the methanol was evaporated by a nitrogen stream followed by re-suspension with the running buffer. The first prototype failed to detect 20% of samples that were positive by LC-MS in the range of 160-480 µg/kg, and were classified as negative or trace level by LFIC. The presence of methanol in the extracts made correct detection of toxins more difficult. The second prototype classified as positive all samples above 160 µg/kg as detected by LC-MS. However, in the second prototype, matrix effects were found to be a major drawback and led to 45% false positives, particularly for mussels, due to compounds in shellfish extracts interfering with the antibodies and reducing the test line intensity. Extraction with a higher percentage of methanol was thought responsible for these matrix effects. Regarding sample migration, both prototypes needed one hour before reading. In an attempt to speed-up sample preparation, a direct digestion of bivalve tissues with sodium hydroxide was evaluated. Low recoveries for esters were found by LC-MS with this hydrolysis technique when compared with conventional hydrolysis of methanolic extracts. While prototype A was not sensitive enough, prototype B had too many false positives to be of use to the shellfish industry or in a monitoring program.

1 Corresponding author: Paulo Vale; Tel. 351-213027000; Fax: 351-213015948; email: pvale@ipimar.pt
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Introduction

Bivalves molluscs from the Portuguese coast are seasonally exposed to *Dinophysis acuminata* and *D. acuta* blooms, which contaminate these shellfish with okadaic acid (OA) and dinophysistoxin-2 (DTX2) toxins above the current EU regulatory limit of 160 µg OA equivalents/kg (European Commission, 2004a). Between the years 2003 and 2006, about 20% of samples tested by LC-MS contained toxins in excess of this limit, and about 40% of the samples contained residual levels between 16-160 µg OA equiv./kg (Vale *et al.*., 2008). The recent Regulation EC 854/2004 requires a laboratory control system to verify food business operators’ compliance with the requirements for the end product at all stages of production, processing and distribution in order to verify that the levels of marine biotoxins do not exceed safety limits (European Commission, 2004b).

Liquid chromatography methods, such as HPLC coupled with fluorescence detection (HPLC-FLD) or mass spectrometry (LC-MS), are very expensive for laboratories processing a limited number of samples, while antibody methods offer the best approach for the development of an easy-to-use, reliable and inexpensive test kit for toxin detection. Some antibodies towards okadaic acid have been reported and used in the commercial ELISA format. A Japanese kit, ‘DSP-Check’, has been exhaustively used to study contamination of Portuguese shellfish (Vale and Sampayo, 1999; Vale *et al.*, 2006). Another type of test for these toxins was developed recently that may be suitable for use without laboratory equipment in the field, and that can be applicable to as few as one sample (Laycock *et al.*, 2006). In aquaculture facilities located far from official control laboratories, the time involved in sample transportation constitutes a key factor in the overall efficiency of the food safety control scheme.

The Jellett Rapid Testing (JRT) lateral flow immunochromatography (LFIC) test for OA group toxins is a competitive immuno-assay on a test strip contained in plastic cassette as shown in Figure 1. Polyclonal antiserum was obtained from rabbits injected with okadaic acid conjugated to a carrier protein via the carboxyl group. Conjugate pads were prepared with mixed antiserum and colloidal gold. The test line on the nitrocellulose membrane consisted of bound okadaic acid and the control line of the same carrier protein used to raise the polyclonal antibodies. To carry out a test, a methanolic extract is diluted with the running buffer, which is supplied with the tests. The composition and dilution with the buffer is designed to provide optimum conditions for the reactions to occur so that the test line is absent or faint with okadaic acid or its analogues at the regulatory
concentration in an extract of 160 µg/kg of whole tissue. Okadaic acid, dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2) were detected similarly with 50% reduction in test line color intensity at 5 nM for the solutions applied to test strips (Laycock et al., 2006). In the absence of toxin in the sample the antibody coated colloidal gold particles bind to okadaic acid on the test line and form a visible line. A visible control line indicates that the sample composition was suitable for immune reactions to occur.

This LFIC test was assessed against LC-MS with several naturally contaminated bivalves from the Portuguese coast.

Materials and methods

Bivalve samples were collected from the Portuguese coast, and belonged to the regular coastal monitoring for marine biotoxins (Figure 2). Species sampled from estuarine/lagunar environment: blue mussel (Mytilus galloprovincialis), common cockle (Cerastoderma edule), clams (Tapes decussatus, Venerupis senegalensis), razor clams (Solen marginatus), oyster (Crassostrea spp.) and the main species from offshore environment: clams (Donax spp. and Spisula solida).

A sample consisted of at least 30 shellfish, except for oysters, where only 12 were used, and razor clams, where 20 shellfish were used. The samples were dissected and whole flesh pooled and homogenised.

