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DEPARTAMENTO DE FARMACOLOGÍA

DESARROLLO DE MÉTODOS BIOQUÍMICOS PARA LA DETECCIÓN DE FICOTOXINAS

Tesis Doctoral

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INFORMA:

Que la tesis doctoral titulada: **"Desarrollo de métodos bioquímicos para la detección de ficotoxinas"**, recogida en la presente memoria, de la que es autora la Licenciada en Ciencias del Mar Dña. **Laura María Pérez Rodríguez**, ha sido realizada bajo su codirección, y cumple las condiciones exigidas para que su autora pueda optar al grado de Doctora por la Universidad de Santiago de Compostela, por lo que da su aprobación para la correspondiente lectura y defensa.

Para que conste a los efectos oportunos, firma la presente en Lugo, a 19 de Septiembre de 2013.

Fdo: Luis Miguel Botana López

Doctor en Farmacia

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Natalia Vilariño del Río, Profesora Titular del Departamento de Farmacología
de la Facultad de Veterinaria

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Fdo: Natalia Vilariño del Río

Doctora en Veterinaria

Fdo: Laura M. Pérez Rodríguez



À mi Madre

À Carlos



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ABREVIATURAS Y ACRÓNIMOS

ABREVIATURAS Y ACRÓNIMOS

Abs	Absorbancia
AChBP	Proteína de unión a acetilcolina (<i>Acetylcholine binding protein</i>)
AD	Ácido domoico
ADHP	10-acetil-3,7-dihidroxifenoxacina
AMPA	Ácido α-amino-3-hidroxi-5-metil-4- isoxazol-propiónico
AMPc	Adenosina 3',5'-monofosfato cíclico
ANTX	Anatoxina-a
AO	Ácido okadaico
AOAC	Asociación de químicos analistas oficiales (<i>Association of Official Analytical Chemists</i>)
ASP	Intoxicación amnésica por consumo de molusco (<i>Amnesic shellfish poisoning</i>)
AZA	Azaspirácido
AZP	Intoxicación por azaspirácido (<i>Azaspiracid poisoning</i>)
CFP	Intoxicación ciguatérica por consumo de pescado (<i>Ciguateric fish poisoning</i>)
DL ₅₀	Dosis Letal 50
DL ₉₉	Dosis Letal 99
DSP	Intoxicación diarreica por consumo de molusco (<i>Diarrhetic shellfish poisoning</i>)
DTX	Dinofisistoxina
EDC	1-etil-3 (dimetilaminopropil) carbodiimida
ELISA	Ensayo por inmunoabsorción ligado a enzimas (<i>Enzyme-Linked ImmunoSorbent Assay</i>)
FAO	Organización de las Naciones Unidas para la agricultura y la alimentación (<i>Food and Agriculture Organization of the United Nations</i>)
GYM	Gymnodimina

HPLC	Cromatografía líquida de alta eficacia (<i>High Performance Liquid Chromatography</i>)
HRP	Peroxidasa de rábano picante (<i>Horseradish peroxidase</i>)
i. p.	Intraperitoneal
Kd	Constante de disociación
LC-MS	Cromatografía líquida acoplada a espectrometría de masas (<i>Liquid Chromatography-Mass Spectrometry</i>)
LC-MS/MS	Cromatografía líquida acoplada a espectrometría de masas en tándem (<i>Liquid Chromatography tandem Mass Spectrometry</i>)
MTT	Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol
nAChR	Receptor nicotínico de acetilcolina (<i>Nicotinic acetylcholine receptor</i>)
NMDA	N-metil-D-aspartato
NSP	Intoxicación neurotóxica por consumo de molusco (<i>Neurotoxic shellfish poisoning</i>)
PE	Ficoeritrina
Pntx	Pinnatoxina
PP1	Fosfatasa de proteína de serina/treonina 1
PP2A	Fosfatasa de proteína de serina/treonina 2A
PSP	Intoxicación paralizante por consumo de molusco (<i>Paralytic shellfish poisoning</i>)
PtTX	Pteriatoxina
QL	Quimioluminiscencia
SPX	Espirólido
STX	Saxitoxina
Sulfo-NHS	Sulfo-N-hidroxisuccinimida
TEF	Factor de toxicidad equivalente (<i>Toxic equivalency factor</i>)
TMB	3,3,5,5' - tetrametilbencidina
YTX	Yessotoxina



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INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Ficotoxinas

Las ficotoxinas son metabolitos naturales sintetizados por microalgas fitoplanctónicas pertenecientes, en su gran mayoría, al grupo de los dinoflagelados. Existen más de 5000 especies de fitoplancton, muchas de ellas capaces de proliferar bajo determinadas condiciones ambientales dando lugar a floraciones algales, que pueden producir cambios en la coloración de la superficie del agua, formando las conocidas mareas rojas [1,2]. De todas esas especies fitoplanctónicas, alrededor de un 2 % producen potentes toxinas capaces de concentrarse en distintas especies (moluscos filtradores, peces, crustáceos, gasterópodos...) llegando al hombre u otros depredadores a través de la cadena trófica. Debido a la ingesta de productos contaminados con toxinas se producen anualmente alrededor de 60 000 intoxicaciones en humanos en todo el mundo, con unos datos de mortalidad del 1,5 % [3]. Además de perjudicar a la salud humana, estos compuestos provocan la muerte de peces, aves o mamíferos [4-7] y afectan gravemente a la economía en el sector de la acuicultura y a la industria conservera [8,9] debido a la clausura temporal de zonas de marisqueo, a la paralización de stocks de peces, o a la desconfianza que los brotes tóxicos generan en los consumidores. Para garantizar la inocuidad de los productos de la pesca y la acuicultura, y evitar de esa forma posibles intoxicaciones, desde hace años se ejecutan de forma rutinaria programas de vigilancia de las zonas de producción. Estos programas responden a una serie de reglamentos y legislaciones en los que se especifican, entre otros, las concentraciones máximas permitidas de cada grupo de toxinas y los métodos aceptados para determinar estas concentraciones [10-13].

En un primer momento las toxinas marinas fueron clasificadas según la sintomatología que provocan en humanos, en los siguientes grupos: intoxicación

paralizante por consumo de molusco (PSP, del inglés *paralytic shellfish poisoning*), intoxicación diarreica por consumo de molusco (DSP, del inglés *diarrhetic shellfish poisoning*), intoxicación amnésica por consumo de molusco (ASP, del inglés *amnesic shellfish poisoning*), intoxicación neurotóxica por consumo de molusco (NSP, del inglés *neurotoxic shellfish poisoning*) e intoxicación ciguatérica por consumo de pescado (CFP, del inglés *ciaguateric fish poisoning*).

Hoy en día la FAO (Food and Agriculture Organization of the United Nations) clasifica las toxinas marinas en función de su estructura química [14].

- Saxitoxina y sus derivados, causantes de la intoxicación paralizante por consumo de molusco (PSP)
- Ácido okadaico y sus derivados, causantes de la intoxicación diarreica por consumo de molusco (DSP)
- Ácido domoico y sus derivados, causantes de la intoxicación amnésica por consumo de molusco (ASP)
- Brevetoxinas, causantes de la intoxicación neurotóxica por consumo de molusco (NSP)
- Azaspirácidos, causantes del envenenamiento por azaspirácido (AZP)
- Iminas cíclicas
- Pectenotoxinas
- Yessotoxinas

Además de los anteriores, existen otros grupos de toxinas, como las ciguatoxinas responsables de la intoxicación ciguatérica por consumo de pescado (CFP); la tetrodotoxina y sus derivados, responsables de la intoxicación por pez globo en Japón; la palitoxina y sus análogos las ostreocinas, ovatoxinas y mascarenotoxinas, cuyos síntomas, similares a los provocados por la

ciguatoxina, se deben a la ingesta de peces y crustáceos asociados a arrecifes coralinos ; o los grupos de toxinas producidos por cianobacterias de agua dulce, como las nodularinas, microcistinas, cilindrospermopsinas o la anatoxina-a, entre otras.

A continuación se describen los grupos de toxinas empleados durante esta tesis doctoral, haciendo mayor hincapié en aquellas para las que se han desarrollado los métodos de detección que se presentan. Así mismo se incluye un resumen de los métodos de detección de ficotoxinas más utilizados y preexistentes a los desarrollados en este trabajo.

1.1.1. Grupos de ficotoxinas

1.1.1.1. Iminas cíclicas

Las iminas cíclicas son un grupo heterogéneo de compuestos macrocíclicos que contienen un radical imina como parte de su estructura cíclica. A este grupo pertenecen espirólidos, gymnodiminas, pinnatoxinas, pteriatoxinas, proroentrólidos y espiro-proroentriminas [15-21].

Estas "toxinas de acción rápida" provocan la muerte de los animales a los pocos minutos (3 - 20 min.) de inyectarles dosis letales de estos compuestos intraperitonealmente; mientras que por debajo de esas dosis, los animales se recuperan por completo sin mostrar ningún tipo de secuelas [22].

Hasta la actualidad no se han correlacionado inequívocamente casos de intoxicación en humanos con la presencia de iminas cíclicas en organismos destinados a consumo humano y no se han establecido límites reguladores para ninguno de los compuestos pertenecientes a este grupo de toxinas. Sin embargo, debido a la alta toxicidad de algunos de estos compuestos durante los bioensayos, algunos polígonos se mantuvieron cerrados por precaución [23], por

lo que es probable que la exposición humana a niveles suficientemente altos de estas toxinas haya sido por el momento mínima.

Espirólidos

Los espirólidos (SPXs), el subgrupo de toxinas más amplio del grupo de las iminas cíclicas, se descubrieron durante una monitorización rutinaria para la detección de toxinas DSP en extractos de las glándulas digestivas de mejillones (*Mytilus edulis*) y vieiras (*Placopecten magellanicus*) de la costa de Nueva Escocia, Canadá, a principios de la década de los noventa [15].

Los dinoflagelados marinos, *Alexandrium ostenfeldii* y *Alexandrium peruvianum*, son los organismos responsables de la producción de SPXs [24-26], y se han descrito en múltiples localizaciones, entre ellas, Canadá [15], Escocia [27], Noruega [28], Dinamarca [29], España [30], Francia [23], Chile [31], Estados Unidos (Golfo de Maine) [32] y el Mar Adriático [33].

Estas toxinas se han encontrado en diversas especies de moluscos filtradores como mejillones, vieiras, navajas, ostras o almejas [15,30,31,34].

Hoy en día se conocen 14 análogos de estos compuestos: espirólido A, espirólido B, espirólido C, espirólido D, espirólido E, espirólido F, espirólido G, espirólido H, espirólido I, 13-desmetil espirólido C, 13,19-didesmetil espirólido C, 27-hidroxi-13,19-didesmetil espirólido C, 13-desmetil espirólido D y 20-metil espirólido G (Figura 1).

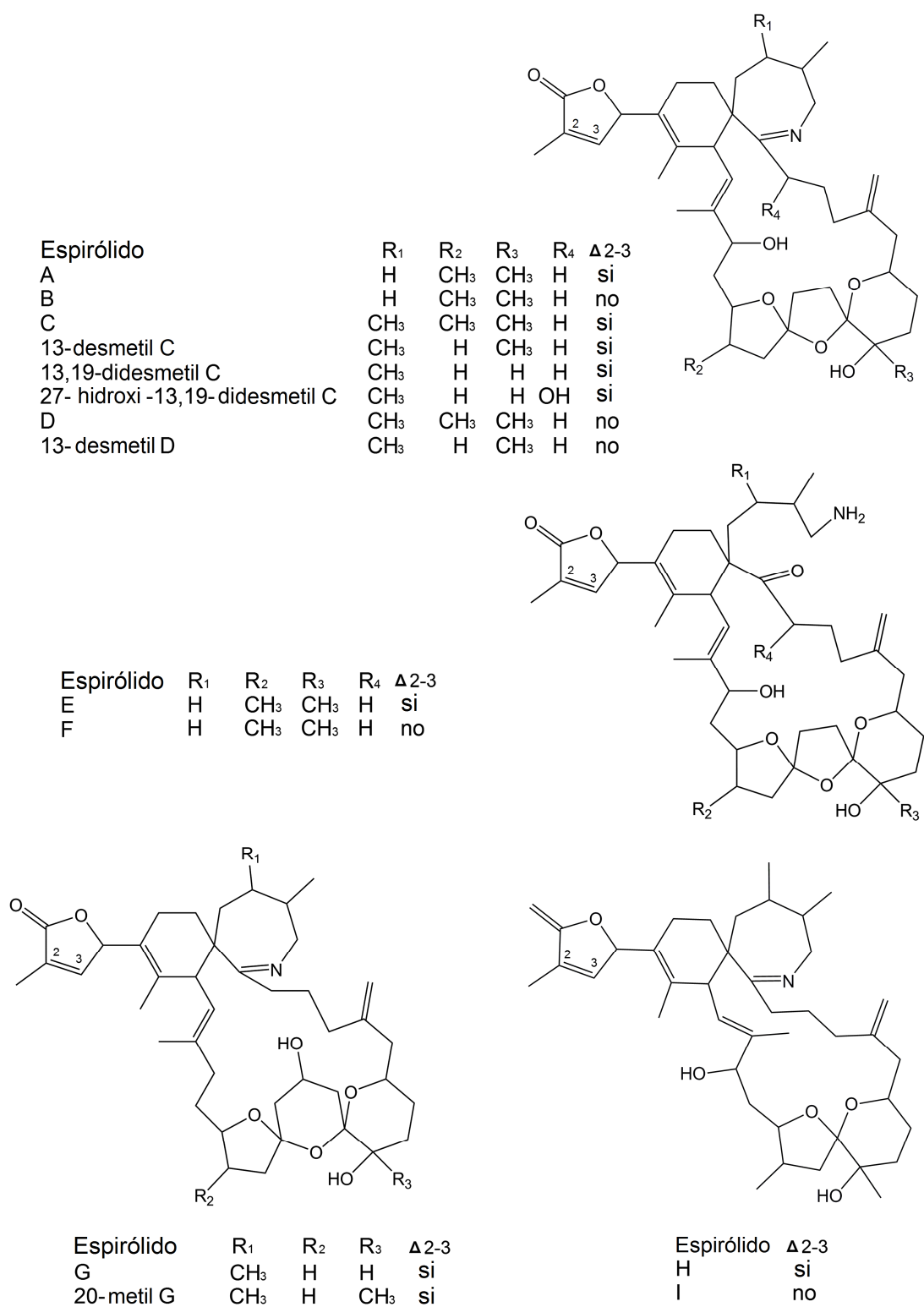


Figura 1. Estructura química de los espirólidos

Los SPXs resultan más tóxicos administrados por vía intraperitoneal (i. p.) que por vía oral, como se ha demostrado en un estudio reciente en el que se obtuvieron valores de DL_{50} de 6.9, 160 y 1000 $\mu\text{g/kg}$ tras la administración del análogo 13-desmetil espirólido C por vía i. p., por sonda intragástrica y mediante la administración oral con comida respectivamente [35].

El grupo funcional imina cíclica parece ser una condición necesaria para la toxicidad de estos compuestos, ya que en análogos como el espirólido E o el espirólido F, en los que este grupo desaparece por la apertura del anillo, la toxicidad es mucho menor [36].

El mecanismo de acción de los SPXs se relacionó inicialmente con los receptores colinérgicos muscarínicos [22], pero hoy en día se ha demostrado que la diana principal de estos compuestos son los receptores colinérgicos nicotínicos, tanto neuronales como musculares, a los que se unen con afinidades subnanomolares (Tabla 1) bloqueando su activación [37].

Tabla 1. Constantes de disociación (K_d) determinadas en ensayos de unión competitivos para el SPX con los subtipos de receptores neuronales ($\alpha 4\beta 2$ y $\alpha 3\beta 2$) y musculares ($\alpha 1_2\beta\gamma\delta$) expresados en células HEK293 [37].

Ligando	K_d , nM		
	$\alpha 1_2\beta\gamma\delta$	$\alpha 4\beta 2$	$\alpha 3\beta 2$
SPX	0.08	0.58	0.021

Aunque no hay evidencias inequívocas de casos de intoxicación en humanos debido al consumo de productos contaminados con estas toxinas, la elevada toxicidad de los SPXs durante los bioensayos y la toxicidad oral, hace que

algunos expertos recomienden el establecimiento de un límite regulador para estos compuestos de 400 µg/kg en la carne de molusco para salvaguardar la salud de los consumidores [35,38].

Gymnodiminas

En 1994, los extractos de ostras (*Tiostrea chilensis*) del Estrecho de Foveaux, en Nueva Zelanda, mostraron una toxicidad neurológica inusual durante un bioensayo rutinario para la detección de toxinas lipofílicas [39]. Durante ese mismo período de tiempo, se observaron floraciones de *Gymnodinium cf. mikimotoi*, por lo que al nuevo compuesto aislado de las ostras contaminadas se le denominó gymnodimina (también llamada gymnodimina-A) [17]. Hoy en día al dinoflagelado planctónico productor de gymnodimina se le conoce como *Karenia selliformis* [40].

Las gymnodiminas (GYMs) son, junto con los SPXs, el subgrupo mejor caracterizado de las iminas cíclicas descritas hasta el momento. Estos compuestos han sido identificados en Túnez [41,42], Sudáfrica [43], Australia [44], Nueva Zelanda [45], China [46], Norte América y Europa [47]. Su amplia distribución puede deberse, en parte, al transporte a través del agua de lastre de los barcos.

Estos compuestos se han encontrado en moluscos filtradores como mejillones, ostras, vieiras, almejas u orejas de mar [45-47].

Además de la GYM-A se han descrito otros tres análogos: GYM-B, GYM-C y recientemente, 12-metilgymnodimina (Figura 2) [48-50]. Esta última se encontró en el dinoflagelado *Alexandrium peruvianum*, organismo causante de la producción de SPXs, lo cual sugiere una posible vinculación genética entre ambos compuestos.

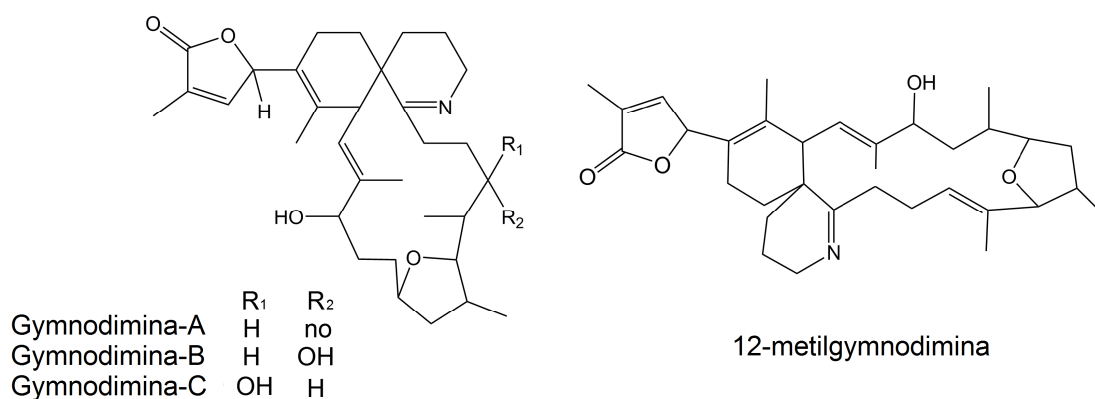


Figura 2. Estructura química de las gymnodiminas

Los estudios de toxicidad realizados en ratones indican que la GYM-A es más tóxica administrada por vía intracerebrovascular ($DL_{50}=3 \mu\text{g/kg}$) que por vía i. p. ($DL_{50}=80 \mu\text{g/kg}$) [51] y su toxicidad disminuye aún más al ser administrada con la comida, ya que concentraciones superiores a los $7500 \mu\text{g/kg}$ no causan ningún tipo de toxicidad en animales de laboratorio [52].

