UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
FACULTAD DE VETERINARIA
DEPARTAMENTO DE FARMACOLOGÍA

Ficotoxinas marinas: métodos de detección en extractos de molusco

Tesis doctoral

Eva Fonfría Subirós

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INFORMA:

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Para que conste a los efectos oportunos, firma la presente en Lugo, a 18 de Marzo de 2009.

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ABREVIATURAS

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ADN	Ácido desoxirribonucleico.
APHA	Asociación americana de salud pública (*American Public Health Association*).
ASP	Intoxicación amnésica por consumo de molusco (*Amnesic Shellfish Poisoning*).
CFP	Intoxicación ciguatérica (*Ciguateric Fish Poisoning*).
CM	Carboximetil dextrano.
dcSTX	Decarbamoil saxitoxina.
dcGTX	Decarbamoil gonyautoxina.
dcNEO	Decarbamoil neosaxitoxina.
Da	Dalton.
DSP	Intoxicación diarreica por consumo de molusco (*Diarrhetic Shellfish Poisoning*).
DTX	Dinophysistoxina.
ELISA	Ensayo inmunoabsorbente ligado a enzimas (*Enzyme-Linked ImmunoSorbent Assay*).
FAO	Organización de las Naciones Unidas para la agricultura y la alimentación (*Food and Agriculture Organization* of the United Nations).
FP	Polarización de fluorescencia (*Fluorescence Polarization*).
GTX	Gonyautoxina.
GYM	Gymnodimina.
HPLC	Cromatografía líquida de alta eficacia (*High Performance Liquid Chromatography*).
IFC	Cartucho de microfluidos integrados (*Integrated μ-Fluidic Cartridge*).
IOC	Comisión intergubernamental ocenográfica (*Intergovernmental Oceanographic Comision of UNESCO*).
I_H	Intensidad perpendicular de fluorescencia
I_V	Intensidad paralela de fluorescencia.
K_D	Constante de disociación.
MEKC	Cromatografía electrocinética micelar (*Micellar Electrokinetic Chromatography*)

mP	Unidad de milipolarización.
MU	Unidad ratón (*Mouse Unit*).
LC-MS	Cromatografía líquida acoplada a espectrometría de masas (*Liquid Chromatography-Mass Spectrometry).*
LD_{50}	Dosis letal 50.
LD_{99}	Dosis letal 99.
neoSTX	Neosaxitoxina.
NSP	Intoxicación neurotóxica por consumo de molusco (*Neurotoxic Shellfish Poisoning*).
OA	Ácido okadaico (*Okadaic Acid*).
P	Polarización.
PbTx	Brevetoxina (*Ptychodiscus brevis Toxin*).
PDE	Fosfodiesterasa.
PnTX	Pinnatoxina.
PP1	Fosfatasa de proteina en residuos Serina/Treonina 1.
PP2A	Fosfatasa de proteina en residuos Serina/Treonina 2A.
PSP	Intoxicación paralizante por consumo de molusco (*Paralytic Shellfish Poisoning*).
PtTX	Pteriatoxina.
PTX	Pectenotoxina.
r	Anisotropía.
rpm	Revoluciones por minuto.
RU	Unidad de resonancia (*Resonance unit*).
SPR	Resonancia del plasmón superficial (*Surface Plasmon Resonance*)
SPX	Espirólido.
STX	Saxitoxina.
UNESCO	Organización de las naciones unidas para la educación, la ciencia y la cultura (*United Nations Educational, Scientific and Cultural Organization*)
WHO	Organización mundial de la salud (*World Health Organization*)
YTX	Yessotoxina.

ÍNDICE

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INTRODUCCIÓN

1. INTRODUCCIÓN

1.1 Ficotoxinas marinas

Las ficotoxinas marinas son productos naturales de origen algal, que pueden llegar al hombre u otros depredadores por vía alimentaria a través de moluscos, crustáceos, gasterópodos o peces, en forma de aerosol o por contacto directo con el agua de mar [*1*]. Entre las 5000 especies de microalgas que forman el fitoplancton, sólo un 1 % tienen la capacidad de producir estas toxinas [*2*] pero a pesar de este bajo porcentaje, sus efectos a nivel sanitario, económico y ambiental son altamente dañinos. A escala mundial, estas toxinas producen en humanos 60000 intoxicaciones al año con una mortalidad del 1,5 % [*1*], pérdidas económicas valoradas en centenares de millones de euros/año [*3, 4*] y mortalidades masivas de peces [*5*], aves [*6*] y mamíferos marinos [*7*].

Históricamente, estas toxinas se clasificaron en cinco grandes grupos basados en la sintomatología y en los vectores de transmisión: intoxicación paralizante por consumo de molusco (PSP, paralytic shellfish poisoning), intoxicación diarreica por consumo de molusco (DSP, diarrhetic shellfish poisoning), intoxicación amnésica por consumo de molusco (ASP, amnesic shellfish poisoning), intoxicación neurotóxica por consumo de molusco (NSP, neurotoxic shellfish poisoning) e intoxicación ciguatérica por consumo de pescado (CFP, ciaguateric fish poisoning). Con el tiempo, la aparición de nuevas toxinas y los avances en los estudios farmacológicos de las ya conocidas, hicieron insuficiente esta clasificación promoviendo la creación de conjuntos nuevos: intoxicación por azaspirácidos, yessotoxinas, iminas cíclicas, etc. Desde 2004, expertos de la FAO/WHOI/IOC recomiendan la clasificación de estas ficotoxinas en función de su estructura química [*8*], lo que da lugar a los grupos de: azaspirácidos, brevetoxinas, iminas cíclicas, ácido domoico, ácido okadaico, saxitoxinas, yessotoxinas y pectenotoxinas. En esta tesis se utilizará esta última clasificación, mostrando sin embargo las equivalencias con la nomenclatura sintomatológica, ya que en las leyes europeas vigentes se siguen utilizando los términos PSP y ASP [*9-11*].

En la Unión Europea el método oficial de detección para la mayoría de estas ficotoxinas (a excepción de las amnésicas o grupo del domoico) es el bioensayo en

ratón. Este método es muy eficaz en cuanto a protección de la salud pública se refiere, pero presenta numerosas limitaciones técnicas además de conllevar una serie de problemas éticos y legales derivados de la utilización de animales en el laboratorio [*12*]. Desde 1986 en Europa existe una directiva que regula el uso de animales con fines científicos, el cuál debe ser mínimo y con el menor dolor posible para el animal [*13*], y aboga por la aplicación de las "3 Rs" (Reemplazo, reducción y refinamiento), concepto que estimula el uso racional y ético de los animales y su sustitución siempre que los métodos alternativos proporcionen resultados fiables. De esta forma, el desarrollo de nuevos métodos de detección que garanticen la salud pública sin perjudicar los intereses de la industria acuícola, eliminando o reduciendo el uso de animales es, hoy por hoy, imprescindible.

En la presente tesis doctoral se han desarrollado métodos de detección para los grupos de: iminas cíclicas, saxitoxina y yessotoxina. Todos ellos, además de los grupos del ácido okadaico y las brevetoxinas (utilizados para comprobar interferencias en uno de los métodos), se describirán en profundidad a continuación.

1.1.1 Grupo de la saxitoxina.

El grupo de la saxitoxina es el causante de la intoxicación paralizante por consumo de molusco (PSP), una de las más peligrosas y extendidas del mundo. Cada año se contabilizan a nivel mundial cerca de 2000 intoxicaciones, con una mortalidad del 15% [*1*]. En términos medioambientales, se ha relacionado este grupo con varios episodios mortales, entre ellos, la muerte de focas monje en la costa sahariana [*14*] y la muerte de ballenas jorobadas en la bahía de Cape Cop [*15*].

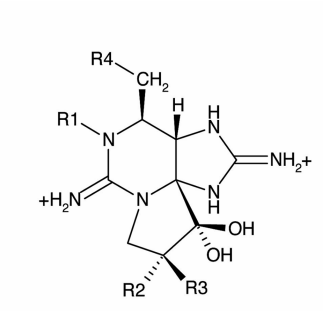
La primera referencia a este síndrome data del s. XVIII durante las expediciones a las costas de la Colúmbia Británica del capitán George Vancouver. Desde entonces, los episodios tóxicos se han ido produciendo con una frecuencia y distribución mayor, habiéndose presentando en Estados Unidos, México, Chile, Costa Rica, Guatemala, Sudáfrica, Índia, Tailandia, Japón, Taiwan, Australia, Nueva Zelanda, el Reino Unido, Noruega, Italia, España y un largo etc. [*16, 17*].

Este grupo está formado por más de una veintena de neurotoxinas hidrosolubles, siendo las más conocidas la saxitoxina (STX) (nombre derivado de la almeja

Saxidomus giganteus de donde se aisló por primera vez en 1957 [*18*]) y las gonyautoxinas (GTX) (nomenclatura derivada de *Protogonyaulax* y *Gonyaulax,* antiguos nombres del género de dinoflagelados *Alexandrium,* uno de sus organismos productores [*19*]). Todas ellas comparten un esqueleto común de tetrahidropurina, pero difieren en las combinaciones de hidroxilos y sulfatos en cuatro posiciones de la molécula (R1-4) (Figura 1). En función de las sustituciones en R4, estas toxinas se dividen en cuatro subgrupos: carbamadas (saxitoxina, neosaxitoxina y gonyautoxinas 1-4), sulfocarbamadas (gonyautoxinas 5-6 y C1-4), decarbamadas (dc-) y deoxidecarbamadas (do-). El radical presente en R4 también es determinante en la toxicidad del compuesto, siendo las toxinas carbamadas las más tóxicas, seguidas por las decarbamadas y las sulfocarbamadas (no existen datos de toxicidad referentes a los análogos deoxidecarbamados debido a las pequeñas cantidades de que se dispone).

Estas toxinas pueden ser producidas por tres géneros de dinoflagelados distintos: *Alexandrium* (entre ellos *A. catenella, A. tamarense, A. minutum, A. andersoni, A. excavatum, A. ostenfeldii, A. tamiyavanichii* y *A. fundyense)*, *Gymnodinium (G. catenatum)* y *Pyrodinium (P. bahamense* var. *compressum)* [*16, 20-23*] así como también por algunas algas verde-azules como *Anabaena circinalis*, *Cylindrospermopsis raciboskii, Aphanizomenon flos-aquae* y *Lyngbya wollei* [*24-27*]

Aunque recientemente se ha descubierto que la saxitoxina y derivados son capaces de unirse a diversas proteínas, como canales de calcio, canales de potasio, saxifilina y óxido nítrico sintasa [*28*], su diana más conocida y estudiada son los canales de sodio dependientes de voltaje. Estas toxinas se unen específicamente ($K_D \sim 2$ nM [*1*]) al llamado sitio 1 de la subunidad α [*29, 30*], situado en la cara externa del canal, bloqueando el flujo de sodio e impidiendo la generación y propagación de los potenciales de acción en las células excitables [*31, 32*]. Esta interacción a nivel molecular se traduce a escala macromolecular en varios síntomas: en intoxicaciones leves, hormigueo y entumecimiento de labios, boca, cara y cuello, náuseas, dolor de cabeza y vértigo, y en intoxicaciones moderadas y severas, parestesia en brazos y piernas, incoordinación motora, dificultades respiratorias, incoherencia en el habla, parálisis muscular creciente y finalmente insuficiencia respiratoria que puede llegar a causar la muerte [*33*].



	Toxina	R1	R2	R3	R4	Toxicidad (MU/µmol)
Carbamadas	STX	H	H	H	$OCONH_2$	2483
	neoSTX	OH	H	H		2295
	GTX1	OH	H	OSO_3-		2468
	GTX2	H	H	OSO_3-		892
	GTX3	H	OSO_3-	H		1584
	GTX4	OH	OSO_3-	H		1803
Sulfocarmadas	GTX5 = B1	H	H	H	$OCONHSO_3$-	160
	GTX6 = B2	OH	H	H		-
	C3	OH	H	OSO_3-		15
	C1	H	H	OSO_3-		239
	C2	H	OSO_3-	H		33
	C4	OH	OSO_3-	H		143
Decarbamadas	dcSTX	H	H	H	OH	1274
	dcNEO	OH	H	H		-
	dcGTX1	OH	H	OSO_3-		-
	dcGTX2	H	H	OSO_3-		1617
	dcGTX3	H	OSO_3-	H		1872
	dcGTX4	OH	OSO_3-	H		-
Deoxidecarbamadas	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO_3-		-
	doGTX3	H	OSO_3-	H		-

Figura 1. Estructura y toxicidad relativa de las toxinas PSP. Valores de toxicidad expresados en unidades ratón (MU, mouse units). 1 unidad ratón es la cantidad de toxina necesaria para matar un ratón de 20 gramos en 15 min [*34*].

Actualmente, en la Unión Europea el límite máximo permitido de estas toxinas en moluscos destinados al consumo humano es de 800 µg STX equivalentes/kg de carne de molusco (cuerpo entero o partes comestibles) [*9*] y el método de referencia para detectarlas es el bioensayo en ratón [*10*]. Este método consiste en extraer la toxina del molusco contaminado hirviendo con HCl e inyectar intraperitonealmente 1 ml del sobrenadante del extracto a un conjunto de ratones de peso establecido. El tiempo que transcurre entre la inoculación y el último aliento del animal, se registra y se interpola en la tabla de Sommer, que contiene la calibración para poder determinar la toxicidad en MU en función del tiempo de muerte del ratón [*35*]. El límite de

detección de esta técnica es aproximadamente 400 µg de toxina/kg de carne de molusco (la mitad del límite permitido), con una precisión de ± 20 %. Las principales interferencias se deben a un exceso de iones sodio (disminuyen la toxicidad aparente de estas toxinas), un exceso de metales (el zinc provoca unos síntomas parecidos, pero retrasa la muerte del animal) o un pH demasiado ácido (puede dar lugar a artefactos debido a acidosis) [*36*]. Además, este método también presenta desventajas inherentes, como un elevado coste (hay que alimentar y mantener una población de ratones con un peso determinado), carencia de selectividad (no distingue entre las distintas toxinas) y un conjunto de problemas éticos y legales ya mencionados.

Para intentar paliar estas deficiencias, en las últimas décadas se han propuesto distintos métodos de detección para estas toxinas: cromatográficos [*37, 38*], electroforéticos [*39*], inmunológicos [*40-42*], radiactivos [*43*], fluorimétricos [*44*], electrofisiológicos [*45*], etc. pero hasta la fecha sólo uno, la cromatografía líquida de alta eficacia (HPLC) con detección fluorescente, ha sido validado y reconocido oficialmente como método alternativo al bioensayo [*11*].

1.1.2 Grupo de las brevetoxinas

El grupo de las brevetoxinas (PbTx) es el causante de la intoxicación neurotóxica por consumo de molusco (NSP). Estructuralmente, estas toxinas liposolubles se dividen en dos clases: A y B. La primera consta de un esqueleto flexible de 10 anillos tipo éter y está formada por las toxinas PbTx-1, 7 y 10, mientras que la segunda tiene una estructura rígida de 11 anillos y la constituyen los congéneres PbTx-2, 3, 5, 6, 8, 9, 11, 12, 13 y 14 (Figura 2) [*46*].

Las estructuras de las principales toxinas de cada grupo, PbTx 1 y PbTx 2, se determinaron a principios de los 80 a partir de floraciones del dinoflagelado *Karenia brevis* (anteriormente llamado *Ptychodiscus brevis* y *Gymnodinium breve*, de donde provienen la abreviatura y el nombre respectivamente [*47*]) [*48, 49*], el principal organismo productor de brevetoxinas, aunque no el único. Estudios recientes muestran que también pueden estar producidas por *Chattonella marina* [*50*], *Chattonella antiqua* [*51*], *Fibrocapsa japonica* [*52*] y *Heterosigma akashiwo* [*53*].

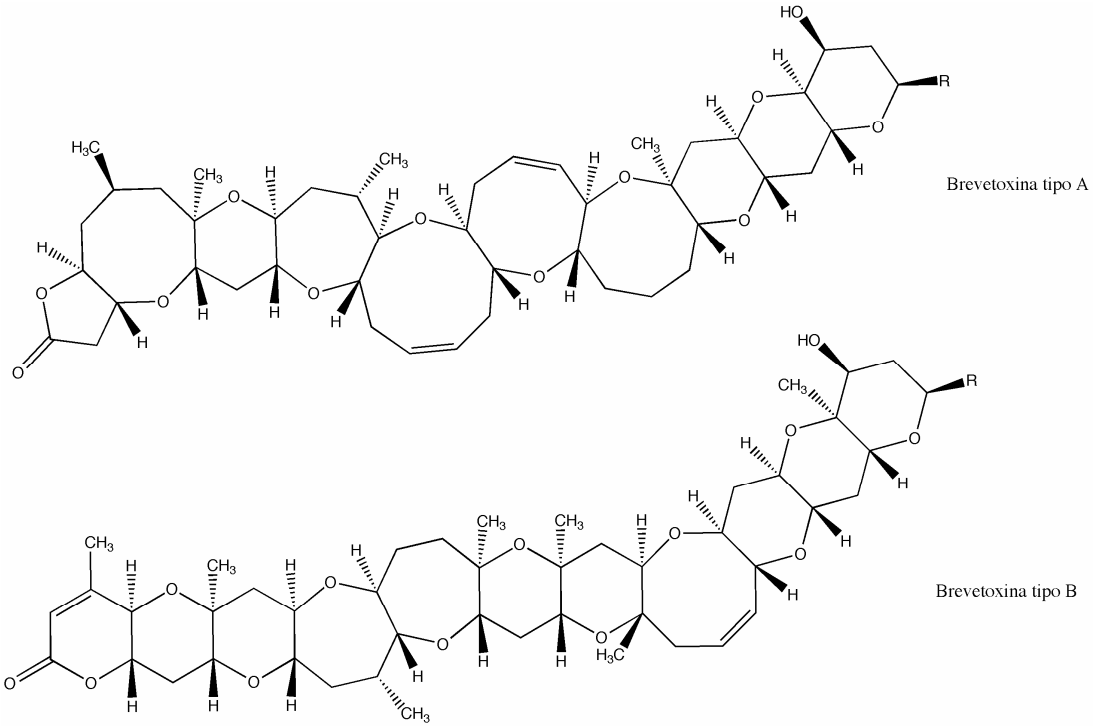


Figura 2. Estructura química general de los tipos A y B de brevetoxinas.

Hasta la década de los 90, este síndrome estaba limitado al golfo de México (donde las primeras referencias se remontan al s. XVII [*54*]) y la costa este de Estados Unidos, donde se creía que era endémico, pero hoy en día existen datos de un episodio tóxico en Nueva Zelanda [*55, 56*], por lo que no se descarta su posible aparición en otros lugares.

Referente a su mecanismo de acción, las brevetoxinas son sustancias despolarizantes, ya que actúan en los canales de sodio dependientes de voltaje provocando una entrada masiva de este catión en las células, tanto por apertura directa de los canales como por inhibición de los sistemas de inactivación [*1*]. Experimentos con sinaptosomas de cerebro de ratas demuestran que estas toxinas se unen específicamente al sitio 5 de la subunidad alfa de estos canales con una estequiometria 1:1 y una K_D entre 1-50 nM [*1, 57, 58*]

Además de por vía oral, las brevetoxinas pueden producir intoxicación por exposición dérmica e inhalación. En el primer caso, los síntomas aparecen entre los treinta minutos y las tres horas y duran unos pocos días, presentando náuseas,

vómitos, diarrea, dolor abdominal, sudoraciones, cambios de temperatura, hipotensión, arritmias, hormigueo, entumecimiento, dilatación de pupilas, parestesia en cara y extremidades, calambres y dolor muscular severo. En los otros casos, suceden irritaciones respiratorias y conjuntivales, catarros, rinorrea y broncoconstricción [*17, 59*].A día de hoy, no se han registrado casos mortales en humanos, sin embargo se ha relacionado estas toxinas con distintos episodios mortales de peces [*60*], aves [*61*] y mamíferos marinos [*62*]. Estudios en ratones muestran que estas toxinas son más tóxicas por vía intraperitoneal (para PbTx-2 por ejemplo LD_{50} = 200 μg/kg) que por vía oral (PbTx-2 LD_{50} = 6600 μg/kg) [*63*].

Actualmente el método oficial para detectar brevetoxinas es el procedimiento propuesto por APHA [*64*], basado en la extracción de las toxinas con éter dietílico seguido por bioensayo en ratón. En este caso la unidad ratón se define como la cantidad de extracto tóxico que mata el 50 % de los ratones inyectados en 930 minutos [*65*]. El límite máximo permitido son 200 MU/kg de carne de molusco, lo que equivale a 0,8 mg PbTx-2/kg. [*66, 67*].

Además del bioensayo, existen diversos métodos para detectar brevetoxinas, entre ellos ensayo inmunoabsorbente ligado a enzimas (ELISA) [*68, 69*], radioinmunoensayo [*70*], cromatografía líquida acoplada a espectrometría de masas (LC-MS) [*71*], ensayos de receptor [*72*] y cromatografía electrocinética capilar micelar (MEKC) con detección fluorescente inducida por láser [*73*].

1.1.3 Grupo del ácido okadaico.

El grupo del ácido okadaico es el causante de la intoxicación diarreica por consumo de molusco (DSP). El primer episodio de este tipo de intoxicación del que se tiene referencia se produjo en Holanda en 1961[*74*]. Desde entonces, se han documentado casos en prácticamente todos los continentes, siendo los países europeos y Japón los más afectados [*75*].

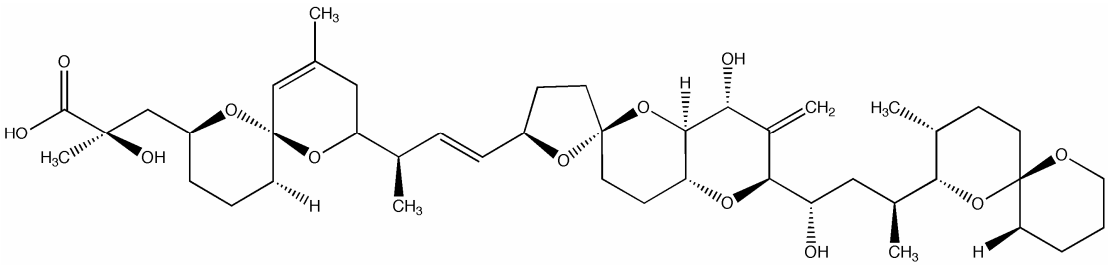


Figura 3. Estructura química del ácido okadaico.

Este grupo está formado por un conjunto de toxinas lipofílicas en las que se incluyen el ácido okadaico (OA), una molécula poliéter de 38 carbonos con un grupo carboxilo y cuatro hidroxilos (figura 3), y sus derivados, entre ellos las dinophysistoxinas (DTX) [*76*]. El primero debe su nombre a una de las esponjas marinas de donde se aisló originalmente, *Halichondria okadai* [*77*], mientras que las segundas, lo deben a uno de los generos de dinoflagelados que las producen, *Dinophysis* spp. A parte de los organismos *Dinophysis fortii*, *D. mitra*, *D. rotundata*, *D. tripos*, *D. acuta*, *D. norvegica*, *D. acuminata* y *D. sacculus* [*78, 79*], el OA y las DTXs también pueden estar producidas por las microalgas *Prorocentrum lima*, *P. hoffmannianum*, *P. maculosum* (*P. concavum), P. belizeanum, P. faustiae y P. areanarium* [*80-85*].

Los principales síntomas de la intoxicación que producen son diarrea, naúseas, vómitos, y dolores abdominales. Estos trastornos gastrointestinales suelen aparecer durante las primeras cuatro horas desde la ingestión del molusco contaminado y desaparecer al cabo de tres días [*86*]. Hasta el momento no se ha registrado ningún episodio letal en humanos ni tampoco en aves, peces ni mamíferos marinos.

El OA y sus análogos DTX-1 y DTX-2 son inhibidores específicos de las serina/treonina proteina fosfatasas 1 y 2A (PP1 y PP2A respectivamente) [*87-89*]. Estas fosfatasas representan dos de las cuatro enzimas principales de defosforilación de proteinas en los residuos Ser/Thr de las células eucariotas y están involucradas en múltiples procesos de regulación celular, tales como metabolismo, contracción muscular, división celular, transporte intracelular transmembranal, síntesis proteica, etc. [*90, 91*]. El efecto diarreogénico del ácido okadaico y las dinophysistoxinas se atribuye a la acumulación de proteínas fosforiladas que controlan la secreción de sodio en las células intestinales y a la inhibición de la defosforilación de elementos

del citoesqueleto que pemiten la permeabilidad de solutos, lo que resulta en una pérdida pasiva de fluidos [*86, 92*].

Estudios en ratones muestran que el ácido okadaico es más tóxico por vía intraperitoneal (LD_{50} en el rango 192-225 μg/kg [*93*]) que por vía intragástrica (LD_{50} 1-2mg/kg [*94*]). Sin embargo, esta toxicidad intraperitoneal es menor que la que presentan otros tipos de toxinas lipofílicas, como la yessotoxina o las iminas cíclicas, por lo que la presencia de otras toxinas lipofílicas en moluscos puede dar lugar a falsos positivos en la determinación de toxinas DSP mediante bioensayo en ratón.

Actualmente en la Unión Europea el límite máximo permitido para ácido okadaico y dinophysistoxinas (junto a pectenotoxinas) es de 160 μg de OA equivalentes/kg [*9*]. Para determinar estas toxinas la regulación EC nº 2074/2005 [*10*] establece que se pueden usar distintos tipos de bioensayo (en rata o ratón), que difieren en la porción analizada (hepatopáncreas o cuerpo entero) y en los disolventes empleados en el proceso de extracción, así como varios métodos alternativos, como el HPLC con detección fluorimétrica, el LC-MS, inmunoensayos y ensayos de inhibición de fosfatasas, si se demuestra que son tan efectivos como los métodos biológicos.

Además de todas estas técnicas, las toxinas de este grupo también se pueden detectar por MEKC [*95*] y mediante biosensores basados en la resonancia del plasmón superfical (SPR, del inglés Surface Plasmon Resonance) [*96*].

1.1.4 Grupo de la yessotoxina.

La yessotoxina (YTX) se aisló por primera vez en 1986 del hepatopáncreas de la vieira *Patinopecten yessoensis* (de donde proviene el nombre) en la bahía japonesa de Mutsu [*97*]. Un año después se determinó su estructura planar [*97*] y en 1996 su configuración absoluta [*98, 99*]. La molécula de YTX consta de 11 anillos contiguos tipo éter de diferentes tamaños, una cadena acílica insaturada de 9 carbonos y dos grupos sulfatos (figura 4). Actualmente se conocen 36 derivados identificados y caracterizados mediante estudios de resonancia magnética nuclear y/o cromatografía líquida acoplada a espectrometría de masas [*100*].

