# Comparative Evaluation of Enzyme-Linked Immunoassay and Reference Methods for the Detection of Shellfish Hydrophilic Toxins in Several Presentations of Seafood

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ARTICLE

# Comparative Evaluation of Enzyme-Linked Immunoassay and Reference Methods for the Detection of Shellfish Hydrophilic Toxins in Several Presentations of Seafood

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A comparative study was conducted to determine the feasibility of enzyme-linked immunosorbent assays (ELISAs) for the detection of amnesic shellfish poisoning (ASP) and paralytic shellfish poisoning (PSP) toxins in nine naturally contaminated species in fresh, frozen, boiled and canned fish and shellfish. PSP and ASP were analyzed in 138 shellfish samples (mussels, clams, barnacles, razor shells, scallops and cockles) and anchovies by mouse bioassay (MBA) and high performance liquid chromatography with ultraviolet detection (HPLC−UV), respectively. Results were compared with toxin concentrations obtained using two commercial competitive ELISAs, saxitoxin and ASP kits. Immunoassays were able to quantify toxins in different matrices showing excellent Pearson's correlation coefficients ($r$ = 0.974 for saxitoxin ELISA and $r$ = 0.973 for ASP ELISA) and to detect PSP and ASP with a lower limit of detection (LOD), namely, 50 $\mu$g saxitoxin equivalent/kg shellfish meat for PSP and 60 $\mu$g/kg domoic acid in shellfish flesh for ASP, than the reference methods (350 $\mu$g saxitoxin equivalent/kg shellfish meat and 1.6 mg/kg domoic acid in shellfish flesh, respectively). These results suggest that the ELISA method could be used as screening systems in a variety of species without matrix interference.

**KEYWORDS: Paralytic shellfish poisoning; ELISA; amnesic shellfish poisoning; mouse bioassay; high performance liquid chromatography with ultraviolet detection (HPLC−UV)**

## INTRODUCTION

World aquaculture has grown noticeably in the last 50 years. Spain is the world's third largest producer of mussels and the main supplier to the European market, with more than 209 thousand tons in 2007 (*1*). However, aquaculture-related sectors, producers, the canning industry and depuration plants are affected by toxic episodes (*2*). These toxic episodes are caused by many types of small non-proteinaceous compounds, called phycotoxins, which become a public health concern when contaminated shellfish are consumed. Phycotoxins are produced by microalgae, such as dinoflagellates and diatoms, which under favorable environmental conditions can multiply into dense blooms that sometimes produce a change in color of the seawater (popularly known as red tides). While harmful algal blooms (HABs) are natural phenomena that have occurred throughout recorded history, during the last seventeen years such events have increased in frequency, intensity and geographic distribution on a global scale (*3*), with a corresponding increase in the impact on public health and economic activity (*2*). Several important toxic episodes recorded around the world were caused by PSP and ASP. Moreover, in 2005 in Galicia (NW Spain) the presence of diarrhetic shellfish poisoning (DSP) toxins led to the closure of more than 50% in some of the mussel harvesting areas (*2*). Although some advances have been made regarding processed shellfish (*2*, *4*), in general once shellfish is contaminated, mitigation strategies are relatively limited.

Paralytic shellfish poisoning (PSP) is caused by several toxins of which the parent compound is saxitoxin (**Figure 1A**). This has a legal limit of 800 $\mu$g saxitoxin equivalents/kg shellfish meat ($\mu$g saxitoxin equiv/kg shellfish meat) (*5*). In recent decades, the mouse bioassay (MBA) has been used to protect consumers' health, and it is now the reference method in the EU for detecting PSP toxins (*6*). Nevertheless, it has some disadvantages: intraperitoneal toxicity has little relation to oral toxicity, and the sensitivity of MBA is relatively low. Moreover, the results are affected by the test conditions, such as animal strain, extract dilution, matrices, and sample preparation (*7*, *8*). To solve this problem, an alternative method that applies HPLC with fluorescence detection (HPLC-FLD) (*9*), was adopted by the Association of Official Analytical Chemists (AOAC), as an official first action method (*10*) and has been approved by the EU as an alternative method (*11*). However, the HPLC-FLD method is time-consuming and expensive and also requires very well trained staff, but its main disadvantage is lack of standards