Estudios recientes demuestran que estas toxinas se unen con gran afinidad a los receptores nicotínicos, compartiendo así diana con los SPXs [37,51].

No se conocen casos de toxicidad en humanos por la presencia de estas toxinas en alimentos y no se han establecido límites reguladores para las GYMs, sin embargo, en base a la toxicidad provocada por estos compuestos en los animales de laboratorio, algunos investigadores recomiendan establecer un límite máximo de 18 mg/kg en carne de molusco [53].

Pinnatoxinas

Las pinnatoxinas (PnTXs) (Figura 3) son un grupo de ficotoxinas aisladas inicialmente del bivalvo *Pinna attenuata* en el sur de China en 1990 [54].

El dinoflagelado *Vulcanodinium rugosum*, aislado en aguas del Mediterráneo [55], ha sido identificado como el organismo productor de estos compuestos en

Australia, Nueva Zelanda y Japón [56]. Recientemente se han encontrado PnTXs en muestras de Noruega [57] y Canadá [58].

Las PnTXs, además de encontrarse en los bivalvos *Pinna muricata*, *Pinna attenuata* y *Pteria penguin*, se han aislado de otras ostras, navajas y mejillones [57-60].

Cinco años después de su aislamiento se caracterizó la estructura molecular de la PnTX A [21], seguida de los análogos PnTX B, PnTX C y PnTX D [18,59,61]. Más tarde, en Australia y Nueva Zelanda, se descubrieron la PnTX E, PnTX F y PnTX G [60].

Se ha demostrado mediante estudios realizados en ratones que consumieron voluntariamente PnTX F, que este compuesto es el más tóxico de entre todas las iminas cíclicas estudiadas hasta ahora, con una DL_{50} de 50 $\mu\text{g/kg}$ [62]. La elevada toxicidad oral de la PnTX F suscita gran preocupación debido a la posibilidad de que este compuesto pueda tener efectos adversos en los consumidores de moluscos, aunque hasta hoy no se ha relacionado de forma inequívoca con casos de intoxicación humana.

Hasta hace poco tiempo la única información acerca del mecanismo de acción de las pinnatoxinas era la participación de la PnTX A en la activación de los canales de calcio [54]. Sin embargo, recientemente se ha llevado a cabo un estudio que demuestra que la PnTX A, al igual que otros miembros de las iminas cíclicas, actúa como bloqueante de los subtipos de receptores nicotínicos neuronales y musculares [63].

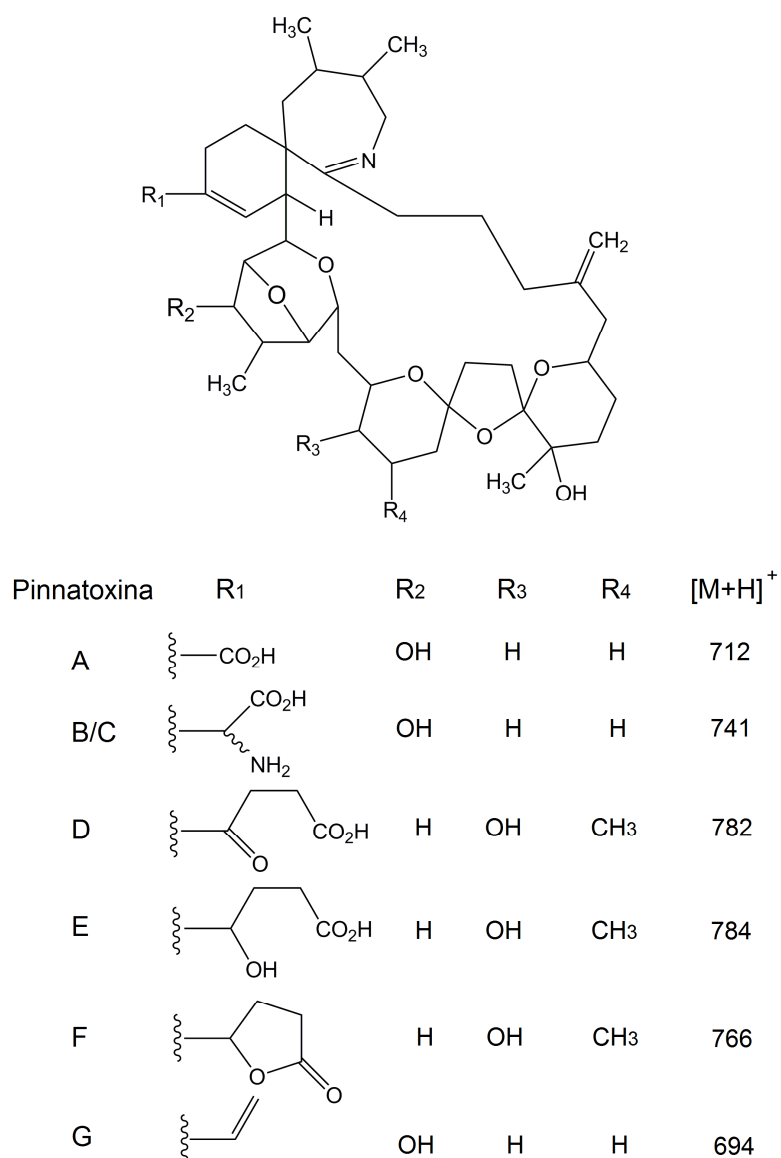


Figura 3. Estructura química de las pinnatoxinas

Pteriatoxinas

Las pteriatoxinas (PtTXs) se aislaron en 2001 de extractos de las vísceras de la ostra *Pteria penguin* [19]. Hasta hoy se han descrito tres análogos (PtTX A, PtTX B y PtTX C) (Figura 4) y se ha estudiado su toxicidad en ratones mediante inyección i. p. obteniendo valores de DL₉₉ de 100 µg/kg y 8 µg/kg para la PtTX A y para una mezcla 1:1 de PtTX B y PtTX C respectivamente [19].

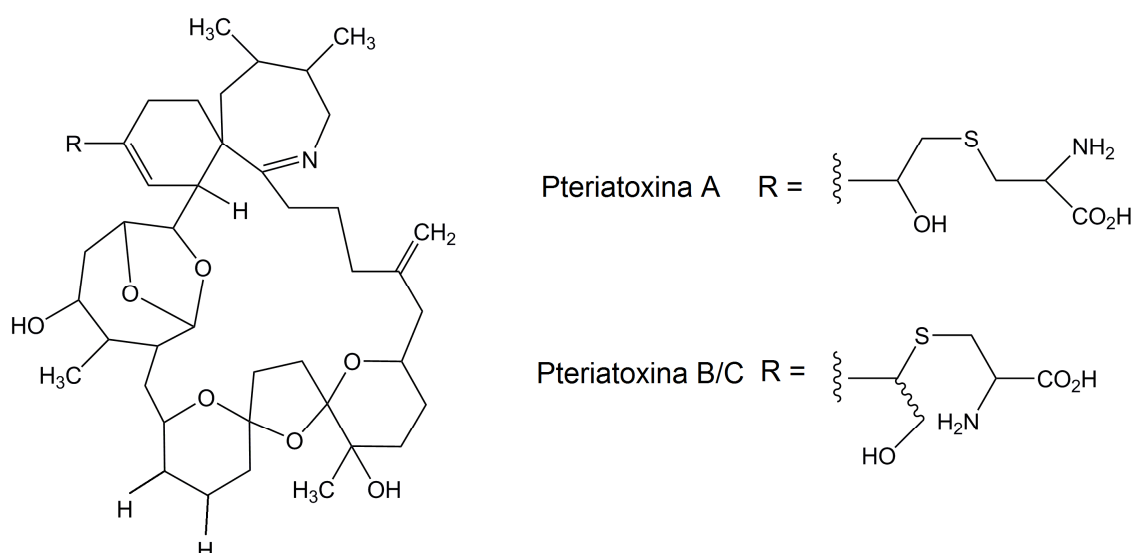


Figura 4. Estructura química de las pteriatoxinas

La toxicidad oral y los mecanismos de acción de estas toxinas se desconocen por el momento.

Prorocentrólidos

El prorocentrólido A (Figura 5) se aisló por primera vez en 1988 en la isla de Sesoko, en Okinawa, del dinoflagelado *Prorocentrum lima* [20]. Más tarde, se describió el prorocentrólido B (Figura 5), aislado del dinoflagelado *Prorocentrum maculosum* [64].

Aunque no se tienen muchos datos sobre su toxicidad y mecanismo de acción, se sabe que los prorocentrólidos producen una respuesta rápida cuando se inyectan en los ratones, provocándoles la muerte poco después de la administración [64].

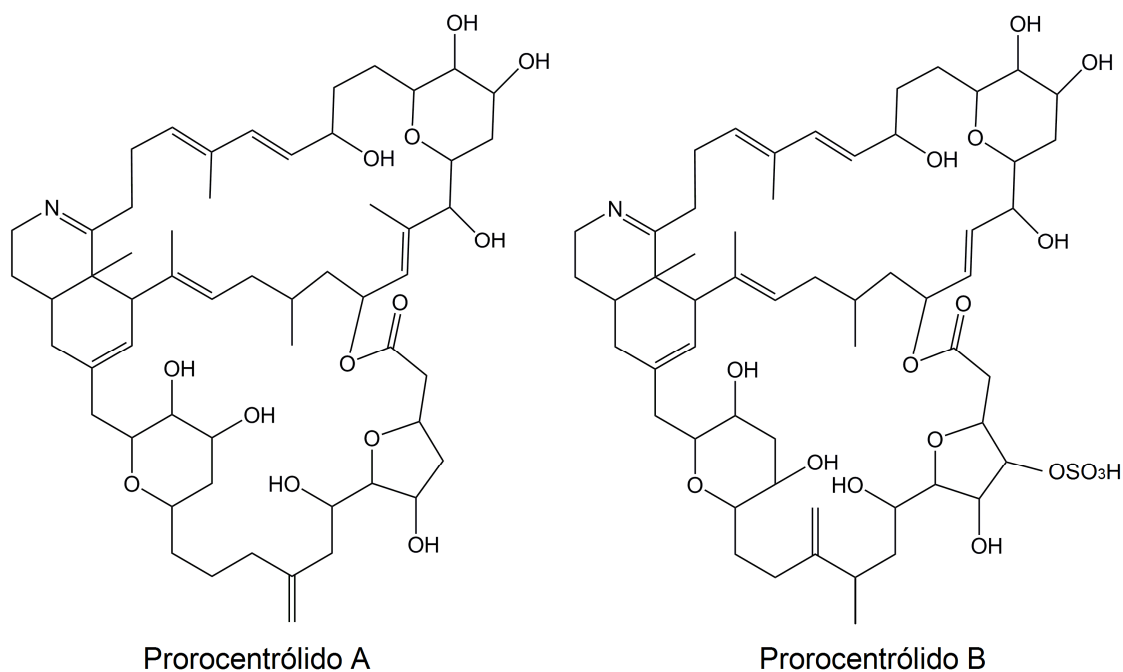


Figura 5. Estructura química de los prorocentrólidos

Espiro-prorocentriminas

La espiro-prorocentrimina (Figura 6) es una toxina polar liposoluble aislada de una cepa bentónica, *Prorocentrum sp.* en Taiwan [16]. Al igual que las toxinas descritas anteriormente, contienen una imina cíclica en su estructura, sin embargo su toxicidad, tras ser inyectada i. p., es mucho más baja ($DL_{99}=2500 \mu\text{g/kg}$) [16].

No existen datos acerca de su mecanismo de acción.

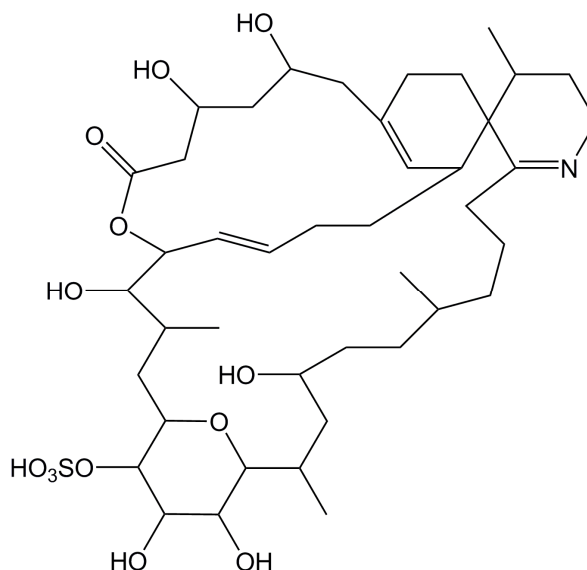
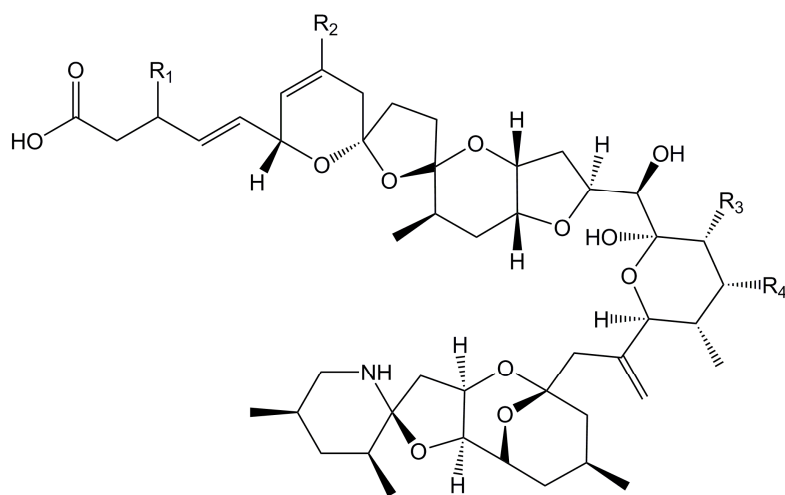


Figura 6. Estructura química de la espiro-prorocentrimina

1.1.1.2. Azaspirácidos

Los azaspirácidos (AZAs) (Figura 7), responsables del nuevo síndrome de intoxicación por azaspirácido (AZP, del inglés, *azaspiracid poisoning*), fueron descubiertos en 1995 tras la intoxicación de varias personas en Holanda debido a la ingesta de mejillones contaminados procedentes de Irlanda [65,66]. Los síntomas observados en las personas afectadas (nauseas, diarrea o vómitos) eran similares a los provocados por el ácido okadaico y las dinofisistoxinas, causantes de la intoxicación diarreica. Sin embargo, en los análisis de los moluscos contaminados se observaron niveles traza de toxinas DSP [65,67]. La estructura química del principal compuesto encontrado en los moluscos contaminados, el AZA-1, se propuso inicialmente en 1998 [66], pero, años más tarde, se demostró, mediante la realización de estudios sintéticos, que aquella primera estructura era errónea [68,69] y se propuso la verdadera estructura química de estas toxinas [70-74].



	R ₁	R ₂	R ₃	R ₄
AZA-1	H	H	CH ₃	H
AZA-2	H	CH ₃	CH ₃	H
AZA-3	H	H	H	H
AZA-4	OH	H	H	H
AZA-5	H	H	H	OH
AZA-6	H	CH ₃	H	H
AZA-7	OH	H	CH ₃	H
AZA-8	H	H	CH ₃	OH
AZA-9	OH	CH ₃	H	H
AZA-10	H	CH ₃	H	OH
AZA-11	OH	CH ₃	CH ₃	H
AZA-12	H	CH ₃	CH ₃	OH
AZA-13	OH	H	H	OH
AZA-14	OH	CH ₃	H	OH

Figura 7. Estructura química de algunos azaspirácidos

En 1997 en la isla de Arranmore, sucedió otro caso de intoxicación en humanos debido a moluscos contaminados y se descubrieron dos nuevos análogos, el 8-metilazaspirácido y el 22-dimetilazaspirácido, llamados AZA-2 y AZA-3 respectivamente [75].

El dinoflagelado *Azadinium spinosum* es el organismo productor de estas toxinas [76], que han sido localizadas en distintos países de Europa [66,75,77-79], en el noroeste de África [80], en América [31,81] y en Japón [82].

Aunque las intoxicaciones humanas se han producido debido a la presencia de AZAs en mejillones, estas toxinas se han encontrado en otros moluscos filtradores como vieiras, ostras, berberechos, almejas o navajas, y en algunas especies de crustáceos y esponjas [31,75,77,78,82-88].

Hoy en día se han descrito más de 20 análogos [77], de los cuales el AZA-1, el AZA-2 y el AZA-3, son los más importantes debido a su toxicidad y a su mayor frecuencia de aparición [31,66,78-82,88].

Tras realizarse estudios de toxicidad en ratones se demostró que los análogos AZA-1, AZA-2 y AZA-3, son los más tóxicos con valores de DL_{50} (tras la administración i. p.) de 200 $\mu\text{g/kg}$, 110 $\mu\text{g/kg}$ y 140 $\mu\text{g/kg}$ respectivamente [66,75], seguidos de el AZA-4 ($DL_{50} = 470 \mu\text{g/kg}$) y el AZA-5 ($DL_{50} = 1000 \mu\text{g/kg}$) [89]. Durante otras investigaciones se observó que el AZA-1 provocaba daños en diversos órganos en animales de laboratorio (estómago, intestino delgado, hígado, bazo...) e incluso que causaba la aparición de tumores [90-92].

Aunque por el momento se desconoce el mecanismo de acción de los AZAs, se ha demostrado que estas toxinas inducen alteraciones morfológicas y en el citoesqueleto de actina en diferentes líneas celulares [93,94]; alteran el sistema E-cadherina en células epiteliales [93]; aumentan los niveles de AMPc (adenosina 3',5'-monofosfato cíclico) y de calcio citosólico [95,96]; provocan la activación de las caspasas en células de neuroblastoma humano y en neuronas neocorticales [97,98]; aumentan los niveles de transcripción de algunos genes implicados en la síntesis de colesterol y ácidos grasos en linfocitos humanos tratados con AZAs [99], o generan un aumento en los niveles nucleares de la

forma activa fosforilada de la quinasa c-Jun-N-terminal [100-102], entre otros efectos *in vitro*.

Basándose en los datos de toxicidad en humanos obtenidos durante los episodios tóxicos, y asumiendo un consumo diario de 250 g de carne de molusco y un peso medio de 60 kg por persona, las regulaciones europeas han establecido un límite de 160 µg de equivalentes de AZA por kg de carne de molusco destinado a consumo humano [10]. Este límite se ha establecido también en Chile y Nueva Zelanda [103].

Por otro lado, en base a los datos de toxicidad *i. p.* obtenidos durante los bioensayos, en algunos países como Irlanda, Noruega, Alemania o Reino Unido, en donde se utilizaba la cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS, del inglés *Liquid Chromatography tandem Mass Spectrometry*), como método complementario a los ensayos *in vivo*, se establecieron los siguientes factores de toxicidad equivalentes (TEF, del inglés *toxic equivalency factor*): (AZA-1 = 1.0, AZA-2 = 1.8, AZA-3 = 1.4, AZA-4 = 0.4 y AZA-5 = 0.2) para convertir la concentración de AZAs a equivalentes de toxicidad de AZA-1 [104].

1.1.1.3. Ácido okadaico

El ácido okadaico (AO), aislado originariamente de esponjas del género *Halichondria* [105], y sus análogos, las dinofisistoxinas (DTXs) forman el grupo del ácido okadaico, también llamadas toxinas diarreicas o DSP (del inglés *diarrhetic shellfish poisoning*) (Figura 8). Dentro de las DTXs se diferencian los derivados "libres" (DTX-1 y DTX-2) y los esterificados (DTX-3 en moluscos y DTX-4, DTX-5, DTX-6 y ésteres diol en microalgas) [106-109].

