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Inhibition Equivalency Factors for Dinophysistoxin-1 and Dinophysistoxin-2 in Protein Phosphatase Assays: Applicability to the Analysis of Shellfish Samples and Comparison with LC-MS/MS

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Supporting Information

ABSTRACT: The protein phosphatase inhibition assay (PPIA) is a well-known strategy for the determination of diarrheic shellfish poisoning (DSP) lipophilic toxins, which deserves better characterization and understanding to be used as a routine screening tool in monitoring programs. In this work, the applicability of two PPIAs to the determination of okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), and their acyl ester derivatives in shellfish has been investigated. The inhibitory potencies of the DSP toxins on a recombinant and a wild PP2A have been determined, allowing the establishment of inhibition equivalency factors (IEFs) (1.1 and 0.9 for DTX-1, and 0.4 and 0.6 for DTX-2, for recombinant and wild PP2A, respectively). The PPIAs have been applied to the determination of OA equivalent contents in spiked and naturally contaminated shellfish samples. Results have been compared to those obtained by LC-MS/MS analysis, after application of the IEFs, showing good agreements.

KEYWORDS: *protein phosphatase 2A, protein phosphatase inhibition assay, okadaic acid, dinophysistoxin-1, dinophysistoxin-2, liquid chromatography−tandem mass spectrometry*

INTRODUCTION

Okadaic acid (OA) and its analogues dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) are lipophilic phycotoxins produced mainly by dinoflagellates of the genera *Dinophysis* and *Prorocentrum*.[1] Their chemical structure is composed of a polyketide backbone containing furan and pyran-type ether rings and an α-hydroxycarboxyl function, the difference between analogues being only the number or the position of the methyl groups.[2] When incorporated in shellfish, these phycotoxins are accumulated mainly in the digestive gland and are responsible for the diarrheic shellfish poisoning (DSP) syndrome, which causes gastrointestinal disturbances such as diarrhea, nausea, vomiting, and abdominal pain.[3]

OA and DTXs are known inhibitors of protein phosphatases (PP1 and PP2A), enzymes that play an important role in protein dephosphorylation in cells.[4] These toxins bind to the receptor site of the enzyme, blocking its activity, and as a consequence they favor hyperphosphorylation of proteins that control sodium secretion and of cytoskeletal or junctional moieties that regulate solute permeability, causing sodium release and a subsequent passive loss of fluids responsible for the diarrheic symptoms.[5] Moreover, it has been demonstrated to be an additional tumor promoter in mouse skin carcinogenesis.[6]

Because of their implications on public health, the Regulation (EC) No. 853/2004 in Europe has established a maximum permitted level of 160 μg of OA equivalents/kg shellfish meat.[7] Although it is possible to use the mouse bioassay (MBA)[8] until December 31, 2014, the Commission Regulation (EU) No. 15/2011 has recently established that liquid chromatography−tandem mass spectrometry (LC-MS/MS) should be applied as the reference method for the determination of lipophilic toxin contents in shellfish.[9] This regulation was applied from July 1, 2011, and the LC-MS/MS method will replace the MBA in 2015. These regulations also accept the use of other chemical methods, as well as immunoassays and functional assays, such as the protein phosphatase inhibition assay (PPIA), as alternatives or supplements to the LC-MS/MS method, provided that they can determine OA, DTX-1, DTX-2, and their esters, that they fulfill the method performance criteria (they should be validated and successfully tested under a recognized proficiency test scheme), and that their implementation provides an equivalent level of public health protection.

The development of rapid, sensitive, and low-cost methods for the detection of DSP toxins is necessary to guarantee shellfish safety and protect human health. The PPIA is an interesting method for the simple, fast, sensitive, and robust determination of DSP toxin contents in shellfish. Colorimetric PPIAs using PP in solution have been developed.[10−17] In most of the works, OA has been used as model DSP toxin,[10,11] and only in a few studies has the inhibitory potential of OA analogues or derivatives been evaluated and none of them have used high-quality certified reference materials.[12−17] The establishment and use of toxicity equivalent factors (TEFs) for toxic compounds of a same group in alternative methods for marine toxin detection is necessary to guarantee consumer protection in monitoring programs, as they allow a better estimation of the toxic potential of a mixture of toxins with different potency.[18,19]

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Apart from the use of the PPIA for quantitative purposes, this assay is a promising screening tool to be run in parallel to the official control methods in monitoring programs. For example, PPIA could be used to screen DSP toxins in hydrolyzed shellfish samples, reducing the instrumental analytical requirements and still protecting public health. Nevertheless, it requires in-depth characterization and performance evaluation before its approval and routine use. With this aim, we have evaluated the practical application of the PPIA to the analysis of shellfish contaminated with DSP toxins. First, the different inhibitory potencies of OA, DTX-1, and DTX-2 on a recombinant and a wild PP2A have been determined, and the corresponding inhibition equivalency factors (IEFs) have been established. Definition of IEFs is important to characterize the performance of the PPIA, but it is also crucial to get comparable results with the reference LC-MS/MS method. Afterward, both PPIAs have been applied to the determination of DSP toxin contents in mussel samples spiked with OA, DTX-1, and/or DTX-2 and in naturally contaminated shellfish (mussels, cockles, clams, and razor clams) samples. Results have been compared with those obtained by LC-MS/MS analysis after the application of the IEFs.