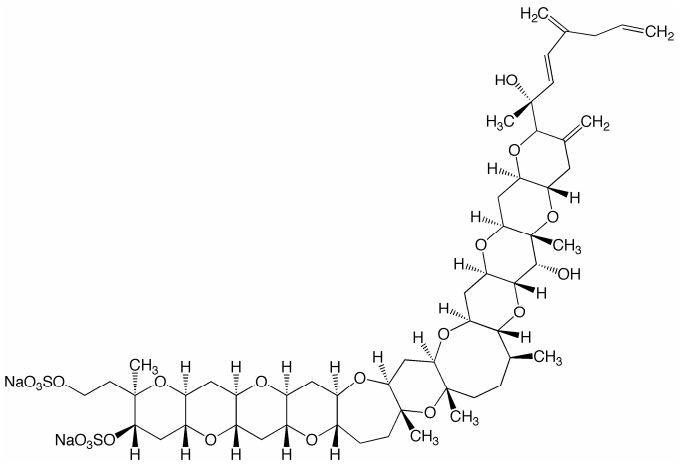


Figura 4. Estructura química de la yessotoxina.

Desde su aparición en Japón en la década de los 80, se han registrado casos de YTXs en moluscos de Noruega, Chile, Nueva Zelanda, Australia, Canadá, Rusia, Italia y España [*59, 100*]. Actualmente se sabe que esta toxina puede ser producida por tres dinoflagelados distintos: *Protoceratium reticulatum* (anteriormente *gonyaulax grindley*) [*101*]*, Lingulodinium polyedrom* (anteriormente *gonyaulax polyedra*) [*102*] y *Gonyaulax spinifera* [*103*].

Inicialmente, las yessotoxinas se clasificaron dentro del grupo de toxinas diarreicas (DSP), debido a su coexistencia y coextracción con ácido okadaico y dinophysistoxinas en moluscos contaminados. Estudios posteriores demostraron que la YTX no inducía diarrea [*104*] ni inhibía la fosfatasa PP2A, diana principal de las toxinas DSP [*105*]. Estos resultados promovieron su exclusión del conjunto DSP para pasar a formar un grupo de toxinas propio.

A nivel molecular su mecanismo de acción aún no está completamente dilucidado, pero existen estudios que revelan que la YTX modula la homeostasis del calcio en linfocitos humanos [*106*], disminuye los niveles intracelulares del adenosina monofosfato cíclico a través de la activación de fosfodiesterasas celulares (PDEs) en estas mismas células [*107*], produce apoptosis en células de neuroblastoma [*108*] y en células humanas HeLa por activación de caspasas [*109*] e induce la disrupción del sistema E-caterina- catenina en células epiteliales [*110*].

Su toxicidad en humanos es desconocida, así como sus efectos medioambientales. Estudios toxicológicos en ratones muestran que su LD_{50} al inyectarse intraperitonealmente es de 100 μg/Kg [*105*]. Oralmente, sin embargo, dosis de 10 mg/kg no son letales para los roedores [*111*]. Esta diferencia, hace que la yessotoxina sea la causa de muchos falsos positivos en la detección de toxinas DSP.

El nivel máximo permitido en la Unión Europea de esta toxina en molusco, cuerpo entero o partes comestibles, es 1 mg de YTX equivalentes/kg [*9*] y los métodos establecidos para detectarla son los mismos que para las toxinas del grupo del okadaico [*10*], aunque con limitaciones en el uso de disolventes.

Este grupo de toxinas puede ser detectado mediante HPLC con detección fluorimétrica [*112*], LC-MS [*113*], ELISA [*114*], polarización de fluorescencia [*115*] y biosensores basados en espejos resonantes [*116, 117*], entre otros.

1.1.5 Grupo de las iminas ciclícas

Este grupo está formado por un conjunto heterogéneo de compuestos macrocíclicos que contienen un radical imina en su estructura: gymnodiminas, espirólidos, pinnatoxinas, pteriatoxinas, prorocentrólidos y espiro-prorocentriminas. Se trata de toxinas de acción rápida con un acusado efecto "todo o nada", donde los ratones inyectados intraperitonealmente con concentraciones tóxicas pero no letales de estas sustancias son capaces de recuperarse sin mostrar secuelas [*118*]. Actualmente no existe ningún caso de intoxicación en humanos ni ningún tipo de regulación, siendo los únicos métodos para detectarlas el bioensayo en ratón y LC-MS [*119-122*].

Gymnodiminas

La primera referencia histórica de la gymnodimina (GYM) (Figura 5), la encontramos en 1994 en Nueva Zelanda, donde controles rutinarios para la detección de toxinas liposolubles presentaron una toxicidad neurológica inusual [*122*] Durante el mismo período, se observó un florecimento de *Gymnodinium* cf. *mikimotoi*, por lo que el compuesto bioactivo que se aisló de ostras contaminadas, se denominó gymnodimina [*123*]. Posteriormente, ese dinoflagelado fue identificado como

Gymnodinium selliforme y finalmente renombrado como *Karenia selliformis* [*124*]. En la actualidad este organismo es el único productor conocido de estas toxinas.

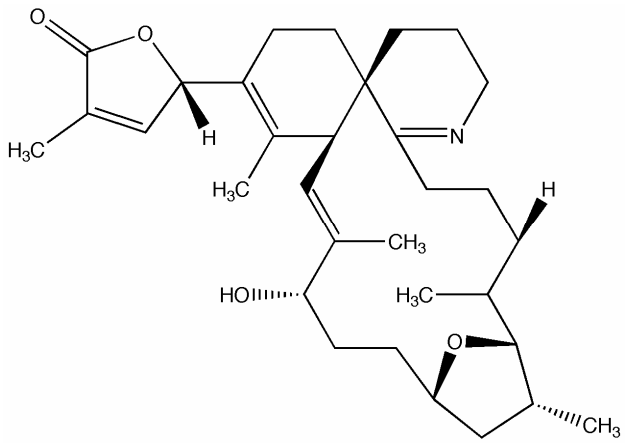


Figura 5. Estructura química de la gymnodimina-A.

A parte de Nueva Zelanda, los únicos países donde se ha registrado presencia de gymnodimina en moluscos han sido Túnez [*125*] y Australia [*126*].

La toxicidad en humanos de este compuesto es desconocida, pero estudios en ratones muestran que la gymnodimina es mucho más tóxica por vía intraperitoneal (LD_{50} en el rango 80-96 μg/kg peso corporal [*127, 128*]) que por vía oral, donde cantidades de 7500 μg/kg suministrados con la comida no provocan ningún signo de toxicidad [*127*]. Actualmente se conocen dos análogos más, las gymnodiminas B y C [*129, 130*], ambas aisladas de cultivos de *Karenia selliformis*. Estudios recientes en ratones muestran que la GYM-B presenta una LD_{50} por vía intraperitoneal de 800 μg/kg [*128*]. No existen datos para GYM-C.

La diana principal de estas ficotoxinas son los receptores colinérgicos nicotínicos, tanto musculares como neuronales, a los que se unen con afinidades nanomolares [*127, 128*].

Espirólidos.

Al igual que la gymnodimina, los espirólidos (SPX) se detectaron por primera vez debido a falsos positivos en controles rutinarios de toxinas lipofílicas [*131*]. Este caso se produjo en las costas de Nueva Escocia (Canadá) en 1991 y desde entonces se ha detectado en moluscos de Noruega [*132*], España [*133*] y Francia [*134*], aunque

no se descarta su aparición en otros países ya que se han identificado cepas de *Alexandrium ostenfeldii*, hasta el momento único organismo conocido productor de estas toxinas [*135*], en aguas noruegas [*119*], italianas [*120*], danesas [*136*], estadounidenses [*137*], neozelandesas [*138*] y escocesas [*139*].

La estructura de los primeros congéneres (espirólidos B y D) fue dilucidada en 1995 [*140*] y su nombre fue debido al "espiro" átomo que une el anillo cíclico de siete miembros que contiene el radical imina con el anillo ciclohexano y el sistema cíclico triéter [*141*]. En la actualidad se conocen doce análogos: A, B, C, D, E, F, G, 13-desmetil C, 13,19-didesmetil C, 27-hidroxi-13,19-didesmetil C, 13-desmetil D y 20-metil G [*118, 119, 121, 142*] (Figura 6). Estudios toxicológicos muestran que estos compuestos presentan mayor toxicidad por vía intraperitoneal que por vía oral [*143*], siendo, por ejemplo, las LD_{50} de una mezcla de espirólidos con una elevada proporción de 13-desmetil espirólido C 40 μg/kg y 1 mg/kg, respectivamente [*131*].

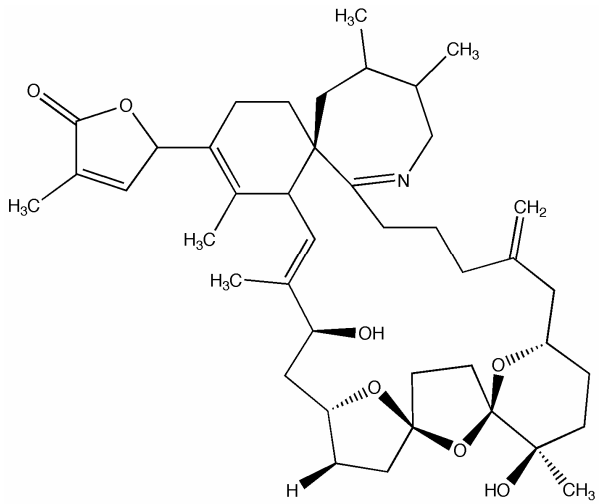


Figura 6. Estructura química del 13-desmetil espirólido C.

Referente a su mecanismo de acción, estudios iniciales sugirieron que se trataba de sustancias bloqueantes de receptores colinérgicos muscarínicos [*131*]. Actualmente también se conoce que actúan sobre receptores nicotínicos [*144*], compartiendo así diana con las gymnodiminas.

Pinnatoxinas

El subgrupo de las pinnatoxinas (PnTX) está formado por cuatro análogos (A, B, C y D), todos ellos aislados de la almeja *Pinna muricata* en Japón (figura 7) [*145-147*].

Existe la hipótesis que no se trata de toxinas producidas directamente por dinoflagelados sino que son el resultado del metabolismo de estas almejas sobre una ficotoxina parental [*118*].

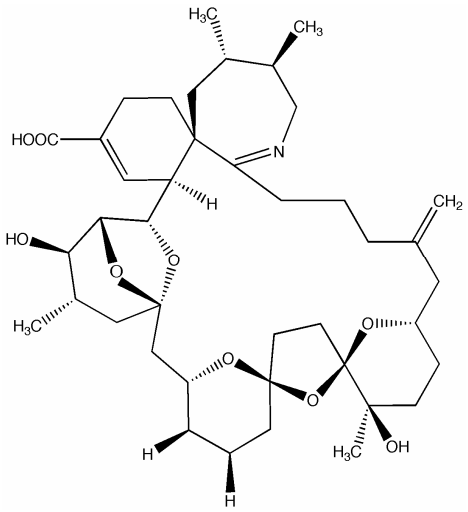


Figura 7. Estructura química de la pinnatoxina A.

Intraperitonealmente (utilizando ratones), estas toxinas presentan LD_{99} entre 22 y 400 μg/kg [*148*]. No existen datos sobre su toxicidad oral.

Aunque estudios iniciales con extractos de *Pinna attenuata*, sugirieron la acción de estos compuestos como activadores de canales de calcio [*149*], no se pudo identificar ninguna pinnatoxina como parte de esos extractos, siendo su modo de acción todavía desconocido [*143*].

Pteriatoxina

Al igual que las pinnatoxinas, las pteriatoxinas, PtTX, (de las que se conocen tres análogos: A, B y C) sólo se han detectado en moluscos y su origen es desconocido (figura 8). El nombre de estas toxinas proviene de la ostra *Pteria penguin*, de donde se aislaron en 2001 [*150*]. Aunque esta especie está ampliamente distribuida, sólo han aparecido pteriatoxinas en Japón [*143*]. Existe la hipótesis que *Pteria penguin* y *Pinna muricata* acumulan las mismas toxinas precursoras pero las metabolizan de forma distinta [*118*].

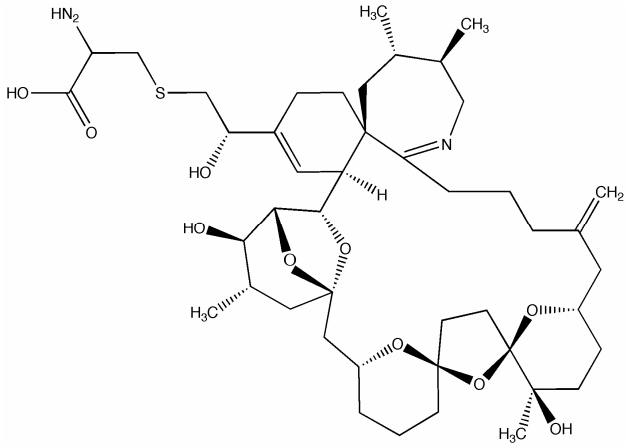


Figura 8. Estructura química de la pteriatoxina A.

La toxicidad intraperitoneal en ratón, LD_{99}, varia entre los 8 y los 100 μg/kg peso corporal [*150*]. No existen datos de toxicidad oral ni de su mecanismo de acción.

Prorocentrólidos

Los prorocentrólidos, A y B, son las iminas cíclicas más grandes, con una masa molecular alrededor de 1000 Da (figura 9). Aislados de *Prorocentrum lima* [*151*] y *Prorocentrum maculosum* (anteriormente *Prorocentrum concavum*) [*152*] respectivamente, a menudo se encuentran junto a ácido okadaico y derivados.

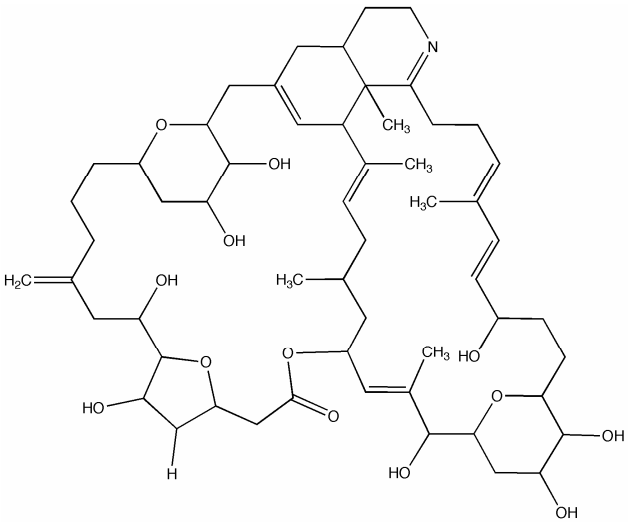


Figura 9. Estructura química de prorocentrólido A.

No existen datos fiables sobre la toxicidad de estos compuestos ni sobre su mecanismo de acción.

Espiro-prorocentrimina

Esta toxina (figura 10), única de su subgrupo, fue aislada de una cepa bentónica de *Prorocentrum* sp. en Taiwan. [*153*].

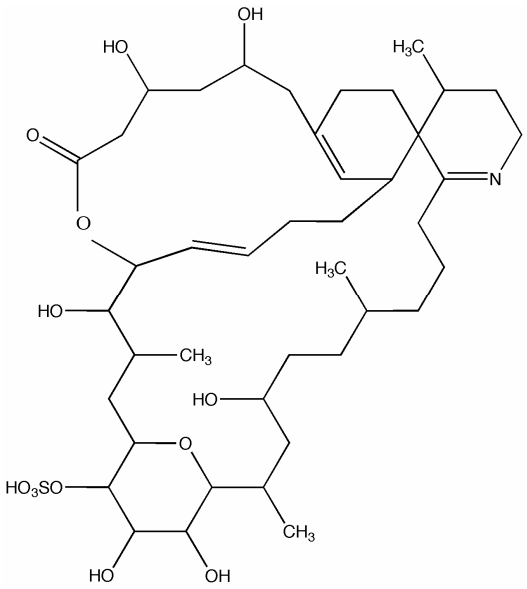


Figura 10. Estructura química de la espiro-prorocentrimina.

El único dato de toxicidad existente es su LD_{99} por vía intraperitoneal (en ratones), que equivale a 2500 μg/kg [*153*]. No existen datos sobre su mecanismo de acción.

1.2 Métodos de detección de ficotoxinas

Los métodos de detección de ficotoxinas, en función de la naturaleza de la información obtenida, pueden clasificarse en métodos de "ensayo" y métodos de "análisis" [*154, 155*].

El término "ensayo" se refiere a aquellos métodos que proporcionan un valor del contenido total de toxinas basado en la medición de una respuesta única, biológica o bioquímica, que comprende la actividad de todas las toxinas presentes en la muestra. La determinación de la toxicidad se lleva a cabo a partir de una curva dosis-respuesta que se realiza con una toxina representativa del grupo que se quiere determinar, expresando la toxicidad total en equivalentes de esa toxina. Dentro de esta categoría, se incluyen ensayos *in vivo*, bioensayos en ratón, rata u otros, y ensayos *in vitro*, de inhibición enzimática, de receptor, celulares, electrofisiológicos, inmunoensayos, etc.

Por el contrario, los métodos de "análisis" son aquellos en los que se realiza una separación, identificación y cuantificación individual de las toxinas existentes en una muestra. Esta cuantificación requiere la calibración previa del equipo instrumental con patrones de concentración conocida para cada una de las toxinas. La respuesta instrumental se convierte a valores de toxicidad a partir de factores de conversión específicos para cada toxina y la toxicidad total se determina como la suma de las toxicidades individuales. En este grupo encontramos métodos químicos como el HPLC asociado a detección fluorimétrica o colorimétrica, el LC-MS, la electroforesis capilar, etc.

En esta tesis se desarrollaron métodos de detección tipo "ensayo" utilizando técnicas relativamente recientes: la polarización de fluorescencia y los biosensores basados en el fenómeno óptico de la resonancia del plasmón superficial.

1.2.1 Polarización de fluorescencia

Fundamentos teóricos

La luz presenta dualidad onda-corpúsculo. Considerada como onda, consiste en un campo eléctrico y un campo magnético perpendiculares entre sí, que oscilan de forma sinusoidal a medida que se propagan a través del espacio. La relación entre la frecuencia y la longitud de onda viene definida por $\lambda\upsilon = c$, donde λ es la longitud de onda (distancia entre cresta y cresta de una onda), υ la frecuencia (número de oscilaciones completas por segundo) y c la velocidad de la luz en el vacío. Desde el punto de vista corpuscular, la luz está constituida por partículas llamadas fotones. La energía de cada fotón viene determinada por la ecuación $E = h\upsilon$, donde h es la constante de Planck y υ la frecuencia. Combinando las dos ecuaciones se observa que la energía y la longitud de onda son inversamente proporcionales [*156*].

Cuando un átomo o una molécula absorben un fotón, los electrones que se encuentran en orbitales de menor energía (estado basal o fundamental) utilizan la energía de la luz para acceder a orbitales desocupados de mayor energía (estado excitado). Si los espines (momentos intrínsecos de rotación) de los electrones del estado excitado y el estado fundamental son opuestos, el estado excitado se denomina estado singulete (en contraposición al estado excitado triplete, donde los

dos espines de ambos estados son iguales). Se denomina fluorescencia a la emisión de un fotón producida por el retorno de un electrón desde un estado singulete a su estado basal (siendo la fosforescencia el retorno desde un estado triplete). Al tener los espines opuestos, la transición del electrón entre los dos estados se produce rápidamente, por lo que la media del tiempo transcurrido entre la excitación y la emisión (tiempo de vida) en fluorescencia es del orden de nanosegundos [*157*]. El fotón emitido presenta una longitud de onda mayor que el fotón absorbido, puesto que su energía es menor al perder parte en el proceso.

La teoría de la polarización de fluorescencia (FP) fue descrita por primera vez por Jean Perrin en 1926 [*158*] y desarrollada por Gregorio Weber y colaboradores en 1950 [*159*]. El principio físico de esta teoría se basa en la observación de pequeñas moléculas fluorescentes en solución. Si se excitan estas moléculas con luz plana polarizada (el vector eléctrico vibra sólo en un plano) emitirán luz polarizada siempre que permanezcan estacionarias (sin moverse) durante su tiempo de vida fluorescente. Por el contrario, si durante ese período las moléculas rotan o voltean, la luz emitida será despolarizada (emitirán en planos distintos al de excitación). La polarización de la luz que emite una molécula fluorescente en estas condiciones es proporcional a su tiempo de relajación rotacional (tiempo que tarda en girar un ángulo de 68.5º), que a su vez, está relacionado con la viscosidad (η), la temperatura (T), el volumen molecular (v) y la constante de los gases (R), según la ecuación de Stokes [*160*]:

$$\text{Tiempo de relajación rotacional} = \frac{3 \cdot v \cdot \eta}{R \cdot T}$$

Si la viscosidad y la temperatura se mantienen constantes, la polarización está directamente relacionada con el volumen molecular (es decir, el tamaño molecular). Si la molécula es muy grande, sus movimientos son muy lentos con lo cuál, la luz emitida permanece mayoritariamente polarizada. Sin embargo, para moléculas pequeñas, donde la velocidad de rotación es más rápida, la luz emitida será en gran parte despolarizada. De este manera, si se produce unión entre una molécula fluorescente pequeña con otra molécula de mayor tamaño, podremos observar un cambio en los valores de polarización y seguir su interacción de forma eficiente (figura 11).

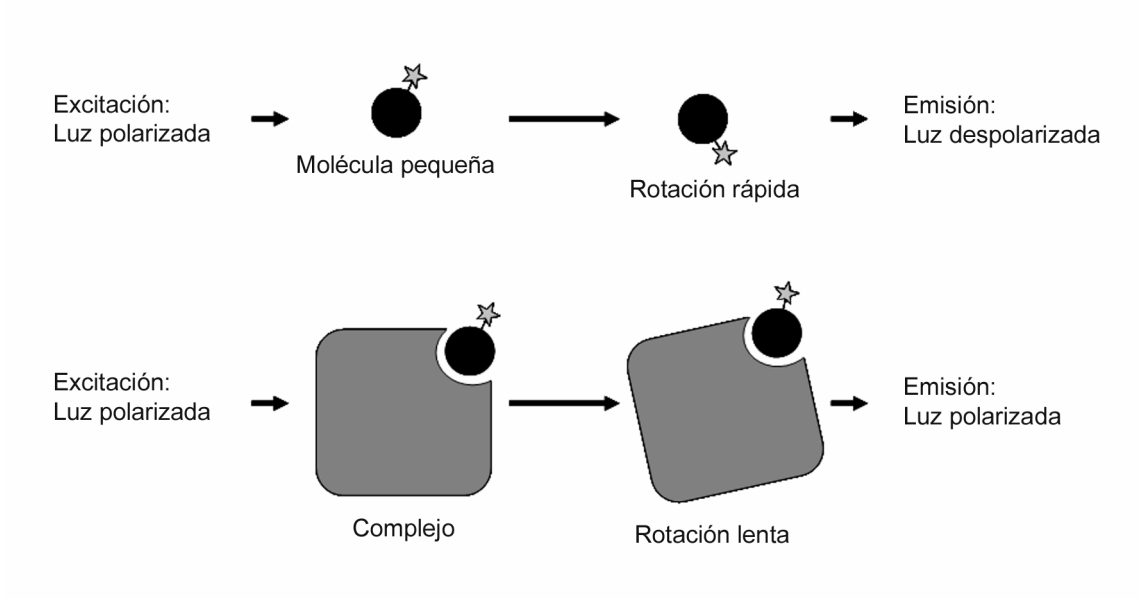


Figura 11. Esquema de las diferencias de FP entre moléculas pequeñas y complejos grandes.

Teóricamente la polarización de la fluorescencia se calcula mediante la ecuación:

$$P = \left[\frac{I_V - G \cdot I_H}{I_V + G \cdot I_H} \right]$$

Donde I_V es la intensidad de fluorescencia medida con filtros de polarización vertical para la excitación y la emisión (denominada intensidad paralela) y I_H es la intensidad de fluorescencia medida con filtros de polarización vertical para la excitación y horizontal para la emisión (denominada intensidad perpendicular). El valor de polarización (P), es un número adimensional (como ratio de intensidades de luz) y a menudo se expresa en unidades de milipolarización (mP).

Asociada a la polarización de fluorescencia en la bibliografía se puede encontrar el término "anisotropía" de fluorescencia (r). Ambos vocablos derivan de la medición de las intensidades verticales y horizontales, y aunque sus definiciones son distintas (la polarización se define como la fracción de luz linealmente polarizada mientras que la anisotropía es la razón entre la componente polarizada y la intensidad de luz total), se trata de conceptos matemáticamente relacionados e intercambiables [*161*]:

$$r = \frac{2 \cdot P}{3 - P}$$

Aplicaciones

Los ensayos de polarización de fluorescencia proporcionan información sobre la orientación molecular, la movilidad y los procesos que los modulan, incluyendo interacciones receptor-ligando [*162*], antígeno-anticuerpo [*163*], proteína-proteína [*164*], proteína-DNA [*165*], proteólisis [*166*] y fluidez membranal [*167*], por lo que su uso, desde las primeras aplicaciones en los años 70, se ha extendido a múltiples áreas científicas (clínica, bioquímica…).

En el campo de la seguridad alimentaria, se han desarrollado métodos basados en la polarización de fluorescencia para la detección de micotoxinas en cereales [*168-170*] y ficotoxinas en moluscos [*115*], entre otros.

Equipo instrumental

El equipo usado para medir fluorescencia y FP fue un lector de placas Chameleon^{TM} de la casa comercial Hidex. Este aparato utiliza una lámpara de xenón que produce luz de amplio espectro para producir la excitación y fotomultiplicadores para detectar la emisión. Las longitudes de onda deseadas tanto para la excitación como para la emisión se determinan mediante filtros (figura 12).

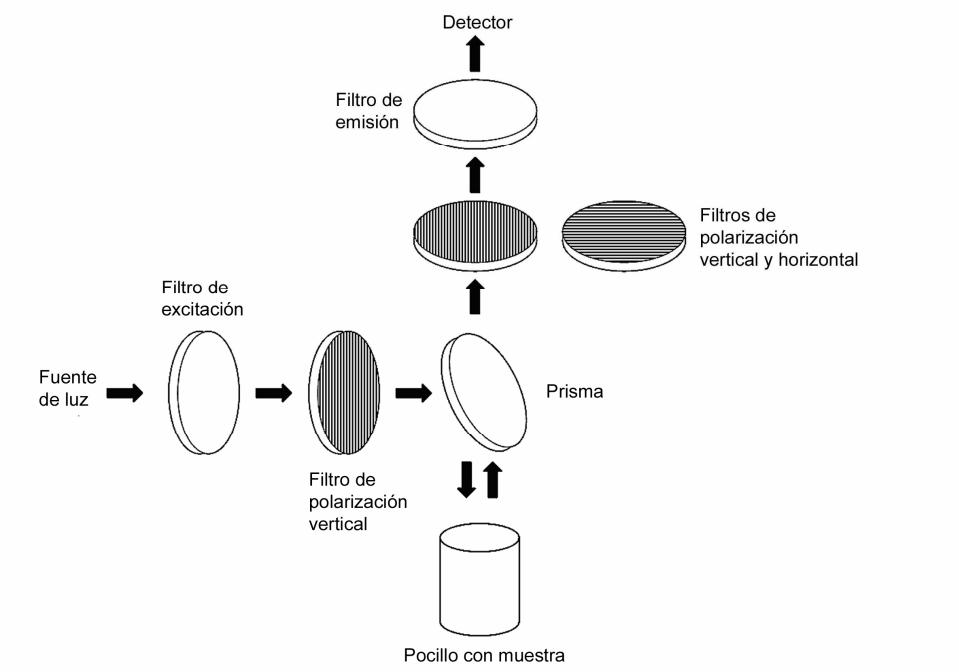


Figura 12. Diagrama del sistema óptico del Hidex Plate Chameleon para la medición de FP.