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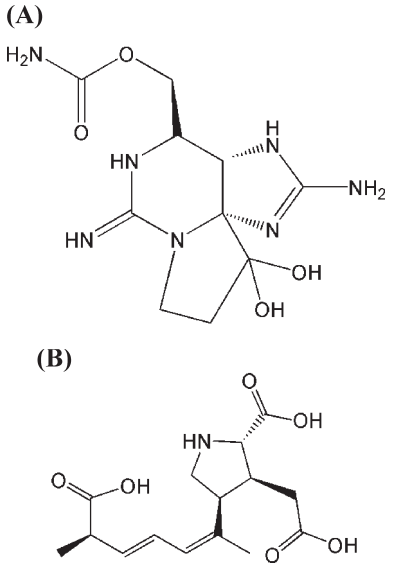


**Figure 1.** Chemical structures of saxitoxin (**A**) and domoic acid (**B**).

[i.e., *N*-sulfocarbamoyl saxitoxin B2 (GTX6), C3, C4; and decarbamoyl-gonyautoxins 1, 4, (dcGTX1, dcGTX4)] for the correct identification and quantification of all the PSP toxins. Therefore, the HPLC-FLD seems unsuitable for surveillance programs requiring a high sample throughput at low cost.

To provide more rapid and economic alternatives, a range of methods has been described, such as immunochemical techniques and enzyme-linked immunosorbent assays (ELISAs) (*12*−*16*). Currently, there is a commercially available competitive ELISA for the quantification of PSP toxins in shellfish, which is able to detect PSP toxin-positive samples with greater sensitivity at lower levels (50 $\mu$g saxitoxin equiv/kg shellfish meat) than the MBA (350 $\mu$g saxitoxin equiv/kg shellfish meat) (*17*, *18*).

Domoic acid (**Figure 1B**) is a naturally occurring neurotoxin produced, among other marine algae, by diatoms of the genus *Pseudonitzschia* that can be accumulated by filter feeding shellfish, causing amnesic shellfish poisoning (ASP) in human consumers (*19*). In Europe, the legal limit was established at 20 mg/kg domoic acid in shellfish flesh to avoid health problems (*5*). There are no ethical concerns regarding the analysis for detecting domoic acid, because animals are not used for this purpose. HPLC−UV (*20*) is used in most countries for official control for ASP toxins. Nevertheless, this technology is expensive with high maintenance costs and requires highly skilled operators and a well-established laboratory infrastructure. Hence, immunological techniques have also been developed for domoic acid detection at lower concentrations than the tolerance level. An ELISA for domoic acid, described by Garthwaite et al. (*21*) and commercially available, has been approved as an AOAC Official Method for detection of domoic acid in shellfish and as an official alternative method by EU legislation (*22*). Several authors have reported the advantages of immunosorbent assay technology applied to the detection of phycotoxins (*15*, *16*, *23*, *24*). However, the studies carried out to develop and validate these screening techniques used only a few naturally contaminated or spiked shellfish samples for ASP toxins, or a lot of samples but in only one type of presentation for PSP toxins. The feasibility of these tests has not yet been evaluated with naturally contaminated matrices in different forms of presentation, such as fresh, frozen, canned and boiled, in an end-user laboratory. Although EU legislation mainly refers to raw shellfish, nevertheless, recent EFSA scientific opinion on marine biotoxins in shellfish includes among other recommendations that "further data on the effect of processing on levels of saxitoxin-group toxins in shellfish are needed" (*25*). In this context, we have started to perform studies focusing on this subject (*4*). Therefore, in this paper, we have analyzed saxitoxin and its derivatives in nine different shellfish samples using a commercial PSP ELISA kit and the traditional MBA. In addition, six different matrices in different presentations were analyzed for domoic acid by a commercial ASP ELISA kit and by HPLC−UV. Samples tested included naturally contaminated ones and three from proficiency tests. Data obtained using the two reference and ELISA methods were compared to assess if ELISA technology may be suitable for the routine screening of PSP and ASP toxins, in different shellfish matrices in various presentations.