Estas toxinas, presentes hoy en día en todos los continentes [110-115], deben su aparición a dinoflagelados de los géneros *Dinophysis* y *Prorocentrum*

[116,117] y causan toxicidad en los humanos debido a su acumulación en las glándulas digestivas de moluscos filtradores como mejillones, vieiras, ostras, berberechos, navajas o almejas, así como en algunos crustáceos y gasterópodos [118-122].

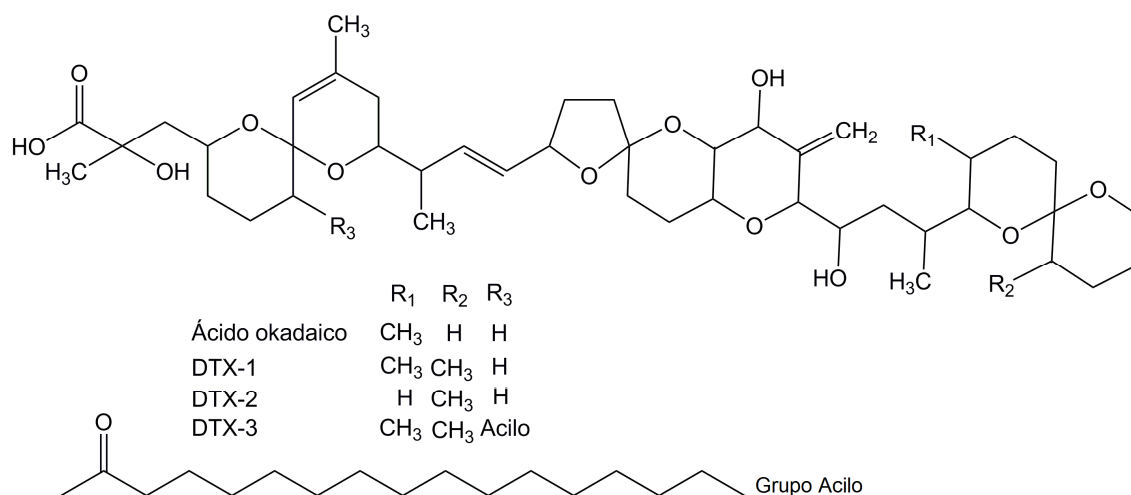


Figura 8. Estructura química del ácido okadaico y las dinofisistoxinas.

El AO resulta más tóxico administrado de forma i. p. que por vía oral, como se ha demostrado en estudios realizados en ratones, en los que se obtuvieron $DL_{50} = 192-225 \mu\text{g/kg}$ y $DL_{50} = 1-2 \text{ mg/kg}$, tras la administración i. p. y oral respectivamente [123,124].

En cuanto a su mecanismo de acción, se ha demostrado que estos compuestos inhiben las fosfatasa de proteína de serina/treonina 1 (PP1) y 2A (PP2A) [125-127], importantes en el control de funciones celulares en células eucariotas [128]. En otros estudios se ha descrito la actividad como promotor tumoral y genotóxica del AO [129-131].

Actualmente el límite máximo permitido para el AO en la Unión Europea, junto con las DTXs y las pectenotoxinas, es de $160 \mu\text{g}$ de equivalentes de AO/kg [10].

Límites similares se han establecido en países como Chile, Nueva Zelanda o Australia [103].

1.1.1.4. Yessotoxinas

La yessotoxina (YTX) (Figura 9) se aisló por primera vez en 1986 de las glándulas digestivas de la vieira *Patinopecten yessoensis*, en la Bahía de Mutsu (Japón) [132]. Desde entonces, esta toxina producida por dinoflagelados de los géneros *Protoceratium reticulatum* [133], *Lingulodinium polyedrum* [134] y *Gonyaulax spinifera* [135], se ha encontrado en Noruega [136], Italia [137], España [138], Rusia [139,140], Nueva Zelanda y Chile [141].

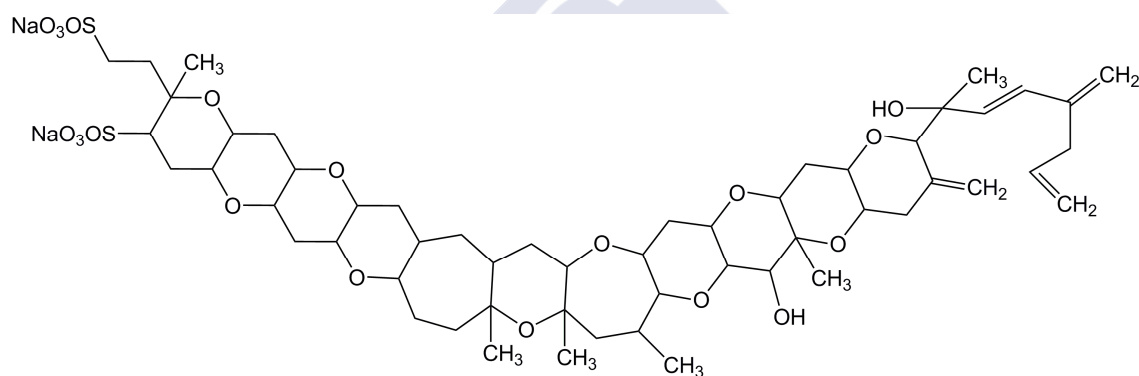


Figura 9. Estructura química de la yessotoxina.

Además de concentrarse en las glándulas digestivas de las vieiras, las YTXs se han encontrado en otros moluscos como mejillones y ostras, y recientemente en gasterópodos [122,142-145].

En un principio la YTX se incluyó dentro del grupo de las toxinas diarreicas, ya que se extraía de moluscos contaminados junto con el AO y las DTXs. Hoy en día, tras comprobarse que este compuesto no produce diarrea ni actúa inhibiendo fosfatasa de proteínas (principal diana del AO), la YTX se ha clasificado en un grupo aparte.

El mecanismo de acción de las YTXs no está del todo claro, sin embargo algunas investigaciones han demostrado que estos compuestos modulan la homeostasis del calcio en linfocitos humanos [146]; disminuyen el nivel de AMPc intracelular mediante la activación de fosfodiesterasas [147]; inducen apoptosis en células de neuroblastoma [148] y células Hela humanas mediante la activación de distintas isoformas de caspasas [149]; y provocan la rotura selectiva del sistema E-cadherina-catenina en células epiteliales [150].

Hasta hoy no se han dado casos de intoxicación en humanos, sin embargo, durante los bioensayos, estas toxinas provocan la muerte de los animales tras la administración de 100 µg/kg por vía i. p.. La toxicidad oral de estos compuestos es mucho menor, ya que la concentración más alta administrada (10 mg/kg) no produce la muerte de los ratones. [151].

Actualmente en Europa se ha establecido un límite regulador para este grupo de toxinas, de 3.75 mg de equivalentes de YTX por kg de carne de molusco [152].

1.1.1.5. Ácido domoico

El primer envenenamiento documentado provocado por ácido domoico (AD) (Figura 10) ocurrió en 1987 en el este de Canadá tras el consumo de mejillones contaminados [153]. Desde entonces, este compuesto se ha detectado en Estados Unidos y en gran número de países europeos [154-159].

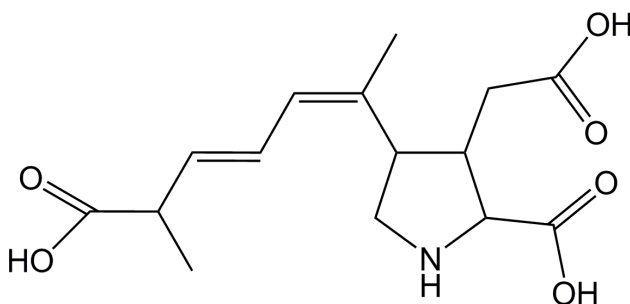


Figura 10. Estructura química del ácido domoico.

El AD es un aminoácido cíclico hidrosoluble, producido principalmente por el alga roja *Chondria armata* [160,161] y diatomeas de los géneros *Pseudo-Nitzschia* [153,162,163] y *Nitzschia* [164,165].

Además de acumularse en mejillones, este compuesto se ha aislado de navajas, almejas, vieiras, crustáceos o peces, como las anchoas y las caballas [164,166-169].

El AD y sus isómeros son los responsables del síndrome ASP (del inglés, *amnesic shellfish poisoning*) cuyos síntomas pueden ser gastrointestinales (nauseas, vómitos, diarrea, dolores abdominales...) o neurológicos (pérdida de memoria y, en los casos más graves, coma y muerte). Este compuesto es un análogo del glutamato y se une a los receptores de éste produciendo un estado de hiperexcitabilidad seguido de un efecto neurotóxico. Los principales receptores sobre los que actúa el AD son el receptor de NMDA (N-metil-D-aspartato), y los no-NMDA (receptores de AMPA (ácido α -amino-3-hidroxi-5-metil-4-isoxazol-propiónico) y de kainato) [170,171].

El límite establecido en Europa para este grupo de toxinas es de 20 mg de AD por kg de carne de molusco [10], correspondiente con los límites establecidos en otros países como Canadá, Estados Unidos, Perú, México, Australia o Nueva Zelanda [14,103].

1.1.1.6. Saxitoxina

La saxitoxina (STX) (Figura 11) fue el primer compuesto de la familia de las toxinas paralizantes, en aislarse en 1957 de la almeja *Saxidomus giganteus* [172].

El grupo de las toxinas PSP está formado por más de 20 neurotoxinas, que se subdividen en: sulfocarbamadas, decarbamadas, deoxidecarbamadas y

carbamadas, siendo estas últimas (entre las que se encuentra la STX) las más tóxicas.

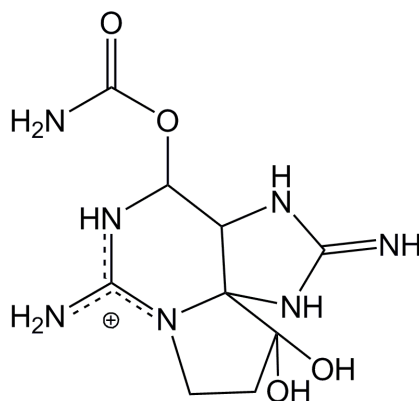


Figura 11. Estructura química de la saxitoxina

Desde la primera intoxicación en humanos producida de forma inequívoca por la ingesta de mejillones contaminados con estas toxinas en 1927 [173], el número de incidentes tóxicos se ha ido extendiendo por todo el planeta [174-179].

Tanto los dinoflagelados de los géneros *Alexandrium*, *Gymnodinium* y *Pyrodinium* [180-182] como las cianobacterias *Aphanizomenon flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei* y *Cylindrospermopsis raciborskii* [183-186] son los responsables de la producción de estas toxinas.

Los mariscos asociados a casos de intoxicación provocados por las toxinas paralizantes o PSP, son principalmente bivalvos filtradores como mejillones, almejas, y en menor proporción, ostras, vieiras y berberechos [14]. Pero además de los anteriores moluscos, estas toxinas se han encontrado en orejas de mar, en distintas especies de crustáceos, en estrellas de mar y en peces como el pez globo, las caballas, peces de agua dulce, etc [187-192].

En cuanto a su mecanismo de acción, se sabe que estos compuestos se unen de forma específica y reversible al sitio 1 de la subunidad α de los canales de sodio dependientes de voltaje [193], inhibiendo su apertura y como consecuencia, disminuyendo o impidiendo la propagación de los impulsos nerviosos, lo que provoca síntomas tales como un entumecimiento o picazón leve, hasta la parálisis respiratoria completa, llegando a provocar la muerte.

Actualmente en la Unión Europea (UE) y en países como Australia, Nueva Zelanda, Chile, Canadá, México, Marruecos, Perú, Singapur, Estados Unidos, Uruguay o Venezuela, existe un límite máximo permitido de 800 μg de equivalentes de STX por kg de carne de molusco [10,103].

1.1.1.7. Anatoxina-a

La anatoxina-a (ANTX) (Figura 12) es una amina secundaria bicíclica de bajo peso molecular que fue descrita por primera vez en la década de los setenta [194]. Esta toxina la sintetizan cianobacterias de los géneros *Anabaena* [195], *Aphanizomenon* [196], *Cylindrospermum* [197], *Microcystis* [198], *Oscillatoria* [197], *Planktothrix* [199] y *Raphidiopsis* [200]. Dos décadas más tarde, se aisló un compuesto similar a la ANTX de una cianobacteria perteneciente al género *Oscillatoria*, la homoanatoxina-a [201].

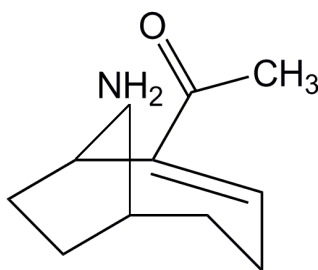


Figura 12. Estructura química de la anatoxina-a

En cuanto a su mecanismo de acción, la ANTX es un potente agonista de los receptores colinérgicos nicotínicos a los que se une con gran afinidad, de manera que se puede producir un bloqueo neuromuscular, que en pocos minutos causa la muerte por parálisis respiratoria [202,203]. De hecho, tras realizarse un estudio en ratones a los que se les administraron dosis letales de ANTX por vía i. p. (250 µg/kg), los animales murieron en un período de 2-5 minutos, por lo que a este compuesto se le consideró como un "factor de muerte rápida" [194].

Otros estudios realizados *in vitro* mostraron la citotoxicidad de la ANTX sobre distintas líneas celulares como linfocitos, timocitos o células renales [204,205].

Aunque han sido varios los casos de intoxicaciones en humanos producidos por cepas tóxicas de cianobacterias (por vía oral mediante el agua de bebida, por inhalación o por contacto con la piel durante actividades recreativas, o por vía intravenosa durante el tratamiento de pacientes sometidos a hemodiálisis) [206-208], hasta hoy la ANTX no se ha relacionado con ningún episodio tóxico en humanos. Sin embargo, la proliferación masiva de cianobacterias neurotóxicas productoras de ANTX en la orilla de lagos y ríos, ha provocado la muerte de distintas especies de animales silvestres en Canadá [209], Escocia [210], Francia [211,212], Kenia [213] o Nueva Zelanda [214]. Recientemente se ha detectado la presencia de otras cianotoxinas, como las microcistinas, en ambientes desérticos, y aunque en ese estudio no se detectó ANTX, los investigadores no descartan la presencia de esta toxina en ambientes terrestres [215].

No se han establecido límites reguladores para ANTX, sin embargo algunos expertos sugieren establecer un máximo de 1 µg/L de ANTX en el agua potable [216], y en Nueva Zelanda, el Ministerio de Sanidad ha propuesto un valor aceptable de 6 µg/L de ANTX en el agua destinada a consumo humano [217].

1.1.2. Métodos de detección de ficotoxinas

Los métodos desarrollados para la detección de ficotoxinas se pueden dividir en métodos analíticos y métodos no analíticos [218].

Los métodos analíticos de detección de toxinas se basan en sus propiedades físico-químicas. En estas técnicas, se realiza una separación, identificación y cuantificación de las toxinas en función de una respuesta instrumental proporcional a la concentración de cada una de las toxinas presentes en la muestra. Dicha cuantificación se realiza una vez que se halla calibrado el equipo instrumental con patrones de concentración conocida de cada una de las toxinas a analizar. En este grupo encontramos métodos químicos como el HPLC (cromatografía líquida de alta eficacia, del inglés *High Performance Liquid Chromatography*) asociado a la detección fluorimétrica, colorimétrica o ultravioleta, el LC-MS (cromatografía líquida acoplada a espectrometría de masas, del inglés *Liquid Chromatography-Mass Spectrometry*), la electroforesis capilar, etc.

Los métodos no analíticos, proporcionan un valor del contenido total de toxinas mediante la medición de una respuesta, biológica o bioquímica, normalmente referida a la totalidad o al conjunto de los distintos análogos presentes en la muestra. La toxicidad se determina mediante una curva dosis-respuesta realizada con el compuesto de referencia y generalmente se expresa en equivalentes de dicha toxina. Dentro de esta categoría, se incluyen ensayos *in vivo* como los bioensayos en ratón, rata u otros, y ensayos *in vitro*, como los ensayos de unión a receptor, ensayos celulares, inmunoensayos...

Los ensayos *in vivo* o bioensayos, han sido los métodos oficiales utilizados para la detección de la mayoría de las ficotoxinas (excepto para el grupo del ácido domoico) tanto en Europa [10] como en otras partes del mundo, incluyendo

países como Canadá, Japón, Chile, México, Australia, Argentina, Nueva Zelanda, Singapur, Marruecos, Panamá, Estados Unidos, Uruguay, Venezuela... durante muchos años [103]. Estas técnicas consisten en la administración a un animal de la muestra a analizar o un extracto de la misma y en la observación de los síntomas que presenta el animal y/o el tiempo que tarda en morir, parámetro a través del cual se cuantifica la toxicidad del compuesto [219]. La administración de la muestra por vía oral es la que mejor simula el modo de intoxicación en humanos, sin embargo durante la administración i. p., las condiciones pueden ser más controladas y las funciones asociadas al tracto gastrointestinal, que podrían afectar a la toxicidad, no se tienen en cuenta.

A pesar de que estos métodos han demostrado su eficacia en la protección de la salud pública durante décadas [220], estas técnicas resultan poco específicas, tienen una baja sensibilidad y están sujetas a variaciones, ya que requieren el mantenimiento y control de grandes reservas de ratones en las que parámetros como la cepa, la edad, el sexo o el estado general del animal pueden afectar a la supervivencia de los individuos y por tanto a los resultados del ensayo [220]. Además de las limitaciones técnicas, los bioensayos resultan poco éticos debido al gran número de animales sacrificados cada año con fines experimentales, por esta razón, desde 1986 en Europa se aboga por su reducción o reemplazo [221].

Recientemente, la legislación europea ha dado un plazo de tres años (desde enero de 2011) para sustituir el bioensayo por el LC-MS/MS como método de referencia para la detección de toxinas lipofílicas [13]. Este método analítico, altamente sensible y específico, permite la detección y cuantificación inequívoca de compuestos que hayan sido caracterizados. Sin embargo, los equipos necesitan ser calibrados con estándares certificados de las toxinas que se vayan a analizar, muchos de ellos no disponibles a día de hoy en el mercado, por lo que la detección de nuevos compuestos o de aquellos para los que no haya

estándares, se perdería con estas técnicas. Además de requerir equipos costosos y personal especializado, otro de los inconvenientes de los métodos analíticos es la necesidad de obtener factores de equivalencia tóxica (TEF) de cada análogo para convertir los resultados analíticos a valores de toxicidad equivalentes de la toxina de referencia de cada grupo [222], ya que estos TEF no existen para todos los grupos, ni para todos los análogos pertenecientes a un grupo.

De los métodos analíticos existentes hasta ahora, el HPLC con detección por fluorescencia para las toxinas PSP [223], el HPLC con detección ultravioleta para el ácido domoico y sus análogos [224], y el LC-MS para toxinas lipofílicas [225] se han validado oficialmente y presentado como alternativa real al bioensayo [11-13].

La necesidad de reducir o reemplazar los bioensayos, junto con la creciente distribución de los incidentes tóxicos y la aparición de nuevos análogos, hacen cada vez más necesario el desarrollo de técnicas complementarias a los métodos analíticos que proporcionen métodos de cribado capaces de determinar la presencia/ausencia de compuestos tóxicos, reduciendo el procesado de muestras mediante métodos analíticos más costosos o laboriosos, con los que se analizarían únicamente aquellas muestras de las que se requiera una cuantificación exacta.