En la práctica, los componentes ópticos del instrumento afectan al paso de la luz por lo que a la ecuación de FP, se le añade un factor de corrección, denominado factor-G, que se determina empíricamente siguiendo los protocolos del fabricante. Siendo la ecuación final de trabajo:

$$mP = 1000\left[\frac{I_V - G \cdot I_H}{I_V + G \cdot I_H}\right]$$

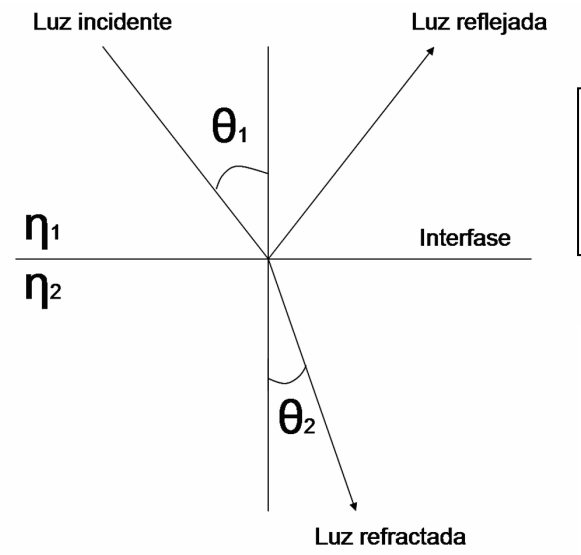
1.2.2 BIOSENSOR BASADO EN SPR

Fundamentos teóricos

Un biosensor es un dispositivo analítico que incorpora un elemento sensor de origen biológico o biomimético, acoplado a un sistema de transducción capaz de transformar la señal biológica producida por la interacción específica entre el elemento sensor y un compuesto de interés, en una señal electrónica cuantificable.

Los biosensores pueden clasificarse en función de múltiples parámetros, la naturaleza del elemento sensor, el tipo de interacción entre éste y el compuesto de interés, el sistema de transducción, etc. En esta tesis se emplearon biosensores de afinidad basados en el fenómeno óptico de la resonancia del plasmón superficial (SPR). Esta tecnología permite el seguimiento de la interacción entre dos moléculas, una de ellas (ligando) inmovilizada en la superficie de un chip sensor y la otra (analito) en flujo continuo sobre esta superficie, en tiempo real sin necesidad de marcaje.

El fenómeno óptico de la resonancia del plasmón superficial tiene lugar en la interfase de dos medios transparentes de diferente índice de refracción bajo determinadas condiciones. En la interfase entre dos medios transparentes de distinto índice de refracción, la luz que viene del medio con el índice más alto es parcialmente reflejada y parcialmente refractada, siguiendo la ley de Snell (Figura 13) [*171*].



Ley de refracción de Snell

$n_1 > n_2 \quad n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

Figura 13. Esquema de los fenómenos ópticos de refracción y reflexión que se producen en la interfase de dos medios transparentes de distinto índice de refracción.

Por encima de un cierto ángulo crítico, la luz no se refracta, sino que se refleja totalmente. A este proceso se le denomina reflexión interna total. Cuando ésto ocurre, el componente del campo electromagnético de la luz incidente es capaz de penetrar una cierta distancia (del orden de nanómetros) en el medio de índice de refracción más bajo, generando una onda evanescente, cuya intensidad se reduce exponencialmente a medida que se aleja de la interfase (figura 14).

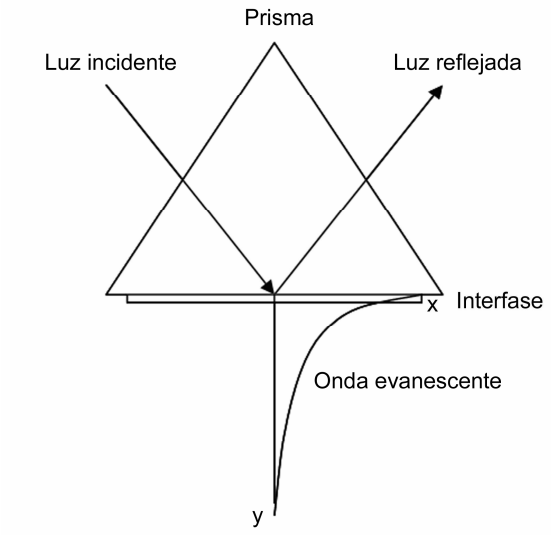


Figura 14. Esquema de la propagación y caída exponencial de la onda evanescente en el índice de refracción más bajo, en condiciones de reflexión interna total. En el eje x intensidad del campo evanescente y en el eje y la distancia desde la interfase entre los dos medios.

En condiciones de reflexión interna total, si la interfase entre los medios se encuentra recubierta por una fina lámina de metal, y la luz incidente es monocromática y p-polarizada (el componente del vector eléctrico es paralelo al plano de incidencia), la onda evanescente se propagará a través del metal excitando los electrones libres de la superficie y convirtiéndolos en "plasmones" (oscilaciones colectivas de electrones) de superficie. Esto provoca un descenso muy grande en la reflectividad del metal ya que la energía de la luz incidente es "absorbida" por los electrones. Este fenómeno es conocido como la resonancia de plasmón superficial y tiene lugar para un determinado ángulo de luz incidente (ángulo SPR o ángulo de resonancia) [*172-174*].

El ángulo de resonancia está determinado por varios factores: la longitud de onda de la luz incidente, las características del metal y el índice de refracción de los medios de ambas caras de éste. En los biosensores comerciales, la longitud de onda, el metal y el índice de refracción del medio donde incide la luz, se mantienen constantes, por lo que el determinante principal es el índice de refracción del medio por donde se propaga la onda evanescente (la cara no iluminada). Este índice depende de la masa de las moléculas unidas a la superficie metálica, por lo que si el analito transportado por la fase móvil a lo largo de una celda de flujo se une al ligando inmovilizado en la superficie, la masa variará cambiando el índice de refracción local y en consonancia, el ángulo de resonancia. Este cambio de ángulo se puede monitorizar en tiempo real a través de la medición de la intensidad de luz reflejada (figura 15 a-b).

Las variaciones del ángulo de resonancia se expresan en unidades arbitrarias RU (unidades de resonancia), donde 1 RU corresponde a un cambio en el ángulo de 0.0001º [*175*]. Representando RUs en función del tiempo, se obtiene un sensograma que nos ilustra el progreso de la interacción en tiempo real (figura 15 c).

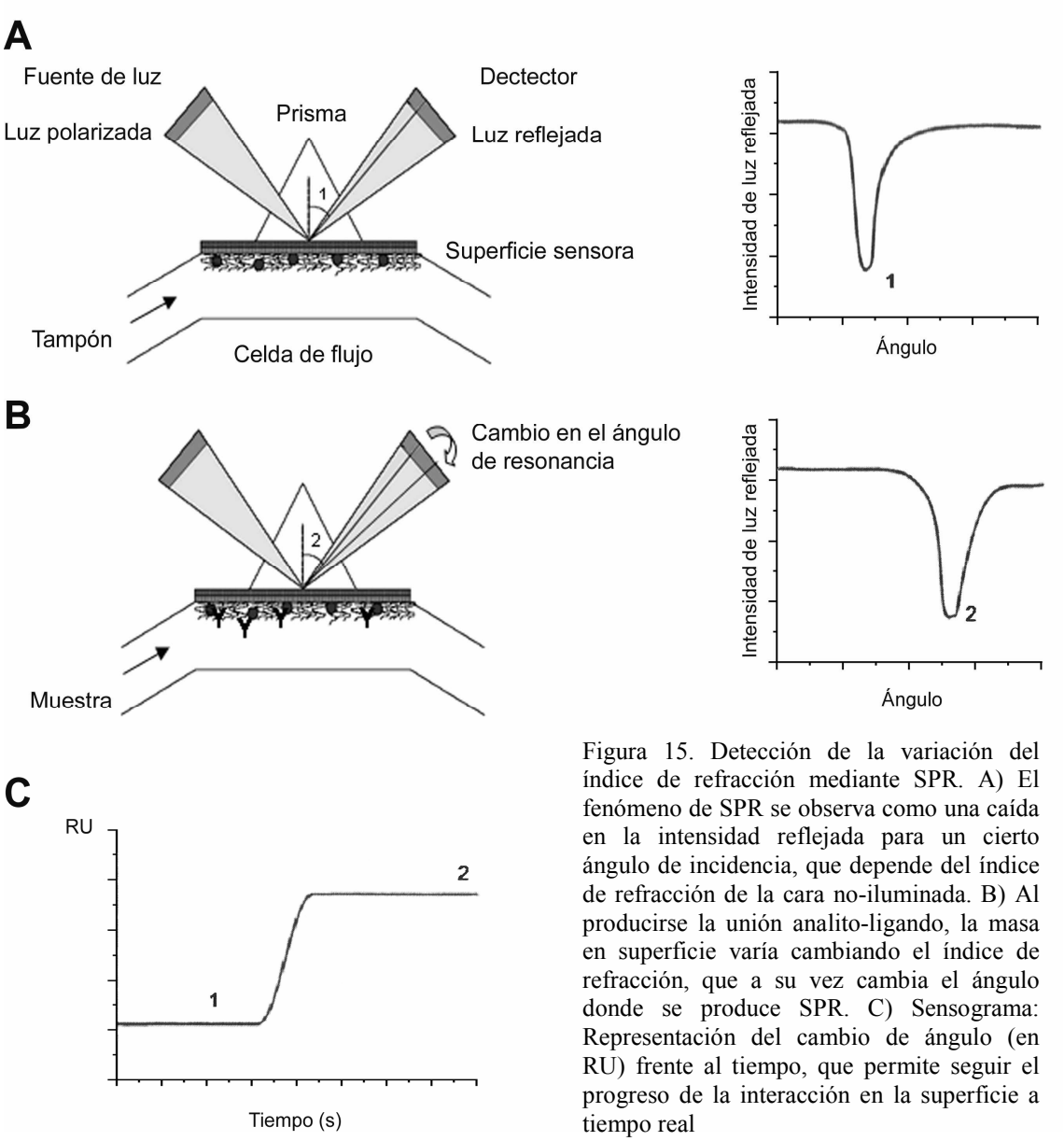


Figura 15. Detección de la variación del índice de refracción mediante SPR. A) El fenómeno de SPR se observa como una caída en la intensidad reflejada para un cierto ángulo de incidencia, que depende del índice de refracción de la cara no-iluminada. B) Al producirse la unión analito-ligando, la masa en superficie varía cambiando el índice de refracción, que a su vez cambia el ángulo donde se produce SPR. C) Sensograma: Representación del cambio de ángulo (en RU) frente al tiempo, que permite seguir el progreso de la interacción en la superficie a tiempo real

Una vez se ha preparado el chip sensor con el ligando inmovilizado en la superficie, el sensograma típico que se obtiene en un biosensor basado en SPR durante un ciclo de análisis consta de cuatro etapas: línea basal, asociación, disociación y regeneración (figura 16). El ciclo empieza mediante la inyección de tampón en flujo continuo para establecer la línea basal que servirá de punto de referencia. En la etapa de asociación, el tampón es reemplazado por la muestra. Si se produce la interacción analito-ligando, el sensograma mostrará un perfil de subida, típico de la unión entre dos moléculas. En la etapa siguiente, de disociación, se vuelve a inyectar tampón en un flujo constante. Si la interacción tiene una alta afinidad el valor de unión (respuesta) se mantendrá, mientras que si K_D es alta, el sensograma mostrará una

caída progresiva. Por último, en la etapa de regeneración, se expone la superficie a condiciones drásticas (de pH, sales, etc.) para eliminar todo el analito unido al ligando (sin dañar este último) y preparar la superficie para un nuevo ciclo. Si la regeneración es óptima, la línea basal posterior a esta etapa es la misma que al empezar el ciclo.

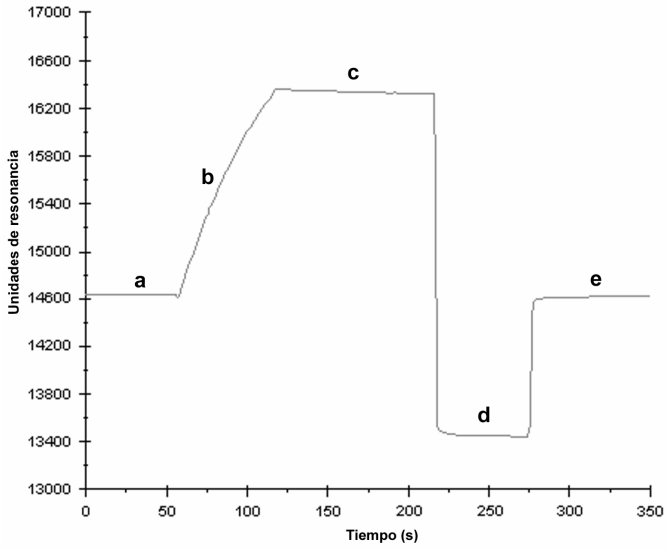


Figura 16. Ciclo de análisis de un sensograma: a) línea basal. b) etapa de asociación. c) etapa de disociación. d) etapa de regeneración. e) línea basal.

La cuantificación de la interacción puede realizarse de varias formas. En esta tesis se utilizaron los valores de unión relativos (incremento de RUs entre un punto preestablecido de la etapa de disociación y un punto preestablecido de la línea basal) para la interacción anticuerpo-STX y la pendiente de la curva de asociación para la unión YTX-PDE 1.

Aplicaciones

Aunque el fenómeno SPR era conocido desde la década de los cincuenta [*176*] no fue hasta 1990 que el primer biosensor basado en esa tecnología (de la casa comercial Biacore ®) salió al mercado [*174*]. Desde entonces, la aplicación de esta técnica se ha utilizado en numerosas áreas científicas (proteómica, microbiología, virología, bioquímica, patología, inmunología…) [*177, 178*]

En el campo de la seguridad alimentaria, esta técnica se utiliza para la detección de micotoxinas [*179*], ficotoxinas [*96, 180*], bacterias [*181*], antibióticos [*182*], aditivos [*183*] y drogas veterinarias [*184*], entre otros analitos.

Equipo instrumental

En la presente tesis se utilizaron dos biosensores distintos de la casa comercial Biacore: Biacore Q y Biacore X. Ambos aparatos comparten los elementos esenciales de los dispositivos Biacore: el chip sensor, el sistema de microfluidos y el sistema óptico [*173, 174*] pero difieren en sensibilidad, automaticidad y otras características instrumentales, como se describe a continuación.

Chip sensor

El chip sensor presenta dos elementos fundamentales: una superficie de cristal recubierta por una fina lámina de oro (común en todos los chips e imprescindible para generar el fenómeno de SPR) y por encima de ésta, un recubrimiento especial (diferente en función del tipo chip (tabla 1) capaz de proporcionar un entorno adecuado para distintos tipos de interacción. Para facilitar su manejo y protección, estos elementos se encuentran insertados en una estructura de plástico rectangular capaz de deslizarse al interior de una funda (figura 17).

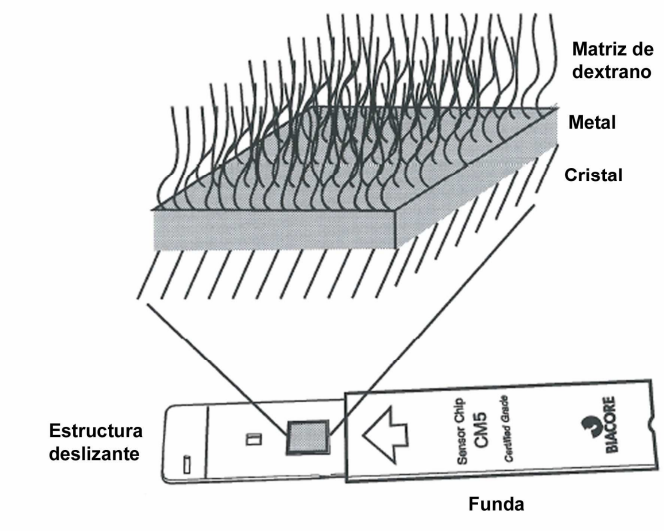


Figura 17. Ilustración esquemática de la estructura de un chip sensor CM5.

Chip sensor	Tipo de superficie	Uso/aplicación
CM5	Matriz de carboximetil dextrano	Uso general
CM4	Matriz de carboximetil dextrano con niveles de carboximetilación menores que CM5	Niveles de inmovilización bajos
CM3	Matriz de carboximetil dextrano con cadenas de dextrano más cortas que CM5	Moléculas grandes
C1	Carboxilada, sin matriz de dextrano	Aplicaciones donde la matriz interfiere.
SA	Matriz de carboximetil dextrano con estreptavidina inmovilizada	Captura de ligandos marcados con biotina.
NTA	Matriz de carboximetil dextrano con ácido nitrilotriacético inmovilizado	Captura de ligandos marcados con polihistidina
L1	Matriz de carboximetil dextrano con modificaciones lipofílicas	Captura de liposomas
HPA	Hidrofóbica	Captura de lípidos monocapa
Au	Sin recubrimiento	Diseño propio de superficies

Tabla 1. Características principales de los distintos tipos de chips sensores comercializados por la empresa Biacore.

Para cada diseño experimental, en función del tipo de chip y de las características del ligando, la estrategia de inmovilización es distinta, y aunque existen protocolos de inmovilización pre-establecidos [*173*], este procedimiento debe optimizarse para cada tipo de ensayo. En la presente tesis se utilizaron chips CM5 en los que se inmovilizaron directamente por medio de una unión amina dos clases de fosfodiesterasas distintas y chips CM5 con saxitoxina inmovilizada mediante un brazo espaciador de jeffamina (ver sección Publicaciones).

Sistema de microfluidos

Cuando el chip sensor es insertado por primera vez en el biosensor, la cara de cristal es presionada contra el prisma cristalino de la unidad óptica, mientras que la cara que contiene la matriz, entra en contacto con un cartucho de microfluidos integrado (IFC). De esta manera, se forman varias celdas de flujo (el número de celdas depende del modelo Biacore), con un volumen que varia en función del biosensor entre los 20

y los 100 nl. La IFC controla el flujo continuo de tampón o muestra sobre la superficie sensora en estas celdas lo que permite exponer el ligando a una concentración constante de analito y establecer de forma precisa y repetitiva el tiempo de contacto entre las dos moléculas [*173*].

Sistema óptico

Como se ha descrito anteriormente, los biosensores Biacore se basan en el fenómeno óptico de la resonancia del plasmón supeficial. La fuente de luz utilizada en estos aparatos es un diodo que emite luz con una longitud de onda cercana a la región del infrarrojo, la luz reflejada se monitoriza mediante diodos bidimensionales y el ángulo de resonancia es calculado por el software mediante complejos algoritmos de interpolación [*173*].

Diferencias entre modelos Biacore: X y Q.

Aunque ambos son capaces de detectar y cuantificar uniones bimoleculares en tiempo real, existen varias diferencias entre ellos (resumidos en la Tabla 2), ya que el Biacore X fue diseñado principalmente para ser usado en investigación, siendo sus principales características la versatilidad y la sensibilidad, mientras que el Biacore Q fue creado para la determinación cuantitativa y cualitativa de analitos en productos alimentarios.

Características	**Biacore X**	**Biacore Q**
Tipo de información	Detección/Concentración/ Datos cinéticos	Detección/Concentración
Cargado de la muestra	Manual	Automática
Capacidad de muestras	-	Dos placas de 96 pocillos + 20 viales
Volumen de muestra	Volumen inyectado + 20 µl	Volumen inyectado + 30 µl
Volumen de inyección	5-100 µl	5-325 µl
Número de celdas	2	4
Detección de moléculas por análisis directo	> 180 Da	> 1000 Da
Tª de análisis	4-40 ºC	25 ºC

Tabla 2. Características principales de los biosensores Biacore X y Biacore Q.

OBJETIVOS

2. OBJETIVOS

Las ficotoxinas marinas representan un grave problema sanitario, económico y social a nivel mundial. Cada año las intoxicaciones en humanos debido a este tipo de toxinas se cuentan por miles y las perdidas económicas por centenares de millones. Para la gran mayoría de estos compuestos, el método oficial de determinación es el bioensayo en ratón. Este método presenta numerosas limitaciones de carácter técnico (sensibilidad, especificidad y rapidez) así como también una serie de problemas éticos y legales derivados del uso de animales en investigación. El objetivo general de esta tesis es el desarrollo de nuevos métodos de extracción, detección y cuantificación de ficotoxinas marinas en moluscos capaces de solventar los inconvenientes del bioensayo, salvaguardando la salud pública sin perjudicar los intereses económicos de la industria acuícola. De forma específica, este objetivo general puede concretarse en:

a) Desarrollar un método de detección y cuantificación de toxinas PSP en extractos de molusco utilizando un anticuerpo anti-GTX2/3 mediante un biosensor basado en el fenómeno óptico de la resonancia del plasmón superficial.

b) Estudiar la viabilidad de utilizar los biosensores SPR en la detección y cuantificación de yessotoxina a partir de su unión a fosfodiesterasas.

c) Desarrollar un método de detección y cuantificación de gymnodiminas y espirólidos en extractos de molusco basado en la competición de estos compuestos y la α-bungarotoxina por la unión a receptores nicotínicos de acetilcolina utilizando la técnica de la polarización de fluorescencia.

PUBLICACIONES

3. PUBLICACIONES

En este apartado de la memoria se describe la parte experimental y se presentan y discuten de forma específica los resultados obtenidos en esta tesis doctoral. Constituyen la sección un total de cuatro publicaciones, divididas en dos bloques en función de la técnica utilizada.

Bloque A. Estudios realizados con biosensores basados en el fenómeno óptico de la resonancia del plasmón superficial.

Los biosensores basados en SPR permiten la detección y monitorización de uniones moleculares en tiempo real sin necesidad de marcaje. En esta tesis se utilizaron para detectar y cuantificar toxinas PSP y YTX:

Artículo 1. **Paralytic Shellfish Poisoning detection by surface plasmon resonance-based biosensors in shellfish matrixes**. E. S. Fonfría, N. Vilariño, K. Campbell, C. Elliott, S. A. Hauguey, B. Ben-Gigirey, J. M. Vieites, K. Kawatsu, L. M. Botana. Artículo publicado en *Analytical chemistry 79 (2007): 6303-6311*. La intoxicación paralizante por consumo de molusco es una de las más extendidas y peligrosas del mundo [*1*]. En este artículo se presenta un nuevo método de detección y cuantificación de estas ficotoxinas (en el rango 150-4000 ng/kg de molusco) mediante un ensayo de inhibición, donde las toxinas en solución procedentes de una muestra de molusco contaminado compiten con STX inmovilizada en un chip CM5 por la unión a un anticuerpo monoclonal anti-GTX2/3. El método de extracción más apropiado, en términos de interferencia de matriz y recuperación de toxina, fue determinado mediante el estudio de cinco protocolos de extracción en cinco especies de molusco. La mejor extracción se utilizó en el análisis de 14 muestras naturales, comparando los resultados obtenidos en el biosensor con los dos métodos oficiales de detección de toxinas PSP: bioensayo en ratón y HPLC. Los resultados del biosensor mostraron una sobreestimación de toxina respecto a los otros métodos, pero la correlación de las muestras negativas fue óptima, avalando su uso como método de criba.

Artículo 2. **Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin**. E. S. Fonfría, N. Vilariño, M.R. Vieytes, T. Yasumoto, L. M. Botana. Artículo publicado en *Analytica chimica acta 617 (2008): 167-170*. La presencia de yessotoxina en moluscos, debido a sus características tóxicas y lipofílicas, representa una de las fuentes más importantes de falsos positivos en la detección de toxinas diarreicas por bioensayo en ratón. La detección de esta toxina de forma individual resulta esencial para evitar pérdidas innecesarias a la industria acuícola. En este artículo se presenta un método de detección y cuantificación de YTX por unión directa a la fosfodiesterasa 1 (PDE 1), inmovilizada en un chip CM5, utilizando biosensores basados en SPR. Anteriormente esta unión se había descrito usando biosensores basados en espejos resonantes [*116, 117, 185*]. Esta tecnología difiere con los biosensores de SPR en el sistema óptico, en la estructura de inmovilización y en el sistema de inyección de la muestra. Los resultados obtenidos muestran que en el mismo rango de detección (3-12 μM), dentro de los límites de regulación marcados por la Unión Europea utilizando un método de extracción adecuado, los biosensores de SPR reducen el tiempo de análisis 10 veces.

Bloque B. Estudios realizados mediante polarización de fluorescencia.

La polarización de fluorescencia permite la detección y monitorización de uniones moleculares en solución y en tiempo real. En esta tesis se utilizaron para detectar y cuantificar GYM y SPX:

Artículo 3. **Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization**. N. Vilariño, E. S. Fonfría, J. Molgó, R. Aráoz, L. M. Botana. Artículo aceptado en la revista *Analytical chemistry*, en prensa. Gymnodiminas y espirólidos pertenecen al grupo de ficotoxinas de las iminas cíclicas [*118*]. Actualmente, su toxicidad en humanos es desconocida y no existe ningún tipo de regulación para ellas. Sin embargo, estas toxinas presentan una toxicidad intraperitoneal en ratón extremadamente alta, dando lugar a falsos positivos en la detección de toxinas lipofílicas por bioensayo. Además, la alta toxicidad oral de los espirólidos en animales de laboratorio ha provocado una corriente de opinión favorable a su regulación entre la comunidad científica actual. En este artículo se presenta un método de detección y cuantificación de gymnodiminas y espirólidos en el rango nM basado en la capacidad de estos compuestos de competir con la α-

bungarotoxina marcada con Alexa Fluor 488 por la unión a receptores nicotínicos de *Torpedo marmorata.* La especificidad del ensayo se estudió mediante la aplicación del método a tres ficotoxinas lipofílicas: yessotoxina, ácido okadaico y brevetoxina-2. Ninguna de ellas interfirió en la interacción α-bungarotoxina-receptor. También se desarrolló un método de extracción de estas toxinas en mejillón. Utilizando acetona, n-hexano y cloroformo se obtuvieron porcentajes de recuperación aceptables para ambos compuestos en muestras de mejillón contaminadas de forma artificial, siendo el rango de cuantificación de GYM-A y 13-desmetil SPX C en este molusco de 50-2000 μg/kg y 70-700 μg/kg de carne, respectivamente.