Two different batches of LFIC prototypes were acquired from Jellett Rapid Testing Ltd. (Nova Scotia, Canada), designated here as Prototype A and Prototype B, and assayed with different solvent extractant and sample preparation.

Prototype A assessment

Prototype A was assessed with bivalve samples harvested during 2005, and involved minimal sample preparation with dilution of the methanolic extract after alkaline hydrolysis for OA and DTX2 esters with the running buffer provided with the kit.

LFIC assay

Tissue homogenates (5 g) were extracted with 20 ml 80% aqueous methanol. Aliquots of 2 ml of supernatant were hydrolysed with 250 µl 1.0 M NaOH (aq) for 45 min at 40°C, neutralised with 250 µl 1.0 M HCl (aq), and 125 µl of this mixture were diluted with 400 µl running buffer solution and 100 µl transferred to the test strips.
Tissue homogenates (5 g) were extracted with 20 ml 80% aqueous methanol. Aliquots of 2 ml of supernatant were hydrolysed with 400 µl 1.0 M NaOH (aq) for 45 min at 40°C and neutralised with 425 µl 1.0 M HCl (aq). This mixture was washed with 2 ml of hexane, extracted twice with 2 ml of dichloromethane, the combined dichloromethane fractions dried in a vacuum evaporator, resuspended in 500 µl 80% methanol and 2.5 µl injected into the LC-MS system.

**Prototype B assessment**

Prototype B was assessed with bivalve samples harvested from September 2006 until April 2007, and involved evaporation by nitrogen stream of the residual methanol in the extract after alkaline hydrolysis, followed by resuspension with the running buffer.

**LFIC assay**

Tissue homogenates (1 g) were extracted with 3 ml methanol. Aliquots of 1 ml of supernatant were hydrolysed with 125 µl 2.5 M NaOH (aq) for 40 min at 70°C, neutralised with 125 µl 2.5 M HCl (aq). An aliquot of 25 µl was dried with a nitrogen stream in an Eppendorf tube, resuspended with 100 µl running buffer solution and the whole contents transferred to the test strips.

**LC-MS preparation**

Tissue homogenates (2 g) were extracted with 18 ml 90% aqueous methanol. Aliquots of 2 ml of supernatant were hydrolysed with 200 µl 2.5 M NaOH (aq) for 40 min at 70°C and neutralised with 210 µl 2.5 M HCl (aq). This mixture was washed twice with 2 ml of hexane, filtered by a disposable 0.22 µm filter, and 5.0 µl injected into the LC-MS system.

**LC-MS analysis**

LC-MS analysis for Prototype A assessment

The final extracts were separated for 9 minutes on a Merck Lichrospher-100 RP-18 column (5 µm, 125 x 2 mm) protected by a guard column packed with the same material (4 x 4 mm) using a mobile phase of acetonitrile:0.05% acetic acid (65:35, v/v) at a flow rate of 200 µL/min. The LC effluent was diverted to waste during the first 2 minutes of separation and then was introduced into the mass spectrometer. Selected ion monitoring (SIM) was used to record the signals from the [M-H]⁺ ions at: m/z 803.5 (for OA and DTX2) and 817.5 (DTX1). All LC-MS equipment used (in-line degasser, quaternary pump, autosampler, column oven and 1946A single-quadrupole mass detector) was from 1100-Series from Hewlett-Packard (HP).

For calibration, an OA certified reference material (CRM-OAb) from the Institute for Marine Biosciences (NRC, Canada) was used. A working solution of 0.96 µg/ml in methanol was used to
generate a 3-point calibration curve every 6-12 samples by variable injection volumes (0.5, 2.5, 5.0 µl). Due to the close structural relation between OA and DTX2, peak area response was assumed to be identical to OA, and later confirmed with a non-commercial standard.

LC-MS analysis for Prototype B assessment
Separation and LC-MS conditions were the same as above. Extraction with dichloromethane was not carried and the extracts contained high levels of NaCl from the hydrolysis reagents. In order to correct for signal suppression in the MS system, calibration standards were also prepared with a similar concentration of NaCl by diluting a stock solution of 0.96 µg OA/ml in pure methanol with a solution consisting of 2.5 M aqueous NaCl and methanol (1:5 v/v). Four calibration solutions were prepared (31-241 ng/ml).

Trials of sample preparation by LC-MS
In order to compare different hydrolysis procedures a similar solvent to tissue ratio was used.
Tissue hydrolysis:
Tissue homogenate (1 g) was hydrolysed with 125 µl 2.5 M NaOH for 40 min at 70ºC and then neutralised with 125 µl 2.5 M HCl. Extraction was performed with 3 ml of methanol. The supernatant was filtered and injected in the LC-MS system.