En las últimas décadas se han desarrollado diversos ensayos *in vitro*, como los ensayos celulares, los inmunoensayos o los ensayos de receptor con el fin de proporcionar métodos fiables para la detección de ficotoxinas.

Los ensayos celulares, estudian los efectos que una toxina o un grupo de toxinas ejercen sobre la funcionalidad celular de células *in vitro*. Entre estos métodos se encuentran, por un lado, los que determinan la viabilidad de las células tras la exposición a las toxinas; y por otro, aquellos que tratan de evaluar una función

particular. Entre los que determinan la viabilidad celular, se encuentran algunos métodos como los desarrollados mediante observaciones morfológicas de cultivos primarios de hepatocitos de rata o células de neuroblastoma para detectar ácido okadaico, dinofisistoxina-1, yessotoxina, saxitoxina, tetrodotoxina, palitoxina, pectenotoxina-2 o brevetoxina-3 [226-228]; los que evalúan la integridad en las membranas celulares de fibroblastos para la detección de maitotoxina y ácido okadaico [229]; los que producen alteraciones del citoesqueleto, como la despolimerización de filamentos de actina usando células de neuroblastoma, o alteraciones en la actividad enzimática en células KB para detectar ácido okadaico [230,231]; en los que se observa la activación de vías apoptóticas, como los métodos desarrollados para la detección de yessotoxinas en células HeLa, en las que se produce la activación de varias isoformas de caspasas, o en células MCF-7 por la acumulación del fragmento de E-caderina [149,232]; o aquellos que determinan la funcionalidad mitocondrial para el estudio de la supervivencia o proliferación celular mediante el uso de indicadores de viabilidad como el MTT (bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol) o el Alamar Blue [233-236]. Recientemente se han prevalidado dos métodos citotóxicos para el descarte de falsos positivos durante los bioensayos. El primero de ellos para la detección de ácido okadaico, azaspirácido-1 y pectenotoxina-2 usando células hepáticas (HepG2) e intestinales (Caco2) humanas y células de neuroblastoma de ratón (Neuro2a); y el segundo para la detección de ciguatoxina en pescado usando células de neuroblastoma [237-239].

Algunos de los métodos desarrollados para la evaluación de la funcionalidad celular incluyen aquellos diseñados para la detección de neurotoxinas como las PSP, mediante cambios en el equilibrio iónico usando técnicas de patch-clamp [240,241]; aquellos en los que se observan cambios en el potencial de membrana, mediante ensayos fluorescentes realizados en microplacas para la

detección de brevetoxinas, toxinas PSP y ciguatoxinas [242-244]; o los basados en las medidas de la concentración de calcio en el citosol usando marcadores fluorescentes para la detección de toxinas PSP y ácido domoico [245].

El uso de cultivos celulares permitiría reemplazar a los animales vivos en los ensayos de toxicidad, sin embargo ciertos inconvenientes como la variabilidad inherente a la obtención y mantenimiento de cultivos celulares, o el hecho de que muchos laboratorios carezcan de las instalaciones apropiadas para su realización, hacen difícil su validación y su uso como técnica de análisis rutinario. Otra de las desventajas, es que generalmente la respuesta de los métodos basados en el uso de células no es lo suficientemente específica como para identificar un grupo definido de toxinas, ya que una gran variedad de compuestos tóxicos presentes en la muestra podría causar la muerte celular o una alteración funcional concreta. De hecho, en alguna ocasión extractos de las glándulas digestivas de mejillón que resultaron negativos durante el bioensayo provocaron citotoxicidad en un cultivo celular [246]. A pesar de esto, se han desarrollado ensayos funcionales que introducen un método de confirmación de que la toxicidad se debe a un mecanismo de acción característico de la toxina a estudiar, como es el caso de la palitoxina, en el que la inhibición de la citotoxicidad inducida por la toxina mediante el uso de anticuerpos anti-palitoxina o de ouabaina, un potente inhibidor de la bomba de Na^+/K^+ a la que se une la palitoxina, se ha usado para proporcionar técnicas con mayor especificidad [236,247]

Los ensayos inmunológicos se basan en el uso de anticuerpos capaces de interaccionar con las toxinas. A pesar de la dificultad a la hora de obtener anticuerpos anti-toxina, debido a la falta de toxinas puras y a los propios efectos tóxicos que provocan la muerte del animal tras inmunizaciones repetidas, los inmunoensayos se han utilizado durante décadas para el desarrollo de métodos

de detección de toxinas. Los inmunoensayos más frecuentes son aquellos realizados en formato ELISA (Ensayo por inmunoabsorción ligado a enzimas, del inglés *Enzyme-Linked ImmunoSorbent Assay*) en los que la unión toxina-anticuerpo se detecta mediante la reacción de una enzima ligada al anticuerpo y un sustrato de la enzima que genera una señal medible. Hoy en día hay ELISAs disponibles para la detección de casi todos los grupos de toxinas incluyendo ácido okadaico, saxitoxina, brevetoxinas, azaspirácidos, ácido domoico, yessotoxinas, ciguatoxinas, palitoxinas o tetrodotoxinas [248-255]. De entre todos los ELISAs para detectar ficotoxinas destaca el desarrollado para el ácido domoico, validado a nivel internacional y método oficial en Europa [256].

Además del ELISA convencional, otra forma de detectar la interacción toxina/anticuerpo son los biosensores, en los que la parte biológica (bien sea un anticuerpo, una enzima, una célula...) sensible al analito, está ligada a un transductor, que transforma la interacción del componente detector biológico con el analito en una señal eléctrica cuantificable.

Los inmunosensores, son biosensores que detectan la presencia del analito mediante la unión de éste a un anticuerpo específico, y se han utilizado para desarrollar métodos de detección en el campo de las toxinas. La gran mayoría de inmunosensores son métodos indirectos, en los que la toxina contenida en la muestra a analizar, compete con toxina inmovilizada sobre una superficie, por la unión al anticuerpo. Hoy en día existen múltiples inmunosensores para la detección de distintos grupos de toxinas, incluyendo los biosensores electroquímicos desarrollados para la detección de ácido domoico, brevetoxina, ácido okadaico, o tetrodotoxina [257-261], o biosensores ópticos, como los biosensores basados en la resonancia de plasmón superficial desarrollados para la detección de toxinas PSP, ácido okadaico, ácido domoico o yessotoxina [262-

267] o el desarrollado recientemente para la detección simultánea de toxinas PSP, ácido domoico y ácido okadaico [268].

Los inmunoensayos proporcionan grandes ventajas en términos de sensibilidad, estabilidad y selectividad. Además, resultan más económicos que otros métodos y son fáciles de llevar a cabo, por lo que no requieren personal especializado. Sin embargo, el inconveniente de estas técnicas es que, con frecuencia, la correlación entre el perfil de reactividad cruzada de un anticuerpo con los distintos análogos de un grupo de toxinas, y el potencial tóxico de estos compuestos no es perfecta, salvo en casos excepcionales como es el de un inmunoensayo desarrollado para la detección de toxinas DSP, en el que se utilizó un anticuerpo monoclonal cuya reactividad cruzada con AO, DTX-1 y DTX-2 se correlacionaba con la toxicidad de estos tres compuestos [269].

Otras técnicas utilizadas para la detección de toxinas, pero menos comunes que los inmunoensayos, son los métodos que usan la interacción de las toxinas con sus dianas biológicas, como es el caso de los ensayos basados en la unión a receptor.

En los últimos años se han desarrollado distintos métodos basados en unión a receptor para la detección de distintos grupos de toxinas. Algunos de estos métodos son los desarrollados para la detección de yessotoxinas e iminas cíclicas usando la fluorescencia de polarización [270,271], los métodos para la detección de ácido okadaico usando la PP2A en placa o mediante biosensores electroquímicos [272-275], o los biosensores de espejos resonantes y biosensores basados en la resonancia de plasmón superficial para la detección de yessotoxinas [263,276,277].

Existen otros ensayos de receptor desarrollados hace años para la detección de brevetoxinas y toxinas paralizantes o PSP mediante técnicas de radiactividad

[278,279]. Este tipo de métodos se siguen usando hoy en día con propósitos de investigación [280,281], sin embargo el uso de compuestos radiactivos, que pongan en peligro la seguridad de los operarios, presenta muchos inconvenientes a la hora de utilizar este tipo de técnicas en laboratorios de análisis rutinario.

Los ensayos basados en unión a receptor no requieren el aislamiento de todos los análogos de un grupo de toxinas, para realizar una estimación de la presencia de toxinas en la muestra, sino una toxina representativa del grupo. Además, la probabilidad de que exista una correlación entre la detección en el ensayo y la toxicidad, es mayor que en los inmunoensayos, ya que en este caso se usan las propias dianas biológicas de las toxinas. Sin embargo, estas dianas no siempre están disponibles, y para algunos grupos de toxinas ni siquiera se han descrito, por lo que estas técnicas sólo pueden desarrollarse, por el momento, para un número limitado de compuestos tóxicos.

Durante esta tesis se han desarrollado ensayos basados en unión a receptor e inmunoensayos para la detección de ficotoxinas empleando técnicas luminométricas (quimioluminiscencia, fluorescencia y colorimetría), y la tecnología Luminex, que combina el uso de microesferas con la citometría de flujo. Ambas técnicas, junto con las proteínas utilizadas para el desarrollo de los métodos, se describen a continuación.

1.2. Metodología

1.2.1. Técnicas luminométricas

Quimioluminiscencia, fluorescencia y colorimetría

La quimioluminiscencia (QL) se define como la emisión de radiación electromagnética (normalmente en la región del espectro visible o del infrarrojo cercano) producida por una reacción química [282].

Las reacciones quimioluminiscentes (Figura 13) se clasifican en función del mecanismo que las genere en:

- *Quimioluminiscencia directa*: Se parte de dos reactivos, normalmente un sustrato (A) y un oxidante (B) que reaccionan para formar un producto (P*). Parte de ese producto pasa a un estado electrónicamente excitado, que al relajarse hasta el estado fundamental, emite un fotón ($h\nu$). A veces, en estas reacciones, se requiere un catalizador (C) que disminuya la energía de activación y aumente el rendimiento cuántico de la reacción quimioluminiscente (Figura 13).

- *Quimioluminiscencia indirecta*: El mecanismo es el mismo que en el caso de la QL directa, sin embargo, en este caso para que se origine la emisión del fotón, el producto de la reacción requiere la presencia de un fluoróforo (F*) al cual le transfiere energía, de forma que éste se excita y emite un fotón al regresar a su estado fundamental (Figura 13).

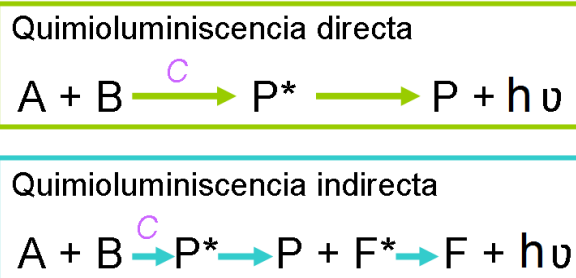


Figura 13. Mecanismos de las reacciones quimioluminiscentes

La intensidad de la emisión producida en los procesos luminiscentes puede expresarse como:

$$I_{QL} = \Phi_{QL} \frac{-dA}{dt}$$

donde I_{QL} es la intensidad de emisión quimioluminiscente (fotones/segundo), Φ_{QL} el rendimiento cuántico (número de fotones / número de moléculas reaccionantes) y $(-dA/dt)$ la velocidad de la reacción.

Aunque el fenómeno de la QL se conoce desde hace mucho tiempo, su uso en aplicaciones analíticas es relativamente reciente. Una de las primeras aplicaciones de la QL como técnica analítica se llevó a cabo durante la década de los sesenta en un estudio realizado para analizar el uso de ciertas sustancias como el luminol o la lucigenina como indicadores volumétricos [283]. Desde entonces el uso de estas técnicas en química, biomedicina, alimentación, medioambiente y toxicología, se ha ido incrementando progresivamente [284-289], y muchas investigaciones se han centrado en el desarrollo de productos quimioluminiscentes para aplicaciones de diagnóstico clínico, pudiendo reemplazar el uso de radioisótopos [290].

do de fondo [295]. Este aumento de la absorbancia se produce al aumentar la incubación del sustrato con la enzima (Figura 10). Este método ofrece una ventaja frente a los ensayos colorimétricos al no requerir una incubación de entre 15 y 60 minutos.

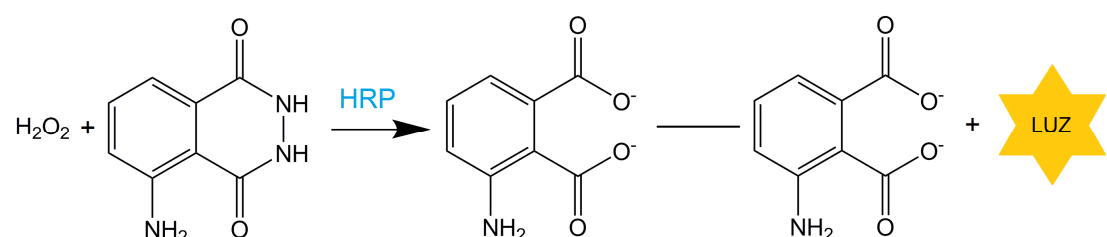


Figura 14. Reacción quimioluminiscente del luminol. La enzima HRP cataliza la reacción del peróxido con el luminol, y se origina un producto excitado, el 3-aminoftalato, que pierde energía en forma de radiación electromagnética (luz) y regresa a su estado fundamental.

Los métodos quimioluminiscentes requieren una instrumentación básica bastante sencilla, ya que el sistema óptico no requiere una fuente externa de excitación [297]. Además, la QL ofrece mayor rango de medida y mayor sensibilidad que la fluorescencia o la colorimetría, ya que se considera una técnica de campo oscuro, y por tanto, en ausencia de niveles altos de luz de fondo se reduce el ruido, lo cual permite mejorar los límites de detección [298]. Por otra parte,

resulta una herramienta útil a la hora de desarrollar métodos de alto rendimiento, ya que permite trabajar con volúmenes pequeños de muestra [299]. Sin embargo deben considerarse ciertas limitaciones, como el hecho de que la emisión de luz quimioluminiscente no es constante sino que varía con el tiempo; la señal alcanza un máximo que se produce tras la mezcla de los reactivos, y después cae progresivamente hasta la línea base [297]. Otro de los inconvenientes de las técnicas quimioluminiscentes como métodos de análisis, es que los lectores de luminiscencia o luminómetros no están disponibles en todos los laboratorios como lo están los de absorbancia o fluorescencia, y en la mayoría de los casos la instrumentación necesaria para realizar estas medidas, sobretodo cuando se requiere gran sensibilidad, resulta algo más costosa que los espectrofotómetros o los fluorímetros [294]. A pesar de esto, hoy en día existen lectores más avanzados que pueden realizar los tres tipos de medidas.

En los ensayos colorimétricos se origina un producto de reacción coloreado que absorbe luz en el espectro visible, siendo la densidad óptica del mismo, proporcional a la cantidad de producto. En el mercado existen numerosos sustratos que interaccionan con las distintas enzimas empleadas en estas técnicas. Las enzimas más utilizadas en los métodos colorimétricos son la peroxidasa y la fosfatasa alcalina [300], ambas estables, económicas y disponibles tanto libres como conjugadas con otras moléculas como los anticuerpos. El sustrato utilizado para los ensayos colorimétricos fue el *1-Step Ultra TMB* de la casa comercial Thermo Scientific. El TMB (3, 3',5',5'-tetrametilbencidina) es el cromógeno más utilizado de los sustratos empleados para la peroxidasa en este tipo de técnicas ya que es el que mayor sensibilidad proporciona. En este caso, la peroxidasa (HRP) cataliza la reacción de oxidación del sustrato (TMB), originando un producto de color azul con una absorción máxima a 370 nm y 652 nm. Tras 30 minutos de incubación, la reacción se detiene al añadir un ácido, en nuestro caso ácido sulfúrico 2M, y el producto azul

se torna amarillo con una absorción máxima a 450 nm (Figura 15) [301]. Los resultados finales de la lectura colorimétrica se reflejan numéricamente mediante valores de absorbancia (Abs), que se obtendrán a la longitud de onda adecuada para el producto de la reacción, en este caso 450 nm.

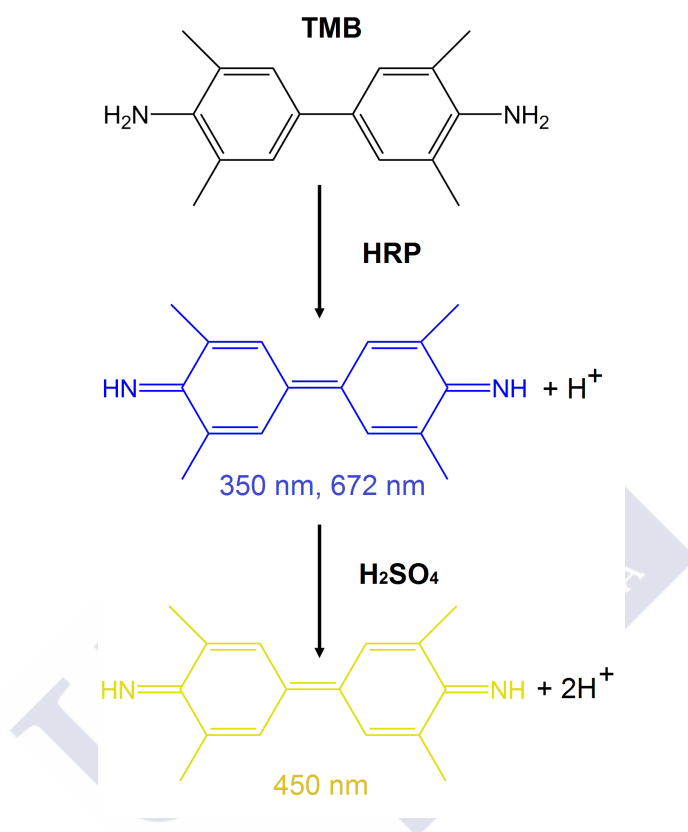


Figura 15. Reacción de la peroxidasa y el TMB

Los métodos colorimétricos son los más utilizados debido a su simplicidad y a su bajo coste, sin embargo estas técnicas tienen menor sensibilidad y menor rango de medida que los métodos quimioluminiscentes o fluorescentes.

En los ensayos fluorescentes, la enzima convierte el sustrato en un producto de reacción que emite fluorescencia cuando se excita a una determinada longitud de onda, siendo las unidades relativas de fluorescencia (fotones de luz emitidos), proporcionales a la cantidad de producto fluorescente.

Existen diversos sustratos en el mercado para la realización de ensayos fluorescentes. En nuestro caso se utilizó el *QuantaRed Enhanced Chemifluorescent HRP Substrate* de la casa comercial Thermo Scientific. Este sustrato contiene potenciadores químicos que aumentan la fluorescencia y la sensibilidad proporcionada por la ADHP (10-acetil-3,7-dihidroxifenoxacina). La ADHP reacciona con la peroxidasa HRP en presencia de peróxido de hidrógeno para producir resorufina, una sustancia soluble y altamente fluorescente con longitudes de onda de excitación y emisión máximas a 570/585 nm [302] (Figura 16).