Artículo 4. **Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization in shellfish matrixes**. E. S. Fonfría, N. Vilariño, J. Molgó, R. Aráoz, L. M. Botana. Artículo enviado a la revista *Analytica chimica acta,* en revisión. El método de detección de gymnodiminas y espirólidos mediante polarización de fluorescencia se basa en la competición de estas moléculas con la α-bungarotoxina Alexa Fluor 488 por la unión a receptores nicotínicos de *T. marmorata* [*186*]. En este artículo se presenta un protocolo de extracción adecuado para detectar y cuantificar GYM y 13-desmetil SPX C en diferentes especies de molusco utilizando la polarización de fluorescencia. Para este estudio, almejas, berberechos, mejillones y vieiras fueron extraídos usando un protocolo de acetona, n-hexano y cloroformo. La interferencia de estas matrices con la fluorescencia de la α-bungarotoxina o la unión de ésta al receptor nicotínico fue menor del 11 % y el porcentaje de recuperación de GYM y 13-desmetil SPX C en muestras contaminadas artificialmente empleando el mismo método de extracción fue del 90 %, con variaciones entre especies. El rango de detección de GYM y 13-desmetil SPX C en todas ellas fue de 50-2000 μg/kg y 70-700 μg/kg de carne de molusco, respectivamente.

Artículo 1. Paralytic Shellfish Poisoning detection by surface plasmon resonance-based biosensors in shellfish matrixes.

E. S. Fonfría, N. Vilariño, K. Campbell, C. Elliott, S. A. Hauguey, B. Ben-Gigirey, J. M. Vieites, K. Kawatsu, L. M. Botana

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Paralytic Shellfish Poisoning Detection by Surface Plasmon Resonance-Based Biosensors in Shellfish Matrixes

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The detection of paralytic shellfish poisoning (PSP) toxins in contaminated shellfish is essential for human health preservation. Ethical and technical reasons have prompted the search for new detection procedures as an alternative to the mouse bioassay. On the basis of the detection of molecular interactions by surface plasmon resonance (SPR) biosensors, an inhibition assay was developed using an anti-GTX2/3 antibody (GT13-A) and a saxitoxin-CM5 chip. This assay allowed for quantification of saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), gonyautoxin 2,3 (GTX2/3), decarbamoyl gonyautoxin 2,3 (dcGTX2/3), gonyautoxin 5 (GTX5), and C 1,2 (C1/2) at concentrations from 2 to 50 ng/mL. The interference of five shellfish matrixes with the inhibition assay was analyzed. Mussels, clams, cockles, scallops, and oysters were extracted with five published methods. Ethanol extracts and acetic acid/heat extracts (AOAC Lawrence method) performed adequately in terms of surface regeneration and baseline interference, did not inhibit antibody binding to the chip surface significantly, and presented STX calibration curves similar to buffer controls in all matrixes tested. Hydrochloric acid/heat extracts (AOAC mouse bioassay method) presented surface regeneration problems, and although ethanol−acetic acid/dichloromethane extracts performed well, they were considered too laborious for routine sample testing. Overall the best results were obtained with the ethanol extraction method with calibration curves prepared in blank matrix extracts. STX recovery rate with the ethanol extraction method was 60.52 ± 3.72%, with variations among species. The performance of this biosensor assay in natural samples, compared to two AOAC methods for PSP toxin quantification (mouse bioassay and HPLC), suggests that this technology can be useful as a PSP screening assay. In summary, the GT13-A-STX chip inhibition assay is capable of PSP toxin detection in ethanol shellfish extracts, with sufficient sensitivity to quantify the toxin in the range of the European regulatory limit of 80 μg/100 g of shellfish meat.

Paralytic shellfish poisoning (PSP) is a worldwide, algal-derived group of toxins that can cause serious food poisoning. The neurotoxins responsible for PSP are saxitoxin (STX) and its analogues. More than 24 compounds have been identified that differ in combinations of hydroxyl and sulfate substitutions located at 4 sites of a 3,4,6-trialkyl tetrahydropurine backbone.[1−6] All the compounds of this group share the same mechanism of action, the inhibition of the voltage-gated sodium channel in excitable cells.[7,8] The blockage of neuronal transmission induces neurological symptoms in humans such as perioral paresthesia, dizziness, paralysis, and even respiratory arrest and death.[9,10] The threat to human health resulting from consumption of PSP-contaminated shellfish has led to the implementation of shellfish and seawater monitoring programs in many countries. In regards to this, shellfish monitoring by the mouse mortality bioassay[11,12] is accepted by many countries as the official method to detect PSP

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toxins. Analytical limitations of the method and ethical issues have stimulated the search for alternative techniques. Other biological methods, such as cytotoxicity tests or electrophysiological assays, and chemical methods, such as HPLC, have been developed to detect PSP toxins;[13−17] however, none of them has replaced the mouse bioassay to date.

Nonradioactive technologies aimed at detecting the interaction between biomolecules have been developed in the last two decades. Among them, the SPR-based biosensors, which use the phenomenon of surface plasmon resonance to detect molecular interactions, have been adapted to food contaminant analysis.[18] In the present study, the interference of mollusk matrixes extracted by a wide range of methods with a STX−anti-gonyautoxin 2,3 (GTX2/3) inhibition assay developed for SPR-biosensors were evaluated. Five different mollusk species, mussel (*Mytilus galloprovincialis*), cockle (*Cerastoderma edule*), clam (*Venerupis* spp.), scallop (*Pecten maximus*), and oyster (*Ostrea edulis*), were extracted with five extraction methods already reported in the literature in order to select the optimal conditions for the inhibition assay. Four parameters of the SPR-based inhibition assay were considered to evaluate the adequacy of the extraction method to be used in this assay: regeneration quality, baseline interference, inhibition of antibody binding signal, and STX calibration curve. Finally, the performance of the assay with natural samples extracted with the method of choice was compared to other PSP detection techniques.

METHODS

Materials. The HBS (Hepes buffered saline) was from Biacore AB (Uppsala, Sweden), and a Supelclean LC-18 SPE cartridge and a Millex-FG13, fluoropore PTFE (pore size 0.2 μm) filter were from Sigma (Madrid, Spain). Saxitoxin diacetate (STXda, 201.3 μg/mL), saxitoxin dihydrochloride (STXdh, 24.2 μg/mL), decarbamoyl saxitoxin (dcSTX, 20.4 μg/mL), neosaxitoxin (neoSTX, 25.23 μg/mL), decarbamoyl neosaxitoxin (dcNEO, 10.4 μg/mL), gonyautoxin 2,3 (GTX2/3, 46.6 μg/mL and 15.4 μg/mL, respectively), decarbamoyl gonyautoxin 2,3 (dcGTX2/3, 40.2 μg/mL and 11.3 μg/mL, respectively), gonyautoxin 1,4 (GTX1/4, 43.6 μg/mL and 14.4 μg/mL, respectively), gonyautoxin 5 (GTX5, 24.7 μg/mL), and C1,2 (C1/2, 54.2 μg/mL and 16.6 μg/mL, respectively) were purchased from NRC-CNRC (Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada). Hydrochloric acid, acetic acid, dichloromethane, ethanol, methanol, and sodium hydroxide were from reagent grade commercial sources.

Anti-GTX2/3 antibody (GT13-A) was a gift of Dr. Kawatsu.[19] The saxitoxin chip (STX-chip) was produced by activation of a CM5 chip (Biacore AB) using an amine coupling kit from Biacore AB (Uppsala, Sweden). A mixture (1:1, v/v) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) was applied for 30 min to the surface of a CM5 chip (Biacore AB, Uppsala, Sweden). After removal of excess solution, 20% Jeffamine (2,2-(ethylenedioxy)-bis(ethylamine)) in borate buffer was added for 1 h, followed by immobilization of STX diacetate onto the amine surface via an amino−amino coupling. The chip surface was then deactivated by exposure to ethanolamine (1 M) for 30 min. The sensor chip surface was washed with deionized water, dried with a stream of nitrogen, and stored desiccated at 4 °C when not in use. The equipment used for binding detection was a Biacore Q biosensor (Biacore AB, Uppsala, Sweden).

PSP free shellfish for interference studies and calibration purposes was purchased fresh from the market on the same day an experiment was to be performed, except for scallops that were frozen. Mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), clams (*Venerupis* spp.), and scallops (*Pecten maximus*) were purchased in Lugo, Spain, in July, August, and September, 2006, and oysters (*Ostrea edulis*) were purchased in La Coruña, Spain, in October, 2006. Natural samples for PSP content testing were provided homogenized and frozen by the European Union Community Reference Laboratory on Marine Biotoxins in collaboration with ANFACO-CECOPESCA.

Detection of Antibody Binding in the SPR-Based Biosensor Biacore Q. A 1 mg/mL stock of the anti-GTX2/3 (GT13-A) antibody was diluted 1:400. The extracts were diluted in HBS as required and mixed with the antibody in a 1:2, 1:3, or 1:4 antibody (Ab):final volume ratio before injection in the biosensor. The duration of the sample injection (contact time of sample with chip surface) was 1 min at a flow rate of 25 μL/min. The antibody bound to the chip surface was removed before the next sample injection, a step called regeneration, by injection of 0.1 M NaOH for 1 min at 25 μL/min. Reporting points were 5 s before injection (baseline, Figure 1A (point 1)) and 30 s after the injection was finished (response, Figure 1A (points 2)), binding signal = (response − baseline). STX diacetate was used unless stated otherwise.

Extraction Methods. Five extraction methods were tested for interference with the PSP-detection assay. The protocols described below are modifications of four methods previously published. For all extractions performed in this study more than 100 g of shellfish meat were homogenized with a blender and a fraction of the homogenate was used for extraction as indicated in the methods. Method 1 (Lawrence[20]): A 5 g amount of mussel, clam, cockle, or oyster meat (whole body) homogenates or 5 g of scallop muscle or muscle plus gonad homogenates were used for extraction. After the addition of 3 mL of 1% acetic acid, the mixture was homogenized by vortexing and the sample was boiled for 5 min in a water bath. The tubes were placed for 5 min in an ice bath and then centrifuged at 3600*g* for 10 min at room temperature. The supernatant was saved, and the pellet was extracted again with 3 mL of 1% acetic acid. The two supernatants were mixed, and the final volume was brought up to 10 mL with H_2O. A Supelclean LC-18 SPE cartridge was preconditioned with 4 mL of methanol

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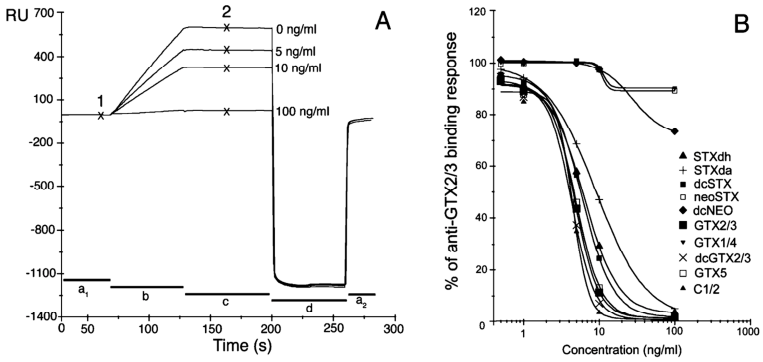

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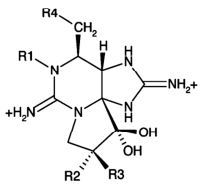

R1	R2	R3	R4:	R4:	R4:-OH	R4:-H
H	H	H	STX	GTX5	dcSTX	doSTX
OH	H	H	neoSTX	GTX6	dcneoSTX	-
OH	OSO_3^-	H	GTX1	C3	dcGTX1	-
H	OSO_3^-	H	GTX2	C1	dcGTX2	doGTX2
H	H	OSO_3^-	GTX3	C2	dcGTX3	doGTX3
OH	H	OSO_3^-	GTX4	C4	dcGTX4	-

Figure 1. Detection of PSP toxins by a GT13-A−STX chip inhibition assay in a SPR-based biosensor. (A) Sensogram showing cycles of sample analysis of antibody control (0 ng/mL STX) and 5, 10, and 100 ng/mL STX in a Biacore Q biosensor. The parts of the sensogram are identified as (a_1) baseline previous to sample injection, (b) binding (antibody/extract injection), (c) dissociation (HBS injection), (d) regeneration (0.01 M NaOH injection), and (a_2) baseline postsample injection/regeneration. Reporting points are marked as 1 (baseline) and 2 (response). (B) Inhibition curves of STXda, STXdh, dcSTX, GTX2/3, GTX1/4, dcGTX1/2, GTX5, neoSTX, dcNEO, and C1/2. Concentrations of 0.5, 1, 5, 10, and 100 ng/mL of each toxin were injected in the biosensor after mixing with the antibody in a 1:2 antibody/final volume ratio. The results are expressed as percentage of binding signal displayed by antibody alone. A calibration curve was obtained for all toxins using a four parameter fit. (C) PSP toxin structure.

followed by 4 mL of H_2O. Then 1 mL of extract was loaded into the cartridge and washed with 2 mL of H_2O. The sample was collected from the time the extract was loaded and the final volume adjusted to 4 mL with H_2O. Finally, after a 1:10 dilution with HBS, the extract was filtered through a Millex-FG13, fluoropore PTFE filter, pore size 0.22 μm. The estimated duration was 1 h and 20 min.

Method 2 (AOAC,[12]): A 10 g amount of mussel, clam, cockle, or oyster meat (whole body) homogenates or 10 g of scallop muscle or muscle plus gonad homogenates were used for extraction. After the addition of 10 mL of 0.1 M HCl, the pH should be between 2 and 4, preferably 3. If necessary, the pH was adjusted with HCl. Then the sample was boiled in a water bath for 5 min and cooled down at room temperature. The sample pH was checked again, and it should be between 2 and 4. The sample was then diluted to a final volume of 20 mL with 0.1 M HCl, mixed until homogeneous, and allowed to settle. The liquid was removed and centrifuged at 1560*g* for 5 min at room temperature. The supernatant was filtered through a paper filter, diluted 1:40 in HBS, and filtered through a PTFE filter, pore size 0.22 μm. The estimated duration was 2 h.

Method 3 (AOAC-SPE): This method was a modification of method 2 that included a cleanup step through SPE C18 cartridges after the filtration through a paper filter. The estimated duration was 2h 20 min.

Method 4 (Garthwaite,[21]): Mollusk meat (whole body) of mussel, clam, cockle, and oyster or muscle or muscle plus gonad of scallop were homogenized with a blender. A 1 g amount of homogenate was extracted with 5 mL of 90% ethanol and centrifuged at 3000*g* for 10 min at room temperature. The supernatant was saved, and the pellet was extracted again with 3 mL of 90% ethanol. The supernatants were pooled together, and the final volume was adjusted to 10 mL with 90% ethanol. After a 1:8 dilution in HBS, the extract was filtered through a PTFE filter, pore size 0.22 μm. The estimated duration was 45 min. When natural samples were analyzed, 10 g of homogenate was extracted with 50 mL of 90% ethanol, the second extraction was with 30 mL, and the final volume was adjusted to 100 mL.

Method 5 (USDC,[22]): A 5 g amount of hepatopancreas homogenate (mussel, clam, cockle) was extracted with 20 mL of 80% ethanol:1% acetic acid (1:4). The pH should be between 3 and 4, and if necessary the pH is brought down with acetic acid. After centrifugation at 4330*g* for 15 min at room temperature, the supernatant was saved and the pellet was extracted again with 10 mL of 80% ethanol:1% acetic acid (1:4). The supernatants were pooled together, evaporated, and diluted with 0.03 N acetic acid. The extract was then filtered through glass wool and extracted

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with dichloromethane (1:2) for 12−24 h. The aqueous solution was recovered, evaporated, and dissolved in 0.03 N acetic acid to a final volume of 10 mL. After a 1:40 dilution with HBS, the extract was filtered through a PTFE filter, pore size 0.22 μm. The estimated duration was 2 days.

For all extraction methods, volumes and dilutions were adjusted so that a final concentration of 10 ng/mL of toxin correlates to a concentration of toxin in mollusk of 80 μg/100 g of homogenate.

Analysis of Samples by Mouse Bioassay and HPLC. The extraction of samples for detection of PSP toxins by mouse bioassay and HPLC and the quantification of toxin content by these methods was performed as described by the AOAC protocols AOAC 959.08 (mouse bioassay)[12] and AOAC 2005.06 (HPLC)[23,24]

RESULTS

SPR-Based Biosensor Assay for Detection of PSP Toxins. An inhibition assay was designed for detection of STX using the anti-GTX2/3 antibody GT13-A and a STX chip. STX in solution is detected by competition for binding to the GT13-A antibody with the STX immobilized to the chip surface. The presence of STX in solution, previously mixed with the GT13-A antibody, inhibited binding of the antibody to the STX chip surface (Figure 1A). Optimization of antibody concentration, antibody:final volume ratio, flow rate, injection time, and regeneration preceded testing of inhibition by other PSP toxins. Final optimized conditions of the biosensor assay are described in the methods section. Regeneration of the chip surface to remove bound antibody returned the baseline to initial values (Figure 1A, levels a_1 and a_2). The chip surface was very stable under these assay conditions, allowing for more than 500 sample injections in one flow cell. The optimized assay could detect concentrations of STX between 2 and 50 ng/ mL (Figure 1B), with the highest sensitivity around 5−10 ng/mL, depending on the antibody:final volume ratio. Actually, three ratios were tested 1:2, 1:3, and 1:4 with slightly higher sensitivity as the dilution of the antibody was increased. Since the performance of the assay with shellfish matrixes presented some problems with the two higher dilutions (see next section), all the results shown in this work were obtained with a 1:2 ratio of antibody/final volume unless stated otherwise.

The ability of this inhibition assay to detect PSP toxins other than STX was also studied. An inhibition curve was obtained using several concentrations of STXda, STXdh, dcSTX, neoSTX, dcNEO, GTX2/3, GTX1/4, dcGTX2/3, GTX5, and C1/2 (Figure 1B). This assay did not recognize neoSTX, GTX1/4, or dcNEO, the toxins hydroxylated at the R1 site (Figure 1C). However, it did detect all the other analogues tested. The IC_{50} values of the PSP toxins in these experimental conditions were 9.08 ng/mL for STXda, 6.01 ng/mL for STXdh, 5.68 ng/mL for dcSTX, 4.48 ng/mL for GTX2/3, 4.05 ng/mL for dcGTX2/3, 4.63 ng/mL for GTX5, and 4.21 ng/mL for C1/2. The sensitivity of the assay was optimized for matrix interference experiments, choosing the conditions that allowed for detection of PSP toxins from concentrations of 15 μg/100 g of meat to 400 μg/100 g of meat with the highest slope of the calibration curve around values of 40−80 μg of STX/100 g of meat (Figure 4).

Table 1. Quality of Regeneration after Extract Injection[a]

extraction method	mussel	cockle	clam	scallop muscle	scallop muscle + gonad	oyster
Lawrence	+++	+++	+++	+++	+++	+++
AOAC SPE	+	+++	+++	+++	+++	+++
AOAC	+	+++	++	+++	+++	+++
Garthwaite	+++	+++	+++	+++	+++	+++
USDC	+++	+++	+++			

[a] After extract or extract plus antibody injection, the chip surface was regenerated with 0.1 M NaOH for 1 min at a 25 μL/min flow rate. If regeneration is adequate, the baseline value before extract injection and after regeneration should be equal. This table reports the performance of regeneration. Key: +++, all experiments regenerated adequately with 0.1 M NaOH for 1 min; ++, one or more experiments did not regenerate adequately with 0.1 M NaOH for 1 min; +, none of the experiments regenerated adequately with 0.1 M NaOH for 1 min ($n = 3$).

Interference of Shellfish Matrixes with the Biosensor Assay. Four parameters of the sensogram (Figure 1A) were evaluated for assay performance in shellfish matrixes. The extraction method determined to have the best performance for all species included in the study was evaluated for toxin recovery.

Regeneration of Chip Surface after Exposure to Mollusk Extract. Three GT13-A antibody dilutions (1:2, 1:3, and 1:4) in the antibody:extract mixture were tested for regeneration quality. The experiments consisted of injection of HBS:extract (extract alone) or GT13-A:extract followed by regeneration with 0.1 M NaOH for 1 min at 25 μL/min. For each analysis cycle in the biosensor, the regeneration was considered adequate if baseline values were the same before sample injection and after one regeneration following sample injection. Table 1 shows the results obtained for regeneration with extracts of five shellfish species. Only extracts from two of the five extraction procedures presented problems with regeneration of the chip surface, i.e., the AOAC extracts obtained from mussels and clams. In those cases, following repeated injection of regeneration solution (0.1 M NaOH), the surface was completely restored without apparent damage to the chip surface. As the need for repeated chip surface regenerations is not suitable for routine analytical methods, when such data were found these have been reported as low performance (+) of regeneration in Table 1. As expected, increasing amounts of extract did not improve the regeneration, and the data obtained showed that effective surface regeneration became more problematical.

Extract Interference with Baseline. The extent of extract interference with the baseline has been reported in Table 2 as the binding signal observed following the injection of each extract alone. Only the results for a 1:2 ratio of antibody:extract mixture are shown; however, the results where similar for ratios 1:3 and 1:4, showing a higher signal with an increasing amount of extract present. Baseline interference was found to be highest for AOAC extracts in most species tested. The other methods yielded extracts with low baseline interference in most species. The exception was the mussel extracts obtained with the Lawrence method.

(23) AOAC official method 2005.06. In *AOAC Official Methods of Analysis*, 18th ed.; AOAC International: Gaithersburg, MD, 2006.
(24) Ben-Gigirey, B.; Rodriguez-Velasco, M. L.; Villar-Gonzalez, A.; Botana, L. M. *J. Chromatogr.*, A **2007**, *1140*, 78−87.

Table 2. Interference of Shellfish Extracts with Baseline[a]

extraction method	mussel	cockle	clam	scallop	scallop muscle + gonad	oyster
Lawrence	87.5 ± 22.0	9.8 ± 6.2	13.9 ± 3.5	6.2 ± 0.8	7.6 ± 0.1	6.9 ± 2.0
AOAC SPE	294.7 ± 16.1	92.9 ± 55.9	45.6 ± 19.8	5.9 ± 0.4	6.9 ± 1.0	161.5 ± 53.7
AOAC	187.6 ± 88.3	203.5 ± 65.3	108.5 ± 25.3	27.8 ± 3.9	41.4 ± 2.7	297.9 ± 59.3
Garthwaite	−6.5 ± 4.6	5.7 ± 0.9	−0.8 ± 1.8	−5.2 ± 1.2	−5.0 ± 1.7	−2.4 ± 1.2
USDC	27.7 ± 10.1	8.8 ± 1.0	19.3			

[a] Data are shown as binding signal in RU of HBS/extract mixed in a 1:2 ratio (no antibody present) ($n = 3$; except for scallop muscle + gonad, $n = 2$).

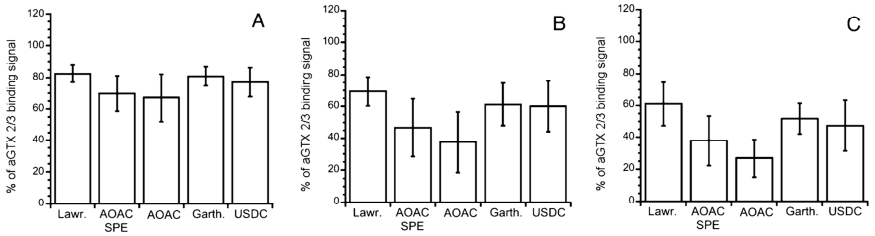

Figure 2. Effect of antibody/extract ratio on antibody binding to the STX chip surface in mussels. Binding signal of GT13-A antibody mixed with mussel extract in (A) 1:2, (B) 1:3, and (C) 1:4 ratios was expressed as percentage of antibody binding signal in the absence of extract (mean ± SEM, $n = 3$; except for AOAC methods, $n = 2$).

Inhibition of Antibody Binding Signal in the Presence of Mollusk Extract. In order to be able to perform an inhibition assay for detection of PSP toxins in shellfish extracts, the different matrixes should not interfere with binding of the antibody to the chip surface. Antibody binding signal in the presence of extract was expressed as percentage of control in HBS, and this value was calculated as follows: $((\text{Ab:extract}_{\text{Ri}})/(\text{Ab:HBS}_{\text{Ri}})) \times 100$, where $\text{Ab:extract}_{\text{Ri}}$ = binding signal obtained after injection of Ab:$\text{extract}_{\text{Ri}}$ − binding signal obtained after injection of HBS:$\text{extract}_{\text{Ri}}$, Ri is the dilution ratio and $\text{Ab:HBS}_{\text{Ri}}$ = binding signal obtained after injection of $\text{Ab:HBS}_{\text{Ri}}$. Again, three ratios of the antibody: extract mixture were tested for all species. In mussel and cockle extracts, the increase of extract fraction induced a higher inhibition of antibody binding; this effect was particularly strong in mussels (Figure 2). Mussel AOAC extracts induced a substantial inhibition of antibody binding in the absence of toxin, even when used at a 1:2 dilution. On the contrary, the extracts obtained by Lawrence and Garthwaite extraction methods did not interfere at all with antibody binding in clams, scallops, and oysters and inhibited binding slightly in cockles and mussels (Figure 3). The extracts obtained by the USDC extraction method performed similarly to Lawrence and Garthwaite extracts. However, since the extraction procedure is too long for routine sample processing, further testing of this method was discontinued. There was no statistical significance among the different extraction methods (ANOVA); however, due to the high variability often encountered in biological samples, the experiments were probably not powered enough to detect statistical differences. When the three dilution ratios were compared within the same extraction method in mussels, the higher dilutions performed significantly different (ANOVA paired with Tukey multiple comparison post-test) than the 1:2 dilutions.

STX Calibration Curves with Mollusk Extracts Using the GT13-A−STX Chip Inhibition Assay. The inhibition assay was performed with a 1:2 dilution of antibody in extract due to the reduced binding of antibody at higher ratios in mussel extracts. The extracts were contaminated with increasing amounts of STX and then mixed with a 1:400 dilution of GT13-A antibody stock. Calibration experiments with AOAC and AOAC SPE extracts were not repeated three times in all species due to the poor quality of the regeneration after extract injection. Percentage of antibody binding was calculated as follows: $((\text{Ab:extract}_{\text{STX}})/(\text{Ab:extract}_0)) \times 100$, where Ab:extract_0 = (binding signal obtained after injection of Ab:extract mixture in the absence of toxin) − (binding signal obtained after injection of extract alone), and $\text{Ab:extract}_{\text{STX}}$ = (binding signal obtained after injection of Ab:extract mixture containing a known concentration of STX) − (binding signal obtained after injection of extract alone). In mussels and oysters, the Lawrence and Garthwaite extracts presented calibration curves very similar to the HBS controls; however, the AOAC and AOAC SPE methods were considerably shifted from controls (Figure 4parts A and F). In clam, cockle, oyster, and scallops, calibration curves for all extracts were very similar to controls, except for the Lawrence method that yielded a calibration curve slightly shifted to the right in scallops, both for muscle alone or muscle plus gonad (Figure 4). With the use of a four parameter fit, the IC_{50} was calculated for each of these calibration curves. The IC_{50} values obtained are presented in Table 3. Most matrixes produced good calibration curves at least with two extraction methods, with IC_{50} close to 10 ng/mL of STX, which is equivalent to the 80 μg/100 g legal limit. Overall these results highlight the Garthwaite extraction method as the best candidate to be used in all five species with acceptable results: the AOAC extracts had regeneration problems, the USDC was too laborious for routine sample testing, and Lawrence extracts induced a statistically significant deviation of the calibration curve from the control in scallops.