Extract hydrolysis:
Tissue homogenate (1 g) was extracted with 3 ml of methanol. A 1.0 ml aliquot of the supernatant was hydrolysed as above. The hydrolysate was filtered and injected in the LC-MS system.

Results and discussion
The materials and methods for the manufacture of the test strips were essentially the same for both prototype batches used in this paper. Concentrations of buffer solution components varied only slightly. The main differences between the prototypes were in sample preparation. The methanol concentration for extraction and to limit residual methanol on the test strip were critical in achieving the required sensitivity.

Although DTX1 did not occur in the bivalves used for these studies, and is uncommon in Portuguese shellfish, nevertheless its presence was always screened. The OA equivalents reported always refer to the sum of OA and DTX2 obtained after hydrolysis. Samples tested during 2005 or 2006/2007 were contaminated by OA alone or simultaneously with OA and DTX2 (data not shown). In bivalve species such as common cockle (*Cerastoderma edule*), clams (*Tapes decussatus*, *Venerupis senegalensis*, *Spisula solida*), razors (*Solen marginatus*), oyster (*Crassostrea* spp.) acyl esters comprise greater than 96% of the toxin fraction, while in mussels (*Mytilus* spp.) and Donax clams (*Donax* spp.), a similar proportion of free and esterified toxins can
commonly exist. The maximum contribution of total DTX2 in the first set of species is commonly
40% of the total of toxins found; while in the second set of species this proportion might increase
during the decay of the *Dinophysis acuta* blooms (Vale, 2004, 2006).

Prototype A – raw data:
One hundred samples were tested with the first batch of JRT prototypes and results are
summarised in Figure 3. Overall, 9% of the results corresponded to false positive assays (traces),
i.e., samples with low levels of toxins that were classified as containing OA analogues at unsafe
levels. Although the theoretical limit of detection (LOD) for this test was reported as 80 µg/kg
(Laycock *et al.*, 2006), the most serious drawback found with this prototype, was a high percentage
(20%) of false negatives, i.e., samples containing toxins in excess of the current maximum
allowable level in the EU of 160 µg OA equivalents/kg but not detected with the test. All samples
with OA group toxins levels between 480 up to 6590 µg OA equiv./kg were classified as positive.
From past experience, these samples were judged as capable of inducing from mild to severe
intoxications (Vale *et al.*, 2008). Overall, an experimental high LOD was observed with the protocol
employed with this prototype, compromising a correct detection of positive samples. According to
the kit producer, this was attributable to the residual methanol in the final extract. The second
batch of prototypes, was assayed resorting to prior evaporation of methanol.

Prototype B
JRT raw data:
With the second JRT batch 450 samples were tested and no false negatives were found (Figure 4).
All samples above 160 µg OA equiv./kg were correctly detected and classified as positive (n=25).
The most detrimental drawback with the protocol employed with this prototype, was a high
percentage of false positives (45% in total). From these, 37% in the range below 50 µg OA
equiv./kg, i.e., the cut-off programmed in the method, and 79% in the range of 50-160 ug/kg

The detection of too many false positives is cumbersome, particularly when low levels of toxins
occurred. This will require food business operators' to unnecessarily further test by a confirmatory
method in a centralised laboratory a high percentage of the samples. Extracts of bivalves from
clams (*Tapes decussatus, Venerupis senegalensis*) and oysters (*Crassostrea* spp.) gave fewer
false positives (Table 1, Figure 3). Cockles (*Cerastoderma edule*) and the clams *Scrobicularia
plana* gave low percentages of false positives in the south and SW coasts, but these were higher
from the NW coast. Mussels (*Mytilus* spp.) had the highest percentage of false positives, which
also seemed dependent on geographic location. The clams *Donax* spp. had a high percentage of
false positives from the south and SW coast. No commercial harvest of this offshore species takes
place on the NW coast, so no data are available for this species at this location. Comparison
between mussels and cockles from Aveiro lagoon, shows false positives might appear from
autumn through spring (Figure 5).

The use of 100% methanol, although suited for OA and DTX2 esters, might contribute
simultaneously to higher recovery of interfering compounds. As the shellfish homogenate is ca.
80% water, both the initial solvent composition and the sample/solvent ratio are important for the
efficiency of a single extraction. Therefore the 5g + 20 mL 80% MeOH in the prototype A equates
to a final solvent strength of ca 67% MeOH, whereas in the prototype B the extraction with 1g +
3mL MeOH equates to a solvent strength of ca 79% MeOH. A commercial ELISA kit (DSP-
Check) was used successfully in the Portuguese monitoring program between 2003 and 2005 for
measuring these toxins. It used an 80% aqueous methanol followed by hexane washing and liquid
partitioning into dichloromethane, followed by vacuum concentration (Vale et al., 2006). For an
efficient extraction of acyl esters, increase of the methanol concentration in the extraction solvent
was recommended by McNabb et al. (2005). In 2006, a 90% methanol extraction (final solvent
strength of ca 83%) was adopted in order to improve recovery of OA and DTX2 acyl esters in
shellfish samples from the Portuguese monitoring program for lipophilic toxins led to a high
percentage of false positives, despite the continued use of the liquid-liquid clean-up, leading to
discontinue the use of this ELISA kit in the monitoring programme (unpublished data).