MATERIALS AND METHODS

Reagents and Materials. Certified calibration solution of okadaic acid (NRC CRM-OA, 14 300 μg/L), dinophysistoxin-1 (NRC CRM-DTX1, 15 100 μg/L), and dinophysistoxin-2 (NRC CRM-DTX2, 7800 μg/L) in methanol (MeOH) were kindly provided by the Institute for Marine Biosciences of the National Research Council (Halifax, Canada). The recombinant protein phosphatase 2A (PP2A) catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France). Commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (New York, NY). The activity of the stock solutions was between 766 and 1025 U/mL for PP2A from GTP Technology and between 5720 and 7491 U/mL for PP2A from Upstate Biotechnology, 1 U being defined as the amount of enzyme required to hydrolyze of 1 nmol *p*-nitrophenyl phosphate (*p*-NPP) in 1 min at room temperature. Components of buffers and *p*-NPP were purchased from Sigma (Tres Cantos, España). For LC-MS/MS analysis, gradient-grade MeOH, formic acid, and hypergrade acetonitrile (ACN) for LC-MS were purchased from Merck (Darmstadt, Germany). Ammonium formate (≥99.995%), sodium hydroxide pellets (≥99%), and hydrochloric acid 37% for analysis were purchased from Sigma-Aldrich (St. Louis, MO), Riedel-de Haën (Seelze, Germany), and Panreac (Barcelona, Spain), respectively. All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, MA).

Samples. Fourteen samples obtained from the European Union Reference Laboratory for Marine Biotoxins (EU-RL-MB) in Vigo, Spain, and that had been analyzed for the collaborative Interlaboratory Validation Study of the "EU-Harmonised Standard Operation Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS" (EU-RL-MB SOP),[20] were used in this work. They corresponded to seven materials distributed as blind duplicates of different species of molluscs naturally contaminated: raw wedge shell clam homogenate (*Donax trunculus*), raw razor clam homogenate (*Ensis acuatus*), raw mussel homogenate (*Mytilus edulis*), raw stripped venus (*Chamelea gallina*), two cooked mussel homogenates (*Mytilus edulis*), and raw cockle homogenate (*Cerastoderma edule*).

Lipophilic Toxin Extraction. For lipophilic toxin extraction, the "EU-Harmonised Standard Operation Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS" (EU-RL-MB SOP) procedure was followed.[20] First, tissue homogenate (2 g) was weighed in a 50-mL polypropylene centrifuge tube. MeOH (100%, 9 mL) was added, and the sample was homogenized by vortex-mixing for 3 min at maximum speed level (~2500 laps/min). Extract was centrifuged at 2000*g* for 10 min at ca. 20 °C, and the supernatant was transferred to a 20-mL volumetric flask. The extraction of the residual tissue pellet was repeated with 100% MeOH (9 mL), and the sample was homogenized for 1 min with a high-speed homogenizer Ultraturrax T25 (IKA-Labortechnik). After centrifugation under the same conditions previously applied, the supernatant was transferred and combined with the supernatant from the previous extraction, and the total extract was made up to 20 mL with 100% MeOH in a volumetric flask. Extracts were passed through 0.2 μm cutoff nylon syringe filters (Whatman), and were directly injected onto the LC-MS/MS system. For extracts to be tested with PPIA, samples were evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, MA) under nitrogen at room temperature, and the residues were resuspended in the corresponding buffer.

Sample Hydrolysis. To determine the total OA and DTX content, an alkaline hydrolysis was performed before LC-MS/MS analysis and PPIA.[20,21] For the hydrolysis, NaOH at 2.5 M (125 μL) was added to the extract (1.25 mL), the mixture was homogenized in a vortex mixer for 0.5 min, and heated at 76 °C for 40 min in a Multi-BlockHeater from Lab-line Instruments, Inc. (Maharashtra, India). After cooling to room temperature, HCl at 2.5 M (125 μL) was added for neutralization and the sample was homogenized by vortex-mixing for 0.5 min. The hydrolyzed extract was then filtered through 0.2 μm cutoff nylon syringe filters (Whatman). As described for crude extracts, hydrolyzed extracts were directly analyzed by LC-MS/MS, while for PPIA they were evaporated under nitrogen and resuspended in the corresponding buffer to the desired concentration.

Colorimetric PPIA. The PPIA was performed as described previously[17,22] but three different experiments were carried out: (1) evaluation of the inhibitory potencies of DSP toxins and the IEFs of DTXs, (2) determination of DSP toxin contents in spiked mussel samples, and (3) determination of DSP toxin contents in naturally contaminated shellfish samples. To this purpose, 50 μL of blank mussel sample solution at 12.5 mg/mL spiked with OA, DTX-1, or DTX-2 standard solutions at different concentrations ranging from 1.6 to 100.0 μg/L (for experiment 1 and for OA calibration curves of experiments 2 and 3), 50 μL of blank mussel sample solution at 12.5 mg/mL spiked with OA at 160 μg/L, DTX-1 at 166 μg/L and/or DTX-2 at 176 μg/L (for experiment 2) or 50 μL of naturally contaminated shellfish sample solution at different concentrations ranging from 1.6 to 12.5 mg/mL (for experiment 3) were added into microtiter wells containing 100 μL of PP2A solution (recombinant from GTP Technology or wild from Upstate Biotechnology) at 1.25 U/mL. Then, 50 μL of 25 mM *p*-NPP solution were added and after 1-h incubation at 22 °C in the dark, the absorbance at 405 nm was measured with an automated multiwell scanning spectrophotometer (Biotek, Synergy HT, Winooski, VT). Samples were prepared in a buffer solution containing 30 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.4. PP2A, and *p*-NPP solutions were prepared in the same buffer, also containing 2 mM DTT and 0.2 mg/mL BSA. Assays were performed in triplicate. In the analysis of naturally contaminated shellfish samples and in the evaluation of the inhibitory effect of DSP toxin mixtures, OA calibration curves using 12.5 and 6.3 mg/mL of mussel matrix, for recombinant and wild PP2A, respectively, were always performed in parallel for the precise toxin quantification. The DSP toxin calibration curves obtained by PPIA were analyzed with SigmaPlot software package 10.0 and fitted to sigmoidal logistic four-parameter equations:

$$y = y_0 + \frac{a}{1 + (x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point, and b is the slope at the inflection point.

LC-MS/MS Analysis. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was applied following the "EU-Harmonised Standard Operation Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS" (EU-RL-MB SOP).[20] Analyses were conducted on an Agilent 1200 LC (Agilent Technologies, Santa Clara, CA) coupled with a 3200 QTRAP mass

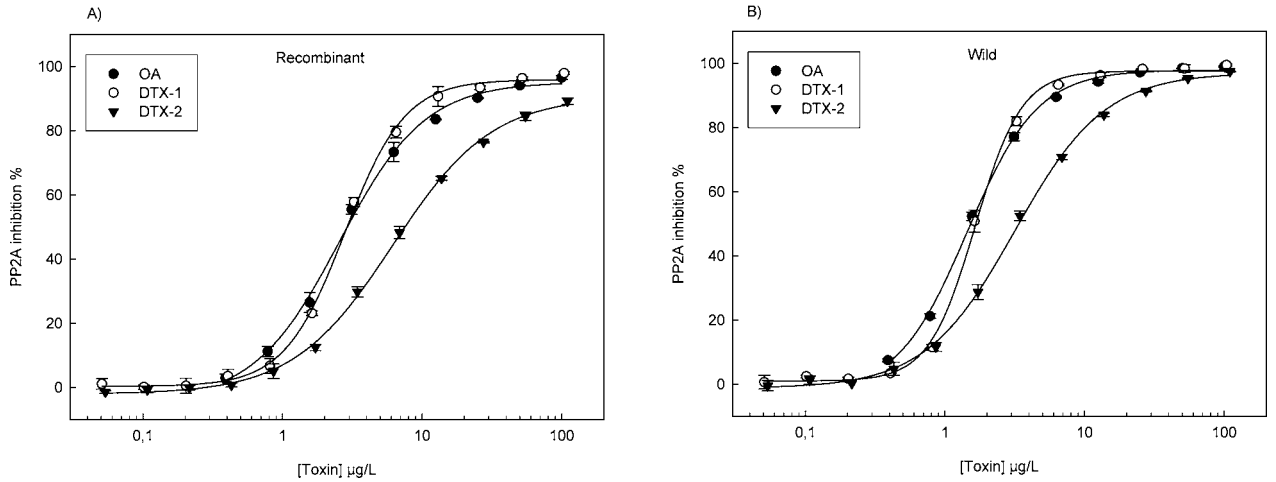

Figure 1. Dose–response curves for the inhibition of recombinant (A) and wild (B) PP2A by OA, DTX-1, and DTX-2. Inhibition is expressed as percentage of the control (no toxin); x values refer to initial toxin concentrations.

Table 1. Curve Parameters Derived from the Sigmoidal Logistic Four-Parameter Fitting for the Inhibition of PP2As by OA, DTX-1, and DTX-2

toxin	enzyme	IC_{50} (µg/L)	IEF	working range IC_{20} – IC_{80} (µg/L)	equation	R^2
OA	recombinant	2.93	1.0	1.2–8.4	$y = -2.0 + (96.1/(1 + x/2.7)^{-1.5})$	0.9994
	wild	1.54	1.0	0.7–3.5	$y = -0.7 + (98.4/(1 + x/1.5)^{-1.8})$	0.9998
DTX-1	recombinant	2.90	1.1	1.4–6.0	$y = 0.3 + (95.5/(1 + x/2.7)^{-2.0})$	0.9996
	wild	1.66	0.9	0.9–2.8	$y = 0.9 + (96.7/(1 + x/1.6)^{-2.6})$	0.9996
DTX-2	recombinant	7.54	0.4	2.2–29.7	$y = -2.1 + (92.8/(1 + x/5.6)^{-1.2})$	0.9996
	wild	3.38	0.6	1.1–9.2	$y = -1.3 + (98.4/(1 + x/2.9)^{-1.4})$	0.9996