Feasibility of Using a Calibration Curve in HBS Instead of Toxin-Free Matrix Extracts. With the focus on the Garthwaite

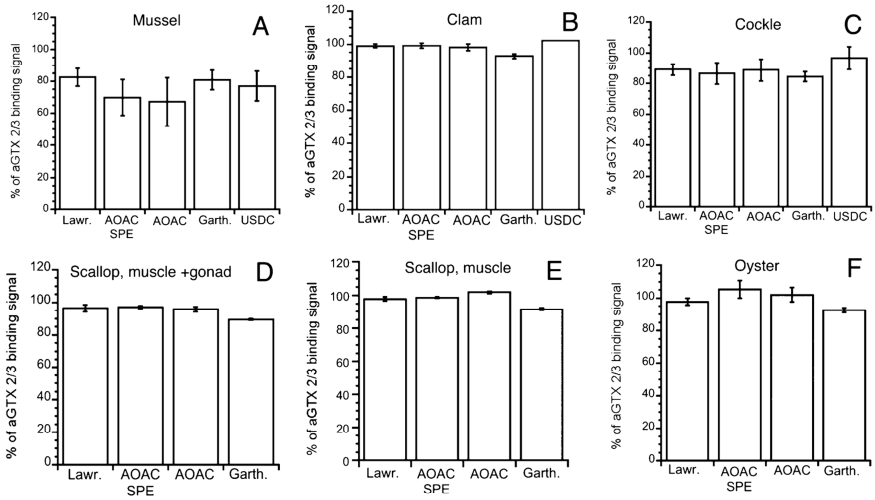

Figure 3. Interference of extracts from several mollusk species with anti-GTX2/3 antibody binding signal. Binding signal of GT13-A antibody mixed with mollusk extract in a 1:2 ratio was expressed as percentage of antibody binding signal in the absence of extract. Five shellfish species were tested (for most of species whole body was used unless stated): (A) mussel, *Mytilus galloprovincialis*; (B) clam, *Venerupis* spp.; (C) cockle, *Cerastoderma edule*; (D) scallop muscle and gonad, *Pecten maximus*, (E) scallop muscle, and (F) oyster, *Ostrea edulis* (mean ± SEM, $n = 3$; except for scallop muscle + gonad, $n = 2$). Antibody binding signal was between 250 and 300 RU (282.42 ± 13.94 RU, mean ± SEM, $n = 20$).

Table 3. IC_{50} Values of STX in the Inhibition Assay for Four Extraction Methods in Several Species and HBS Control[a]

	IC_{50} (ng/mL)					
	mussel	clam	cockle	scallop	scallop muscle + gonad	oyster
control HBS	9.62 ± 1.31	9.14 ± 1.27	7.61 ± 1.29	9.71 ± 1.71	12.30 ± 0.56	8.73 ± 0.93
Lawrence	9.82 ± 1.73	10.48 ± 1.64	7.70 ± 1.33	17.11 ± 6.53	22.89 ± 5.66	8.95 ± 0.84
AOAC SPE	1.62	9.80 ± 2.88	8.35 ± 1.94	9.82 ± 1.30	13.10 ± 0.58	6.29 ± 2.35
AOAC	5.57	9.02 ± 1.73	8.10 ± 2.05	9.29 ± 1.47	12.09 ± 0.46	2.89 ± 1.13
Garthwaite	7.95 ± 1.04	10.26 ± 0.74	6.36 ± 1.12	9.08 ± 1.03	12.87 ± 1.24	9.12 ± 0.65

[a] STX IC_{50} was calculated using the four parameter fit of calibration curves from Figure 4. Values are expressed in ng/mL.

extraction method, the next phase of the study compared the values of STX concentration obtained for shellfish extracts spiked with 5 and 10 ng/mL STX using both matrix and HBS calibration curves. Table 4 shows that for the three matrixes analyzed, the concentration of STX was overestimated when using the HBS calibration curve vs the extract curve. In mussel and scallop extracts, the accuracy of the concentration of STX calculated with the matrix calibration curve was very good; however, in cockles there was a slight underestimation of the amount of toxin.

Recovery Rates with the Garthwaite Extraction Procedure. The recovery rate of STXdh in spiked mollusk meat was analyzed in mussel, cockle, and scallop (muscle and gonad). The samples were spiked with 40 or 80 μg/100 g of meat, before or after the extraction procedure in order to calculate the recovery rate. Three samples were extracted for each species. Recovery rates for the three species are shown in Table 5. In order to improve the analysis time, the possibility of eliminating the second extraction with 90% ethanol of the Garthwaite method was tested. The recovery was 7−8% lower than with two extractions.

Analysis of Natural Samples by SPR-Biosensor Assay and Comparison with the Mouse Bioassay and HPLC. Fourteen natural samples of mussel, clam, cockle, and scallop were analyzed by the biosensor assay using a calibration curve in extract. In order to have a more representative sample, 10 g of shellfish homogenate were extracted with 90% ethanol (see methods section). The toxin concentration was calculated using the binding signal of the mixture of antibody:sample extract after subtraction of the binding signal of sample extract alone. When the concentration of toxin was out of the quantification range of the curve, the sample was diluted until the binding signal was in the high slope section of the calibration curve. The results of quantification of PSP toxins by the SPR-biosensor assay in these 14 samples are shown in Table 6, where they can be compared to the quantification obtained by mouse bioassay and HPLC. The content of toxin detected by the biosensor assay was about 5 times higher than the amount of toxin detected by the mouse bioassay. The overestimation versus HPLC was slightly higher since the toxin content detected by HPLC was in general lower than detection

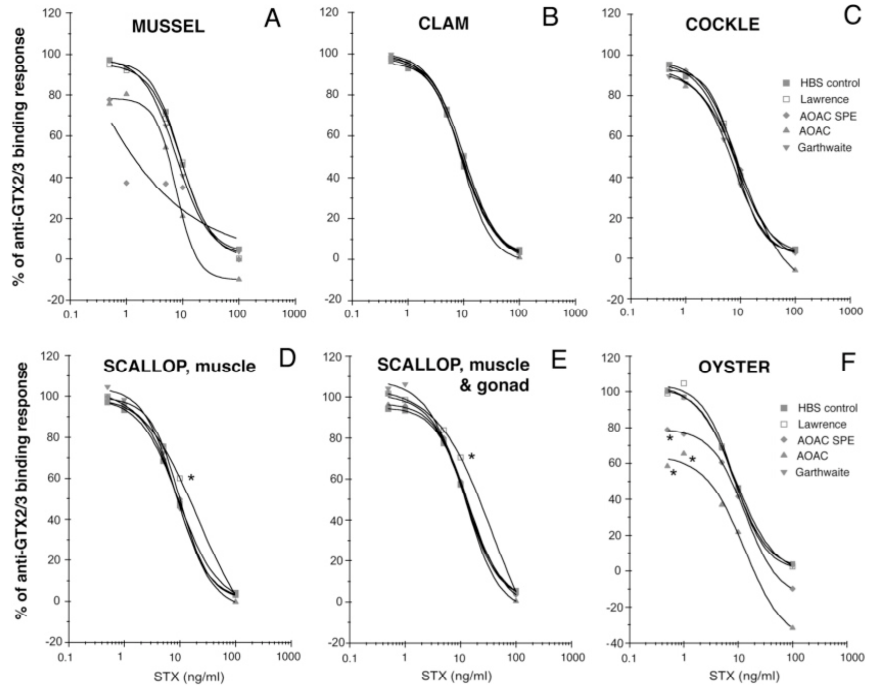

Figure 4. STX calibration curves in the presence of shellfish extracts. STX was diluted at 0.5, 1, 5, 10, and 100 ng/mL in shellfish extracts to obtain calibration curves that are compared with controls in HBS. The extracts were mixed with GT13-A in a 1:2 final antibody dilution and injected over a STX chip. Five extraction methods were tested for (A) mussel ($n = 3$; except for AOAC methods, $n = 1$), (B) clam ($n = 3$; except for AOAC methods, $n = 2$), and (C) cockle ($n = 3$; except for AOAC methods, $n = 2$), and four extraction methods for (D) scallop muscle ($n = 3$), (E) scallop muscle and gonad ($n = 2$), and (F) oyster ($n = 3$). Data are expressed as percentage of antibody binding response. Calibration curves were fit by a four parameter equation. The value of χ was <5 in all fits except for AOAC methods in mussels and oysters (∗statistically significant, ANOVA paired with Dunnett multiple comparison post-test).

Table 4. Comparison of STX Concentrations Calculated Using Calibration Solutions in HBS or in Garthwaite Extracts for Three Matrixes[a]

		STX concentration (ng/mL)		
spikcd STX (ng/mL)	calibration curve	mussel calcd	cockle calcd	scallop calcd
5	matrix extract	5.36 ± 0.23	4.59 ± 0.03	5.69 ± 0.72
	buffer	7.69 ± 0.55	5.81 ± 0.26	7.45 ± 1.53
10	matrix extract	10.19 ± 0.86	8.72 ± 0.06	9.7 ± 0.10
	buffer	12.56 ± 1.24	9.96 ± 0.95	12.64 ± 0.82

[a] Mussels, cockles, and scallops were extracted with the Garthwaite method. The extracts were contaminated with 5 or 10 ng/mL STX, mixed in a 1:2 ratio with GT13-A antibody and injected in the biosensor for analysis. The concentration was calculated using a four parameter fit of the HBS and matrix extract calibration curves (mean ± SEM, $n = 3$).

Table 5. Recovery Rate of STX in Shellfish Extracted with the Garthwaite Method[a]

	recovery rate (%)		
spiked amount (μg/100 g)	mussel	cockle	scallop
80	49.8 ± 9.5	70.1 ± 7.5	60.3 ± 5.1
40	51.3 ± 9.5	74.9 ± 6.7	56.7 ± 3.5

[a] Mussels, cockles, and scallops were extracted with the Garthwaite method. Part of the homogenized meat was spiked with 40 or 80 μg of STX/100 g of meat and extracted. Another part of noncontaminated meat was spiked after extraction with an amount of STX equivalent to 40 or 80 μg/100 g of meat. The extracts were mixed in a 1:2 ratio with GT13-A antibody and injected in the biosensor for analysis. The amount of STX was calculated using a four parameter fit of a calibration curve in matrix extract. Recovery was calculated as the percent fraction of toxin in postextraction-contaminated samples that were recovered in the pre-extraction-contaminated samples (mean ± SEM, $n = 3$).

by the mouse bioassay. All the samples that had a content of toxin below 80 μg/100 g of shellfish meat, the current regulatory limit in many countries, by the biosensor bioassay were also negative by the mouse bioassay.

DISCUSSION

The SPR-biosensor assay proposed for detection of PSP toxins consists of an inhibition of antibody binding to a STX chip surface by PSP toxins in solution. After optimization, the assay allows for quantification of STX in the range of 15−400 μg/100 g of shellfish meat. Most countries have an action limit for PSP toxins of 80 μg/100 g of shellfish meat, which is more than 5 times higher than the lower limit of detection of this assay. In regards to other toxin analogues, this inhibition assay detects dcSTX, GTX2/3, dcGTX2/3, GTX5, and C1/2 with slightly higher sensitivity than STX. However, the cross-reactivity of this GT13-A antibody is low for neoSTX, GTX1/4, dcNEO, and presumably any other PSP toxin

Table 6. Measurement of PSP Toxin Content in 14 Samples by SPR-Biosensor Assay, Mouse Bioassay, and HPLC[a]

		content of PSP toxin (μg/100 g of meat)			
species	sample no.	SPR-biosensor	mouse bioassay	HPLC	toxin profile by HPLC
mussel	082/06	low	negative		
mussel	CRL/06/P/07	34.9	14.3	7.9	dc-STX (0.04743 μg/mL) GTX5 (0.03398 μg/mL) *C1,2 (low)
mussel	CRL/06/P/08	569	173.0	94.6	dc-GTX2,3 (0.1279 μM/L) dc-STX (0.2379 μM/L) C1,2 (1.2201 μM/L) GTX5 (0.7289 μM/L) dc-Neo (0.1182 μM/L) *GTX6, possibly C3,4
mussel	047/06	82.3	68.0	14.8	dc-STX (0.0260 μg/mL) GTX5 (0.0564 μg/mL) *GTX2,3 <L.Q, STX<L.Q, GTX6, *possibly dc-Neo and C1,2
mussel	049/06	0.6	negative	<LOQ	*GTX2,3<L.Q
mussel	059/05	5870	1160.0	291.8	dc-GTX2,3 (0.2494 μg/mL) dc-STX (0.3557 μg/mL) GTX 5 (1.4129 μg/mL) *GTX6 (high), C1,2 (high), dc-Neo
mussel	084/05	1860	456.0	113	dc-GTX2,3 (0.0573 μg/mL) dc-STX (0.1372 μg/mL) GTX5 (0.8006 μg/mL) *GTX6, C1,2
mussel	CRL/05/P/01	546	134.0	129.2	GTX2,3 (0.0941 μg/mL) STX (0.1161 μg/mL)
mussel	CRL/05/P/02	low	negative	<LOQ	
clam	057/05	507	84.0	32.4	dc-GTX2,3 (0.0208 μg/mL) dc-STX (0.0197μg/mL) GTX5 (0.4347 μg/mL) *GTX6 (high), C1,2 (high), dc-Neo
clam	CRL/06/P/03	1994	302.0	300.1	dc-GTX2,3 (0.8036 μg/mL) dc-STX (0.1971 μg/mL)
scallop	045/06	43.3	36.0	16.8	GTX2,3 (0.0380 μg/mL) STX (0.0027 μg/mL)
scallop	083/06	low	negative		
cockle	062/05	129.9	43.0	8.95	dc-GTX2,3 (0.0096 μM/L) dc-STX (0.0051 μM/L) C1,2 (0.2480 μM/L) GTX5 (0.1128 μM/L) *GTX6, possibly C3,4

[a] Fourteen blinded samples were analyzed for PSP toxin content by biosensor assay after extraction with the Garthwaite procedure. The same samples were also analyzed by the AOAO methods 959.08 (mouse bioassay) and 2005.06 (HPLC). The results of total toxin content obtained by the three methods are expressed in μg/100 g of meat. The toxin profile detected by HPLC is shown in the last column (*, amount of toxin not quantified due to unavailability of standard at the moment of the analysis).

hydroxylated at the site R1. Because there are many STX analogues, poor cross-reactivity with some of them is a common feature of the antibodies developed against PSP toxins.[21,25−29] Further work is in progress to develop binders with improved cross-reactivity to overcome this problem.

SPR-based biosensor assays consist of two steps of equal importance if they are to be used for routine sample analysis. During the binding step the antibody binds to the surface generating a binding signal, and during the regeneration step the antibody bound to the surface is removed, leaving the surface ready for another analysis. A good regeneration is essential for repeated analysis using the same chip surface, as well as surface stability after regeneration or extract injection. The STX−CM5 chip surface is very stable in these assay conditions, with more than 500 analysis cycles being performed on the same chip cell without any apparent loss of antibody binding activity. The quality of regeneration excluded the AOAC hydrochloric acid extraction as a candidate for the sample extraction method to be used in this assay.

When comparing different matrixes, mussel extracts always performed worse than any other species tested within the study. In the cases of cockle, scallop, oyster, and clam extracts, the Ab/extract ratio did not affect the quality of the results generated; however, it was an important parameter for mussel extracts. In mussel extracts, the 1:3 and 1:4 final dilutions of the antibody in extract caused pronounced reduction in antibody binding and therefore were not suitable for the inhibition assay. In conclusion,

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the 1:2 antibody:extract ratio can be used and satisfactory results were obtained in all matrixes tested.

A comparison of the five extraction methods concluded that the Garthwaite ethanol extraction method was the most suitable for SPR-based biosensor assays. The AOAC hydrochloric acid extracts had substantial regeneration problems in some species. The USDC ethanol:acetic acid/dichloromethane method was more laborious for routine testing than any other method tested. The Lawrence acetic acid extracts had higher baseline interference in mussels than the Garthwaite extracts, and in scallops the calibration curves were significantly shifted to the right versus the control. Therefore, the Garthwaite method was chosen for further studies of recovery and calibration in buffer.

It is not uncommon during a toxic outburst that toxin-free samples are difficult to obtain and also that samples from different species are received at the same time in the lab. Therefore, it would be very convenient to be able to quantify the amount of PSP toxin in many samples without the need of gathering a toxin-free sample of each species for calibration purposes. Since interference with the baseline was minimal in Garthwaite extracts, the possibility of using a buffer calibration curve for sample analysis was investigated. The amount of toxin and the variability of measurements were higher when quantified with a calibration curve in buffer than in a matrix extract. However, the quantifications with both calibrations are quite close, and an overestimation of toxin amount would not pose a risk for human health, although it would cause unnecessary economic losses to the aquaculture industry.

In regards to recovery rates; although the recovery has been reported to be good for hydrophilic toxins using the method of extraction with ethanol,[21] this GT13-A antibody can only recognize a fraction of the toxin spiked in shellfish homogenate. The elimination of the second ethanol extraction from this protocol would save approximately 12 min; however, the recovery of toxin is lower than with two extractions and therefore it is not recommended. Since recoveries are lower than 90%, it is believed that the inclusion of a correction factor to calculate toxin concentration may be needed. The evaluation of a recovery correction factor is complicated by the facts that there was a high variability among species, that recovery rates may vary also with the toxin analogue,[20,30] and that spiking of samples for recovery studies does not always reflect what happens with incurred material.

Another way of evaluating recoveries is to quantify the amount of toxin in incurred samples by other PSP detection methods and compare the results to those obtained by the biosensor assay. Fourteen samples were analyzed by the biosensor assay, the mouse bioassay, and Lawrence's HPLC method. Although from the recovery data an underestimation of toxin content would be expected with the biosensor assay, there is actually an approximately 5-fold overestimation of the amount of toxin present in the samples versus mouse bioassay and HPLC. Several causes might be responsible for this overestimation, such as toxin profile of the sample or presence of PSP analogues not detected by the mouse bioassay (analogues without toxic activity) or HPLC (only 14 standards available out of more than 20 analogues already described) but reactive with the GT13-A antibody. All the samples which content of PSP toxins was under the action limit when quantified by the biosensor assay, were also negative when quantified by the mouse bioassay. This result supports the value of this biosensor assay as a screening method that would help to reduce the use of other more expensive, time-consuming, even ethically questionable methods, without putting at risk human health protection. On the other hand, although all samples contaminated with PSP toxins were detected by the biosensor assay, there were two samples that when quantified by the biosensor assay had a content of toxin above the regulatory limit but by the mouse bioassay the amount of toxin was below the regulatory limit. As discussed before, the overestimation of the amount of toxin would have a negative economic impact on the aquaculture sector. However, considering that these samples do have toxin when tested by mouse bioassay, the limit of quantification of the mouse bioassay (40 μg/100 g of meat) and its high variability (20%), it is at least questionable if these samples could actually be considered false positives. Overall, the biosensor assay performed adequately as a screening assay for these 14 samples. When looking at the toxin profile of these 14 samples, we observed that there is none or very low amounts of the toxin analogues that would not be detected by this biosensor assay. Unfortunately, samples with a higher content of R1 hydroxylated toxins were not available at the moment and although the heterogeneous nature of most PSP toxic outbursts might reduce the problem of cross-reactivity with some analogues, the performance of this assay with samples containing R1 hydroxylated toxins will be studied as soon as these samples are made available to us.

In summary, matrix effects on SPR-biosensor inhibition assays using the GT13-A antibody and a STX-chip depend on the sample extraction method. Extractions with ethanol or acetic acid yield acceptable results in terms of regeneration, baseline interference, antibody binding, and calibration curves. The ethanol extraction would be the method of choice based on assay performance and sample analysis time. The GT13-A−STX chip biosensor assay is capable of PSP toxin detection at concentrations 5 times lower than the regulatory limit, and its performance with natural shellfish samples supports its use as a screening method for PSP toxin detection.

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Artículo 2. Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin.

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Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin

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ABSTRACT

Yessotoxin (YTX) is a disulfated polyether toxin produced by marine dinoflagellates. Although there is no clear evidence that YTX is toxic to humans, it is a major cause of false positives in DSP toxin detection by mouse bioassay. We developed a new detection and quantification method for yessotoxin using a BiaCore X Surface plasmon resonance (SPR)-based biosensor. The assay is based in the interaction of YTX with phosphodiesterase enzymes (PDE), one of its cellular targets. The injection of several YTX concentrations (3–12 μM) over immobilized PDE I, showed a dose dependent binding signal, which K_{obs} (observed rate constant) allowed us to obtain a calibration curve with a linear fit. The detection of yessotoxin using SPR-based biosensor allows the quantification of the toxin with an automated and repetitive method at concentrations in the range of the 1 mg kg^{-1} European regulatory limit.

1. Introduction

The increased occurrence of phycotoxins in coastal seawaters is a public concern, since they can pose a serious threat to human health and cause severe economic losses to aquaculture. One of these phycotoxins is yessotoxin (YTX, Fig. 1), which was first isolated from the hepatopancreas of the scallop *Patinopecten yessoensis* in Japan [1]. Nowadays yessotoxin is spread worldwide, having been described in Japan, Italy, Norway, New Zealand and Chile [2]. Initially, YTX was classified in the DSP toxin group because of its lipophilic nature and because it often coexists with these toxins [2]. Subsequent

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Abbreviations: SPR, surface plasmon resonance; DSP, diarrhetic shellfish poisoning; YTX, yessotoxin; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; HBS-EP, hepes buffered saline; NHS, *N*-hydroxysuccimide; EDC, ethyl-*N*-(dimethylaminopropyl)carbodiimide; HPLC, high pressure liquid chromatography; LC–MS, liquid chromatography–mass spectrometry.
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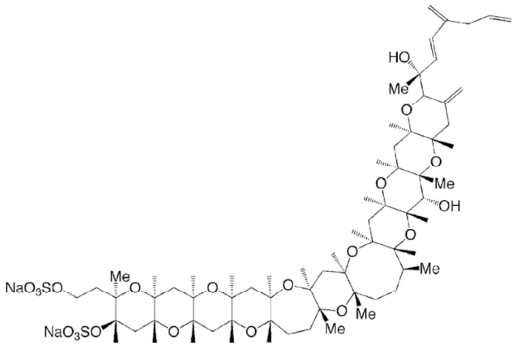

Fig. 1 – Yessotoxin structure.

studies demonstrated that YTX does not induce diarrhoea [3] and is not a PP2A inhibitor, a well-known target of DSP toxins [4]. Therefore, in many countries yessotoxin has been placed in a separate phycotoxin group for regulatory purposes, with a maximum permitted level of 1 mg of YTX equivalents kg^{-1} of shellfish [13].

Although yessotoxin toxicity to humans is unknown, oral administration to mice does not induce toxic effects [4]. However, its intraperitoneal lethality is the strongest among all lipophilic phycotoxins (0.1 mg kg^{-1}) [4] and its presence in shellfish may lead to a high overestimation of toxicity when the mouse bioassay is used for DSP toxin detection. Currently, YTX can be detected by several analytical methods, including HPLC with fluorimetric detection [5], LC–MS [6], enzyme-linked immunosorbent assays (ELISA) [7], fluorescence polarization [8] and resonant mirror-based biosensor [9]. In this work, we present a detection method using a surface plasmon resonance (SPR)-based biosensor. SPR-based biosensor detection offers the advantages of biomolecular interaction monitorization in real-time without the need of fluorescent labels and it is an automated system that improves accuracy and reliability versus other biosensors.

Although the exact mechanism of action of YTX is still unclear, the activation of cellular phosphodiesterases by this toxin has been recently described (PDEs) [10] and a direct interaction between YTX and PDEs was demonstrated using a resonant mirror biosensor (IAsys) [11]. Using this interaction, we developed a new and fast method for YTX detection and quantification in SPR-based biosensors (BiaCore X).

2. Materials and methods

2.1. Chemicals

YTX was purified by Dr. T. Yasumoto [1]. CM5 sensor chips, HBS-EP (Hepes buffered saline) and amine coupling kit were supplied by BiaCore AB (Uppsala, Sweden). HCl was purchased from Panreac Quimica SA (Barcelona, Spain). Phosphodiesterase I (PDE I, type IV from *Crotalus atrox*), phosphodiesterase 3′,5′-cyclic-nucleotide-specific (PDEs, from bovine brain), dimethyl sulfoxide (DMSO), sodium acetate and Tween-20 were from Sigma Chemical Co. (Madrid, Spain). Phosphate buffered saline solution (PBS/T) was: 8.2 mM Na_2HPO_4, 137 mM NaCl, 1.5 mM KH_2PO_4, 3.2 mM KCl, pH 7.4, plus 0.05% Tween-20 (Sigma). PBS/T was degassed and filtered through a Millex® GP 0.22 μm pore size filter from Millipore Corporation (Bedford, MA, USA). PB was: 8.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.7.

2.2. PDE immobilization

Sensor surface activation and ligand immobilization were performed using PBS/T as running buffer at a flow rate of 5 μL min^{-1} and 25 °C. The activation of the CM5 dextran matrix was carried out by injection over the chip surface of a freshly prepared mixture of equal volumes of 75 mg mL ^{1} EDC and 11.5 mg mL^{-1} NHS for 10 min. PDE I or PDEs were dissolved in 10 mM acetate buffer, pH 5.0 at a final concentration of 0.25 mg mL^{-1}, and injected over the chip for 14 min. Deactivation of the remaining NHS active esters was achieved by injection of 1 M ethanolamine-HCl, pH 8.5, for 5 min. After injection of 10 mM HCl for 1 min, the surface was ready for binding experiments (supplementary material Figure 4S).

2.3. Association experiments and chip regeneration

The interaction of YTX with immobilized phosphodiesterase was monitored at 25 °C, using HBS-EP as running buffer at a flow rate of 10 μL min^{-1}. Serial dilutions of yessotoxin stock (5 mM in DMSO) were prepared in HBS-EP at 3, 5, 7, 10 and 12 μM concentrations and injected for 2 min. Control solutions with a matching concentration of DMSO were injected in the same way and subsequently subtracted from YTX response signal. Regeneration of the surface between injections was carried out with a single 1 min pulse of 10 mM HCl.

Since YTX dissociates almost completely, we used the association phase to quantify the YTX-PDE I interaction. A pseudo-first-order association rate constant K_{obs} (observed rate constant) was determined using the 1:1 Langmuir association model of the BiaEvaluation software (BiaCore), from the binding data of each toxin concentration.

3. Results and discussion

Two different phosphodiesterases, PDE I from *C. atrox* and PDEs from bovine brain were assayed to determine the better ligand for yessotoxin in our conditions. Both PDE I or PDEs activated surfaces showed binding activity after injection of YTX. However, at low yessotoxin concentrations, the PDE I sensor chip had a higher sensitivity and therefore the PDE I-CM5 chip was used for further assay optimization. Two running buffers were tested for optimum binding: a phosphate buffer (PB) and a HEPES buffered saline (HBS-EP). Binding of YTX to the PDE I chip was lower in PB than in HBS-EP. The regeneration solution (10 mM HCl) allowed for a complete regeneration of the sensor surface with retention of binding activity at least for 50 cycles of regeneration.