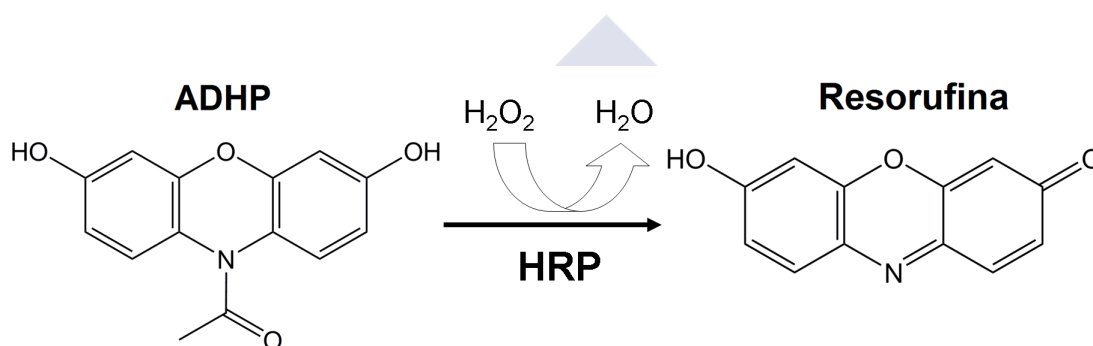


Figura 16. Reacción de la ADHP con la peroxidasa HRP en presencia de peróxido de hidrógeno, que origina un compuesto soluble y altamente fluorescente, la resorufina.

Las ventajas de la fluorescencia frente a la colorimetría y a la quimioluminiscencia son: en primer lugar, la sensibilidad y la amplia selección de fluoróforos para marcado fluorescente disponibles en el mercado, y en segundo lugar, la estabilidad de las moléculas fluorescentes frente a los sustratos quimioluminiscentes [299]. Sin embargo, estos métodos están sujetos a ciertos factores que reducen o aumentan la señal inespecíficamente como son la dispersión de la luz o "light scattering" al entrar en contacto con moléculas, partículas en suspensión o la superficie de la microplaca; la autofluorescencia o "background" que puede producirse por los propios componentes de la muestra, de los diluyentes o del material de la placa; el apagamiento o "quenching" provocado por la absorción de la emisión debido al oxígeno disuelto; o el

blanqueamiento o "bleaching" en donde la señal disminuye debido a un exceso en el tiempo de excitación [300]. Además de estos fenómenos, existen otro tipo de interferencias que pueden distorsionar las lecturas de fluorescencia, como son las variaciones de temperatura, la estabilidad de la fuente de luz o el ancho de la banda de excitación [291].

Los ensayos en placa realizados durante la tesis se optimizaron utilizando medidas de quimioluminiscencia, fluorescencia y colorimetría, con el fin de ofrecer una técnica con distintas modalidades de detección que se adecue a las necesidades de cada laboratorio, y el equipo utilizado para realizar las medidas fue un lector de placas multi-modal, el Synergy 4 de la casa comercial Biotek, que incluye las tres modalidades de detección.

1.2.2. Tecnología de multidetección xMap de Luminex

El sistema Luminex es una plataforma de detección basada en el uso de microesferas y los principios de la citometría de flujo. Las microesferas son partículas de poliestireno de 5,6 micras de diámetro, marcadas internamente con dos fluorocromos, de manera que estableciendo un ratio de ambos en cada microesfera, se forman hasta un total de 100 poblaciones (o regiones) distintas (Figura 17).

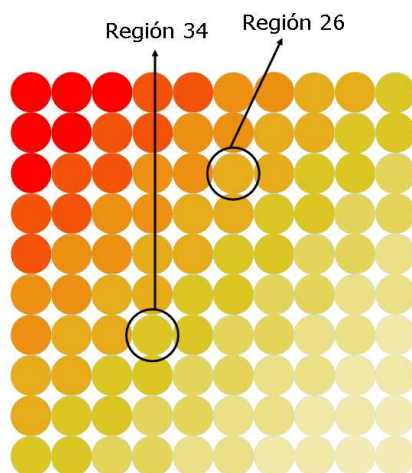


Figura 17. Poblaciones de microesferas

Modificando la superficie de estas microesferas se pueden unir a ellas distintas moléculas (nucleótidos, péptidos, proteínas, receptores, polisacáridos, lípidos...) [303] que permiten detectar, de forma específica un analito. La detección de este analito se realiza midiendo la intensidad de fluorescencia proporcionada por un marcador fluorescente, generalmente ficoeritrina, unido a un anticuerpo o a otra molécula. Combinando los distintos tipos de microesferas, recubierto cada tipo con una molécula detectora específica de un analito concreto y diferente de las demás, se pueden detectar, de forma simultánea, distintos analitos en una misma muestra. Un ejemplo de la modificación de la superficie de las microesferas se muestra en la figura 18, en la que una proteína se une de forma covalente a la superficie de una microesfera carboxilada mediante la modificación química de ésta con EDC (1-etil-3(dimetilaminopropil)carbodiimida) y sulfo-NHS (sulfo-N-hidroxisuccinimida). Los grupos carboxilo situados en la superficie de las microesferas reaccionan con el sulfo-NHS en presencia de una carbodiimida, como la EDC, originando un éster inestable que se rompe fácilmente en presencia de grupos amina para dar lugar a un enlace amida. De esta forma aquellas toxinas que posean aminas primarias en su estructura podrán inmovilizarse sobre la superficie de estas microesferas mediante la modificación química de ésta con EDC/sulfo-NHS.

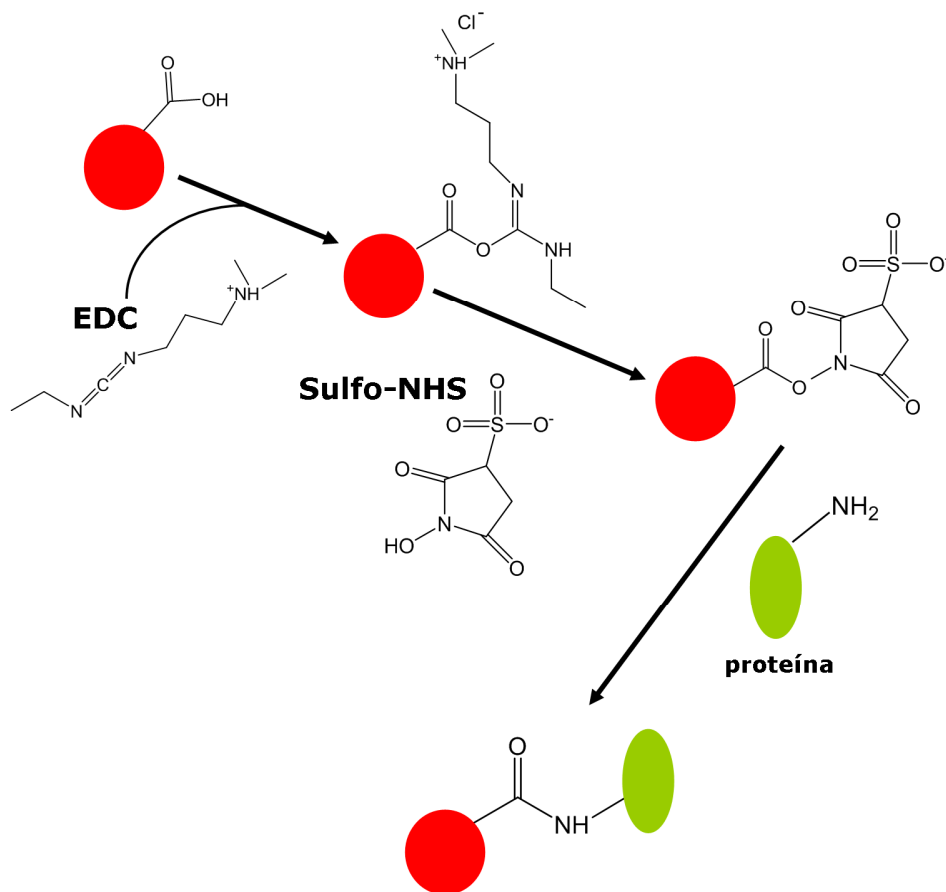


Figura 18. Inmovilización de proteínas sobre la superficie de microesferas carboxiladas. Los grupos carboxilo se activan mediante EDC y sulfo-NHS para formar un enlace amida estable en la superficie que permite la unión covalente de proteínas.

El equipo instrumental, Luminex 200, está formado por dos láseres, un sistema de fluidos, cuatro detectores y un procesador de la señal digital en tiempo real capaz de distinguir cada uno de los distintos tipos de microesferas.

Las microesferas entran en una celda, y gracias al sistema de fluidos son alineadas. Al pasar por el detector de fluorescencia, los fluorocromos internos de las microesferas y el marcador fluorescente utilizado en el ensayo, son excitados por dos láseres de longitudes de onda de 635 y 532 nm respectivamente. El láser de 635 nm excita los fluorocromos de la microesfera, lo que permite su identificación, y el láser de 532 nm excita el marcador fluorescente (Alexa Fluor 532 o ficoeritrina (PE)), detectando así la cantidad de analito presente en la

muestra (Figura 19). El espectro de emisión, único para cada grupo de microesferas, posibilita el análisis de hasta 100 analitos en una sola muestra. Aunque actualmente no se ha publicado ningún estudio en el que se analizaran 100 compuestos, existen kits comerciales para la detección simultánea de hasta 42 analitos [304].

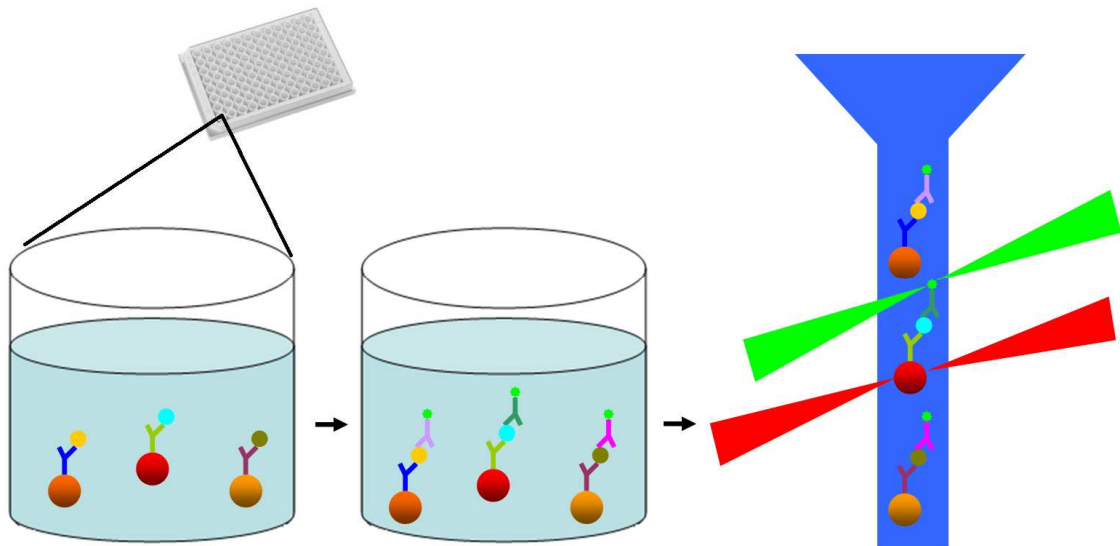


Figura 19. Ensayo de multidetección usando la tecnología Luminex

La primera vez que se usó la citometría de flujo para el análisis de inmunoensayos usando microesferas fue en 1977 [305], y dos años más tarde se propuso el uso de diferentes tamaños de microesferas para la detección simultánea de diferentes analitos [306]. Desde entonces, estos métodos se han usado en distintos tipos de inmunoensayos [307], ensayos ligando-receptor [308] o ensayos enzimáticos [309].

Aunque las principales aplicaciones de este tipo de técnicas son para diagnóstico clínico (testado de alérgenos, diagnóstico de enfermedades autoinmunes, fibrosis quística, enfermedades infecciosas, virus respiratorios...) [310], esta plataforma permite desarrollar métodos de multidetección aplicados a otros campos como la genética, mediante el análisis de ácidos nucleicos [311] o la alimentación, en el

que se han desarrollado técnicas de detección de proteínas [312], antibióticos [313], hidrocarburos aromáticos policíclicos [314], virus [315], bacterias [316], micotoxinas [317] y recientemente ficotoxinas de los grupos de las PSP, ácido domoico y ácido okadaico [318,319].

La posibilidad de la detección simultánea, junto con la velocidad y la sensibilidad, proporcionan a estas técnicas el potencial necesario para reemplazar a otros métodos de detección usados hasta ahora tanto en diagnóstico clínico como en investigación [320,321]. Además, este tipo de técnicas usan pequeños volúmenes de muestra lo que facilita la reducción de costes, y ofrece grandes ventajas con respecto al ELISA convencional [322].

Para desarrollar los métodos de detección de iminas cíclicas y azaspirácidos presentados en esta tesis, se utilizaron los receptores nicotínicos de acetilcolina incluidos en las membranas del órgano eléctrico de la raya *Torpedo marmorata*, una proteína de unión a acetilcolina procedente del gasterópodo *Lymnaea stagnalis*, y un anticuerpo monoclonal anti-azaspirácido, el 8F4. Todas ellas se describen brevemente a continuación.

1.3. Proteínas detectoras

Las proteínas empleadas en el desarrollo de los métodos de detección, se eligieron por su interacción con las toxinas de interés.

La alta afinidad de algunos grupos de toxinas como las iminas cíclicas o la ANT-X por ciertas proteínas como los receptores nicotínicos de acetilcolina o la proteína de unión a acetilcolina, permitió desarrollar ensayos basados en la unión a receptor para la detección de estos compuestos [271,323-325]. Por otro lado, la producción de anticuerpos capaces de detectar aquellos grupos de toxinas cuya

diana biológica se desconoce hasta el momento, como es el caso de los azaspirácidos, hizo posible el desarrollo de métodos capaces de detectar estos compuestos [250].

Los receptores nicotínicos de acetilcolina (nAChR, del inglés *nicotinic acetylcholine receptor*), son proteínas de membrana pertenecientes a la familia de los canales iónicos activados por ligando. Existen dos subtipos de nAChRs: los neuronales y los musculares. Todos ellos son estructuras pentaméricas. Los subtipos de receptores musculares se componen de 4 subunidades distintas con una estequiometría $\alpha_1\beta\gamma\delta$, y se encuentran tanto en la unión neuromuscular de vertebrados como en los órganos eléctricos de ciertas especies marinas, incluyendo la raya eléctrica *Torpedo marmorata* [326].

Estos receptores son las moléculas transductoras de la señal química en eléctrica en la unión neuromuscular y se consideran dianas terapéuticas en muchas enfermedades relacionadas con desórdenes del sistema nervioso central como la esquizofrenia, la adicción a la nicotina o la enfermedad de Alzheimer, entre otras [327,328].

El uso de los receptores musculares presentes en las membranas del órgano del *Torpedo marmorata* (40% de la proteína total) en combinación con α -neurotoxinas como la α -bungarotoxina, procedente del veneno de la especie tailandesa *Bungarus multicinctus*, un antagonista que se une con gran afinidad a los subtipos de nAChRs musculares ($\alpha_1\beta\gamma\delta$), ha permitido desarrollar ensayos competitivos de unión a ligando para la detección de toxinas, proporcionando métodos rápidos, económicos y sencillos, sin necesidad de usar animales [271,323,325].

Los nAChRs se hallan íntimamente asociados a la bicapa lipídica por lo que su purificación y caracterización resulta complicada ya que la extracción de estas

proteínas de su entorno lipídico destruye la estructura cuaternaria de la proteína, y pierde su función [329].

El conocimiento detallado de la estructura de la región extracelular del nAChR y por tanto, del sitio de unión de ligandos colinérgicos, ha sido posible gracias al descubrimiento y a la cristalización de una proteína homopentamérica soluble, la AChBP (proteína de unión a acetilcolina, del inglés *acetylcholine binding protein*). Esta proteína se describió por primera vez en el caracol de agua dulce *Lymnaea stagnalis* en el que se secreta por las células gliales en las sinapsis colinérgicas, donde se cree que modula la interrupción de la transmisión sináptica al unirse a acetilcolina [330,331]. Hoy en día, se ha determinado la estructura de la AChBP identificada en otras especies de moluscos como *Aplysia californica* o *Bulinus truncatus* [332,333].

La AChBP carece de los dominios intracelular y transmembrana presentes en los nAChRs, pero está íntimamente relacionada con el extremo N-terminal de las subunidades α , donde se localiza el sitio de unión de la acetilcolina, por lo que estas proteínas pueden unir a los agonistas y antagonistas competitivos de los nAChRs como la acetilcolina, la nicotina, la d-tubocurarina o la α -bungarotoxina [331]. Por tanto, la AChBP puede usarse como ejemplo del dominio N-terminal de las subunidades α de los nAChRs, resultando una herramienta estructural útil para la identificación de nuevos ligandos de estos receptores [334].

Recientemente se ha demostrado la alta afinidad del 13-desmetil espirólido C y de la gymnodimina-A, por las AChBPs procedentes tanto de *Lymnaea stagnalis* como de *Aplysia californica*, mediante ensayos competitivos de unión a ligando [37], ofreciendo la posibilidad de usar estas proteínas para detectar iminas cíclicas en mariscos.

En el caso de otros grupos de toxinas, en los que el mecanismo de acción es aún objeto de estudio, para poder desarrollar un método no analítico que detecte estos compuestos se puede recurrir al uso de un anticuerpo específico como proteína detectora.

Los anticuerpos son glicoproteínas que se encuentran de forma soluble en la sangre u otros fluidos corporales de los vertebrados, y son empleados por el sistema inmunitario para identificar y neutralizar elementos extraños tales como bacterias, virus, hongos o parásitos [335]. Aunque la estructura general de todos los anticuerpos es muy semejante, una pequeña región del ápice de la proteína varía. A esta parte variable de los anticuerpos es a la que se unen las distintas dianas, conocidas como antígenos [336]. La única parte del antígeno reconocida por el anticuerpo se denomina epítipo.

El anticuerpo utilizado durante esta tesis, el 8F4, es un anticuerpo monoclonal obtenido con un fragmento de la molécula de AZA que contiene un grupo azaspiro con un anillo 2, 9-dioxobiciclononano (Figura 7) [250]. Este anticuerpo fue elegido para el desarrollo del inmunoensayo para la detección de azaspirácidos, por su alta afinidad tanto por el AZA-1 y el AZA-2, como por el fragmento de la molécula citado anteriormente, común a todos los análogos de este grupo de toxinas [250].



OBJETIVOS

2. OBJETIVOS

El trabajo realizado en la presente tesis doctoral tiene como finalidad el desarrollo y optimización de métodos bioquímicos para la detección y cuantificación de iminas cíclicas y azaspirácidos, capaces de solventar los inconvenientes éticos de los bioensayos y menos costosos que los sistemas de detección cromatográficos. Por tanto los objetivos se pueden resumir en tres puntos:

- a) Desarrollar un método de detección y cuantificación de espirólidos y gymnodiminas en extractos de moluscos basado en la competición de estas toxinas con la α -bungarotoxina por la unión a los receptores nicotínicos de acetilcolina, mediante técnicas colorimétricas, fluorescentes y quimioluminiscentes.
- b) Desarrollar un método de detección y cuantificación de toxinas que se unan a los receptores nicotínicos de acetilcolina, basado en la interacción toxina/receptor, utilizando la plataforma Luminex con el fin de incluirlo en el futuro en un sistema de multidetección.
- c) Desarrollar un inmunoensayo para la detección de azaspirácidos mediante el uso de un anticuerpo específico y el sistema Luminex.



PUBLICACIONES

3. PUBLICACIONES

En esta sección de la memoria se presentan los resultados obtenidos durante la tesis doctoral junto con una discusión específica de cada uno de ellos.