spectrometer through a TurboV electrospray ion source (Applied Biosystems, Foster City, CA). Chromatographic separations were performed at 30 °C and 0.2 mL/min on a Luna C8(2) column (50 mm × 1 mm, 3 µm) protected with a SupelcoGuard C8(2) cartridge (4 mm × 2 mm, 3 µm), both from Phenomenex (Torrance, CA). Acidic chromatographic elution was selected with mobile phases 100% water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. For DSP toxins, multiple reactions monitoring (MRM) analysis was accomplished from the precursor ions 803.5 and 817.5 m/z for OA/DTX-2 and DTX-1, respectively. Product ions were common for all DSP toxins, with ions 255.2 m/z monitored for quantification and 113.1 m/z acquired for confirmatory purposes. The mass spectrometer was operated in negative polarity, and compound-dependent parameters for MS/MS detection were tuned on the mass spectrometer through direct infusion of the CRM-OA standard: declustering potential −115 V, entrance potential −12 V (for 803.5 > 255.2) and −10.5 V (for 803.5 > 113.1), collision entrance potential collision energy −64 V (for 803.5 > 255.2) and −68 V (for 803.5 > 113.1), and collision cell exit potential −4 V. Gas/source parameters were also optimized (curtain gas 20 psi; ion spray −4500 V, temperature 400 °C, nebulizer gas 50 psi, heater gas 50 psi). Under these conditions, the LOD and LOQ were 10 and 30 µg/kg OA in shellfish meat, respectively. The quantification curve obtained for OA was used also for the quantification of DTX-1 and DTX-2, because this approach was the recommended by the EU-RL-MB SOP.[20] Analyst software v1.4.2 was used for the entire MS tune, instrument control, data acquisition, and data analysis.

Statistical Analysis. To evaluate differences in the calibration curves for OA, DTX-1, and DTX-2 between recombinant and wild PP2A, the paired t test was used (N = 10). Differences in the results were considered statistically significant at the 0.05 level. Prior to analysis, data were tested for normality; Wilcoxon matched-pairs signed-ranks test was used for non-normally distributed data sets instead of the paired t test.

To evaluate the correlation between the OA equivalent contents in spiked mussel samples determined from the two PPIAs and the expected values, the linear regression model was used. The linear regression model was also used to evaluate the correlation between the OA equivalent contents in naturally contaminated shellfish samples determined from the two PPIAs and the values obtained from the LC-MS/MS analysis after application of the IEFs for each PP2A and the TEFs from EFSA. Differences in the results were considered statistically significant also at the 0.05 level. The SigmaStat software package 3.1 was used for the paired t tests, the Wilcoxon matched-pairs signed-ranks tests, and the linear regressions.

RESULTS AND DISCUSSION

Inhibitory Potencies of DSP Toxins and IEFs of DTXs. Dose–response curves with OA, DTX-1, and DTX-2 (Figure 1) were performed to evaluate the inhibitory potencies of these DSP toxins on the activity of two PP2A enzymes from different origins (recombinant and wild). Toxin dilutions from stock solutions were prepared in a buffer solution containing blank mussel matrix at 12.5 mg/mL. This blank mussel did not contain DSP lipophilic toxins as determined by LC-MS/MS. The 12.5 mg/mL concentration had been previously established as equal to (for wild PP2A) or below (for recombinant PP2A) the maximum loading limit to use in the PPIA to avoid unspecific inhibition from the mussel matrix.[22] In Table 1, the 50% inhibition coefficient (IC_{50}) values, the inhibition equivalency factors (IEFs), and the working ranges (defined between IC_{20} and IC_{80}) are presented together with the equations and the corresponding R^2 values. The IEFs were calculated as the ratios of the IC_{50} for OA to the IC_{50} for DTX-1 or DTX-2, for each enzyme.

Comparing enzyme sources, the wild PP2A was significantly more sensitive to all DSP toxins than the recombinant one (t_{OA}

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Table 2. DSP Lipophilic Toxin Spiking Combinations, OA Equivalent Contents (μg of OA equiv/kg mussel meat) Expected According to the Spiked Concentrations and the IEFs and Determined by PPIA with Recombinant and Wild PP2A[a]

	DSP lipophilic toxin			expected $[OA]_{eq}$		determined $[OA]_{eq}$	
combination	OA	DTX-1	DTX-2	$PP2A_{rec}$	$PP2A_{wild}$	$PP2A_{rec}$	$PP2A_{wild}$
1	+	+	+	404	404	576 ± 3	569 ± 17
2	+	+	−	340	307	314 ± 16	328 ± 3
3	+	−	+	224	257	277 ± 18	273 ± 1
4	−	+	+	244	244	187 ± 9	208 ± 4
5	+	−	−	160	160	172 ± 3	164 ± 1
6	−	+	−	180	147	101 ± 6	127 ± 9
7	−	−	+	64	97	n.d.[b]	63 ± 2
8	−	−	−	0	0	n.d.	n.d.

[a]The + symbol indicates 160, 166, and 176 μg/kg for OA, DTX-1, and DTX-2, respectively; the − symbol indicates 0. [b]n.d. = not detected: <96 μg/kg for $PP2A_{rec}$; <56 μg/kg for $PP2A_{wild}$.

= 3.957, p_{OA} = 0.003; W_{DTX-1} = 53, p_{DTX-1} = 0.004; t_{DTX-2} = 5.125, p_{DTX-2} < 0.001): 1.9-fold lower IC_{50} for OA, 1.7-fold lower IC_{50} for DTX-1, and 2.2-fold lower IC_{50} for DTX-2. Regarding the individual DSP toxins, DTX-1 inhibits both PP2As at approximately the same potency than OA (t_{rec} = 1.258, p_{rec} = 0.240; t_{wild} = 0.311, p_{wild} = 0.763), whereas DTX-2 inhibits both PP2As significantly less (with respect to OA: t_{rec} = 4.502, p_{rec} = 0.001; t_{wild} = 3.610, p_{wild} = 0.006; with respect to DTX-1: t_{rec} = 3.885, p_{rec} = 0.004; t_{wild} = 2.796, p_{wild} = 0.021). Although the IC_{50} values were different for each PP2A, the determined IEFs were similar because the trend was the same: OA ≈ DTX-1 > DTX-2.