## MATERIALS AND METHODS

**Samples.** Naturally contaminated fish and shellfish samples representing the nine most commonly consumed species in Europe (mussels, cockles, barnacles, scallops, clams, oysters, giant cockles, razor shells) (*2*), in different preparations (fresh, frozen, boiled and canned), were tested for PSP toxins ($n$ = 80) and ASP ($n$ = 58, including two samples of anchovies). Although a few clams were from Chile, the samples were mainly of European origin. Some toxic samples were purchased from local harvesters (NW Spain) after obtaining permission for extraction from the Xunta de Galicia (Regional Autonomous Government) through the Dirección Xeral de Recursos Mariños (regional management of marine resources). In addition, three samples were obtained from proficiency tests. For each type of shellfish, samples were processed depending on the presentation: for raw products the whole flesh tissue was removed from the shells and drained in a sieve to remove salt water; canned products were washed with fresh water in order to remove sauces or brine and frozen products were allowed to thaw at room temperature on a sieve. Samples were homogenized with a domestic blender.

**Detection of PSP Toxins Using the AOAC Mouse Bioassay and the Ridascreen Fast PSP Test.** Shellfish homogenate (100 g) was extracted in 0.1 M HCl, adjusted to pH 2.5−4.0, according to the mouse bioassay AOAC extraction procedure (*26*), boiled for 5 min in a boiling water bath and centrifuged at 3000*g* for 10 min. The supernatants were recovered and tested by both MBA and ELISA methods, or stored frozen at −20 °C after MBA prior to ELISA analysis. The MBA was calibrated using saxitoxin dihydrochloride (STXdiHCl) standard supplied by the Canadian National Research Council (CNRC, Halifax, Canada). One-milliliter aliquots of extracts were ip injected into three male mice and observed for 1 h to quantify the toxin, according to the time of death. The limit of detection (LOD) of this technique was calculated, and a value of 350 $\mu$g saxitoxin equiv/kg shellfish meat was obtained, as previously reported (*26*). Shellfish extracts were analyzed using the Ridascreen Fast PSP (R-Biopharm AG, Darmstadt, Germany), a competitive ELISA for the quantitative analysis of saxitoxin and related toxins, based on anti-saxitoxin antibodies that bind PSP toxins with different affinities: saxitoxin 100%, gonyautoxins 2, 3 70%, decarbamoyl saxitoxin 20%, and neo-saxitoxin 12%. The LOD of this assay is 50 $\mu$g saxitoxin equiv/kg. The ELISA was carried out according to the kit user's manual (*27*). Briefly, 50 $\mu$L of six saxitoxin standard solutions with concentrations ranging from 0 to 40 ppb (0, 2.5, 5, 10, 20, and 40 ppb) or diluted shellfish extracts (range 1:40 to 1:640, depending on the toxin concentration of each sample) were added into separate wells. The same volume of diluted enzyme conjugate and anti-saxitoxin antibodies in solution was added into each well. The plate was mixed and incubated for 15 min at room temperature. After washing three times with deionized water, 100 $\mu$L of substrate cromogen was added to each well and incubated for 15 min in the dark. Color reaction was stopped with 100 $\mu$L of $H_2SO_4$ 0.5 M and the absorbance at 450 nm was measured. All standards and samples were tested in duplicate. A calibration curve was constructed using six standards concentrations (0 to 40 ppb). The working range (where the curve was linear showing a regression coefficient $>0.97$) of the calibration curve, usually ranging from 0 to 20 $\mu$g saxitoxin equiv/kg shellfish meat, was used to calculate the toxin concentration.

Mouse bioassays (*26*), were undertaken at ANFACO-CECOPESCA, a center approved by the Consellería do Medio Rural, Xunta de Galicia

**Table 1.** Summary of the Matrices of PSP Toxins and the Preparations Tested[a]

| shellfish | product presentation | *n* | range for PSP toxins ($\mu$g saxitoxin equiv/kg shellfish meat) MBA | ELISA |
|---|---|---|---|---|
| mussel (*Mytilus galloprovincialis*) (*n* = 64) | fresh | 25 | 410−7660 | 310−9000 |
| | frozen | 10 | <350−24540 | 20−20040 |
| | boiled | 2 | <350−590 | 20−390 |
| | homogenized (ref mater[b]) | 2 | 680−2220 | 450−2500 |
| | canned in brine sauce | 25 | 360−780 | 300−1170 |
| small clam (*Donax* spp), clam (*Tapes* spp) (*n* = 5) | fresh | 4 | <350−4510 | 40−4110 |
| | homogenized (ref mater) | 1 | 840 | 930 |
| cockle (*Cardium* spp) (*n* = 3) | fresh | 3 | 420−3980 | 1070−3420 |
| scallops (*Chlamys varia*) (*n* = 1) | fresh | 1 | <350 | 40 |
| barnacle (*Pollicipes cornucopia*) (*n* = 4) | fresh | 4 | 460−620 | 150−660 |
| oyster (*Ostrea* spp) (*n* = 1) | fresh | 1 | <350 | 40 |
| giant cockle (*Acanthocardia tuberculatum*) (*n* = 1) | fresh | 1 | <350 | 30 |
| razor shell (*Ensis* spp) (*n* = 1) | fresh | 1 | <350 | 40 |

[a] *n* = number of total samples for each kind of shellfish. [b] Ref mater: reference material from a proficiency test.