Este apartado está formado por un total de cuatro publicaciones, tres de las cuales están relacionadas con la detección de iminas cíclicas, y una cuarta en la que se describe un método para la detección de azaspirácidos. Además se incluye un anexo, en el que se presenta una prueba preliminar de un ensayo para detectar anatoxina-a, que será publicado dentro de un método de multidetección, junto con otros grupos de cianotoxinas.

Los cuatro trabajos expuestos a continuación se dividen en dos bloques en función de la técnica utilizada.

Bloque I. Diseño y optimización de técnicas luminométricas empleadas en la detección de iminas cíclicas.

Artículo 1. *Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines by Chemiluminescence, Fluorescence, or Colorimetry.* Laura P. Rodríguez, Natalia Vilariño, Jordi Molgó, Rómulo Aráoz, Álvaro Antelo, Mercedes R. Vieytes, Luis M. Botana. Artículo publicado en *Analytical Chemistry* 83 (2011): 5857-5863.

En este artículo se presenta un método de detección de iminas cíclicas basado en la capacidad de los espirólidos y la gymnodimina para competir con la α -bungarotoxina por la unión a los receptores nicotínicos de acetilcolina. El complejo α -bungarotoxina/receptor se inmoviliza en la superficie de una microplaca recubierta por estreptavidina, y se detecta mediante un anticuerpo específico. Finalmente, un anticuerpo secundario marcado con la enzima HRP

ofrece tres posibilidades de detección: quimioluminiscencia, fluorescencia y colorimetría. El rango de detección del ensayo para el 13-desmetil espirólido C es de 5-150 nM, cinco veces más sensible que en el caso del método de polarización de fluorescencia desarrollado anteriormente. Este método es capaz de detectar la gymnodimina, aunque con una sensibilidad diez veces menor que en el caso de los espirólidos, y podría resultar muy útil como método de criba para reducir el número de muestras procesadas mediante bioensayos o métodos analíticos.

Artículo 2. *High-throughput Receptor-Based Assay for the Detection of Spirolides by Chemiluminescence.* Laura P. Rodríguez, Natalia Vilariño, Jordi Molgó, Rómulo Aráoz, Luis M. Botana. Artículo publicado en *Toxicon* 2013 Jul 1. pii: S0041-0101(13)00235-3. doi: 10.1016/j.toxicon.2013.06.015. [Epub ahead of print]

En este estudio se ha optimizado y prevalidado el método descrito en la publicación anterior para la detección de espirólidos. Este método está basado en la competición entre el 13-desmetil espirólido C y la α -bungarotoxina, inmovilizada en una superficie de estreptavidina, por la unión a los receptores nicotínicos de acetilcolina. El protocolo del ensayo se optimizó para microplacas de 384 pocillos que permiten reducir el volumen de reactivos usados por muestra, e incrementar el número de muestras a analizar por placa. El ensayo detecta el 13-desmetil espirólido C en un rango de 5-150 ng/mL y el límite de detección es de 50 μ g/kg en la carne de molusco. El porcentaje de recuperación del método es del 80% y el coeficiente de variación interensayo de un 8%. Este método de quimioluminiscencia en placas de 384 pocillos puede usarse como método de criba de alto rendimiento para la detección del 13-desmetil espirólido C en la carne de los moluscos, lo cual permite reducir el número de muestras procesadas mediante ensayos *in vivo* o métodos analíticos.

Bloque II. Diseño de métodos de detección de iminas cíclicas y azaspirácidos mediante la tecnología Luminex.

Artículo 3. *Development of a Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines Using a Microsphere-Flow Cytometry System.* Laura P. Rodríguez, Natalia Vilariño, Jordi Molgó, Rómulo Aráoz, M. Carmen Louzao, Palmer Taylor, Todd Talley, Luis M. Botana. Artículo publicado en *Analytical Chemistry* 85 (2013): 2340-2347.

El principal objetivo de este trabajo fue desarrollar un método de detección de espirólidos usando el sistema Luminex. Para ello se utilizaron dos proteínas de unión; por un lado, los receptores nicotínicos de acetilcolina procedentes del órgano eléctrico del *Torpedo marmorata*; y por otro, una proteína de unión a acetilcolina procedente del gasterópodo *Lymnaea stagnalis*. Utilizando la competición de las iminas cíclicas con la α -bungarotoxina por la unión a las proteínas, se desarrolló un ensayo de inhibición para la detección de estas toxinas. La cantidad de α -bungarotoxina marcada con biotina unida a la superficie de las microesferas, se detectó usando estreptavidina marcada con ficoeritrina, y la fluorescencia se midió usando el sistema Luminex 200. Ambas proteínas unieron el 13-desmetil espirólido C de forma eficiente, sin embargo el perfil de reactividad cruzada usando el ensayo realizado con los receptores nicotínicos de acetilcolina simula mejor la potencia tóxica relativa de los espirólidos y la gymnodimina. Este método permite detectar el 13-desmetil espirólido C en un rango de 10-6000 $\mu\text{g/kg}$ en la carne de los moluscos, usando un simple protocolo de extracción con acetona, y proporcionando mayor sensibilidad y rango dinámico que otros métodos publicados anteriormente. Este ensayo proporciona un buen sistema de criba y ofrece la posibilidad de incluir a estas toxinas en un futuro ensayo de multidetección.

***Anexo al artículo 3.** *Ensayo basado en unión a receptor para la detección de ANTX mediante la tecnología xMAP de Luminex.*

El método basado en unión a receptor para la detección de iminas cíclicas mediante la tecnología Luminex, se optimizó para la ANTX, comprobando que la proteína de unión a acetilcolina proporcionaba una técnica con mayor sensibilidad que los receptores nicotínicos de acetilcolina, para la detección de esta cianotoxina. Este método ha sido incluido en un ensayo de multidetección en desarrollo.

Artículo 4. *Microsphere-Based Immunoassay for the Detection of Azaspiracids.*

Laura P. Rodríguez, Natalia Vilariño, M. Carmen Louzao, Tobin J. Dickerson, K. C. Nicolaou, Michael O. Frederick and Luis M. Botana. Artículo aceptado en *Analytical Biochemistry*.

En este artículo se presenta un inmunoensayo basado en el uso de microesferas, para la detección de AZAs usando el sistema Luminex. Este método se basa en la competición entre el AZA-2 inmovilizado en la superficie de las microesferas y AZAs libres, por la interacción con un anticuerpo monoclonal específico, el 8F4. En este inmunoensayo de inhibición, la cantidad de anticuerpo unido a las microesferas inmovilizadas con AZA-2 se cuantifica usando un segundo anticuerpo marcado con ficoeritrina, y la fluorescencia se mide con el analizador Luminex. Una simple extracción con metanol o con metanol/acetato proporciona un extracto que no interfiere con el ensayo y unos porcentajes de recuperación de 75.8% y 86.5%, respectivamente. Esta publicación presenta, por primera vez, un método de criba rápido y sencillo capaz de detectar concentraciones de AZAs inferiores al límite regulador establecido en Europa, usando un sistema basado en el uso de microesferas y los principios de la citometría de flujo.

**Bloque I. Diseño y optimización de técnicas
luminométricas empleadas en la detección de
iminas cíclicas**





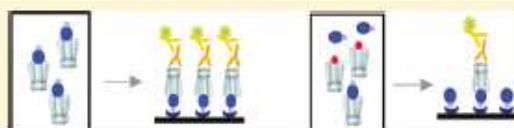
Artículo 1. *Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines by Chemiluminescence, Fluorescence, or Colorimetry.*

Laura P. Rodríguez, Natalia Vilariño, Jordi Molgó, Rómulo Aráoz, Álvaro Antelo,
Mercedes R. Vieytes, Luis M. Botana.

Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines by Chemiluminescence, Fluorescence, or Colorimetry

Laura P. Rodríguez,[†] Natalia Vilarino,[†] Jordi Molgó,[‡] Rómulo Aráoz,[‡] Alvaro Antelo,[§] Mercedes R. Vieytes,[‡] and Luis M. Botana^{*,†}[†]Departamento de Farmacología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain[‡]CNRS, Institut Fédératif de Neurobiologie Alfred Fessard—FRC2118, Laboratoire de Neurobiologie et Développement—UPR3294, 91198 Gif sur Yvette cedex, France[§]CIFGA Laboratorio, Plaza de Santo Domingo, 20, 5ª planta, 27001 Lugo, Spain[‡]Departamento de Fisiología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain

ABSTRACT: The spirolides and gymnodimines are marine phycotoxins included in the group of cyclic imines. The toxicity of these compounds to humans is still unknown, although their toxicity by intraperitoneal injection in rodents is very high. A receptor-based method was developed using the competition of the 13-desmethyl spirolide C with biotin-labeled α -bungarotoxin for binding to nicotinic acetylcholine receptors and the immobilization of the α -bungarotoxin-receptor complex on streptavidin-coated surfaces. The quantification of the immobilized receptor can be achieved using a specific antibody. Finally, after the addition of a secondary antibody labeled with horseradish peroxidase, three alternative substrates of this enzyme generate a chemiluminescent, fluorescent, or colorimetric signal. The assay performs well in shellfish extracts and the detection range is 5–150 nM of 13-desmethyl spirolide C in shellfish extracts, which is at least 5 times more sensitive than the existing fluorescence polarization assay. This assay can also detect gymnodimine, although with 10 times lower sensitivity than the spirolide. The detection of cyclic imines with microplate assays would be useful for screening purposes in order to reduce the number of samples to be processed by bioassays or analytical methods.



Spirolides and gymnodimines are algal toxins discovered in the early 1990s during routine monitoring of bivalve mollusks in Nova Scotia, Canada¹ and New Zealand,² respectively. The spirolides and gymnodimines have been included in the cyclic imine group of marine phycotoxins due to the presence of an imino ring in their macrocyclic chemical structure. Similarly to other cyclic imines such as pinnatoxins, pteriatoxins, prorocentrolides, and spiroporocentrimine, these toxins display a “fast acting toxicity” in mammalian bioassays.^{1,3,4} Spirolides and gymnodimines have been found in Canada, Norway, Italy, Spain, Denmark, France, Chile, New Zealand, and Tunisia.^{2,5–13} *Alexandrium ostenfeldii* and *Alexandrium peruvianum* have been identified as the causative organisms of spirolide toxicity^{14–16} and *Karenia setiformis* of gymnodimine toxicity.^{2,17} Despite some consumer reports of gastric distress and tachycardia during the recurrent spirolide toxic episodes in the Canadian coast, spirolides and gymnodimines have not been unequivocally linked to human intoxications.¹

Several techniques have been used to detect the presence of spirolides in shellfish. As mentioned above, these toxins can be detected by mouse bioassay,¹ which is the most commonly used method to detect marine toxins. However, bioassays have several important drawbacks, such as high numbers of false positives or ethical issues related to the sacrifice of laboratory animals. In the last two decades, several techniques using mass spectrometry have gained popularity for the detection of marine toxins and, in several countries, will soon replace the mouse bioassays as the

official reference method for some marine toxin groups.¹⁸ Mass spectrometry (MS)-based methods have been also widely used for the detection of cyclic imines.^{5,8,11,19–21} Some of the main advantages of these techniques are the unequivocal identification of the different compounds present in a sample and the accuracy of quantification, as well as the possibility of multidetection. However, MS-based methods require the availability of certified standards of every toxin, which are currently not available commercially, as well as highly trained laboratory personnel and high volumes of organic solvents. A third analytical option has been published very recently: a receptor-based assay has been developed for the detection of spirolides and gymnodimines by fluorescence polarization.^{22–24} The fluorescence polarization assay is easy to perform and does not require highly trained laboratory personnel. However, fluorescence polarization is not a technique available in most routine analysis laboratories. The advantage of receptor-based assays is an increased probability of detection cross-reactivity profiles among different compounds of the same group that resemble the toxicity profile. Although routine analysis with biological methods does not usually require certified standards of each compound of the group, these certified solutions are absolutely necessary to accurately determine the

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assay cross-reactivities of the group members and their correlation with toxicity equivalency factors (TEFs).

The aim of this work was to develop a receptor-based method that is easy to perform, more sensitive than the other existing receptor-based methods, and can be adapted to widespread detection techniques, such as chemiluminescence, fluorescence and colorimetry, that, as well as similar biological methods, can be used as a semiquantitative screening tool.

MATERIALS AND METHODS

Materials. Biotin- α -bungarotoxin was obtained from Molecular Probes (Eugene, OR). 13-Desmethyl spirolide C standard solutions were purchased from Laboratorios Cifga (Lugo, Spain) and gymnodimine standard solutions were obtained from NRC-CNRC (Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada). Bovine serum albumin (BSA) and Tween-20 were obtained from Sigma Chemical Co. (Madrid, Spain). Acetone, chloroform, *n*-hexane, ethanol absolute, sodium chloride, and sodium phosphates were obtained from Panreac (Barcelona, Spain). The nAChR-rich membranes from *Torpedo marmorata* were purified as previously described.²⁴ Monoclonal mAb 35 anti-nAChR antibody was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Peroxidase-conjugated goat antimouse IgG, (H+L), QuantaRed Enhanced Chemifluorescent HRP Substrate, SuperSignal ELISA Femto Maximum Sensitivity Substrate, 1-Step Ultra TMB-ELISA, and Reacti-Bind streptavidin-coated 96-well plates were purchased from Thermo Scientific (Rockford, IL). Toxin-free cockles (*Cerastoderma edule*) were purchased from the market in Lugo (Spain). Amicon Ultra-4, PLGC Ultracel-PL membrane, 10-kDa-molecular-weight-cut-off (MWCO) filters were obtained from Millipore Corporation (Bedford, MA). Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH₂PO₄, 8.5 mM Na₂HPO₄, pH 7.0. PBS-BT solution was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20, and PBS-x2BT was PBS supplemented with 0.2% w/v BSA and 0.2% v/v Tween-20.

Solid-Phase Receptor Assay. An inhibition assay was designed based on the competition between α -bungarotoxin (α -BTX), which is a toxin from the venom of the snake *Bungarus multicinctus*, and 13-desmethyl spirolide C for binding to the nAChR from the *T. marmorata* electric organ. The protocol consisted of a first incubation of sample or standard solution with membrane fragments containing nAChRs, followed by a second incubation of this mixture with biotin-labeled α -BTX. This part of the protocol took place in a microtube where 40 μ L of a 1:40 dilution of the nAChR-enriched membrane stock (2 mg/mL of protein) was mixed with 40 μ L of sample or standard solution (containing (or not containing) 13-desmethyl spirolide C) and the mixture was allowed to interact for 30 min. Then, 80 μ L of a 40 nM biotin- α -BTX solution was added to the same microtube. After 30 min, 100 μ L of this mixture were transferred to a streptavidin-coated well (96-well plate) for immobilization of the biotin-labeled α -BTX on the plate surface. The immobilization step proceeded for 1 h and was followed by three washes with PBS-BT. The detection of the immobilized nAChR bound to biotin-labeled α -BTX was achieved using a monoclonal anti-nAChR antibody. One hundred microliters (100 μ L) of a 1:2000 dilution of this primary antibody was added to the well plate and incubated for 1 h. Three washes with PBS-BT preceded the addition of an HRP-labeled antimouse antibody (1:1000) that was allowed to bind for 30 min. All the reagent dilutions were

prepared in PBS-BT, and all the incubations took place at room temperature. Volumes, concentrations, and incubation times were optimized in order to deliver a high-sensitivity assay. The same protocol was used for the detection of gymnodimine with varying durations of the incubation of toxin with nAChR-enriched membranes.

Chemiluminescent, Fluorescent, and Colorimetric Detection. The quantification of the immobilized receptor was performed via the addition of HRP substrates to the well plate. For chemiluminescent detection, 150 μ L of the SuperSignal ELISA Femto Maximum Sensitivity Substrate was added to each well of a streptavidin-coated white plate. The chemiluminescence signal was measured within 5 min of the addition using a Synergy 4 Multi-Mode Microplate Reader from Biotek Instruments (Winnoski, VT). No filters were used for luminescence detection. For fluorescence detection, 200 μ L of the QuantaRed Enhanced Chemifluorescent HRP substrate was added to the wells of a streptavidin-coated black plate for 15 min and the reaction was stopped, following the manufacturer instructions. Fluorescence was measured at an excitation wavelength of 550 nm and an emission wavelength of 600 nm in a Synergy 4 Multi-Mode Microplate Reader. Colorimetric measurements were performed using the commercial kit 1-Step Ultra TMB-ELISA, following the manufacturer instructions. Optical density at 450 nm was detected using transparent plates in a Synergy 4 Multi-Mode Microplate Reader.

Biotin- α -BTX binding response was calculated as follows:

$$\%R = \left(\frac{S_i - S_{min}}{S_{max} - S_{min}} \right) \times 100$$

where %R is the percentage of receptor binding response, S_i the signal obtained for a given condition, S_{max} the maximum signal obtained in control wells containing nAChR and biotin- α -BTX, and S_{min} the minimum signal obtained in control wells containing nAChR and no biotin- α -BTX.

Shellfish Sample Preparation for the Receptor Assay. Two hundred grams (200 g) of cockle meat (whole body) were homogenized with a blender, divided into 4-g aliquots, and stored at -20°C until use. The extraction procedure consisted of the addition of 4 mL of acetone to 1 g of cockle meat homogenate. The mixture was vortexed for 10 s and roller mixed for 15 min at room temperature. After centrifugation at 3500g 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, 60 min). Chloroform layers were pooled together and evaporated. The residue was reconstituted with 4.1 mL of ethanol:PBS (4:6 v/v) and filtered through a 10 kDa MWCO filter (Millipore). An equal volume of PBS-2xBT then was added. The extracts were analyzed on the same day that the extraction was started. In addition, the same cockle homogenate was analyzed by LC-MS for the presence of 13-desmethyl spirolide C, 13, 19-didesmethyl spirolide C, 20-methyl spirolide G, and GYM to be sure that the samples were not contaminated with these toxins and were suitable as blanks for recovery and interference studies. Briefly, 2 g of cockle whole body homogenate were extracted twice with methanol. Toxins were separated in an Acquity Model

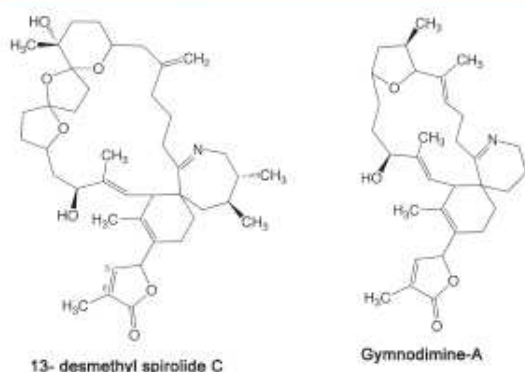


Figure 1. Chemical structure of 13-desmethyl spirolide C and gymnodimine-A.

UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) at 35 °C. The mobile phase consisted of 100% water containing 2 mM ammonium formate and 50 mM formic acid in channel A and acetonitrile:water (95:5) containing 2 mM ammonium formate and 50 mM formic acid in channel B. The analyses were carried out at a flow rate of 0.4 mL/min by running a linear gradient elution, starting with a gradient from 30% B to 90% B in 3 min, followed by a 1.5-min hold at 90% B and decreasing to 30% B over 0.1 min, which was held again for 2 min until the next run. The ESI interface was operated in positive mode and the mass spectrometer was operated in multiple reaction-monitoring (MRM), analyzing the transitions 692.4/674.4 and 692.4/444.4 for the 13-desmethyl spirolide C, 678.5/660.5 and 678.5/430.5 for the 13,19-didesmethyl spirolide C, 706.3/164.2 for the 20-methyl spirolide G, and 508.3/490.3 and 508.3/392.3 for GYM. The cockle matrix had no detectable amount of 13-desmethyl spirolide C (limit of detection (LoD) is 0.60 ng/mL), 13,19-didesmethyl spirolide C (LoD = 0.6 ng/mL, estimated from 13-desmethyl spirolide C data), 20-methyl spirolide G (LoD = 0.6 ng/mL, estimated from 13-desmethyl spirolide C data), or GYM (LoD = 2.14 ng/mL) from natural contamination when they were analyzed by LC-MS.