Few works exist describing the inhibitory potencies of DTXs. Regarding DTX-1, Takai and collaborators[12] reported a 1.6-fold lower dissociation constant for DTX-1 compared to OA for a catalytic subunit PP2A from rabbit skeletal muscle (value comparable to our IEFs calculated from the IC_{50} values). Rivas and co-workers[23] also observed a lower IC_{50} value for DTX-1 with respect to OA for a PP2A purified from the mussel *Mytilus chilensis*, resulting in an IEF of 2.4. On the contrary, Mountfort and collaborators[21] observed a higher IC_{50} value for DTX-1 compared to OA for the same PP2A as the wild used in our work, which could be translated into an IEF of 0.6 for DTX-1. More recently, Ikehara and co-workers[16] obtained an IEF of 0.9 for DTX-1 with a catalytic subunit of recombinant human PP2A (also calculated from the IC_{50} values as described herein), and Smienk and collaborators[24] obtained an IEF of 0.75 for DTX-1 with a PP2A purified from human red blood cells.

Regarding DTX-2, the lower inhibitory potency of DTX-2 with respect to OA is comparable to its reduced acute intraperitoneal toxicity observed in mice, which allowed to establish a relative toxicity equivalency factor (TEF) of about 0.6.[18] This TEF has been adopted by the Panel on Contaminants in the Food Chain for regulated marine biotoxins.[25] The IEFs found in this work are also similar to the IEF reported by Aune and co-workers[18] of 0.48 with a PP2A from human red blood cells. The lower inhibitory potency of DTX-2 on PP2A has been suggested to be due to the 35-methyl group stereochemistry, which would be responsible for unfavorable interactions with Gln122 and His191 residues.[26,27] Nevertheless, Smienk and collaborators[24] have recently reported equal toxicity for DTX-2 and OA.

Several reasons could explain the different IEFs reported in the literature. Differences in enzyme sources, enzyme concentrations, toxin standard purities, enzyme substrates, and buffer compositions results in different IC_{50} values. Consequently, for a clear establishment of IEFs, PPIAs should be carefully controlled and performed simultaneously for all toxins under study.

Regarding the applicability of the PPIAs, taking into account the shellfish matrix loading limit and the attained LODs, the assays developed herein should be able to quantify 96 and 56 μg of OA/kg shellfish meat with recombinant and wild PP2A, respectively, far below the 160 μg of OA equivalents/kg shellfish meat regulation level established by the EU 853/2004.[7] By applying the IEFs obtained in this work, the regulation levels for a sample containing only DTX-1 would be 145 and 178 μg of DTX-1/kg shellfish meat with recombinant and wild PP2A, respectively. The assays are able to quantify 88 and 72 μg of DTX-1/kg shellfish meat with recombinant and wild PP2A, respectively. For samples containing only DTX-2, the regulation levels would be 400 and 267 μg of DTX-2/kg shellfish meat with recombinant and wild PP2A, respectively. The assays are able to detect 176 and 88 μg of DTX-2/kg shellfish meat, with recombinant and wild PP2A, respectively. Consequently, in principle the developed PPIAs should be able to protect human health, regardless of the DSP toxins present in the shellfish sample.

Determination of DSP Toxin Contents in Spiked Mussel Samples. To evaluate the applicability of the developed PPIAs to the analysis of shellfish samples with multi-DSP toxin profiles, an experiment was performed using different OA/DTX-1/DTX-2 combinations. In principle, the experiment was planned to spike 160 μg of each DSP toxin/kg mussel meat. However, preliminary reported DSP toxin concentrations were used in the PPIAs, which slightly varied at the moment of writing the present manuscript after LC-MS/MS confirmation. Nevertheless, the purpose of the assay is not compromised by these slightly higher concentrations (166 μg/kg for DTX-1 and 176 μg/kg for DTX-2). As in the previous experiment, a buffer solution containing blank mussel matrix at 12.5 mg/mL was used for the DSP toxin spiking and the OA calibration curve. In the determination of the DSP toxin contents (μg of OA equiv/kg mussel meat), the inhibition percentage obtained for each spiked mussel sample and the corresponding IC value of the OA calibration curve determined from the sigmoidal logistic four-parameter equation were taken into account. Table 2 shows the DSP toxin combinations used in the spiking, the expected $[OA]_{eq}$, according to the theoretical spiked concentrations and the established IEFs, and the $[OA]_{eq}$ determined by the PPIAs with both enzymes. According to the spiked DSP toxin concentrations and the corresponding IEFs, combinations 7 and 8 were expected as "negatives",

Table 3. OA, DTX-1, and DTX-2 Contents Determined by LC-MS/MS Analysis, Total OA Equivalent Contents Calculated by the Application of the Corresponding TEFs and IEFs Values to the Individual Toxin Quantifications, and Total OA Equivalent Contents Obtained by the PPIAs with the Recombinant and the Wild PP2A[a]