(Regional Autonomous Government) for carrying out mouse bioassays to detect PSP toxins, in accordance with Spanish and EU legislation.

**Determination of ASP Toxins by High Performance Liquid Chromatography with Ultraviolet Detection (HPLC−UV) and by ASP ELISA.** Shellfish samples were blended with an ultrahomogenizer. Amnesic toxins were extracted according to the standard method of Quilliam et al. (*28*). In addition, whole anchovies were used to obtain the methanolic extracts. The Lawrence method (*29*) was also used for HCl sample extractions in this study (data not shown). A small aliquot from each sample was analyzed using ASP ELISA kit (Biosense Laboratories, Bergen, Norway), a direct competitive immunoassay where free domoic acid in the sample competes with domoic acid-conjugated protein coated on plastic wells for binding to anti-domoic acid antibodies in the solution. These antibodies are specific for domoic acid with no cross-reactivities to nontoxic structural analogues, such as kainic acid and L-glutamic acid among others. A previous study suggests that anti-domoic acid antibodies could detect the domoic acid isomers in addition to the domoic acid and epi-domoic acid (*30*). However, the cross-reactivities of anti-domoic acid antibodies have not been tested. Shellfish extracts were diluted stepwise 1:2000, 1:20000, and 1:200000, with 10% methanol in phosphate buffered saline (PBS). To avoid unspecific matrix effects, 0.1% Tween was added prior to analysis. Ten calibration standards were freshly prepared by serial dilution of the certified reference calibration solution NRC-CRM-DA-d in the range of 10000−0.16 pg/mL. The assay was carried out according to the ASP ELISA kit user's manual (*31*). Briefly, 50 $\mu$L of each standard solution or diluted shellfish extracts were added into separate wells. The same volume of diluted enzyme conjugate anti-domoic acid antibodies was added into each well, except for the blank wells. The plate was sealed and incubated for 1 h at room temperature in darkness. After washing four times with 300 $\mu$L of washing buffer, 100 $\mu$L of TMB peroxidase substrate was added to each well and incubated for 15 min in the dark. Color reaction was stopped with 100 $\mu$L of $H_2SO_4$ 0.3 M, and the absorbance at 450 nm was measured. All standards and samples were tested in duplicate. A calibration curve was constructed plotting the absorbance values of the 10 standard dilutions on a linear scale (*y*-axis) against domoic acid concentrations of standard dilutions on a logarithmic scale (*x*-axis). Toxin concentrations of samples were calculated using the valid working range of the calibration curve, usually ranging from 6.5 to 254 pg/mL, with regression coefficients greater than 0.990.

**Data Analysis.** All experiments were carried out in duplicate on independent days. Results are expressed as means. Data were analyzed using Pearson's correlation (*r*) (SPSS, version 17.0), as the estimator of the correlation between both reference and immunological methods.

## RESULTS AND DISCUSSION

**Quantification of PSP Toxins.** To assess the suitability of the ELISA technology as an alternative method, several shellfish matrices were considered in different preparations. A total of 80 samples with a wide range of PSP toxin levels (**Table 1**) were tested by both ELISA and MBA. The ELISA showed higher sensitivity