Data Analysis. All experiments were performed at least three times; moreover, in every experiment, each condition was tested in the solid phase assay in triplicate. The results were expressed as mean ± SEM. [SEM = standard error of the mean.] The calibration curves were fitted using Biaevaluation 3.2 RC1 software (Biacore, Uppsala, Sweden). The four-parameter fit equation was: $y = R_{\infty} - (R_{\infty} - R_0) / (1 + [(x/A_1) \wedge A_2])$, where R_{∞} is the response at infinite concentration, R_0 is the response at zero concentration, and A_1 and A_2 are fitting constants with no chemical significance. The limits of detection (LoD) were calculated using three times the standard deviation (SD) of raw binding signals of the highest point of the calibration curve.

RESULTS

Detection of the 13-Desmethyl Spirolide C and Gymnodimine by a Solid-Phase Receptor-Based Assay. An inhibition assay was designed for the detection of 13-desmethyl spirolide C (see Figure 1), based on the competition for binding to nAChRs with biotin-α-BTX, which is immobilized on a streptavidin-coated surface. Therefore, the presence of the 13-desmethyl spirolide

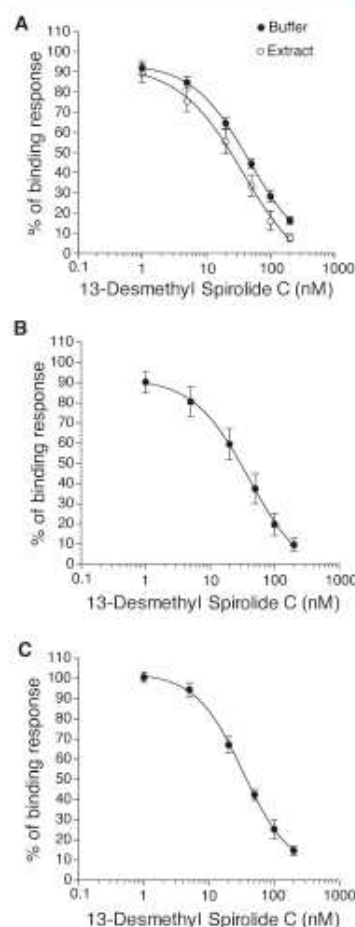


Figure 2. Calibration curves of 13-desmethyl spirolide C in buffer and shellfish extract using the solid-phase receptor-based assay. The spirolide standard was diluted at 1, 5, 20, 50, 100, and 200 nM in PBS-BT containing 20% ethanol or in extract. These solutions were used to perform the solid-phase assay. The data are expressed as a percentage of the maximum α-BTX-binding response. The calibration curves were obtained with a four-parameter fit. (A) Calibration curve obtained with chemiluminescence detection in buffer and cockle extract. The two curves belong to nonmatched experiments (mean ± SEM, $n = 3$ (buffer), and $n = 5$ (shellfish extract)). (B) Calibration curve obtained with fluorescence detection in buffer (mean ± SEM, $n = 4$). (C) Calibration curve obtained with colorimetric detection in buffer (mean ± SEM, $n = 3$).

C in a test solution would reduce the number of receptors immobilized with the biotin-α-BTX on the streptavidin-coated surface. The ability of the assay to detect the 13-desmethyl spirolide C was tested with three different end points: chemiluminescence, fluorescence, and colorimetry. The results demonstrated that the spirolide inhibited binding of the nAChR-enriched membranes to the well surface. Inhibition curves of biotin-α-BTX binding to the receptor were obtained with different concentrations of the spirolide (see Figure 2). The

Table 1. Calibration Curve Parameters, IC_{50} , Dynamic Range, and Estimated Limit of Detection (LoD) of the Receptor-Based Assay for Chemiluminescent, Fluorescent, or Colorimetric Detection^a

Assay end point	Chemiluminescence (buffer)	Fluorescence (buffer)	Colorimetry (buffer)	Chemiluminescence (cockle extract)	Chemiluminescence (gymnodimine, 2 h)
R_{∞}	-13.4	-7.13	-4.68	-2.09	3.85
R_0	92.8	92.2	103	94.1	91.2
A_1	37.5	39.9	33.2	45.9	468.6
A_2	0.874	1.01	1.2	0.987	0.798
χ^2	1.32	1.53	1.32	6.02	1.36
IC_{50} (nM)	31.4 ± 4.9	40.9 ± 8.8	37.4 ± 3.1	47.9 ± 6.6	526.0 ± 38.1
IC_{20} (nM)	8.1 ± 1.1	13.0 ± 1.9	11.9 ± 0.8	13.2 ± 2.7	93.3 ± 7.4
IC_{80} (nM)	100.0 ± 18.1	117.8 ± 22.4	142.2 ± 25.2	179.7 ± 24.6	NA ^b
Limit of detection, LoD (nM)	16.0 ± 4.7	10.2 ± 2.9	15.9 ± 3.6	5.2 ± 1.9	154.0 ± 64.3

^a The data were obtained with 13-desmethyl spirolide C, except for the last column, which corresponds to the gymnodimine calibration curve obtained with 2 h of incubation of toxin with receptor. The parameters of the curve for chemiluminescent detection in cockle extract are also included. The four-parameter fit equation was: $y = R_{\infty} - (R_{\infty} - R_0) / (1 + [(x/A_1) \wedge A_2])$, where R_{∞} is the response at infinite concentration, R_0 is the response at zero concentration, and A_1 and A_2 are fitting constants with no chemical significance. ^b NA indicates that the four-parameter fit did not yield a IC_{50} value for two out of three experiments.

spirolide standard was provided dissolved in methanol. The solvent was removed by evaporation and replaced by 20% ethanol in PBS-BT, because of previous reports on the incompatibility of organic solvents with nAChR-based assays.²⁴ Concentrations of 13-desmethyl spirolide C of 1, 5, 20, 50, 100, and 200 nM were mixed with nAChR-enriched membranes, yielding final concentrations in the wells of 0.25, 1.25, 5, 12.5, 25, and 50 nM, respectively. The calibration curves for the 13-desmethyl spirolide C were very similar for the three end points (see Figures 2A, 2B, and 2C). The IC_{50} of the inhibition curve for the chemiluminescent, fluorescent, and colorimetric assays was calculated for each experiment using the calibration curve (see Table 1). The IC_{50} values were very similar for the three end points with a slightly lower value for the chemiluminescence assay. The dynamic range of the three assays, IC_{20} – IC_{80} , was also very similar (see Table 1). Despite the similarity of the calibration curves, the maximum signal intensity to background ratio was higher for the chemiluminescence assay. The LoD of the assay was estimated in the range of 10–16 nM (see Table 1), with no statistical significance among the three assays. Moreover, the calibration curve of the 13-desmethyl spirolide C using the chemiluminescence assay was not dependent on the duration (30–120 min) of the incubation of toxin and receptor (data not shown).

Calibration curves were also obtained for gymnodimine using the chemiluminescence assay. Following the same protocol, concentrations of gymnodimine of 0.5, 1, 10, 20, 50, 100, 500, 1000, and 2000 nM were mixed with nAChR-enriched membranes, yielding final concentrations of 0.125, 0.25, 2.5, 5, 12.5, 25, 125, 250, and 500 nM, respectively, in the wells. The mixture was incubated for 30 min or 2 h. The calibration curves obtained for both incubation times show that the detection of gymnodimine is time-dependent (see Figure 3). The assay cannot detect gymnodimine efficiently when the first incubation lasts for 30 min. Even after an incubation time of 2 h, the IC_{50} of the assay for gymnodimine is more than 10 times higher than the IC_{50} for the 13-desmethyl spirolide C (see Table 1).

In addition, 10 μ M azaspiracid-2, 10 μ M okadaic acid, and 10 μ M saxitoxin did not affect binding of the receptor to biotin- α -BTX in this receptor-based assay with the chemiluminescence detection (data not shown).

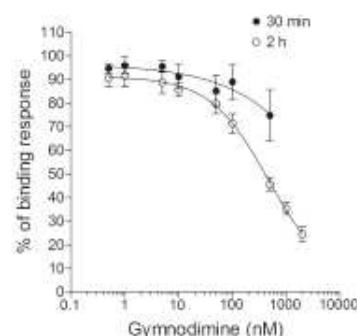


Figure 3. Calibration curves of gymnodimine in buffer using the solid-phase receptor-based assay with chemiluminescence end point. The gymnodimine standard was diluted at concentrations of 0.5, 1, 10, 20, 50, 100, 500, 1000, and 2000 nM in PBS-BT containing 20% ethanol. These solutions were used to perform the solid-phase assay. The first incubation after mixing gymnodimine solutions and nAChR-enriched membranes lasted for 30 min or 2 h. The experiments that generated the two curves were performed in different days. The data are expressed as a percentage of the maximum α -BTX-binding response. The calibration curves were obtained with a four-parameter fit (mean $\pm$ SEM, $n = 3$).

Cockle Matrix Effects on the Solid-Phase, Receptor-Based Assay. The compatibility of this receptor-based assay using streptavidin-coated surfaces with shellfish extracts was analyzed using cockles as the shellfish matrix and chemiluminescence as the assay end point. Whole body homogenates of cockle were extracted as previously published,²² using a first extraction with acetone, a subsequent water/hexane partition, and a final water/chloroform partition. The extract was reconstituted in ethanol/PBS and filtered. Considering the sensitivity of this assay, the samples were diluted in 4.1 mL of ethanol/PBS, the volume of which is 4-fold greater than that in the previously published protocol. Finally, an equal volume of PBS-2xBT was added to the extract. A calibration curve was obtained with 13-desmethyl spirolide C, using the procedure described above. After evaporation

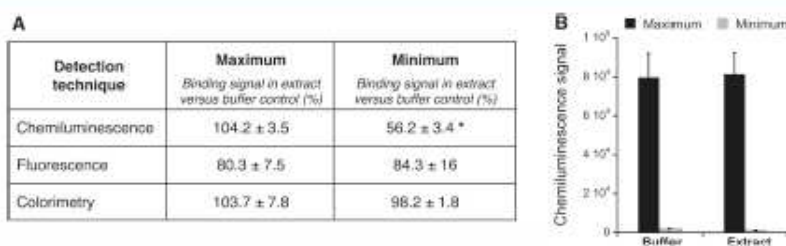


Figure 4. Matrix interference with maximum and minimum binding signals. (A) The binding signal in extract is expressed as a percentage, considering the binding signal in the buffer to be 100% (mean $\pm$ SEM, $n = 5$ (chemiluminescence), $n = 4$ (fluorescence), $n = 2$ (colorimetry)). (B) Binding signal expressed in raw luminescence values for the chemiluminescence assay (mean $\pm$ SEM, $n = 5$).

of the methanol stock, the toxin was dissolved in cockle extract. The calibration curve for the 13-desmethyl spirolide C in shellfish extracts showed a similar IC_{50} value and dynamic range as the calibration curve for this molecule in buffer (see Figure 2A). The IC_{50} value for the 13-desmethyl spirolide C in cockle extracts using this solid-phase assay was 39.9 ± 4.6 nM. The dynamic range of the assay in shellfish extracts was also similar to the data obtained in a buffer (see Table 1). A preliminary estimation of the LoD of the spirolide in cockle extracts yielded a value of 5.2 ± 1.9 nM.

The extract interfered significantly with the minimum binding signal of the assay using a chemiluminescent end point (see Figure 4A). In contrast, in the same experiments, the maximum binding signal was similar in cockle extracts versus buffer controls (see Figure 4A). The raw luminescence data showed a high ratio between the maximum and minimum binding signals, both in buffer and extract (Figure 4B), suggesting that the matrix interference with the minimum binding signal will probably have a negligible effect on the quantification of sample toxicity. Moreover, the fact that the maximum binding in the extract and the buffer is the same indicates that this cockle matrix did not contain any nAChR-binding compounds. The interference of cockle extracts with the maximum and minimum binding signals in the colorimetric and fluorescence assays was also tested. No interference was detected with the colorimetric assay, and although the values of the maximum and minimum binding signals were slightly lower in extracts when using the fluorescent assay, the values obtained in the buffer and the extract were not statistically different (Figure 4A).

The recovery of this extraction procedure was tested by spiking 1 g of cockle homogenate with 283 ng of 13-desmethyl spirolide C (this is an amount that would theoretically yield a concentration of 50 nM in the final extract). In addition, 1 g of nonspiked cockle homogenate was extracted simultaneously and the final extract was spiked with a final concentration of 50 nM spirolide. The recovery of the extraction procedure was calculated using 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] $\times$ 100. The recovery rate for this extraction procedure was $67.8 \pm 1.2\%$ (mean $\pm$ SEM, $n = 3$).

DISCUSSION

The solid-phase receptor-based assay developed in this study is capable of detecting the 13-desmethyl spirolide C with a higher sensitivity than the previously published receptor-based

fluorescence polarization assay.²⁴ This solid-phase assay is based on the immobilization of nicotinic acetylcholine receptors bound to biotin-labeled α -BTX on streptavidin-coated surfaces, followed by receptor quantification using a primary anti-nAChR antibody and a secondary HRP labeled antibody. The use of three end points using different HRP substrates was also explored, yielding very similar calibration curves; however, the chemiluminescence substrate seemed to be more efficiently transformed by HRP, which provided the best signal-to-noise ratio. The IC_{50} value of this solid-phase assay for the 13-desmethyl spirolide C was ~ 35 nM, with slight variations depending on the end point, and lower than the IC_{50} value of the fluorescence polarization assay for the same compound, which had a value of 100 nM.²⁴ Moreover, the solid-phase assay also provides a wider dynamic range than the fluorescence polarization assay. These improvements are probably due to the signal amplification provided by the HRP enzymatic reaction. Other lipophilic toxins that can be extracted simultaneously with the spirolides do not inhibit α -BTX binding to the nAChR. Okadaic acid, yessotoxin, and brevetoxin-2 had been tested previously.²⁴ In this work, okadaic acid was retested to confirm the lack of inhibition in this new assay format and azaspiracid-2 was tested for the first time, also to demonstrate no interference with the assay. Moreover, saxitoxin, which is a hydrophilic toxin, did not inhibit binding of α -BTX to the receptor either. The specificity of the method for cyclic imines among other marine toxins is provided by the competition with α -bungarotoxin for binding to the nAChR and the detection with a monoclonal antibody, which are two highly specific interactions. In addition, although the optimization of the assay was performed with the 13-desmethyl spirolide C, we demonstrated that the assay also detects gymnodimine. The sensitivity of the chemiluminescence assay for gymnodimine was more than 10 times lower than for 13-desmethyl spirolide C. Considering that gymnodimine toxicity (LD_{50} : 80–96 μ g/kg, mouse, i.p.^{13,25}) also is ~ 10 times lower than 13-desmethyl spirolide C toxicity (LD_{50} : 6.9 μ g/kg, mouse, i.p.²⁶), this feature of the assay would be an advantage for total toxicity evaluation of a sample. Moreover, the time dependency of gymnodimine detection can be used to titrate the assay sensibility for gymnodimine and adjust the cross-reactivity of the assay to the relative toxic potency of these two molecules or to future regulations. The interaction of α -BTX with nAChRs from the *T. marmorata* electric organ is also inhibited by the 13,19-didesmethyl spirolide C,^{13,23,24,27} therefore, this solid-phase assay also should be capable of detecting this compound of the cyclic imine group. Unfortunately, the determination of the assay cross-reactivity for the 13,19-didesmethyl spirolide C and other members of the cyclic imine group

will have to wait until certified standards are available for each compound.

The solid-phase chemiluminescence assay allows for the detection of cyclic imines, or any other nicotinic receptor binding molecules that compete with α -bungarotoxin, in cockle extracts. Neither the fluorescent nor colorimetric end points showed any cockle matrix interference. The performance of the assay was similar in buffer and cockle extracts. Actually, the similarity of the data suggests that a calibration curve in the buffer might be used for the determination of spirolide concentration in shellfish extracts. However, a thorough study should be performed, since a calibration curve in the buffer might lead to an overestimation of toxin content. Considering sample dilution during the extraction protocol and the dynamic range of the assay in extract, the assay would detect concentrations of 13-desmethylspirolide C of 40–1000 $\mu\text{g}/\text{kg}$ in shellfish meat. It might be possible to increase the sensitivity by reducing the final sample dilution at the end of the extraction procedure; however, the effects of matrix interference of more-concentrated samples have not been tested in this solid-phase assay and would probably preclude the use of buffer calibration curves. The extraction procedure is adequate for testing the presence of the 13-desmethylspirolide C in cockle, although the recovery in this set of experiments was lower than previously reported,²² possibly because of seasonal variations in cockle matrices. In previous studies, gymnodimine and 13, 19-didesmethyl spirolide C have also been extracted with this procedure in several shellfish species.^{22–24} This method can be used as a semiquantitative assay for the rapid screening of a high number of samples for the presence of spirolides and gymnodimines, and possibly for other nAChR binding compounds.

The toxicity of spirolides and gymnodimines to humans is still unknown. In mice, spirolide and gymnodimine oral toxicity is considerably lower than intraperitoneal toxicity. A safe level of spirolide toxin content in shellfish has been estimated to be ~400 $\mu\text{g}/\text{kg}$ by some authors; this level was estimated to be 18 mg/kg for gymnodimines,^{26,28} which is within the range of our assay. However, the scientific community has not reached a consensus regarding the cyclic-imine-related threat, and the recently published EFSA opinion does not recommend legal regulation of the content of cyclic imines in shellfish, given the lack of evidence of human toxicity and the calculated risk of exposure.²⁹ Although the information currently available regarding cyclic imine toxicity and toxin occurrence point to a low risk for human consumers, the toxicological data are very scarce and an eventual increase in shellfish toxicity, because of an unprecedented toxic bloom, could be a potential threat. Actually, considering the remarkably high affinity of the spirolides for nAChRs,²⁷ even under conditions of very low oral bioavailability, small amounts of the toxin reaching the bloodstream might induce toxic effects. Awareness of a potential change of the current toxicological evaluation of cyclic imines is necessary, in our opinion, to ensure the protection of human health. Periodic monitoring of spirolide levels in shellfish and phytoplankton in coastal waters probably should be considered, to detect an eventual increase in the presence of cyclic imine.

In summary, this novel solid-phase assay for the detection of cyclic imines was designed using the competition of these toxins with α -bungarotoxin (α -BTX) for binding to nAChRs. The assay is sensitive enough to detect concentrations higher than 40 $\mu\text{g}/\text{kg}$ of 13-desmethylspirolide C in shellfish meat and could be used as a semiquantitative screening assay, offering three detection modalities that increase the number of laboratories

capable of performing the assay versus the former fluorescence polarization assay.

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***Artículo 2. High-throughput Receptor-Based Assay for the Detection
of Spirolides by Chemiluminescence.***

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High-throughput receptor-based assay for the detection of spirolides by chemiluminescence

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ABSTRACT

The spirolides are marine toxins that belong to a new class of macrocyclic imines produced by dinoflagellates. In this study a previously described solid-phase receptor-based assay for the detection of spirolides was optimized for high-throughput screening and prevalidated. This method is based on the competition between 13-desmethyl spirolide C and biotin- α -bungarotoxin immobilized on a streptavidin-coated surface, for binding to nicotinic acetylcholine receptors. In this inhibition assay the amount of nAChR bound to the well surface is quantified using a specific antibody, followed by a second anti-mouse IgG antibody labeled with horseradish peroxidase (HRP). The assay protocol was optimized for 384-well microplates, which allowed a reduction of the amount of reagents per sample and an increase of the number of samples per plate versus previously published receptor-based assays. The sensitivity of the assay for 13-desmethyl spirolide C ranged from 5 to 150 ng mL⁻¹. The performance of the assay in scallop extracts was adequate, with an estimated detection limit for 13-desmethyl spirolide C of 50 μ g kg⁻¹ of shellfish meat. The recovery rate of 13-desmethyl spirolide C for spiked samples with this assay was 80% and the inter-assay coefficient of variation was 8%. This 384-well microplate, chemiluminescence method can be used as a high-throughput screening assay to detect 13-desmethyl spirolide C in shellfish meat in order to reduce the number of samples to be processed through bioassays or analytical methods.