		LC-MS/MS			$\sum[OA]_{eq}$				
sample	number	OA	DTX-1	DTX-2	TE $value_{EFSA}$	IE $value_{rec}$	IE $value_{wild}$	$PP2A_{rec}$	$PP2A_{wild}$
raw razor clam	1	n.d.[b]	n.d.	n.q.[c]	22	19	22	n.d.	n.d.
	1H	64	n.d.	n.q.	81	78	81	n.d.	157
	2	n.d.	n.d.	n.q.	22	19	22	n.d.	n.d.
	2H	55	n.d.	n.q.	72	69	72	n.d.	165
raw cockle	3	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	3H	163	n.d.	63	206	194	205	139	188
	4	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	4H	152	n.d.	55	190	180	190	136	266
raw stripped venus	5	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	5H	175	n.d.	50	210	201	210	175	307
	6	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	6H	201	n.d.	45	233	225	233	177	300
raw wedge shell clam	7	30	n.d.	105	98	78	98	n.d.	162
	7H	172	n.d.	131	256	230	255	237	403
	8	30	n.d.	125	110	86	110	n.d.	161
	8H	151	n.d.	138	239	212	238	240	362
raw mussel	9	36	133	n.d.	172	184	159	345	465
	9H	69	275	n.d.	347	374	320	368	499
	10	43	157	n.d.	203	218	187	333	408
	10H	63	187	n.d.	253	271	234	495	565
cooked mussel	11	294	n.d.	n.q.	311	308	311	217	428
	11H	450	n.d.	n.q.	467	464	467	312	1229
	12	284	n.d.	n.q.	301	298	301	194	422
	12H	479	n.d.	n.q.	496	493	496	316	802
	13	178	107	168	386	363	375	289	581
	13H	388	129	252	668	631	655	420	1651
	14	186	135	162	418	399	405	254	443
	14H	430	120	224	684	652	672	459	1199

[a]All values are expressed as μg of toxin/kg mussel meat. [b]n.d. = not detected: <10 μg/kg for LC-MS/MS; <96 μg/kg for $PP2A_{rec}$; <56 μg/kg for $PP2A_{wild}$. [c]n.q. = not quantified: 10 μg/kg $\leq$ concentration $\leq$30 μg/kg for LC-MS/MS.

combinations 1, 2, 3, 4 were expected as "positives", and combinations 5 and 6 were expected as "suspicious". Statistical analysis of all combinations as a whole revealed that there were not significant differences in the OA equivalent contents determined by the two PPIAs with respect to the expected values ($PP2A_{rec}$: $y = 1.191\ x - 25.131$, $R^2 = 0.817$, $p = 0.002$; $PP2A_{wild}$: $y = 1.298\ x - 42.204$, $R^2 = 0.910$, $p < 0.001$).

Regarding "negative" combinations, the control sample without any DSP toxin (combination 8) did not induce any PP2A inhibition, thus confirming that the mussel matrix loading was appropriate for the developed PPIAs. Samples with only DTX-2 (combination 7) slightly inhibited the wild PP2A but did not inhibit the recombinant PP2A. In fact, in the previous section we define 176 μg/kg as LOD of DTX-2 for recombinant PP2A; consequently, the toxin content of this sample was close to the corresponding LOD and thus difficult to detect. Nevertheless, the experimental design shows the suitability of the PPIAs as screening tools able to identify "negative" samples in a simple, fast, inexpensive, and reliable way.

Both PPIAs indicated that combinations 1, 2, 3, and 4 are "positive", as expected. One can appreciate that in the most toxic profile (combination 1), the OA equivalent contents determined by the PPIAs were higher than the expected ones, which may indicate a possible overestimation at high toxin levels. One cannot neglect that in this combination the three toxins are present and thus a synergistic effect could be present. Nevertheless, results indicate that PPIAs are able to identify "positive" samples. In an official monitoring program, a preventive closure of the shellfish harvesting area would be recommended to protect the consumer health; the sample would be also processed by LC-MS/MS to confirm the "positive" result.

Regarding "suspicious" combinations, quantifications derived from the PPIAs also agree with the expected values. Taking into account the obtained results and to use the PPIAs as screening tools, we define a "suspicious range" between 80 and 180 μg/kg of OA equivalent contents, which imply the analysis of the sample by the reference LC-MS/MS method for decision purposes. As we mentioned in the previous paragraph, a result above 180 μg/kg of OA equivalent contents would require the LC-MS/MS analysis for confirmatory purposes.

Determination of DSP Toxin Contents in Naturally Contaminated Shellfish Samples. The PPIAs with recombinant and wild PP2A were applied to the determination of DSP toxins in fourteen naturally contaminated shellfish samples. They corresponded to seven samples analyzed as duplicates: raw wedge shell clam homogenate (*Donax trunculus*), raw razor clam homogenate (*Ensis acuatus*), raw mussel homogenate (*Mytilus edulis*), raw stripped venus (*Chamelea gallina*), two cooked mussel homogenates (*Mytilus edulis*), and raw cockle homogenate (*Cerastoderma edule*). OA