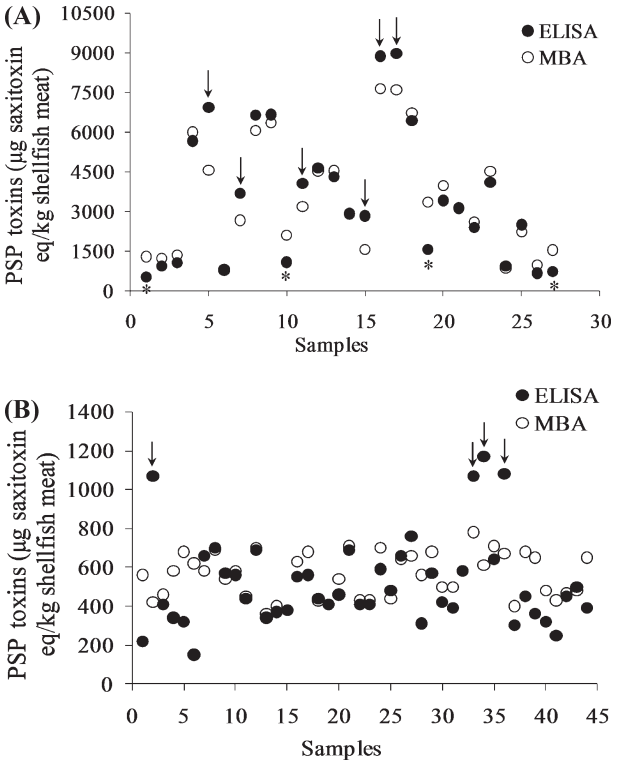


**Figure 2.** Comparison between the concentrations of PSP toxins obtained by ELISA and MBA in natural samples with higher (**A**) and lower (**B**) levels than 800 $\mu$g saxitoxin equiv/kg shellfish meat. Arrows indicate higher values by ELISA than by MBA, * indicate lower values by ELISA than by MBA.

than the MBA, with a LOD of 50 $\mu$g saxitoxin equiv/kg shellfish meat, while the LOD for the MBA was 350 $\mu$g saxitoxin equiv/kg shellfish meat. Moreover, the relative cross-reactivity of anti-saxitoxin antibodies used in this ELISA assay ensures that other saxitoxin analogues, such as neo-saxitoxin, decarbamoyl and gonyautoxins, are also detected. Of the 28 positive samples (>800 $\mu$g saxitoxin equiv/kg shellfish meat) determined by MBA, 18 were completely consistent with the ELISA test, and ten showed some differences. Six samples showed higher concentrations by ELISA than by MBA (**Figure 2A**, arrows), and four samples were lower by ELISA than by MBA (**Figure 2A**, asterisks). Two of these lower samples corresponded to fresh clam and mussel and both had the same origin. The other two samples, canned pickled mussels, gave values very close to the permitted

legal limit (**Table 2**). Although values for these four samples were possibly "underestimated" by ELISA, showing values < 800 $\mu$g saxitoxin equiv/kg shellfish meat, they were much closer to the permitted legal limit, indicating that in such cases confirmation by the reference methods is required. In this context, it should be stressed that the quantitative agreement between the MBA and the PSP-kit is dependent on antibody specificities and the toxin profile in the shellfish (*23*). Therefore, "overestimation" of the toxin content by ELISA in comparison with MBA could be related to the toxin composition in samples, because anti-saxitoxin antibodies also recognize saxitoxin analogues as decarbamoyl-saxitoxin and gonyautoxins 2/3, which are poorly detected by MBA.

Conversely, "underestimation" could be caused by relatively high values of neo-saxitoxin, gonyautoxins 1/4, or *N*-sulfocarbamoyl toxins (B1, B2, C1, C4) in some samples, which have less or no cross-reactivities with the anti-saxitoxin antibodies used in this screening method (*23*). Hence, the toxin profile could affect the PSP value obtained, as was observed in two samples with the same origin (**Figure 2A**, **Table 2**). We suggest that these samples could contain some saxitoxin derivatives that are not easily recognized by the anti-saxitoxin antibodies, thereby producing, in this case, lower values with ELISA than with MBA. The concentrations of toxins reached by ELISA (520−730 $\mu$g saxitoxin equiv/kg shellfish meat) were very close to the cutoff point of 800 $\mu$g saxitoxin equiv/kg shellfish meat, the current official limit. The remaining two samples were in pickled sauce, and the PSP value could be overestimated by MBA, as the sauce used in the canning process may interfere with the MBA. Our very preliminary data suggest that this sauce could interfere with some methods, such as "Lawrence" and ELISA. We are currently undertaking further work in this field.

**Table 2.** Concentration of PSP Toxins Measured by MBA and ELISA in Samples with Inconsistent Results[a]

| | concn of PSP toxins ($\mu$g saxitoxin equiv/kg shellfish meat) | |
|---|---|---|
| samples | MBA | ELISA |
| fresh clam[b] ($n$ = 1) | 1290 | 520 |
| fresh mussel[b] ($n$ = 1) | 1540 | 730 |
| pickled mussels ($n$ = 2) | 820 | 770 |
| | 980 | 670 |

[a] $n$ = number of samples. [b] Samples come from the same origin.