Hydrolysis temperature used in Prototype A followed for precautionary approach a mild
temperature by lack of detailed information on the stability of the ester. This was corrected at the
time of Prototype B, as it was found that the reaction was incomplete at 40°C for 45 min
(Rodrigues, 2007).

Trials of sample preparation for LC-MS

The protocol for assaying Prototype B came with a suggestion from the JRT company of
hydrolysing the homogenated bivalve matrix directly, instead of hydrolysing the methanolic extract,
as commonly performed by research groups around worldwide (Yasumoto et al., 1989; Vale and
Sampayo, 1999; McNabb et al., 2005). The advantage was supposed to be to avoid excessive
tissue dilution. A short trial of hydrolysis carried out directly in tissue homogenates was compared
with the customary preparation of hydrolysates from methanol supernatants, and is shown in
Figure 6. Almost no OA group toxins were detected when hydrolysis was carried out directly in
tissue homogenates, in particular in bivalve species where acyl esters comprise the majority of the
toxin fraction, such as cockles, clams and razor clams. In mussels (Mytilus spp.) and Donax clams
(Donax spp.), where a fair proportion of free toxins commonly exists, toxicity could be detected
when hydrolysis was carried out in homogenates, and was attributed to the free toxins already
present in these bivalve species. Due to the very low recovery of free toxins recognisable by the
antibody, this preparation was discontinued.
Conclusions

Prototype A protocol was incapable of classifying as positive many samples that by LC-MS showed the presence of OA analogues in the range of 160-480 µg/kg. However, highly toxic samples, with the potential to cause human outbreaks of gastroenteritis, were always detected.

Prototype B protocol classified as positive all samples above 160 µg/kg as detected by LC-MS. However, matrix effects with the second prototype were found to be a major drawback and led to false positives, particularly for mussels, due to compounds in shellfish extracts interfering with the antibodies and reducing the test line intensity.

Matrix effects seemed to be geographically and species dependent. Generally, contamination with marine bioactive compounds, such as some commonly studied marine biotoxins, occurs predominantly at the NW coast (Vale et al., 2008). Mussels eliminate several marine biotoxins (eg: okadaic acid group, saxitoxins, yessotoxins) at slower rates than other estuarine species such as cockles, clams, razors and oysters (Artigas et al., 2006; Gomes et al., 2006; Vale, 2006). From offshore species, Donax clams eliminate OA group toxins particularly slowly, but may contain lower concentrations of yessotoxins than the clam *Spisula solida* (Gomes et al., 2006; Vale, 2006). This finding led to the supposition that matrix effects might result from ingested compounds, rather than the species itself.

Further work on this kit is required before it would be fit for purpose, either in end-product testing or in monitoring programmes.

Acknowledgements

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References


Figure captions:

Figure 1. Diagram of Jellett Rapid test.

Figure 2. Localization of main inshore and offshore bivalve production areas along the Portuguese coastline. Est. = Estuary; Lag. = Lagoon; NW = northwest offshore coast; SW = southwest offshore coast. Offshore areas are numbered from north to south.

Figure 3. Detection of OA group toxins in methanolic extracts of bivalves by Prototype A: results grouped according to the current EU regulatory limit and 3 times the limit. Samples were harvested between May 2005 and September 2005. \( N = \text{number of samples}; \ ND = \text{not detected} \)

Figure 4. Detection of OA group toxins in methanolic extracts of bivalves by Prototype B: results grouped according to JRT detection limit (50 µg/kg) and the current EU regulatory limit. Samples were harvested between September 2006 and April 2007. \( ND = \text{not detected} \)

Figure 5. Temporal evolution of contamination by OA group toxins and JRT results from bivalves harvested at Aveiro lagoon (determined with Prototype B).

Figure 6. Concentration of OA group toxins found by LC-MS in different bivalves after: hydrolysis carried out in tissue homogenate prior to methanol extraction (tissue) or, hydrolysis carried in the methanolic extract (extract).
Table 1. Distribution of Prototype B false positives, with OA group toxins levels below 50 µg OA equiv. /kg, by geographic area and species.

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<td>5.0</td>
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<td>35.0</td>
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<td><em>Cerastoderma</em> + <em>Scrobicularia</em></td>
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Fig. 1.

Fig. 2.
Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.