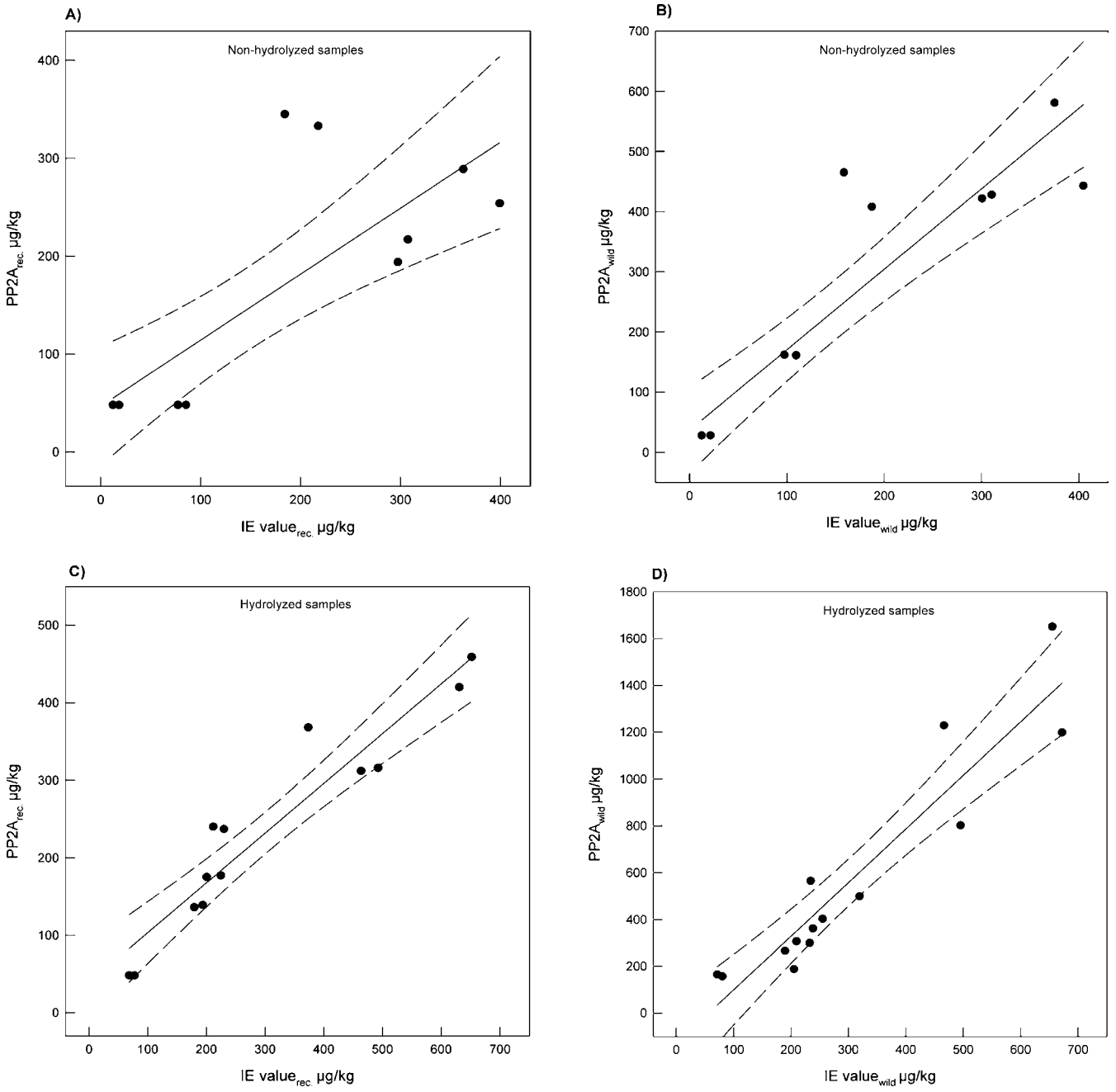

Figure 2. Linear regressions for the correlations of the PPIAs for non-hydrolyzed (A and B) and hydrolyzed (C and D) samples, with the recombinant (A and C) and wild (B and D) PP2As, with respect to the corresponding IE values.

calibration curves were performed in parallel to each quantification analysis because of possible slight differences in the inhibition percentages between assays. As in the previous experiments, a buffer solution containing blank mussel matrix at 12.5 mg/mL was used for the OA calibration curves with recombinant PP2A, but a mussel matrix concentration of 6.3 mg/mL was used with wild PP2A because of the higher unspecific inhibition of hydrolyzed mussel on the activity of this enzyme.[22] In the determination of the DSP toxin contents (μg of OA equiv/kg shellfish meat), the IC_{50} values of the shellfish sample dose−response curves determined from lineal regressions and the IC_{50} values of the OA calibration curves determined from the sigmoidal logistic four-parameter equations were used. In Table 3, PPIA results are compared to those determined by LC-MS/MS analysis, which provided individual OA, DTX-1, and DTX-2 contents. Additionally, total OA equivalent contents ($\sum[OA]_{eq}$) were calculated by applying the TEFs proposed by EFSA[25] and the IEFs obtained in this article for each PPIA to the individual toxin quantifications obtained by LC-MS/MS analysis. For those samples where the toxin content was not detected or not quantified, compromised values corresponding to a half of the thresholds were considered.

In general terms, results derived from the PPIAs agree with those obtained from the application of the TEFs and IEFs to the LC-MS/MS analysis. Moreover, PPIAs with both the recombinant and the wild PP2A provided similar results, and agreement is also observed between duplicates. As LC-MS/MS

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indicates, all samples contain acyl ester derivatives, detectable after hydrolysis. Because hydrolyzed samples always provided higher toxin contents in the PPIAs, it is fair to suggest that the acyl ester derivatives inhibit PP2As less than the corresponding precursor toxins. Nevertheless, they may still be inhibiting and thus contributing to the OA equivalent contents determination in non-hydrolyzed samples.