The rest of the samples ($n$ = 44) were negative by MBA (< 800 $\mu$g saxitoxin equiv/kg shellfish meat), and eight samples had lower levels than the LOD of MBA (350 $\mu$g saxitoxin equiv/kg shellfish meat). Four of the negative samples (**Figure 2B**, arrows) showed inconsistency between the results for ELISA and MBA. Higher toxin concentrations were obtained by ELISA, corresponding to values higher than the permitted legal limit and, therefore, considered not safe for human consumption.

In a few cases, the concentrations obtained by ELISA were "overestimated" or "underestimated", but comparison of the Ridascreen Fast PSP ELISA with the MBA gave a good qualitative agreement between the two methods, for all the different species with a high Pearson's correlation coefficient ($r$ = 0.974), and the following correlation equation: $y = 1.0575x - 57.517$.

Immunoassay techniques offer the advantages of standardized test format, simplicity, low cost and speed. It is worth stressing that the European Food Safety Authority (EFSA) recently recommended the implementation and validation of rapid and cost-effective screening methods for marine biotoxins (*25*, *32*). The high sensitivity of the ELISA method enables detection of PSP toxins in several shellfish species without interference from the matrix effect and applying the AOAC acid extraction procedure (*26*), showing toxic levels far below those that can be detected by MBA. In this context, the EFSA also recommends a more sensitive LOD than that available with current methods. Specifically, the expert panel proposes reducing the legal limit of PSP from 800 to 75 $\mu$g saxitoxin equiv/kg shellfish meat (*25*), which would make ELISA, with an LOD of 50 $\mu$g saxitoxin equiv/kg shellfish meat, a good method for PSP analysis. However, it should be noted that international validation of the ELISA kit for detection of PSP toxins would be necessary in order to use it as an alternative screening method.

Hence, ELISA technology has the important advantages of being simple, rapid and low cost, which make it a good choice for screening, reducing the number of animals used in MBA. This is supported by the excellent correlation coefficient found between both methods ($r$ = 0.974). Nevertheless, the toxin profile of samples should be taken into account when the immunoassay is used for screening purposes, ensuring that samples close to the legal limit will also be tested by MBA, as currently required by Commission Regulation 2074/2005, concerning the Lawrence method: "If the results are challenged, the reference method shall be the mouse bioassay" (*6*).

**Table 3.** Summary of the Matrices of ASP Toxins and the Preparations Tested[a]

| | | | range for ASP toxins (mg/kg domoic acid in shellfish flesh) | |
|---|---|---|---|---|
| shellfish | product presentation | $n$ | HPLC−UV | ELISA |
| mussel (*Mytilus galloprovincialis*) ($n$ = 7) | fresh | 2 | <1.6−2.3 | <0.6−3.9 |
| | frozen | 4 | 3.8−25.1 | 2.5−33.9 |
| | boiled | 1 | 5.0 | 14.6 |
| clams (*Tapes* sp, *Donax* spp, *Protothaca thaca*) ($n$ = 10) | fresh | 2 | 3.7 | 3.1−4.4 |
| | canned | 4 | <1.6 | 1.8−9.0 |
| | frozen | 4 | 5.0−6.0 | 5.0−9.0 |
| scallops (*Chlamys* spp, *Pecten jacobaeus*) ($n$ = 33) | fresh | 4 | <1.6−133 | 0.5−120 |
| | frozen | 8 | <1.6−115 | 1.3−127 |
| | without hepatopancreas | 5 | <1.6−17.0 | 1.6−24.0 |
| | canned | 15 | <1.6−35.9 | 1.3−10.9 |
| | boiled | 1 | 5.0 | 6.2 |
| cockle (*Cardium* spp) ($n$ = 5) | fresh | 3 | 2.63−3.4 | 2.2−4.4 |
| | frozen | 2 | 9.0−22.0 | 11.4−22.0 |
| anchovies (*Engraulis* spp) ($n$ = 2) | frozen | 2 | 79.6−80.3 | 53.9−83.9 |
| razor shell (*Ensis* spp) ($n$ = 1) | frozen | 1 | 10.4 | 11.5 |

[a] $n$ = number of total samples for each kind of shellfish.