The injection of several YTX concentrations (3–12 μM) over the PDE I activated chip showed a dose dependent binding signal (Fig. 2). The interaction between YTX and PDE I seemed

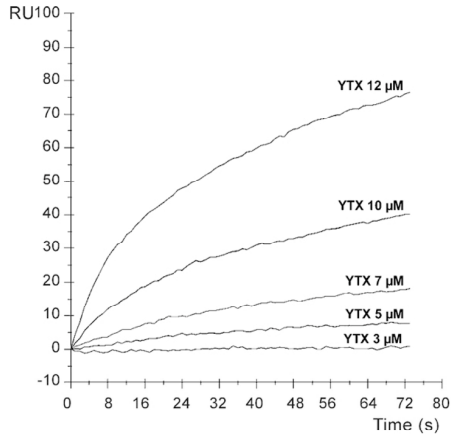

Fig. 2 – Association curves of YTX to immobilized PDE I. YTX was injected over the PDE I-CM5 chip at concentrations of 3, 5, 7, 10 and 12 µM using HBS-EP as running buffer and a flow rate of 10 µL min^{-1}. The association curves of all YTX concentrations were obtained after subtraction of their respective DMSO controls. The time interval from 7 to 81 s used for K_{obs} calculation is represented. Representative of 4 experiments.

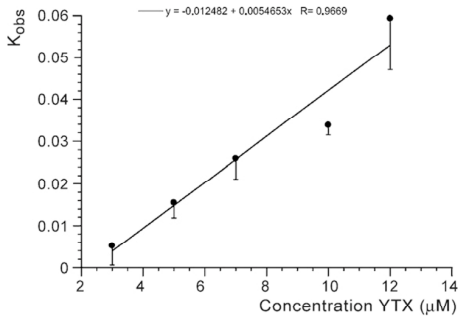

Fig. 3 – Yessotoxin calibration curve. YTX was injected over the PDE I-CM5 chip at concentrations of 3, 5, 7, 10 and 12 µM. The K_{obs} of the association curves (e.g. Fig. 2) was calculated by the BiaEvaluation software and plotted versus toxin concentration (mean ± S.E.M., $n = 4$).

to be reversible, showing a fast, almost complete dissociation after toxin removal (supplementary material Figure 2S-A). This characteristic of the interaction, together with the high bulk effect due to the toxin carrier (DMSO) (supplementary material Figure 2S-A and 2S-B), prevented us from working with absolute binding signal. Since the YTX-PDE I interaction follows a pseudo-first-order kinetics, the K_{obs} of the binding curve was used to quantify YTX. The K_{obs} was calculated by the BiaEvaluation software (BiaCore) between 7 and 81 s after the toxin injection was started. K_{obs} was plotted versus YTX concentration, obtaining a calibration curve with a linear fit, $y = 0.00546x - 0.01248$, $R = 0.9669$, for $n = 4$ experiments (Fig. 3). The range of quantification of this SPR-biosensor assay is similar to that reported previously for the resonant mirror biosensor assay, which allowed for quantification of the toxin around the levels of the current European regulatory limit (1 mg of YTX kg^{-1} of whole body or any part edible) using an acetone extraction with posterior liquid/liquid partition in dichloromethane/H_2O [9]. Moreover, PDE I binds several YTX derivatives [12], susceptible of detection by this assay. Although the assay sensitivity for YTX is similar in both biosensors, the SPR-based biosensor assay has several advantages versus the resonant mirror biosensor assay. Besides being ten times faster than the previous method developed in a resonant mirror-based IAsys biosensor (contact time of the sample was reduced from 20 to 2 min), the SPR-biosensor assay accuracy and reproducibility is improved by the constant flow and semiautomatic injection technologies incorporated in the BiaCore X biosensor.

In summary, this paper reports a new, sensitive and fast assay to detect YTX in the range of the European regulatory limit, considerably improving previously reported detection with biosensor technologies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.01.010.

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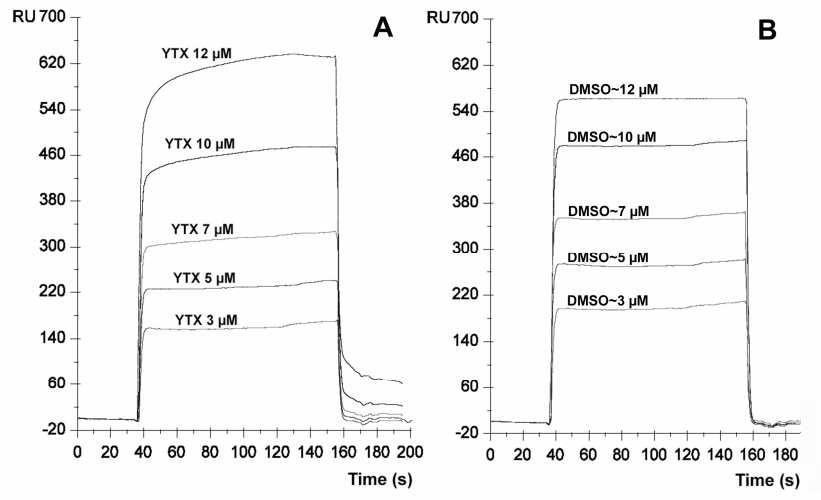

Figure 2S. (A) Association curves of different YTX concentrations without subtraction of DMSO controls and (B) association curves of their DMSO controls.

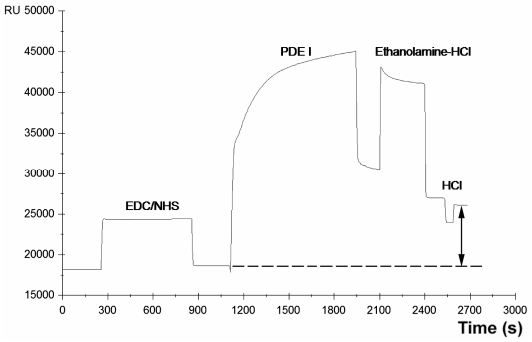

Figure 4S. PDE I immobilization. Activation of the carboxylic groups of CM5 chip was performed with a mixture of EDC/NHS injected over the sensor surface for 10 min. Then, PDE I (0.25 mg/ml) was added for 14 min. The remaining activated sites were then blocked with 1 M ethanolamine-HCl, for 5 min. Finally, 10 mM HCl was injected for 1 min. Bound PDE I is indicated by the arrow.

Artículo 3. Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization.

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Detection of Gymnodimine-A and 13-Desmethyl C Spirolide Phycotoxins by Fluorescence Polarization

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The gymnodimines and spirolides are phycotoxins classified into a heterogeneous group of marine biocompounds called cyclic imines. Although there is no clear evidence of their toxicity to humans, gymnodimines and spirolides are highly toxic to rodents and constitute a source of false positives in lipophilic toxin detection by the mouse bioassay. Using nicotinic acetylcholine receptor-enriched membranes of *Torpedo*, and fluorescent α-bungarotoxin, we developed a fluorescence polarization assay to detect and quantify gymnodimine-A and 13-desmethyl C spirolide. The presence of these cyclic imines in solution inhibited the interaction of fluorescent-labeled α-bungarotoxin with nicotinic acetylcholine receptors in a concentration-dependent manner. The sensitivity of the assay is in the order of nanomolar concentrations of gymnodimine and 13-desmethyl C spirolide. Okadaic acid, yessotoxin, and brevetoxin-2, three lipophilic marine toxins, did not interfere with this assay. A suitable extraction method in shellfish was also developed. The gymnodimine-A and 13-desmethyl C spirolide recovery rates of mussel matrix extraction with acetone/chloroform were 63.6% ± 3.5% and 87.4% ± 5.3%, respectively. In summary, this inhibition assay is capable of gymnodimine-A and 13-desmethyl C spirolide detection in mussel extracts with enough sensitivity and specificity to quantify these toxins in the range of 50−2000 μg/kg and 70−700 μg/kg of shellfish meat, respectively.

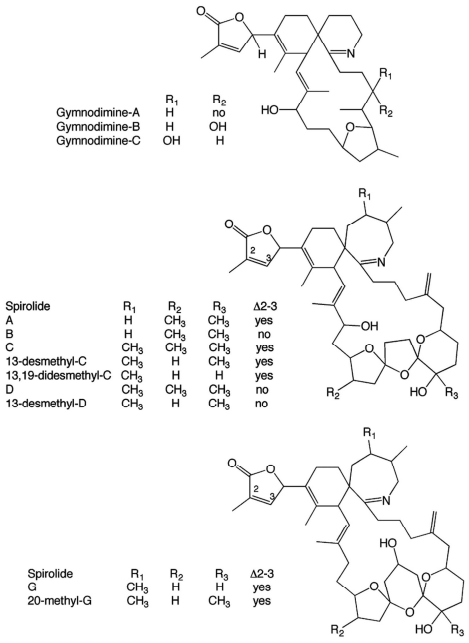

Figure 1. Chemical structures of GYMs and spirolide. GYM-A, usually referred to as GYM, and 13-desmethyl C spirolide were the toxins used in this study.

Gymnodimines (GYMs) and spirolides are marine toxins described for the first time during the first half of the 1990s.[1−3] Both GYMs and spirolides are included in the group of marine toxins known as cyclic imines[4,5] due to the spiro center and the imine function present in their chemical structures (Figure 1). These toxins have been demonstrated to have a worldwide distribution. Thus, GYMs have been detected in shellfish and/or plankton in New Zealand and Tunisia,[3,4,6] and spirolides have been detected in Canada, Denmark, Norway, Adriatic Sea, France, Ireland, Scotland, and Spain[1,7−10] among other locations.

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10.1021/ac900144r CCC: $40.75

GYMs and spirolides are highly toxic when injected intraperitoneally in the mouse bioassay,[4,11] which is a widely used method for the detection of lipophilic toxins in molluscs. The intraperitoneal LD_{50} (dose that is lethal to 50% of test subjects) of gymnodimine-A (GYM-A) is in the range of 80−96 μg/kg,[6,11] and the LD_{50} of spirolides (a mixture predominantly with 13-desmethyl C spirolide) is 40 μg/kg.[12] Recent studies suggest that this value may be even lower for pure 13-desmethyl C spirolide and other analogues.[13] GYM has been demonstrated to be potentially toxic by oral administration, with an estimated LD_{50} of 755 μg/kg when administered by gavage.[11] Initial studies showed that spirolides also have oral toxicity by intragastric administration, with an estimated LD_{50} of 1 mg/kg.[12] Actually, recent data suggest that this value could be lower and that their oral toxicity is also reduced when administered with food.[13] At present, the toxicity of GYMs and spirolides to humans remains unknown. Although toxic algal blooms and toxic shellfish have been recurrently reported in some areas, no human intoxication has been unequivocally linked to spirolide or GYM poisonings.[12]

Neurological symptoms followed by rapid death are displayed after intraperitoneal and oral administrations to mice. Munday et al. suggested that GYM may target the nicotinic acetylcholine receptor (nAChR) at the skeletal neuromuscular junction based on the resemblance of the symptoms induced by intraperitoneal injection of GYM and tubocurarine, as well as by the inhibition of the GYM-induced toxicity by the acetylcholinesterase inhibitor neostigmine.[11] The toxicity of spirolides was initially suggested to be due to a block of muscarinic AChRs based on a reduction of the time of death by atropine, a muscarinic receptor blocker, and the extension of the time to death by physostigmine.[12] Later reports showed an up-regulation of muscarinic and nAChR transcription in rats.[14] Recent studies have provided direct evidence that GYM-A and 13-desmethyl C spirolide target muscular and neuronal nAChR subtypes with high affinity and have demonstrated that both phycotoxins inhibit the specific α-bungarotoxin (α-BTX) binding to nAChRs.[6,15] Current detection methods for GYMs and spirolides include mouse bioassay and liquid chromatography−mass spectrometry (LC−MS)-based detection techniques. GYMs and spirolides were discovered due to the observation of unusual symptoms in mouse bioassays with positive results for lipophilic toxin testing.[2,12] Actually, given the lack of proved human toxicity of GYMs and spirolides, they are often considered as a source of false positives in the mouse bioassay.[16] The most extended methods for the detection of cyclic imines are MS-based techniques,[4,8,9,16−20] with even multitoxin detection approaches allowing the identification of multiple toxins present in the sample (domoic acid, cyclic imines, okadaic acid and derivatives, azaspiracids, yessotoxins, and pectenotoxins).[19,20] Despite the advantage of unequivocal identification of the toxins, routine testing with LC−MS-based techniques would not allow one to detect and quantify new analogues of the toxin that have not yet been characterized, or analogues for which there are no available standards. Therefore, the presence of new possible toxic analogues would be missed with MS detection methods (for a review on marine toxin detection methods see ref 21). Alternative detection techniques based on the quantification of molecular interactions, among them fluorescence polarization, are gaining popularity in the pharmaceutical industry and food contaminant detection field for multiple applications.[22−29] An approach of inhibition of protein−ligand interaction is often used in the detection of small molecules to avoid the need of labeling the analyte.

The current opinion of the scientific community is inclined to consider that there is a need to regulate at least the levels of spirolides given their high intraperitoneal and oral toxicity in order to protect human consumers. In this study we present a fast, nonradioactive, quantitative method for the detection of cyclic imines in shellfish based on the high affinity binding of GYMs and spirolides to nAChRs. This method, that does not require the use of laboratory animals, was designed as an inhibition assay, where the binding of fluorescent α-BTX to the nAChR of the *Torpedo marmorata* electric organ, which is detected by fluorescence polarization, is inhibited by GYM and spirolides. The advantage of using a natural target of the toxins for their detection is that this method would detect any analogue of the group that may interact with nAChRs.

MATERIALS AND METHODS

Materials. Alexa Fluor 488 α-BTX and tetramethylrhodamine α-BTX were purchased from Molecular Probes (Eugene, OR). GYM and 13-desmethyl C spirolide standard solutions were

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purchased from NRC−CNRC (Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada). The GYM-A used for matrix spiking purposes was purchased to Dr. R. Kharrat (Laboratoire des Venins et Toxines, Pasteur Institut Tunisia). Okadaic acid was from Alexix (Laüfelfingen, Switzerland), Yessotoxin was a gift from Professor T. Yasumoto. Brevetoxin-2 (PbTx-2), α-BTX, bovine serum albumin (BSA), Tween-20, and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (Madrid, Spain). Acetone, chloroform, *n*-hexane, methanol, ethanol (96%), sodium chloride, sodium phosphates, and hydrochloric acid were from reagent grade commercial sources. Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, pH = 7.0. PBS−BT solution was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20. Amicon Ultra-4, PLGC Ultracel-PL membrane, 10 kDa molecular weight cutoff (MWCO) filter was from Millipore Corporation (Bedford, MA). Toxin-free mussels (*Mytilus galloprovincialis*) were purchased from the market in Lugo, Spain. The *T. marmorata* were obtained from the Marine Station in Arcachon (France) and kept in artificial seawater for a few weeks in the aquarium of the CNRS animal house in Gif sur Yvette.

Preparation of Membranes Enriched in nAChRs from *T. marmorata* Electric Tissue. Electrocyte membranes rich in nAChRs from *T. marmorata* electric tissue were purified according to procedures previously described[30] with some modifications. The whole membrane purification procedure was performed in a cold room at 4 °C. A freshly dissected electric organ (150 g) was sliced in small pieces with a scalpel and immersed in the Torpedo membrane extraction buffer (TMEB) containing 50 mM Tris−HCl, 3 mM EDTA, and 1 mM EGTA supplemented with a cocktail of protease inhibitors (Complete Roche Diagnostics GmbH, Mannheim, Germany), pH = 7.5. The electric tissue was homogenized in 150 mL of TMEB using a Waring blender (Appareils Scientifiques O.S.I., Paris, France) at maximal speed for 1 min (three times, with 1 min interval). The homogenate was centrifuged at 4000*g* for 10 min at 4 °C (Sorvall, Dupont Instruments, Newtown, CT; rotor GSA), and the supernatant (S1) was collected and filtered through a gauze of 250 μm pore size. The remaining pellet was rehomogenized and centrifuged, and the resulting supernatant (S2) was filtered as described above. Both supernatants, S1 and S2, were pooled and centrifuged at 25 000*g* for 50 min at 4 °C (Sorvall, rotor GSA). The pelleted membranes were resuspended in 100 mL of TMEB and homogenized using a Potter-Elvehjem (glass/Teflon). Sucrose crystals were added to the crude membrane homogenate to a final concentration of 35% sucrose (w/w). An amount of 16 mL of the 35% sucrose membrane suspension was overlaid on top of 10 mL of 43% sucrose solution in TMEB (w/w) in a 30 mL polycarbonate tube. The samples were centrifuged at 106 000*g* during 3 h at 4 °C (Beckman, Palo Alto, CA; rotor 50Ti). The nAChR-rich membranes were collected with a syringe from the interface between the 35% and the 43% sucrose layers. The collected membranes were precipitated by centrifugation at 106 000*g* for 1 h at 4 °C (Beckman, rotor 50Ti). The precipitated membranes were resuspended in 50 mL of TMEB and centrifuged at 106 000*g* for 1 h at 4 °C (Beckman, rotor 50Ti). The membranes were homogenized in 50 mL of 5 mM glycine using a Potter-Elvehjem (glass/Teflon) and centrifuged 106 000*g* for 1 h at 4 °C (Beckman, rotor 50Ti). The washing step was repeated once, and the pelleted membranes were resuspended in 5 mL of 5 mM glycine. Protein concentration was determined by the Bradford method (Bio-Rad, Munich, Germany). The protein concentration of the membrane solution was adjusted to 2.5−3.5 mg mL^{-1} protein. Aliquots of 1 mL were prepared and stored at −80 °C.

Fluorescence Polarization Measurement. Fluorescence intensity and fluorescence polarization (FP) were measured in a Plate Chameleon fluorometer from Hidex (Turku, Finland), with a xenon flash lamp as light source. Alexa Fluor 488 α-BTX fluorescence was measured with an excitation filter of 485 nm and emission filter of 535 nm. For FP measurements, excitation and emission detection were achieved with polarization filters at 485 and 535 nm, respectively. After optimization, the gain was set at a value of 20. Fluorescence intensity was approximately 10 times higher than background. The autofluorescence of the nicotinic receptor membranes and the toxins, including GYM-A, 13-desmethyl C spirolide, okadaic acid, yessotoxin, and PbTx-2, did not interfere with the detection method.

FP (in millipolarization units, mP) was calculated by the following equation:

$$\mathrm{mP} = 1000\left[\frac{I_\mathrm{v} - GI_H}{I_\mathrm{v} + GI_H}\right]$$

where I_V is the fluorescence intensity measured with vertical polarization excitation filters and vertical polarization emission filters (called parallel intensity), I_H is the fluorescence intensity measured with vertical polarization excitation filters and horizontal polarization emission filters (called perpendicular intensity), and G is a correction factor that accounts for the optical components of the instrument that affect the light beam depending on its polarization plane. The value of G was determined by measuring FP in a dilution series of the Alexa Fluor 488 α-BTX. G has a value in the above equation that places the calculated mP of all fluorophore dilutions between 20 and 30. The experimental value obtained for G in our conditions was 0.98.

Initial experiments with tetramethylrhodamine α-BTX were performed using excitation and emission filters at 544 and 616 nm, respectively, a gain of 40, and a G value of 1.

Fluorescence Polarization Competition Assay. α-BTX labeled with Alexa Fluor 488 dye was used to detect binding to the nAChR from *Torpedo* membranes by changes in fluorescence polarization. An inhibition assay was developed, based on binding competition to nAChRs with the fluorescent α-BTX. The problem solution was incubated with nAChR-enriched membrane of *Torpedo*'s electric organ for 2 h. For this purpose 40 μL of the problem solution was added to 40 μL of a 1:20 dilution of the nAChR stock (3.5 mg/mL of protein) in a 96 well plate (black, flat bottom plates, TermoLabsystems, Franklin, MA) and mixed by shaking at 140 rpm for 1 min before the 2 h incubation. Finally, 80 μL of 40 nM Alexa Fluor 488 α-BTX was added to the well, and the mixture was shaken again for 1 min. Fluorescence polarization was measured 30 min after the addition of fluorescent α-BTX. All the incubations took place in the dark at room temperature. To correct for background, vertical and horizontal fluorescence values from

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control wells in which fluorescent α-BTX was omitted were, respectively, subtracted from vertical and horizontal fluorescence values of fluorophore-containing wells, obtaining the I_V and I_H values used in the equation above. The percentage of α-BTX-binding response in a certain condition was calculated as follows:

$$\%BR = \frac{(mP_i - mP_{min})}{(mP_{max} - mP_{min})} \times 100$$

where %BR is the percentage of α-BTX-binding response, mP_i is the mP value for a given condition, mP_{max} is the mP value obtained for control wells containing the nAChR-enriched membranes plus Alexa Fluor 488 α-BTX, and mP_{min} is the mP value obtained for control wells containing only Alexa Fluor 488 α-BTX.

Shellfish Sample Preparation for the Fluorescence Polarization Assay. An amount of 100 g of mussel meat (whole body) was homogenized with a blender, divided in 10 g aliquots, and stored at −20 °C until use. The extraction procedure consisted in the addition of 4 mL of acetone to 1 g of mussel meat homogenate. The mixture was vortexed for 10 s and roller-mixed for 15 min at room temperature. After centrifugation at 3500*g* for 10 min at 4 °C, the supernatant was saved, and the pellet was re-extracted twice with acetone, as described. The combined supernatants were evaporated, and the resulting residue was dissolved in 4 mL of water. The aqueous extract was partitioned against an equal volume of hexane for 30 min. The hexane was then discarded, and the water was extracted three times with chloroform (1:1.5 v/v). Chloroform layers were pooled together and evaporated. The residue was reconstituted with 1 mL of ethanol/PBS (4:6 v/v) and filtered through a 10 kDa MWCO filter (Millipore). Then, an equal volume of PBS with 0.2% BSA and 0.2% Tween-20 was added. The extracts were analyzed on the same day the extraction was started.

Data Analysis. All experiments were carried out at least three times, and in every experiment each condition was tested in the fluorescence polarization assay by triplicate. The results are expressed as mean ± SEM. For statistical significance, the results were analyzed using the Student *t* test. A probability level of 0.05 or less was used for statistical significance. The calibration curves were fitted using Kaleida graph 4.03 for logarithmic fits and BiaEvaluation 3.2 RC1 software (BiaCore) for four-parameter fits. The logarithmic fit equation was $y = a + b \log(x)$. The four-parameter fit equation was $y = R_{hi} - (R_{hi} - R_{10})/(1 + ((x/A_1)\text{^}A_2))$.

RESULTS

Optimization of the Fluorescence Polarization Inhibition Assay. The assay was designed as a competition experiment where nAChR-binding molecules compete with fluorescent α-BTX for binding to the nAChRs. The changes in fluorescence polarization induced by the interaction of the fluorescent α-BTX with the receptor should be inhibited by the presence of other nAChR-binding molecules. Incubation times, volumes, and concentrations for the inhibition assay were optimized using fluorescent α-BTX, the nAChR from the *T. marmorata* electric organ, and unlabeled α-BTX as the competing compound. The aim was to use the lower amount of reagents compatible with a good sensitivity. Two fluorescent α-BTXs, Alexa Fluor 488 α-BTX and tetramethylrhodamine α-BTX, were compared for performance in the Plate Chameleon fluorometer. In our experimental conditions Alexa Fluor 488 α-BTX allowed for a 4 time reduction of the amount of nAChR used per well, and increased the sensitivity of the assay for GYM-A, and therefore it was our fluorophore of choice. The optimized conditions of the final assay are described in the Materials and Methods section.

A screening of the compatibility of this fluorescence polarization inhibition assay with several organic solvents was carried out due to the lipophilic nature of these toxins and the use of receptors contained in membrane fragments. Methanol and DMSO were not compatible with the assay at final concentrations as low as 1.5%. However, the inhibition assay performed adequately in the presence of ethanol concentrations as high as 4.8%.

Detection of GYM-A by the Fluorescence Polarization Inhibition Assay. The ability of the fluorescence polarization inhibition assay to detect GYM-A (Figure 1) in solution was tested based on the competition of the toxin with α-BTX for binding to the nAChRs. The GYM-A standard was provided dissolved in methanol, which had to be evaporated due to solvent interference with the assay. GYM-A was then dissolved with 19.2% ethanol in PBS−BT. Serial dilutions at concentrations of 50, 100, 500, 1000, and 2000 nM GYM-A were prepared in PBS−BT. The final concentrations of GYM-A in the well after addition of the fluorescent α-BTX were 12.5, 25, 125, 250, and 500 nM. The final concentration of ethanol in the well was 4.8% and was maintained constant for all wells. The presence of GYM-A inhibited the change of fluorescence polarization induced by binding of the receptor to the fluorescent α-BTX. The results were plotted as percentage of α-BTX binding to the receptor versus GYM-A concentration in the solution added to the well (this concentration would be equivalent to the concentration in a sample extract prepared for analysis) (Figure 2A). A calibration curve with a logarithmic fit was obtained for these concentrations of GYM-A (Figure 2A), showing an IC_{50} (half-maximal inhibitory concentration) of 391.0 ± 55.3 nM (mean ± SEM, $n = 3$). The quantification range of GYM was between 50 and 2000 nM (12.5 and 500 nM final concentrations in the well), which would allow the detection of extracts containing a concentration of GYM higher than 50 nM. These results demonstrate that this inhibition assay using Alexa 488 α-BTX is capable of detecting nAChR-binding substances.