Taking into account the different behavior between non-hydrolyzed and hydrolyzed samples, we performed statistical analysis of free and total DSP toxin contents separately. The result obtained for sample 10H with the recombinant PP2A was considered discrepant (out from the 95% prediction band), and it was not included in the analysis. Figure 2 shows the linear regressions for the correlations between the OA equivalent contents obtained from the PPIAs and the IE values. As it can be observed, hydrolyzed samples correlate better ($PP2A_{rec}$: $y = 0.642x + 39.067$, $R^2 = 0.899$, $p < 0.001$; $PP2A_{wild}$: $y = 2.290x - 129.43$, $R^2 = 0.875$, $p < 0.001$) than non-hydrolyzed ones ($PP2A_{rec}$: $y = 0.674x + 46.771$, $R^2 = 0.664$, $p < 0.001$; $PP2A_{wild}$: $y = 1.338x + 36.691$, $R^2 = 0.850$, $p < 0.001$). As we mentioned above, the contribution from the acyl ester derivatives, which are inhibiting the enzymes although in a lower extent than the precursor toxins, may be responsible for the worse agreements in the free DSP toxin contents. The same behavior was observed in the comparison of the PPIA results with the TE values (Figure S1, Supporting Information), due to the similarity between the IEFs and the TEFs established by the EFSA.

Comparing enzyme sources, whereas the tendency of the recombinant PP2A is to underestimate the OA equivalent contents, the tendency of the wild PP2A is to overestimate them. The underestimation from the recombinant PP2A together with the higher LODs compared to the wild PP2A would explain that the recombinant PP2A did not detect toxicity in samples 1H, 2H, 7, and 8, whereas the wild PP2A did. The overestimation from the wild PP2A is more evident in the analysis of hydrolyzed samples than in the analysis of the non-hydrolyzed ones. The hydrolysis procedure could be responsible for the overestimation, because unspecific inhibition from blank hydrolyzed samples is higher than from non-hydrolyzed samples for this wild PP2A.[22]

Finally, no trends are observed in the OA equivalent contents determined by the PPIAs regarding the different DSP toxin profiles (OA, DTX-1, and/or DTX-2) of the samples, indicating the good performance of the assays in the analysis of shellfish samples with multitoxin profiles. Furthermore, fit-for-purpose agreement with the reference LC-MS/MS method can be expected during their application within a monitoring program. Despite the under- and overestimations, which require further investigation, results obtained with both PPIAs, especially in the analysis of hydrolyzed samples, correlate well with LC-MS/MS analysis. Using the PPIAs as screening tools and taking into account the previously defined "suspicious" range, we would have identified neither false "negative" nor false "positive" samples, demonstrating that our strategy is appropriate. Because the hydrolysis step is required only for the indirect determination of DSP toxin acyl ester derivatives (acyl esters of other toxins are not regulated to date), hydrolyzed extracts could be analyzed exclusively with PPIA, which means a reduction by a half in the number of samples analyzed by LC-MS/MS.

In summary, the inhibitory potencies of DTX-1 and DTX-2 on two PP2As, one recombinant and one wild, have been compared to that of OA allowing the establishment of the corresponding IEFs. Whereas the inhibition potency of DTX-1 is not significantly different from that of OA, DTX-2 inhibits PP2As markedly less. Regarding the enzyme source, the wild enzyme is slightly more sensitive than the recombinant one. Nevertheless, both PPIAs attain appropriate LODs, regardless of the nature of the enzyme. The developed assays have been applied to the determination of OA equivalent contents in mussel samples spiked with OA, DTX-1, and/or DTX-2. The experimental results have shown a good agreement with the expected OA equivalent contents, calculated from the theoretical toxin concentrations and the corresponding IEFs. Moreover, the experiment has allowed the establishment of a "suspicious range" between 80 and 180 μg/kg of OA equivalent contents, which would require samples to be processed by LC-MS/MS prior to decision making. From our results, confirmatory analysis with the reference method would not even be essential above 180 μg/kg, the PPIA providing a fast response against severe DSP outbreaks. In the analysis of naturally contaminated shellfish samples, OA equivalent contents determined by PPIAs have shown a good agreement with both TE and IE values, demonstrating that the assays can be used as reliable screening tools in monitoring programs.

The developed PPIAs with both recombinant and wild PP2A have shown the ability to detect DSP toxins with good performance. The assessment of the IEFs for the determination of the total OA equivalent contents has contributed to better understand the agreement with LC-MS/MS analysis. The PPIA is an interesting method for the simple, fast, sensitive, robust, and reliable determination of DSP toxin contents in shellfish. The use of this functional assay in monitoring programs for the screening of a high number of samples would substantially decrease economic costs and save time. For example, although crude extracts would still require LC-MS/MS analysis for the quantification of non-DSP-like lipophilic toxins, hydrolyzed samples could be screened with this biochemical tool, reducing the instrumental analytical requirements while providing equivalent protection level of public health. Moreover, PPIA can yet be considered an excellent tool for DSP toxin quantification and research purposes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure 1S: Linear regressions for the correlations of the PPIAs for non-hydrolyzed (A and B) and hydrolyzed (C and D) samples, with the recombinant (A and C) and wild (B and D) PP2As, respect to the corresponding TE values from EFSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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