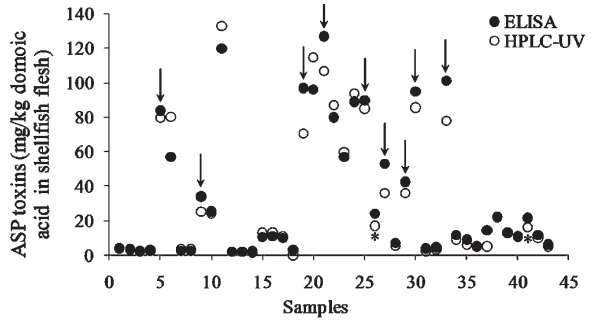


**Figure 3.** Comparison between the concentrations of ASP toxins obtained by ELISA and HPLC−UV in natural samples. Arrows indicate some inconsistent values, * indicate higher values by ELISA than by HPLC−UV.

**Quantification of ASP Toxins.** Naturally contaminated shellfish samples ($n = 58$) with domoic acid content, determined by HPLC−UV, in the range of $< 1.6-133$ mg/kg domoic acid in shellfish flesh and in different preparations (**Table 3**) were analyzed by ASP ELISA and compared with toxin concentrations obtained by the current reference method (HPLC−UV). The LOD was determined as 0.01 mg/kg domoic acid in shellfish flesh for ELISA and < 1.6 mg/kg domoic acid in shellfish flesh for HPLC−UV.

Of the 17 samples determined by HPLC−UV as unsuitable for consumption, all were properly determined by ELISA. Nine samples showed a higher concentration by immunoassay than by HPLC−UV, although these differences between the two methods do not influence the final result, as both methods indicate that the samples are not safe for consumption (**Figure 3**). Two samples (samples 26 and 41, marked with asterisks) had inconsistent results for the two methods, showing concentrations below the legal limit (20 mg/kg domoic acid in shellfish flesh) by HPLC−UV (16 and 17 mg/kg domoic acid in shellfish flesh), while higher values were found using ELISA (22 and 24 mg/kg domoic acid in shellfish flesh, respectively). Nevertheless, an excellent Pearson's correlation coefficient ($r = 0.973$) and correlation equation ($y = 0.96x - 0.4859$) were found between the two methods.

All samples tested were correctly determined by immunoassay, without "false negative" results. The ASP immunoassay was capable of detecting domoic acid at or below the maximum permitted limit in different species of shellfish and in several preparations without matrix interferences. The EFSA recommendations also propose reducing the legal limit of ASP from 20 to 4.5 mg/kg domoic acid in shellfish flesh (*32*). In this sense, the detection limit for ASP ELISA (60 μg/kg domoic acid in shellfish flesh) is more sensitive than the one for HPLC−UV (1 mg/kg domoic acid in shellfish flesh) (*32*). However, there are a few inconsistent results in samples close to the current legal limit. In some cases, ELISA overestimated toxin levels, concurring with a previous work by Kleivdal et al. comparing both methods (*30*). In this case, the authors suggested that the differences obtained could be due to detection by the antibodies of domoic acid isomers in addition to the domoic acid and epi-domoic acid. This fraction of domoic acid isomer toxins is not accounted for by most HPLC methods, including the current reference method. Indeed, if values close to the legal limit are obtained in a routine screening, the HPLC−UV method should be used as required by current legislation: "If the results are challenged, the reference method shall be the HPLC method" (*6*).

As proposed in Directive 86/609 (*33*), the use of immunoassays could help to reduce the number of bioassays within a monitoring system for shellfish toxins, establishing enhanced screening strategies, and thereby contributing to the improvement of food safety.

In summary, we have demonstrated in this study that the PSP ELISA method was able to quantify PSP toxins in nine of the most consumed shellfish species in different preparations without matrix interference. The high correlation coefficient found between MBA and ELISA suggests that the immunoassay could be a helpful tool to determine if a sample would also be positive in the MBA, reducing the number of animals used in routine shellfish monitoring. Similarly, the ASP ELISA was able to detect domoic acid in nine different species in several presentations. The excellent correlation coefficient shows that the matrix effect did not affect the accuracy of the assay, offering a good alternative to other screening methods in monitoring programs. The ELISA methods have a more sensitive detection limit for ASP and PSP toxins. Therefore, if the permitted toxin limits are eventually reduced, these methods could be a good alternative in screening.

## ABBREVIATIONS USED

ASP, amnesic shellfish poisoning; MBA, mouse bioassay; PSP, paralytic shellfish poisoning.

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