Detection of 13-Desmethyl C Spirolide by the Fluorescence Polarization Inhibition Assay. The detection of the 13-desmethyl C spirolide (Figure 1) by the fluorescence polarization assay was tested using a standard solution in which the methanol used as solvent was removed by evaporation. The phycotoxin was then dissolved in 19.2% ethanol in PBS−BT. Serial dilutions were prepared at concentrations of 50, 75, 100, 250, and 500 nM. The final concentrations of spirolide in the well were 12.5, 18.75, 25, 62.5, and 125 nM. A concentration of 4.8% ethanol was kept constant in all wells. The presence of 13-desmethyl C spirolide in the solution also inhibited the binding of fluorescent α-BTX to the nAChR. As before, the results were plotted as percentage of the α-BTX-binding response versus the 13-desmethyl C spirolide concentration and a calibration curve was obtained using a four-

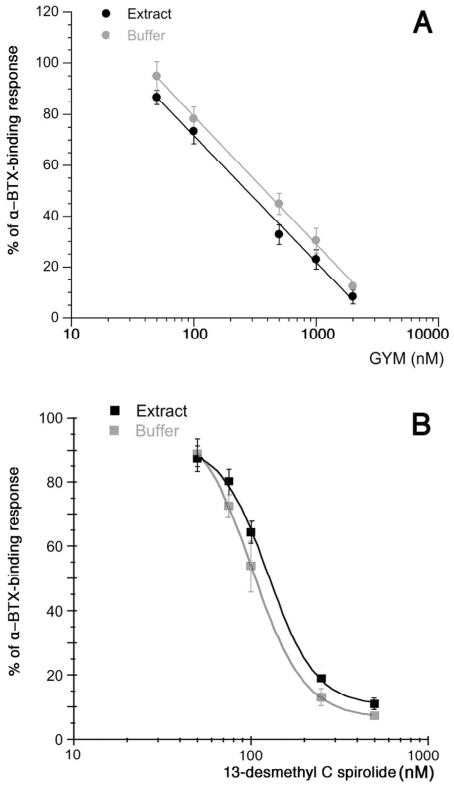

Figure 2. Calibration curves of GYM-A and 13-desmethyl C spirolide in PBS−BT or mussel extracts using the fluorescence polarization inhibition assay. (A) Calibration curve of GYM-A standard in PBS−BT (gray line, $n = 3$, mean ± SEM) and mussel extract (black line, $n = 4$, mean ± SEM). The GYM-A standard was diluted at 50, 100, 500, 1000, and 2000 nM in PBS−BT containing 19.2% ethanol or in extract and added to a well containing a solution of *T. marmorata* nAChR-enriched membranes. After 2 h, Alexa Fluor α-BTX was also added to the well and its binding to receptors was determined by changes in fluorescence polarization. Data are expressed as percentage of maximum α-BTX-binding response. The calibration curve equations were obtained using a logarithmic fit. The parameters of the equation were $a = 180.22$, $b = -50.425$, and $R = 0.99937$ for the curve in buffer and $a = 171.43$, $b = -49.866$, and $R = 0.99742$ for the curve in extract. (B) Calibration curve of 13-desmethyl C spirolide standard in PBS−BT (gray line, $n = 3$, mean ± SEM) or in mussel extract (black line, $n = 4$, mean ± SEM). The spirolide standard was diluted at 50, 75, 100, 250, and 500 nM in PBS−BT containing 19.2% ethanol and added to a well containing a solution of the *T. marmorata* nAChR. After 2 h, Alexa Fluor α-BTX was also added to the well and its binding to the receptor was determined by changes in fluorescence polarization. Data are expressed as percentage of maximum α-BTX-binding response. The equation of the calibration curve was obtained with a four-parameter fit. The parameter values were $R_{hi} = 6.5$, $R_{lo} = 99.8$, $A_1 = 101.3$, $A_2 = 2.9$, and $\chi^2 = 0.28$ for the curve in PBS−BT and $R_{hi} = 10.4$, $R_{lo} = 92.0$, $A_1 = 125.1$, $A_2 = 3.2$, and $\chi^2 = 3.71$ for the curve in extract.

parameter fit (Figure 2B). Calculation of the IC_{50} in these assay conditions yielded a value of 108.2 ± 11.9 nM for 13-desmethyl C spirolide (mean ± SEM, $n = 3$). The range of quantification of 13-desmethyl C spirolide with this assay was from 50 to 500 nM (corresponding to 12.5 and 125 nM final concentrations in the well).

Interference of Lipophilic Phycotoxins with the Fluorescence Polarization Inhibition Assay. The specificity of the fluorescence polarization inhibition assay for the detection of cyclic imines was studied using other lipophilic toxins that can be easily coextracted with GYMs and spirolides. The effect of okadaic acid, yessotoxin, and PbTx-2 on the inhibition assay was tested in the same conditions that were used to obtain the GYM-A and 13-desmethyl C spirolide calibration curves. The solvent of okadaic acid stock solution (1 mM in methanol) was evaporated and replaced by 19.2% ethanol in PBS−BT. The same procedure was performed with the ethanolic 200 μM PbTx-2 stock solution for concentration purposes. In the case of yessotoxin, the stock solution (5 mM in DMSO) was diluted directly in PBS−BT supplemented with 19.2% ethanol. The final concentration of DMSO in the wells (0.6%) was previously shown not to interfere with the assay. A concentration of 30 μM okadaic acid or yessotoxin in the solution added to the well did not interfere with nAChR binding to Alexa Fluor 488 α-BTX. The percentage of binding response was 97.5% ± 1.3% and 100.9% ± 6.0% of α-BTX maximum binding (mean ± SEM, $n = 3$) for okadaic acid and yessotoxin, respectively. These values were not statistically different from maximum binding controls. PbTx-2 was tested at a concentration of 20 μM in the solution added to the well, and the α-BTX-binding response in these conditions was 92.1% ($n = 1$ due to the small amount of brevetoxin available).

Matrix Effect on the Fluorescence Polarization Inhibition Assay. A protocol for GYM-A and 13-desmethyl C spirolide extraction from mussels was optimized in order to be able to use the fluorescence polarization assay to detect the presence of these toxins in shellfish. After testing several alternatives, the toxins were extracted from homogenized mussel meat (whole body) with acetone and subsequently with chloroform (see the Materials and Methods section for a detailed protocol). An intermediate partition with hexane had to be included to remove sample pigments that interfered with Alexa Fluor 488 α-BTX fluorescence. The protocol was initially designed to ensure that a content of GYM-A of 1 mg/kg of shellfish would be in the detection range of the assay in buffer. In order to test the interference of shellfish matrix with the assay, serial dilutions of GYM-A and 13-desmethyl C spirolide were prepared in the mussel extract at the same concentrations of toxin standard used in former experiments. The calibration curves obtained in mussel extract for GYM-A and 13-desmethyl C spirolide are shown in Figure 2, parts A and B (black lines). The interference of the extract with the maximum and minimum binding responses was 9.0% ± 2.0% and 8.1% ± 2.6% ($n = 8$, mean ± SEM), respectively, allowing a range of quantification of the assay in extract for the two toxins similar to the range obtained with the buffer. The IC_{50} values of GYM-A and 13-desmethyl C spirolide in mussel extracts were 281.03 ± 44.2 and 129.3 ± 7.2 nM (mean ± SEM, $n = 4$), respectively.

The recoveries of the two toxins with this extraction procedure were also evaluated. For that purpose 1 g of mussel meat homogenate was spiked with an amount of GYM-A or 13-desmethyl C spirolide equivalent to a final concentration in the extract of 300 or 200 nM, respectively, and then extracted

following the above-described protocol. Noncontaminated extracts were also spiked with the same concentration of toxin once the extraction was finished. The concentration of toxin in the samples contaminated before and after the extraction was calculated using the fluorescence polarization assay. The recovery rate was calculated with the following equation: recovery rate (%) = [amount of toxin detected in the samples contaminated before extraction/amount of toxin detected in the samples contaminated after extraction] × 100. The recovery rates were 63.6% ± 3.5% for GYM-A and 87.4% ± 5.3% for 13-desmethyl C spirolide (mean ± SEM, $n = 4$). In the case of 13-desmethyl C spirolide a standard solution was used for the contamination of the extracts with a 200 nM concentration, and the amount of toxin calculated in these postextraction contaminated samples using the fluorescence polarization assay was 213.4 ± 21.2 nM (mean ± SD, $n = 4$).

DISCUSSION

The fluorescence polarization assay developed in this work for the detection of GYM-A and 13-desmethyl C spirolide is based on the inhibition of α-BTX binding to the nAChR by the presence of GYM-A and spirolides in the solution. After the optimization of assay parameters and extraction methods, the assay allows one to quantify GYM-A and 13-desmethyl C spirolide in the range of 50−2000 μg/kg and 70−700 μg/kg in shellfish meat, respectively. The IC_{50} obtained for GYM-A and 13-desmethyl C spirolide in the same assay conditions suggests that the latter toxin has a higher affinity for the nAChRs than GYM-A, which would be in accordance with the in vivo data that indicate that this spirolide has a higher toxicity than GYM-A.[11,12] At present, these toxins are not still regulated since it has not been demonstrated that they are unsafe for human consumption. However, considering that at least GYM-A has a low oral toxicity in mice, similar to what has been reported for yessotoxin,[11,12,31] we considered that this method should allow detection of this cyclic imine at a level similar to the regulatory limit of 1 mg/kg of shellfish meat established for yessotoxin. On the basis of these cyclic imines acute toxicity and applying several safety factors, Dr. Rex Munday (AgResearch Senior Scientist, Ruakura Research Centre, Hamilton, New Zealand) suggested guidance levels for GYM-A and spirolide content in shellfish to ensure consumer safety in the range of 18 mg/kg for GYM-A and 400 μg/kg for spirolides.[13,32] The suggested values are within the detection range of this fluorescence polarization assay.

The extraction method presented in this work is based on the solubility of these cyclic imines in organic solvents. It has been reported that GYMs are soluble in acetone and ethyl acetate, whereas spirolides are soluble in methanol and chloroform.[33] We developed a method that could be used for both groups of toxins, but focusing on the solubility data of spirolides, since they pose the higher risk to human health. The recovery rates of 63.6% ± 3.5% and 87.4% ± 5.3% for GYM-A and 13-desmethyl C spirolide, respectively, are a reflection of this approach. This difference in the recovery rate is probably related to slight differences in the physicochemical properties of the toxins due to their chemical structure and could vary among the analogues of each group. Unfortunately there are no other purified analogues of these toxins commercially available, so their performance in the assay, including the extraction procedure, will have to be evaluated in the future. With the use of this extraction method, a mussel matrix does not change substantially the range of detection of the fluorescence polarization inhibition assay. Actually, the calibration curves obtained in buffer and mussel extract are fairly close. Hopefully, the same extraction procedure will be suitable for other shellfish species or phytoplankton samples. In fact, although the results obtained in mussels cannot be extrapolated to other molluscs, mussels are usually the species with more matrix effects on marine toxin detection methods, and therefore we expect similar or better results with other species. The performance of the assay with other shellfish matrixes will be part of our future studies. Moreover, although a more extensive evaluation should be done, the results already point to an acceptable variability and accuracy of the method in matrix extracts. Remarkably, the coefficient of variation (CV) of 13-desmethyl C spirolide measurements using this assay in samples contaminated postextraction with a 200 nM concentration of toxin is lower than 10%.

Cyclic imines can be coextracted with other lipophilic toxins including okadaic acid, dinophysistoxins, yessotoxin, or brevetoxins.[20,34,35] In order to evaluate the specificity of this fluorescence polarization assay, we tested α-BTX binding to the receptor in the presence of concentrations of okadaic acid or yessotoxin 15 times higher than the concentration of GYM-A that yielded a total inhibition of the binding response. Another lipophilic toxin, PbTx-2, belonging to the group of brevetoxins, responsible for the neurotoxic shellfish poisoning (NSP), was also tested at a concentration 10 times higher than the GYM-A maximum due to availability issues. None of these three toxins inhibited the interaction between α-BTX and the nAChR. Paralytic shellfish poisoning (PSP) toxins can also co-occur with spirolides, since some strains of the dynoflagellate *Alexandrium ostenfeldii* have been shown to produce both kinds of toxins.[36] Although we have not tested PSP toxins in this fluorescence polarization assay, the method we propose would not detect PSP toxins in shellfish, since they would not be coextracted with cyclic imines because they are not soluble in chloroform.[37]

In summary, this paper reports for the first time a new, specific, and fast assay to detect cyclic imines using their interaction with nAChR. Although these toxins are not still regulated, our assay is sensitive enough to detect concentrations higher than 70 μg/kg in shellfish meat. Moreover, we also present an extraction method in shellfish that does not interfere with the analysis and delivers acceptable recovery rates.

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Artículo 4. Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization in shellfish matrixes.

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Feasibility of Gymnodimine and 13-Desmethyl C Spirolide detection by fluorescence polarization in shellfish matrixes

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Running title: Matrix effect on cyclic imines detection

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Abbreviations: nAChR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; α–BTX, α–bungarotoxin; FP, fluorescence polarization; GYM, gymnodimine; PBS, phosphate buffered saline; SPX, spirolide.

Abstract

The detection of toxins in shellfish through reliable methods is essential for human health preservation and prevention of economic losses in the aquaculture industry. Although no human intoxication has been unequivocally linked to gymnodimines or spirolides, these phycotoxins are highly toxic by intraperitoneal injection causing false positives in lipophilic toxin detection by the mouse bioassay. Based on the detection of molecular interactions by fluorescence polarization, an inhibition assay was developed using fluorescent α-bungarotoxin and nicotinic acetylcholine receptor-enriched membranes of *Torpedo marmorata* to detect gymnodimine and 13-desmethyl C spirolide. Both toxins, classified into the cyclic imine group, inhibit the interaction of α-bungarotoxin with *Torpedo* nicotinic acetylcholine receptors in the nM range. In this study we analyze the matrix effect of four shellfish species on the fluorescence polarization assay. Mussels, clams, cockles and scallops were extracted with acetone and sequentially partitioned with n-hexane and chloroform. The interference of these shellfish extracts with the α-bungarotoxin fluorescence or its binding to the nicotinic acetylcholine receptor was lower than 11%. The average recovery rates of Gymnodimine and 13-desmethyl C spirolide using these solvents were 90.6 ± 7.8 % and 89.6 ± 3.2 % respectively with variations among species. The quantification range of this fluorescence polarization assay for gymnodimine and 13-desmethyl C spirolide in all tested species was 50-2,000 µg/kg and 70-700 µg/kg of shellfish meat, respectively. This assay format can be used to detect gymnodimine and 13-desmethyl C spirolide in shellfish as a screening assay.

Introduction

Gymnodimines (GYM) and spirolides belong to an emerging, heterogeneous group of lipophilic marine toxins called cyclic imines [1] . Since their discovery in the early 1990s in New Zealand [2] and Canada [3] respectively, gymnodimines and spirolides have been demonstrated to have a global distribution. GYM have been also detected in Tunisia [4] and Australia [5] and spirolides have been detected in Norway, Adriatic Sea, Denmark, France and Spain, among other places [6-10] . Worldwide, gymnodimines or/and spirolides have been found as contaminants in clams, oysters, pipis, mussels, cockles and scallops [2, 4-6, 9-14] .

Cyclic imines are easily recognizable by expert laboratory personnel due to their "fast acting toxicity" and their acute threshold response ("all or nothing") in mammalian bioassays. Rapid death is observed after intraperitoneal administration to rodents of these toxins at lethal doses, but animals recover fully and quickly at sublethal doses [3] . The oral and intraperitoneal toxicities of gymnodimines and spirolides were determined by toxicological studies in mice. The intraperitoneal LD_{50} of GYM-A is in the range of 80-96 µg/Kg [15, 16] , while recent studies suggest that the LD_{50} of pure 13-desmethyl C spirolide is 5-8 µg/Kg [17] . Orally, GYM has an estimated LD_{50} of 755 µg/Kg when administered by gavage [16] and pure 13-desmethyl C spirolide has an estimated LD_{50} of approximately 150 µg/Kg by the same administration route [17] .

At present, gymnodimines and spirolides have not been categorically linked to any human intoxication and there is still no legal regulation regarding their presence in shellfish. However, their high intraperitoneal toxicity is an important source of false positives in lipophilic toxin detection by mouse bioassay [11] and the current opinion of the scientific community is in favor of regulating the levels of spirolides given their high oral toxicity in laboratory animals. Developments of fast, specific and reliable detection methods for gymnodimines and spirolides that can be used in shellfish extracts are thus essential to guarantee adequate closure of aquaculture areas, and for human health protection. Currently the only detection methods for GYM and spirolides are the mouse bioassay, and liquid chromatography-mass spectrometry (LC-MS)-based detection techniques [6, 8, 11, 13, 18, 19] . The mouse bioassay is not a specific technique and it entails technical, ethical and legal

problems derived from the use of laboratory animals. LC-MS allows unequivocal identification of toxins, but it could not detect new analogues or well-known analogues for which there are no available standards. Very recently we have developed a fluorescence polarization assay based on the high affinity of these toxins for the nicotinic acetylcholine receptor (nAChR) [20] .

In the field of food contaminants the need to develop detection methods compatible with several food matrixes is very common. In the case of marine toxins, many species of filtering mollusks can become poisonous due to accumulation of the toxins through the trophic chain. In this paper we study the suitability of a gymnodimine/spirolide extraction method for the detection of these toxins in clams, mussels, cockles and scallops using a fluorescence polarization assay. This fluorescence polarization detection method is based on the inhibition of the interaction of fluorescent α-bungarotoxin (α-BTX) with *Torpedo* nicotinic acetylcholine receptors (nAChR) by GYM and spirolides [20] .

Materials and methods

Materials

Alexa Fluor 488 α-BTX was purchased from Molecular Probes (Eugene, Oregon, US). GYM and 13-desmethyl C spirolide standard solutions were purchased from NRC-CNRC (Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada). Bovine serum albumin (BSA) and Tween-20 were from Sigma Chemical Co. (Madrid, Spain). Acetone, chloroform, n-hexane, methanol, ethanol (96%), sodium chloride, sodium phosphates and hydrochloric acid were from reagent grade commercial sources. Phosphate buffered saline solution (PBS) was: 130 mM NaCl, 1.5 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, pH = 7.0. PBS-BT solution was PBS supplemented with 0.1 % w/v BSA and 0.1 % v/v Tween-20. Amicon Ultra-4, PLGC Ultracel-PL Membrane, 10 kDa MWCO filter was from Millipore Corporation (Bedford, MA, USA). Toxin free mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), clams (*Venerupis semidecussatus*) and scallops (*Pecten maximus*) were purchased from the market in Lugo, Spain. The *Torpedo marmorata* were obtained from the Marine Station in Arcachon (France) and kept in artificial seawater for a few weeks in the aquarium of the CNRS-animal house in Gif sur

Yvette. Electrocyte membranes rich in nAChRs from *Torpedo marmorata* electric tissue were purified according to procedures previously described [20] .

Fluorescence polarization measurement

Fluorescence intensity and fluorescence polarization (FP) was measured in a Plate Chameleon fluorometer from Hidex (Turku, Finland), with a xenon flash lamp as light source. Alexa Fluor 488 α-BTX fluorescence was measured with an excitation filter of 485 nm, and emission filters of 535 nm. For FP measurements, excitation and emission detection were achieved with polarization filters at 485 and 535 nm respectively. After optimization, the gain was set at a value of 20. Fluorescence intensity was approximately 10 times higher than background. The autofluorescence of the nicotinic receptor membranes, GYM and 13-desmethyl C spirolide did not interfere with the detection method.

FP (in milli polarization units, mP) was calculated by the following equation:

$$mP = 1000\left[\frac{I_V - G \cdot I_H}{I_V + G \cdot I_H}\right]$$

Where I_V is the fluorescence intensity measured with vertical polarization excitation filters and vertical polarization emission filters (called parallel intensity), I_H is the fluorescence intensity measured with vertical polarization excitation filters and horizontal polarization emission filters (called perpendicular intensity), and G is a correction factor that accounts for the optical components of the instrument that affect the light beam depending on its polarization plane. The value of G was determined by measuring FP in a dilution series of the Alexa Fluor 488 α-BTX. G has a value in the above equation that places the calculated mP of all fluorophore dilutions between 20 and 30. The experimental value obtained for G in our conditions was 0.98.

Fluorescence polarization competition assay

α-BTX labeled with Alexa Fluor 488 dye was used to detect binding to the nAChR from *Torpedo* membranes by changes in fluorescence polarization. An inhibition assay was developed, based on binding-competition to nAChRs with the fluorescent α-BTX. The problem solution was incubated with nAChR-enriched membrane of *Torpedo´*s electric organ for 2 h. For this purpose 40 µl of the problem solution were

added to 40 µl of a dilution of the nAChR stock in a 96 well plate (black, flat bottom plates, TermoLabsystems, Franklin, MA, US). Two stocks of nAChR were used for these experiments, a 3.5 mg/ml of protein stock was diluted 1:20 and a 2.7 mg/ml of protein stock was diluted 1:40. These dilutions were adjusted so that the calibration curves in buffer for the two toxins used were equal for both receptor batches. The contents of the wells were mixed by shaking at 140 rpm for 1 min before the 2 h incubation. Finally, 80 µl of 40 nM Alexa Fluor 488-α-BTX were added to the well and shaken again for 1 min. Fluorescence polarization was measured 30 min after the addition of fluorescent α-BTX. All the incubations took place in the dark at room temperature. To correct for background, vertical and horizontal fluorescence values from control wells in which fluorescent α-BTX was omitted were respectively subtracted from vertical and horizontal fluorescence values of fluorophore containing wells, obtaining the I_V and I_H values used in the equation above. The percentage of α-BTX-binding response in a certain condition was calculated as follows:

$$\% \text{ BR} = \frac{(mP_i - mP_{min})}{(mP_{max} - mP_{min})} \text{ x } 100$$

Where % BR is the percentage of α-BTX-binding response, mP_i is the mP value for a given condition, mP_{max} is the mP value obtained for control wells containing the nAChR enriched membranes plus Alexa Fluor 488-α-BTX, mP_{min} is the mP value obtained for control wells containing only Alexa Fluor 488-α-BTX.

Shellfish sample preparation for the fluorescence polarization assay

One hundred g of mussel, clam or cockle meat (whole body) or 100 g of scallop muscle plus gonad were homogenized with a blender, divided in 10 g aliquots and stored at -20 ºC until use. The extraction procedure consisted in the addition of 4 ml acetone to 1 g of shellfish homogenate. The mixture was vortexed for 10 s and roller mixed for 15 min at room temperature. After centrifugation at 3,500*g* for 10 min at 4 ºC, the supernatant was saved and the pellet was re-extracted twice with acetone, as described. The combined supernatants were evaporated and the resulting residue was dissolved in 4 ml of water. The aqueous extract was partitioned against an equal volume of hexane for 30 min. The hexane was then discarded and the water was extracted three times with chloroform (1:1.5 v/v) for 1 h. Chloroform layers were pooled together and evaporated. The residue was reconstituted with 1 ml ethanol:

PBS (2:3 v/v) and filtered through a 10 kDa MWCO filter (Millipore). Then, an equal volume of PBS with 0.2% BSA and 0.2% Tween-20 was added. The extracts were analyzed on the same day the extraction was started.

Data analysis

All experiments were carried out four times, and in every experiment each condition was tested in the fluorescence polarization assay by triplicate. The results are expressed as means ± SEM. For statistical significance, the results were analyzed using the Student *t* test. A probability level of 0.05 or less was used for statistical significance. The calibration curves were fitted using Kaleida graph 4.03 for logarithmic fits and BiaEvaluation 3.2 RC1 software (BiaCore) for four-parameter fits. The logarithmic fit equation was: $y = a+b\log(x)$. The four-parameter fit equation was: $y = R_{hi} – (R_{hi} - R_{10})/(1+((x/A_1)\hat{}A_2))$

Results

Detection of GYM and 13-desmethyl C spirolide by the fluorescence polarization inhibition assay

The ability of the fluorescence polarization inhibition assay to detect GYM and 13-desmethyl C spirolide in solution was tested based on the competition of these toxins with α-BTX for binding to the nAChRs. GYM and 13-desmethyl C spirolide standards were provided dissolved in methanol, since this solvent interfere with the assay [20] it was evaporated and the toxins were redissolved with 19.2% ethanol in PBS-BT. Serial dilutions of GYM at concentrations of 50, 100, 500, 1,000 and 2,000 nM and of 13-desmethyl C spirolide at 50, 75, 100, 150, 250 and 500 nM were prepared in PBS-BT. The final concentration of toxin in the well after addition of the fluorescent α-BTX was 1/4 of the initial concentration. The final concentration of ethanol in the well was 4.8% and it was maintained constant for all wells. The presence of GYM and 13-desmethyl C spirolide inhibited the change of fluorescence polarization induced by binding of the receptor to the fluorescent α-BTX. The results were plotted as percentage of α-BTX binding to the receptor versus GYM or 13-desmethyl C spirolide concentration in the solution added to the well. Calibration curves with a logarithmic fit and a four-parameter fit were obtained for these concentrations of GYM and 13-desmethyl C spirolide respectively (Fig. 1A and Fig 2A). IC_{50} values obtained in PBS-BT are presented in Table 1. The quantification

range was between 50 nM and 2,000 nM for GYM and 50 to 500 nM for 13-desmethyl C spirolide.

Matrix effect on the fluorescence polarization inhibition assay

An optimized protocol for GYM and 13-desmethyl C spirolide extraction was tested in mussels, clams, cockles, and scallops to study the shellfish matrix effect on the fluorescence polarization assay. The toxins were extracted from homogenized shellfish meat with acetone and subsequently with chloroform (see Material and Methods for a detailed protocol). An intermediate partition with hexane was included to remove sample pigments that interfered with Alexa Fluor 488 α-BTX fluorescence. Serial dilutions of GYM and 13-desmethyl C spirolide were prepared in the extracts from mussels, clams, cockles, and scallops at the same concentrations of toxin standard used in former experiments. The calibration curves obtained in the four shellfish extracts for GYM (Fig. 1B-E) and 13-desmethyl C spirolide (Fig. 2B-E) were very similar to the calibration curves obtained in buffer for both toxins. Actually, there was not statistical significance between measurements obtained in buffer and measurements obtained in individual extracts. The interference of extracts with the maximum and minimum binding responses was lower than 11%, and not statistically significant in most cases (Table 2). For mussels and cockles, although the interference was low, the data were statistically different from buffer controls, suggesting that for routine analysis a calibration curve in shellfish matrix may be advisable. The IC_{50} values of GYM and 13-desmethyl C spirolide in mollusk extracts are presented in Table 1. The fluorescence polarization assay is suitable for detection of these toxins in extracts from mussels, clams, cockles and scallops.

In order to evaluate the precision of the fluorescence polarization assay for detection in shellfish matrixes, extracts from the four species were contaminated with 300 nM GYM, or 200 nM 13-desmethyl C spirolide. The fluorescence polarization assay was used to quantify the amount of toxin present in these extracts. The results of the quantification are shown in Table 3. Importantly, for 13-desmethyl C spirolide the coefficient of variation (CV) was lower than 14% in the four species studied. However, for GYM the CV of the measurements in shellfish extracts was higher than 15% in the four species tested.

Recovery rates

The recoveries of the two toxins were evaluated using this extraction procedure in the same shellfish matrixes. For that purpose 1 g of shellfish meat homogenate was spiked with an amount of GYM or 13-desmethyl C spirolide equivalent to a final concentration in the extract of 300 nM or 200 nM respectively. The extraction was performed following the above described protocol. Non-contaminated extracts were also spiked with the same concentration of toxin once the extraction was finished. The concentration of toxin in the samples contaminated before and after the extraction was calculated using the fluorescence polarization assay The recovery rate was calculated with the following equation: recovery rate (%) = [amount of toxin detected in the samples contaminated before extraction/ amount of toxin detected in the samples contaminated after extraction] x 100. The recovery rates for both toxins were higher than 85% in all the species tested except for GYM in mussels (Table 4). The coefficient of variation of the concentrations calculated for shellfish meat homogenates contaminated before extraction was also lower than 14%, as it can be seen in Table 5. Again, the variability of the GYM results was high.

Discussion

After the optimization of assay parameters and extraction methods, the assay allows to quantify GYM and 13-desmethyl C spirolide in the range of 50-2,000 µg/kg and 70-700 µg/kg of shellfish meat, respectively. This study demonstrates that this fluorescence polarization assay is capable of GYM and 13-desmethyl C spirolide detection in clams, mussels, cockles, and scallops. The interference of these matrixes with this competition assay is minimum and does not change the performance of the method. At present, no regulatory limits have been established for these toxins since it has not been demonstrated that they are unsafe for human consumption. Based on the acute toxicity of these cyclic imines in mice and applying several safety factors, Dr. Rex Munday (AgResearch Senior Scientist, Ruakura Research Centre, Hamilton, New Zealand) suggested guidance levels for GYM and spirolide content in shellfish to ensure consumer safety in the range of 18 mg/kg for GYM and 400 µg/kg for spirolides [17, 21] . The suggested values are within the detection range of this fluorescence polarization assay in all matrixes tested (Figs. 1 and 2).

The IC_{50} values obtained for 13-desmethyl C spirolide in the different shellfish matrixes were very close to the IC_{50} obtained in buffer (PBS-BT). However, the IC_{50} values obtained with GYM showed more variability and were less close to controls than 13-desmethyl C spirolide. The higher variability of the GYM IC_{50} can be related to the use of a logarithmic fit instead of a four-parameter fit, since a slight variation in the x axis will yield a higher concentration variation due to the logarithmic scale. Nevertheless, there are other factors that could be increasing the variability such as possible interactions between toxins and matrixes.

For 13-desmethyl C spirolide the results obtained in extracts from the four mollusk species have an acceptable variability and accuracy. The coefficient of variation (CV) of measurements using this assay in samples contaminated post-extraction with a 200 nM concentration of toxin is lower than 14%. This concentration of the spirolide is equivalent to a concentration in shellfish meat of 276 µg/Kg. Regarding the accuracy of the fluorescence polarization assay, a full evaluation would need more experiments with different laboratories and operators involved. However, in these preliminary data the calculated concentrations of 13-desmethyl C spirolide in contaminated extract with a 200 nM concentration differ less than 10% from that concentration in mussels and clams, and around 20% in cockles and scallops. GYM measurements present more variability and less accuracy than the spirolide. The cause of this variation is unknown at the moment, although as we mentioned above several factors could be responsible. Because the spirolides have a higher toxicity than the GYM it is preferable that the method yields better results with the more dangerous of both molecules.

The extraction method presented in this work was optimized for spirolides based on their solubility in organic solvents. The recovery rates of 13-desmethyl C spirolide are actually higher than 85% for the four species tested. Not surprisingly, the method also extracts GYM. However, in this case as well, the variability of the recovery rates is higher than for the spirolide, showing good recoveries for clam, cockle, and scallop, but considerably lower for mussels. Differences between GYM and 13-desmethyl C spirolide in the recovery rates are probably related to slight differences in the physicochemical properties due to their chemical structure, while differences

between extracts using the same toxin might be caused by the different interactions established between toxins and shellfish matrixes.

In summary, this paper reports a suitable extraction method for GYM and 13-desmethyl C spirolide to detect these toxins by the fluorescence polarization technique using their ability to inhibit fluorescent α-BTX interaction with the nAChR. Our assay is sensitive enough to detect concentrations of these toxins higher than 70 µg/Kg in mussel, clam, cockle, and scallop meat, and the average recovery rates of both toxins are around 90%.

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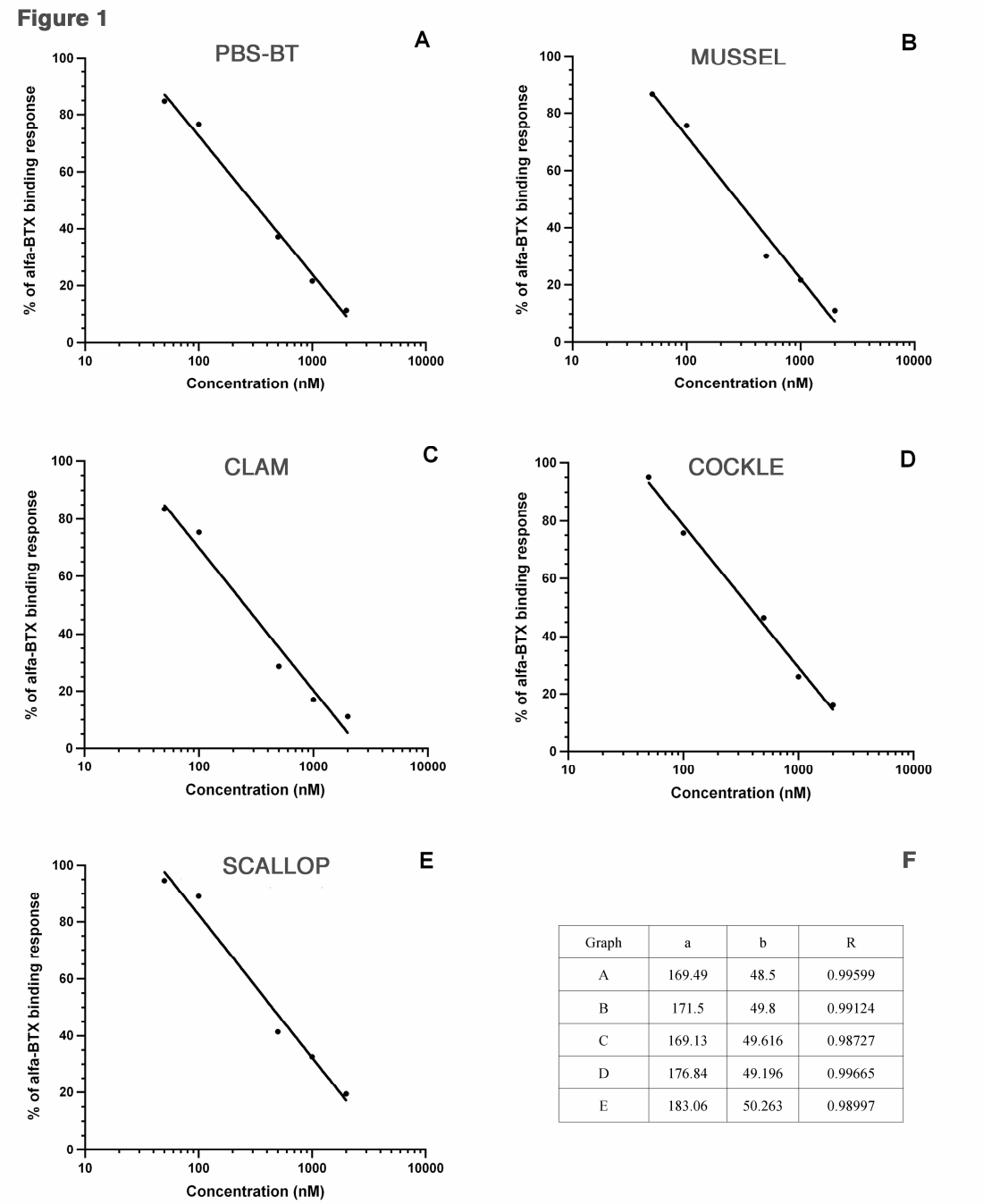
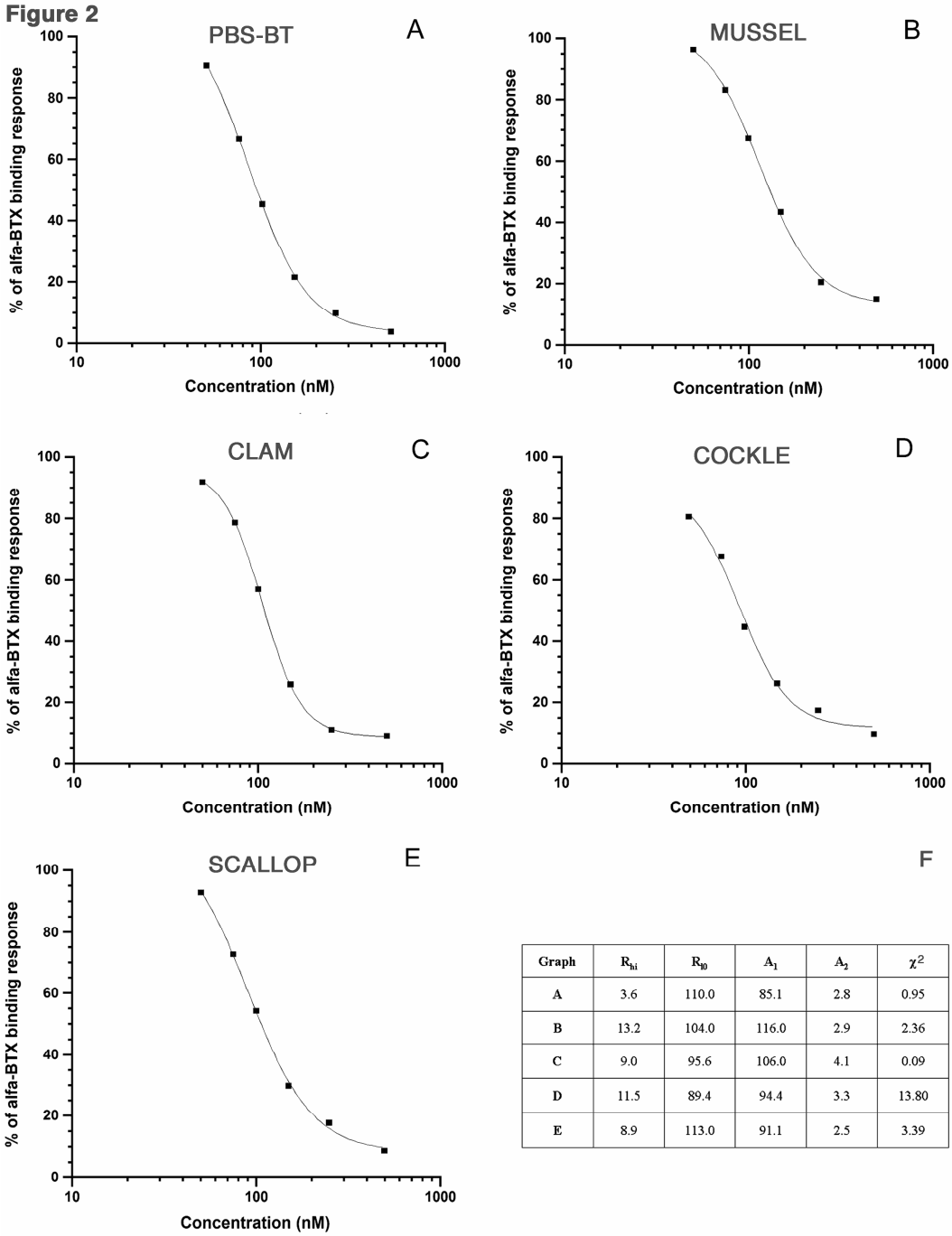
FIGURES.



Graph	a	b	R
A	169.49	48.5	0.99599
B	171.5	49.8	0.99124
C	169.13	49.616	0.98727
D	176.84	49.196	0.99665
E	183.06	50.263	0.98997

Figure 1. GYM calibration curves in PBS-BT and shellfish extracts using the fluorescence polarization assay. (A) PBS-BT buffer, (B) mussel, *Mytilus galloprovincialis* (C) clam, *Venerupis semidecussatus* (D) cockle, *Cerastoderma edule* (E) scallop, *Pecten maximus,* (F) Parameter values of the logarithmic fit. Serial dilutions of GYM were prepared at concentrations of 50, 100, 500, 1000 and 2,000 nM in mollusk extract or in PBS-BT containing 19.2% ethanol and were added to a well containing a solution of *Torpedo marmorata* nAChR-enriched membranes. After 2 h Alexa Fluor α-BTX was also added to the well and its binding to receptors determined by changes in fluorescence polarization. Data are expressed as percentage of maximum α-BTX-binding response. Calibration curves were obtained using a logarithmic fit. Representative experiment of n = 4 for each condition.



Graph	R_{hi}	R_{lo}	A_1	A_2	χ^2
A	3.6	110.0	85.1	2.8	0.95
B	13.2	104.0	116.0	2.9	2.36
C	9.0	95.6	106.0	4.1	0.09
D	11.5	89.4	94.4	3.3	13.80
E	8.9	113.0	91.1	2.5	3.39

Figure 2. 13-desmethyl C spirolide calibration curves in PBS-BT and shellfish extracts using the fluorescence polarization assay. (A) PBS-BT buffer, (B) mussel, *Mytilus galloprovincialis* (C) clam, *Venerupis semidecussatus* (D) cockle, *Cerastoderma edule* (E) scallop, *Pecten maximus*. (F) Parameter values of the four-parameter fit. Serial dilutions of 13-desmethyl C spirolide were prepared at concentrations of 50, 75, 100, 150, 250 and 500 nM in mollusk extract or in PBS-BT containing 19.2% ethanol and were added to a well containing a solution of *Torpedo marmorata* nAChR-enriched membranes. After 2 h Alexa Fluor α-BTX was also added to the well and its binding to receptors determined by changes in fluorescence polarization. Data are expressed as percentage of maximum α-BTX-binding response. Calibration curves were obtained using four-parameter fits. Representative experiment of n = 4 of each condition.

TABLES

	PBS-BT	Mussel	Cockle	Clam	Scallop
13-desmethyl C spirolide (nM)	104.6 ± 9.1	129.3 ± 7.2	98.5 ± 5.5	104.4 ± 2.6	102.8 ± 2.2
Gymnodimine (nM)	351.5 ± 55.6	280.3 ± 44.3	293.1 ± 47.4	360.0 ± 55.1	447.7 ± 39.4

Table 1. IC_{50} of 13-desmethyl C spirolide and GYM calibration curves in shellfish extracts or in PBS-BT. Serial dilutions of 13-desmethyl C spirolide were prepared at concentrations of 50, 75, 100, 150, 250 and 500 nM in mollusk extracts or in PBS-BT. Serial dilutions of GYM were prepared at concentrations of 50, 100, 500, 1,000 and 2,000 nM in mollusk extract or in PBS-BT. Calculation of IC_{50} was performed using a four parameter fit and logarithmic fit for 13-desmethyl C spirolide and GYM respectively (mean ± SEM, n = 4).

	Mussel	Cockle	Clam	Scallop
Maximum binding in PBS-BT	107.0 ± 4.4	98.2 ± 2.2	94.4 ± 3.3	93.6 ± 3.8
Maximum binding in shellfish extract	97.1 ± 3.4*	87.8 ± 1.9*	90.6 ± 3.8	91.8 ± 4.6
Minimum binding in PBS-BT	31.1 ± 1.7	25.1 ± 1.9	30.0 ± 4.8	23.2 ± 1.5
Minimum binding in shellfish extract	28.8 ± 1.7*	26.8 ± 1.2	30.5 ± 5.0	21.9 ±2.1

Table 2. Interference of shellfish extracts with maximum and minimum binding response. Data are shown as binding signal in mP (mean ± SEM, n = 8). The values of the maximum and minimum binding signal in shellfish extract are shown together with the matched controls in buffer (* $p < 0.05$).

	Mussel	Cockle	Clam	Scallop
13-desmethyl C spirolide (nM)	213.4 ± 21.2	163.6 ± 22.2	194.8 ± 12.7	160.2 ± 14.1
Gymnodimine (nM)	341.0 ± 73.3	294.4 ± 94.3	339.9 ± 74.0	359.2 ± 92.4

Table 3. Post-extraction contamination concentrations calculated of 13-desmethyl C spirolide and GYM using calibration curves in shellfish extracts. Mussels, cockles, clams and scallops were extracted using the acetone/chloroform method. The extracts were contaminated with a final concentration 200 nM 13-desmethyl C spirolide or 300 nM GYM. The concentration was calculated using a four parameter fit or a logarithmic fit of the matrix extract calibration curves of 13-desmethyl C spirolide and GYM, respectively (mean ± SD, n = 4).

	Mussel	Cockle	Clam	Scallop
13-desmethyl C spirolide (%)	87.4 ± 5.3	91.5 ± 7.5	86.9 ± 7.7	92.8 ± 7.6
Gymnodimine (%)	62.0± 2.8	97.8 ± 19.2	115.3 ± 12.9	85.5 ± 14.3

Table 4. Recovery rate of 13-desmethyl C spirolide and GYM in shellfish extracted with the acetone/chloroform method. Mussels, cockles, clams and scallops were extracted using the acetone/chloroform method. One g of homogenized meat was spiked with an amount of GYM or 13-desmethyl C spirolide equivalent to a final concentration in the extract of 300 nM or 200 nM, respectively. Non-contaminated extracts were also spiked after extraction with the same concentration of toxin. The concentration of 13-desmethyl C spirolide and GYM was calculated using a four parameter fit or logarithmic fit of the calibration curve respectively. The toxin recovery was calculated as the percent fraction of toxin in postextraction-contaminated samples that were recovered in the pre-extraction-contaminated samples (mean ± SEM, n = 4).

	Mussel	Cockle	Clam	Scallop
13-desmethyl C spirolide (nM)	183.4 ± 13.0	147.3 ± 10.7	168.3 ± 23.2	147.6 ± 17.8
Gymnodimine (nM)	214.3 ± 36.5	262.3 ± 43.7	386.9 ± 94.3	300.3 ± 114.8

Table 5. Pre-extraction contamination concentrations calculated of 13-desmethyl C spirolide and GYM using calibration curves in shellfish extracts. Mussels, cockles, clams and scallops were extracted using acetone/chloroform method. One g of homogenized meat was spiked with an amount of GYM or 13-desmethyl C spirolide equivalent to a final concentration in the extract of 300 nM or 200 nM respectively. The concentration of toxin in the extract was calculated using a four parameter fit and logarithmic fit of the matrix extract calibration curves of 13-desmethyl C spirolide and GYM, respectively (mean ± SD, n = 4).

DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

La detección y cuantificación de ficotoxinas en moluscos destinados al consumo humano a través de métodos rápidos, sensibles, específicos y fiables es un elemento clave para la prevención de intoxicaciones y la reducción de las pérdidas económicas en el sector acuícola derivadas de la detección de falsos positivos (muestras negativas que el método utilizado para la determinación de toxinas detecta como positivas).

En la Unión Europea, así como en la mayoría de países donde existe regulación para la presencia de ficotoxinas en moluscos comestibles, el método oficial de referencia para determinar estos compuestos (a excepción del grupo del ácido domoico) es el bioensayo en ratón. Esta técnica consiste en la inyección intraperitoneal de una muestra previamente extraída (con disolventes adecuados en función de las características hidrofílicas o lipofílicas de las moléculas a determinar) a un conjunto de ratones de peso establecido y en la posterior evaluación de la sintomatología producida y/o el tiempo de supervivencia de los animales. Aunque este método garantiza la protección de la salud pública, presenta una serie de problemas y limitaciones de carácter técnico, ético y legal, inherentes a su realización. Para paliar estas deficiencias, en las últimas décadas se han desarrollado varios métodos de detección para los distintos grupos de toxinas, pero muy pocos han sido reconocidos oficialmente como métodos alternativos a los respectivos bioensayos [*10, 11*].

En esta tesis se presentan tres métodos de detección y cuantificación de ficotoxinas mediante el uso de la polarización de fluorescencia y los biosensores basados en la resonancia del plasmón superficial. Aunque el principio físico de estas técnicas es distinto, ambas permiten la detección y monitorización de uniones moleculares en tiempo real.

En comparación con los bioensayos, las técnicas utilizadas en esta tesis proporcionan dos ventajas importantes: no usan animales (evitando problemas éticos y legales) y son específicas (se trabaja directamente con la molécula con la que interactúan las toxinas, bien sea un anticuerpo o su diana natural, evitando interferencias con otros compuestos). Respecto a otros métodos de detección de ficotoxinas, como el HPLC,

el LC-MS, los ensayos inmunoabsorbentes o los radioinmunoensayos, la polarización de fluorescencia y los biosensores basados en SPR no utilizan cantidades ingentes de disolventes orgánicos (éstos pueden ser necesarios en la extracción de la muestra, pero no durante su detección en el equipo donde los tampones de trabajo son mayoritariamente acuosos), no requieren pasos extra de lavado (evitando la posible pérdida de analito) y no utilizan moléculas radiactivas (evitando el peligro y los residuos que eso conlleva).

De forma individual, los biosensores basados en SPR destacan por no necesitar purificaciones exhaustivas de la muestra (la luz no penetra en la muestra, por lo que se pueden utilizar soluciones coloreadas o fluidos orgánicos), trabajar con volúmenes pequeños de muestra (habitualmente menores a 100 µl, en nuestros ensayos se utilizan alrededor de 50 µl), su rapidez (el tiempo de un ciclo completo de análisis, asociación-disociación-regeneración, habitualmente es inferior a los 10 minutos, siendo en nuestros ensayos de detección de YTX y toxinas PSP menor a 6 minutos), su precisión (el flujo y el tiempo de exposición del analito al ligando son controlados de forma precisa por el aparato) y, sus sistemas de autoevaluación integrados (los sistemas Biacore constan de una opción de autodiagnóstico para determinar pérdidas u obstrucciones en celdas y jeringas además de comprobar la calidad de la señal a partir de un protocolo prediseñado). Las principales desventajas de esta técnica derivan de la inmovilización de una de las moléculas a una estructura sólida, lo que puede dar lugar a orientaciones inadecuadas del ligando, las cuales ocluyan el sitio de unión o dificulten el acceso del analito al mismo. Las posibles uniones inespecíficas a la superficie del chip son otro problema frecuente en este tipo de técnica. Una vez solventados o normalizados estos problemas, la sensibilidad y la calidad de cada ensayo depende de la naturaleza de las moléculas que interaccionan y de su afinidad.

En el caso de las toxinas del grupo de la saxitoxina, al no poder utilizar su diana directa (canales de sodio dependientes de voltaje), el método de detección fue desarrollado con un anticuerpo anti-GTX2/3. Empleando las condiciones adecuadas, este anticuerpo permite la detección de STX, dcSTX, GTX2/3, dcGTX2/3, C1/2 y GTX5 a concentraciones cinco veces menores al límite permitido por la Unión Europea (800 µg/kg de carne de molusco), pero presenta una baja reactividad

cruzada con los análogos GTX1/4, dcNEO y neoSTX. Sin embargo, esta característica, común en muchos anticuerpos anti-PSP [*40-42, 187, 188*], no lo excluye de ser un buen método de criba, ya que los episodios tóxicos de estas toxinas son muy heterogéneos, presentándose muchas toxinas a la vez.

En el caso de la yessotoxina sí es posible utilizar una diana celular (la PDE 1), y aunque la afinidad entre ambas moléculas sólo permite la cuantificación en el rango μM, es suficiente para su detección dentro de los límites europeos (1 mg YTX/kg de molusco).

Referente a las características individuales de la polarización de fluorescencia, destacan principalmente su homogeneidad (la interacción entre las moléculas tiene lugar realmente en solución), su rapidez (el tiempo de lectura de una placa de 96 pocillos es menor a 10 minutos) y sensibilidad (su límite de detección se sitúa en el rango sub-nanomolar). La principal desventaja reside en el marcaje fluorescente de uno de los componentes del ensayo. Habitualmente las reacciones de marcaje se producen en los radicales más reactivos de la molécula, que a su vez suelen ser los radicales por donde la molécula reacciona con otros compuestos, haciendo a veces incompatible el marcaje con la interacción.

En el método desarrollado para la detección de gymnodiminas y espirólidos, la molécula fluorescente es la α-bungarotoxina (marcada fluorescentmente de forma comercial) por lo que ni el receptor nicotínico ni las toxinas se encuentran modificados, lo que permite su unión de forma libre. En este ensayo, la gymnodimina-A y el 13-desmetil espirólido C pueden detectarse en el rango nM, lo que se traduce en un rango de μg/kg de molusco (utilizando el protocolo de extracción apropiado), adecuado tanto para garantizar la salud de los consumidores en caso de una futura regulación [*63, 143*] como para su detección en caso de falsos positivos.

De forma conjunta, aunque ninguno de los tres métodos de detección descritos en esta tesis puede sustituir de manera total a los bioensayos, sí pueden utilizarse como métodos de criba o de descarte/confirmación en caso de falsos positivos, reduciendo

el número de animales sacrificados y el uso de métodos más caros, lentos, inespecíficos y ética y legalmente cuestionables.

CONCLUSIONES

5. CONCLUSIONES

Los resultados obtenidos en esta tesis doctoral permiten establecer las siguientes conclusiones:

1. El método de detección de toxinas PSP basado en el uso de un biosensor SPR, un anticuerpo anti-GTX2/3 y un chip de STX, puede ser utilizado como método de criba en muestras naturales si se realiza la extracción con etanol al 90% ya que el ensayo es capaz de detectar toxinas PSP a una concentración 5 veces menor que el límite máximo permitido por la Unión Europea en moluscos destinados al consumo humano.

2. Los biosensores SPR permiten la detección de YTX en el mismo rango que los biosensores de espejos resonantes, dentro de los límites establecidos por la Unión Europea, pero con un tiempo de análisis de muestra 10 veces menor.

3. La inhibición de la unión entre la α-bungarotoxina marcada fluorescentemente y los receptores nicotínicos de acetilcolina, por parte de gymnodimina A y 13-desmetil espirólido C, puede usarse para detectar y cuantificar la presencia de estas toxinas en moluscos mediante polarización de fluorescencia si se utiliza una extracción acetona/hexano/cloroformo.

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6. BIBLIOGRAFÍA

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RESUMEN

Las ficotoxinas marinas son productos naturales de origen algal, que pueden llegar al hombre u otros depredadores por vía alimentaria a través de moluscos, crustáceos, gasterópodos o peces, en forma de aerosol o por contacto directo con el agua de mar. Aunque sólo un 1% de las microalgas que forman el fitoplancton tienen la capacidad de producirlas, sus efectos a nivel sanitario y económico son altamente dañinos, provocando al año miles de intoxicaciones en humanos y millones de pérdidas en la industria acuícola en todo el mundo. En este contexto, la detección, monitorización y regulación de dichas toxinas, resulta esencial para salvaguardar la salud humana sin perjudicar los intereses industriales. En la Unión Europea, el método oficial de detección para la mayoría de estas toxinas es el bioensayo en ratón, pero este método presenta numerosos inconvenientes técnicos, éticos y legales, derivados del uso de animales en el laboratorio. En la presente tesis doctoral, se han desarrollado métodos de detección en extractos de molusco para tres grupos de ficotoxinas distintos: iminas cíclicas, saxitoxina y yessotoxina, empleando dos técnicas relativamente recientes: la polarización de fluorescencia y los biosensores basados en el fenómeno óptico de la resonancia del plasmón superficial (SPR, del inglés surface plasmon resonance). Ambas técnicas permiten la detección y monitorización de uniones moleculares en tiempo real, proporcionando respecto al bioensayo dos ventajas importantes: no usan animales y son altamente específicas. Los resultados obtenidos permiten concluir: 1) El ensayo desarrollado para las toxinas del grupo de la saxitoxina basado en el uso de un biosensor SPR, un anticuerpo anti-gonyautoxina2/3 (un análogo de la saxitoxina) y un chip de saxitoxina, puede ser utilizado como método de criba en muestras naturales si se emplea una extracción con etanol al 90 % ya que permite la detección de estas toxinas a una concentración 5 veces menor que el límite máximo permitido por la Unión Europea. 2) Los biosensores SPR permiten la detección de yessotoxina en el mismo rango que los biosensores de espejos resonantes (dentro de los límites establecidos por la Unión Europea), pero con un tiempo de análisis de muestra 10 veces menor y 3) La inhibición de la unión entre la □-bungarotoxina marcada fluorescentemente y los receptores nicotínicos de acetilcolina, por parte de gymnodimina y espirólidos (toxinas pertenecientes al grupo de las iminas cíclicas); puede utilizarse para detectar y cuantificar la presencia de estas toxinas en moluscos mediante polarización de fluorescencia si se utiliza una extracción de la muestra con acetona/hexano/cloroformo.