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1. Introduction

In the early 1990s a new class of macrocyclic imines, known as spirolides (Fig. 1), was identified in toxic extracts

of the digestive glands of mussels and scallops from the Atlantic coast of Nova Scotia, Canada (Hu et al., 1995). The marine dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum* have been described as the causative organisms producing spirolides (Cembella et al., 1999, 2000; Touzet et al., 2008), and have been found at multiple locations worldwide, including Canada, Scotland, Norway, USA (Gulf of Maine), Denmark, Adriatic Sea, Spain, France and Chile (Hu et al., 1995; John et al., 2003; Aasen et al., 2005; Anderson et al., 2005; Ciminiello et al., 2006; MacKinnon et al., 2006; Villar Gonzalez et al., 2006; Amzil et al., 2007; Álvarez et al., 2009). In spite of the worldwide appearance of spirolide producing algal blooms, no episodes of human intoxication have been reported,

Abbreviations: BSA, Bovine serum albumin; α -BTX, α -bungarotoxin; CR, cross-reactivity; CV, coefficient of variation; HRP, horseradish peroxidase; i.p., intraperitoneal; LC-MS, liquid chromatography–mass spectrometry; LoD, limit of detection; MBA, mouse bioassay; MWCO, molecular-weight-cutoff; nAChR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; SD, standard deviation; SEM, standard error of the mean.

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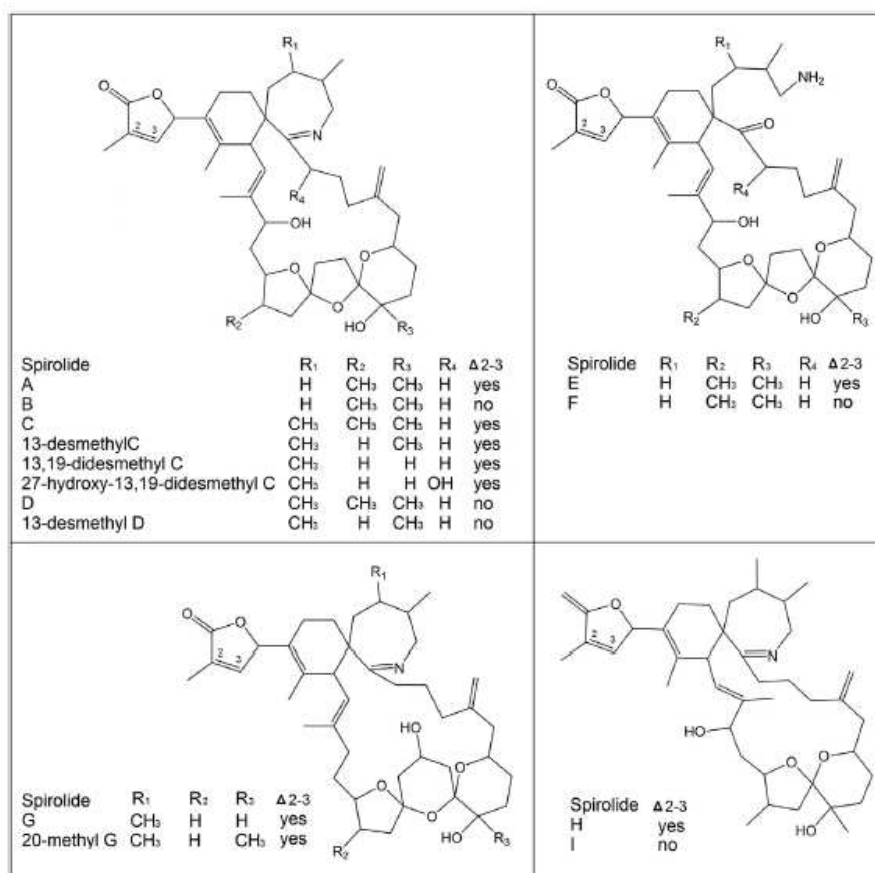


Fig. 1. Chemical structure of spirolides.

probably in part because the presence of spirolides is detected by the mouse bioassay (MBA) used for many years to protect humans from lipophilic toxins.

The cyclic imine moiety found in the structure of these compounds is believed to be responsible for the toxic effects in experimental animals (Hu et al., 1996). This moiety is also present in other toxins, like pinnatoxins, pteriatoxins, spiro-prorocentrimine, prorocentrolides and gymnodimine (Torrigoe et al., 1988; Seki et al., 1995; Uemura et al., 1995; Lu et al., 2001; Takada et al., 2001a, 2001b), which belong together with the spirolides to the group of marine toxins known as cyclic imines. The mechanism of action of the spirolides is most likely related to the inhibition of the nicotinic acetylcholine receptor (nAChR), because representative molecules of the spirolides and gymnodimine have been shown to bind to the orthosteric site of the nAChR with high affinity and to block its activation (Kharat et al., 2008; Bourne et al., 2010).

The methods more frequently used to detect cyclic imines have been the MBA and liquid chromatography-mass spectrometry (LC-MS) techniques (Hu et al., 2001; Richard

et al., 2001; Stirling, 2001; Biré et al., 2002; Aasen et al., 2005; Sleno and Volmer, 2005; Ciminiello et al., 2006; Villar Gonzalez et al., 2006; Fux et al., 2007). The MBA consists of the intraperitoneal (i.p.) injection of shellfish extracts to mice. This technique has some disadvantages, among them the number of laboratory animals sacrificed every year for the purpose of marine toxin detection. The detection and quantification of marine toxins by LC-MS methods also has some drawbacks, it requires certified standards of these compounds, in many cases non-existent, and the detection of new analogues/toxins would be lost with these techniques. Furthermore this method is expensive and requires highly qualified personnel. The development of alternative techniques based on molecular interactions to detect marine toxins is necessary to provide high-throughput screening methods that allow a reduction of the number of samples to be tested by other, more expensive and/or ethically questionable methods. Recently, two receptor-based methods have been developed to detect gymnodimines and spirolides by fluorescence polarization and chemiluminescence using the competition between

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these cyclic imines and α -bungarotoxin (α -BTX), a toxin from the venom of the snake *Bungarus multicinctus*, for binding to nAChRs (Vilariño et al., 2009; Rodríguez et al., 2011).

The aim of this work was to optimize a previously reported solid-phase receptor-based chemiluminescent assay for high-throughput screening of the presence of cyclic imines in shellfish, and characterize its performance in terms of cross reactivity, matrix interference, stability, recovery and repeatability.

2. Materials and methods

2.1. Materials

Biotin-labeled α -bungarotoxin was obtained from Molecular Probes (Eugene, OR). 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C and 20-methyl spirolide G standard solutions were purchased from Laboratorio Cifga (Lugo, Spain). Bovine serum albumin (BSA) and Tween-20 were obtained from Sigma Chemical Co. (Madrid, Spain). Acetone, chloroform, *n*-hexane, ethanol, sodium chloride, and sodium phosphates were obtained from Panreac (Barcelona, Spain). The *Torpedo marmorata* membranes rich in $\alpha_1\beta\gamma\delta$ nAChRs were purified as previously described (Vilariño et al., 2009) and stored at -80°C in one-use aliquots (stable for at least two years). Monoclonal anti-nAChR antibody mAb 35 was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Peroxidase-conjugated goat anti-mouse IgG, (H + L), SuperSignal ELISA Femto Maximum Sensitivity Substrate and Reacti-Bind streptavidin-coated 384-well plates were purchased from Thermo Scientific (Rockford, IL). Toxin-free scallops (*Pecten maximus*) were purchased from the market in Lugo (Spain). Amicon Ultra-4, PLGC Ultracel-PL membrane, 10-kDa-molecular-weight-cutoff (MWCO) filters were obtained from Millipore Corporation (Bedford, MA). 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, and PBS-x2BT was PBS supplemented with 0.2% w/v BSA and 0.2% v/v Tween-20.

2.2. Solid-phase receptor-based assay

The receptor-based assay consisted of an inhibition assay based on the competition between α -BTX and some cyclic imines for binding to the nAChR from the *T. marmorata* electric organ. The protocol started with a first incubation of sample or standard solution with purified membrane fragments enriched with nAChRs, followed by a second incubation of this mixture with biotin-labeled α -BTX. This part of the protocol took place in a microtube where 20 μL of a 1:100 dilution of the nAChR-enriched membrane stock (1.5 mg mL^{-1} , previously sonicated) were mixed with 20 μL of sample or standard solution, and allowed to interact for 30 min in order to increase assay sensitivity. Then, 40 μL of 5 nM biotin-labeled α -BTX were added to the same microtube. After 30 min, 50 μL of this mixture were transferred to a streptavidin-coated well (384-well plate) for immobilization of the biotin-labeled

α -BTX on the microplate surface. The immobilization step proceeded for 1 h and was followed by three washes with PBS-BT. The detection of the nAChR bound to immobilized biotin-labeled α -BTX was carried out using the monoclonal anti-nAChR antibody mAb 35. Fifty microliters of a 1:2000 dilution of mAb 35 were added to the well plate. After a 1 h incubation with the anti-nAChR antibody, three washes with PBS-BT preceded the addition of a HRP-labeled anti-mouse antibody (1:1000 dilution) that was allowed to bind for 30 min. All the reagent dilutions were prepared in PBS-BT, and all the incubations took place at room temperature. The quantification of the immobilized receptor was achieved by the addition of 75 μL of the SuperSignal ELISA Femto Maximum Sensitivity Substrate to each well. The chemiluminescence signal was measured within 5 min of the addition using a Synergy 4 Multi-Mode Microplate Reader from Biotek Instruments (Winnoski, VT). Biotin- α -BTX binding response was calculated as follows: $\% R = \{(S_i - S_{\min}) / (S_{\max} - S_{\min})\} \times 100$, where $\% R$ is the percentage of α -BTX binding response, S_i the signal obtained for a given condition, $S_{\max}$ the signal obtained in control wells containing nAChR and biotin- α -BTX, and $S_{\min}$ the signal obtained in control wells containing nAChR and no biotin- α -BTX. The background signal in control wells with nAChRs and no biotin- α -BTX was similar to the binding signal in wells with high concentrations of α -BTX-competing molecules.

2.3. Preparation of shellfish samples

Three hundred grams (300 g) of scallop meat (muscle and gonad) were homogenized with a blender, divided in 4 g aliquots, and stored at -20°C until use. The extraction procedure was performed as previously described (Vilariño et al., 2009). Briefly, the extraction started with the addition of 4 mL acetone to 1 g of scallop 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, 60 min). Chloroform layers were pooled together and evaporated to dryness, and the residue was reconstituted with 4.1 mL of ethanol: PBS (4:6 v/v) and filtered through a 10 kDa MWCO filter (Millipore). Finally an equal volume of PBS-2xBT was added. The scallop homogenate used in this study was analyzed by LC-MS/MS as described before (Rodríguez et al., 2011) and did not contain detectable amounts of 13-desmethyl spirolide C (limit of detection (LoD): 0.6 ng mL^{-1}), 13,19-didesmethyl spirolide C (LoD: 0.6 ng mL^{-1}) or 20-methyl spirolide G (LoD: 0.6 ng mL^{-1}), which guarantees the adequacy of this sample as blank.

2.4. Data analysis

All experiments were performed at least three times; and, in every experiment, each condition was tested in

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triplicate. The results were expressed as mean $\pm$ SEM. The calibration curves were fitted using GraphPad Prism software (La Jolla, CA). The four-parameter fit equation was: $y = \{ \text{Min} + (\text{Max} - \text{Min}) / 1 + 10 \exp ((\log \text{IC}_{50} - x) n_H) \}$, where Min is the signal in the absence of ligand, Max is the maximum binding signal at saturating ligand, $\log \text{IC}_{50}$ is the concentration when the response is halfway between Min and Max and n_H is the Hill Slope. Statistical analysis was performed using ANOVA ($p < 0.05$), with Tukey multiple comparisons post-test when required.

3. Results

3.1. Optimization of spirolide-detection by a receptor-based assay in 384-well plates

The receptor-based inhibition assay was designed using the competition between α -BTX and 13-desmethyl spirolide C for binding to the nAChR. The characteristics of a pre-existent chemiluminescence inhibition assay for the detection of 13-desmethyl spirolide C were improved using 384-well microplates. After optimization, the volumes of all reagents used during the assay were reduced to a half of the volumes used in the previously published 96-well plate assay (Rodríguez et al., 2011) (see methods section). Additionally, the concentration of biotin-labeled α -BTX was reduced to 5 nM. The final assay provided a good sensitivity for 13-desmethyl spirolide C (Fig. 2A) with an IC_{50} of 36.4 ± 2.7 nM and a limit of detection (LoD) of 6.2 ± 1.9 nM (Table 1). The LoD was calculated using three times the standard deviation (SD) of raw binding signals of the highest point (lower toxin concentration) of the calibration curve.

3.2. Cross reactivity profile of the competition assay

The cross-reactivity of the assay for the detection of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C

Table 1

IC_{50} , dynamic range (IC_{10} – IC_{90}) and limits of detection (LoD) of the solid-phase assay for 13-desmethyl spirolide C in buffer and scallop extract. The IC_{10} , IC_{50} , IC_{90} and LoD values were obtained from the experiments that generated Fig. 2A using a four parameter fit equation. For IC_{50} calculation the concentration that corresponded to half of the response between maximum and minimum (background) binding was considered. The same criterion was followed for IC_{10} and IC_{90} (mean $\pm$ SEM, $n = 9$ (shellfish extract) or $n = 6$ (buffer)).

	Buffer	Scallop
IC_{10} (nM)	10.6 ± 1.4	9.3 ± 0.7
IC_{10} (ng mL ⁻¹)	7.3 ± 1.1	6.4 ± 0.5
IC_{50} (nM)	36.4 ± 2.7	36.7 ± 1.8
IC_{50} (ng mL ⁻¹)	25.1 ± 1.8	25.3 ± 1.2
IC_{90} (nM)	168.1 ± 20.2	241.1 ± 32.2
IC_{90} (ng mL ⁻¹)	115.9 ± 13.9	166.3 ± 22.2
LoD (nM)	6.2 ± 1.9	9.8 ± 2.2
LoD (ng mL ⁻¹)	4.2 ± 1.4	6.8 ± 1.5

and 20-methyl spirolide G was evaluated by comparing IC_{50} values of the calibration curves obtained for the three molecules within the same assay. Stock solutions of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, and 20-methyl spirolide G were provided in methanol. Methanol was evaporated and the toxins were reconstituted with 20% ethanol in PBS-BT (PBS-BT/EtOH). Serial dilutions were prepared in a concentration range of 1–500 nM. The calibration curves obtained for these cyclic imines demonstrated that this assay detects the presence of the three spirolides (Fig. 3). The cross-reactivity of this competition assay for the three compounds was calculated using the IC_{50} values obtained from the calibration curves (Table 2). Considering 13-desmethyl spirolide C as reference compound, cross-reactivity was calculated as follows: % Cross-reactivity (% CR) = (IC_{50} of 13-desmethyl spirolide C / IC_{50} of spirolide) $\times$ 100. The cross-reactivity of the assay was higher for the 13,19-didesmethyl spirolide C, while the detection of 20-methyl spirolide G was slightly less efficient than the 13-desmethyl spirolide C detection (Table 2).

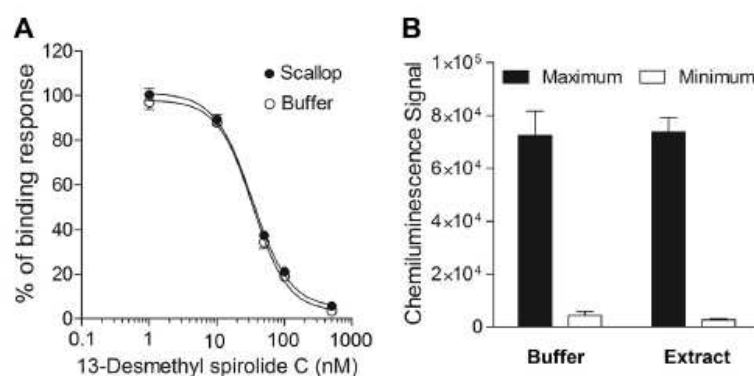


Fig. 2. Effect of scallop matrix on the detection of 13-desmethyl spirolide C using the chemiluminescence receptor-based assay. (A) Calibration curves of 13-desmethyl spirolide C in buffer and scallop extract using the receptor-based assay. Serial dilutions of 13-desmethyl spirolide C were prepared at concentrations of 1, 10, 50, 100 and 500 nM in scallop extract or in buffer (PBS-BT/EtOH). The data are expressed as percentage of the maximum α -BTX-binding response. The calibration curves were obtained with a four-parameter fit. The two curves belong to non-matched experiments (mean $\pm$ SEM, $n = 6$ (buffer), and $n = 9$ (shellfish extract)). (B) Maximum and minimum binding signals for the chemiluminescence assay in buffer and scallop extract expressed in relative luminescence units. The data belong to non-matched experiments (mean $\pm$ SEM, $n = 6$ (buffer), and $n = 9$ (shellfish extract)).

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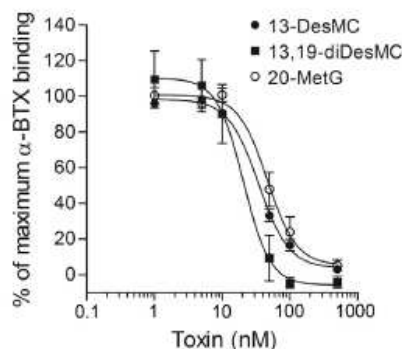


Fig. 3. Cross-reactivity profile for the detection of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C and 20-methyl spirolide G using the chemiluminescence receptor-based assay. Calibration curves of 13-desmethyl spirolide C (13-DesMC), 13,19-didesmethyl spirolide C (13,19-diDesMC) and 20-methyl spirolide G (20-MetG) were obtained using the competition assay. Calibration solutions were prepared at concentrations of 1, 5, 10, 50, 100 and 500 nM in PBS-BT/EtOH for the three toxins. The data are expressed as a percentage of the maximum α -BTX-binding response. The calibration curves were obtained with a four-parameter fit. The three curves belong to matched experiments (mean $\pm$ SEM, $n = 3$).

3.3. Scallop matrix effect on the chemiluminescence inhibition assay

The compatibility of the assay with shellfish extracts was analyzed using scallops (*P. maximus*) as shellfish matrices. The scallop extracts were prepared by extraction of meat homogenates with acetone, a subsequent water/hexane partition and a final water/chloroform partition. In order to explore the stability of 13-desmethyl spirolide C in acetone, water and chloroform, the extraction procedure was interrupted at each step and samples of acetone, water (pre and post hexane partition) and chloroform extracts were contaminated with 13-desmethyl spirolide C and stored at 4–8 °C overnight. These samples were analyzed the next day by LC–MS as previously described (Rodríguez et al., 2011) with no significant degradation of 13-desmethyl spirolide C in any of these conditions (Table 3). Consequently, the extraction procedure was interrupted overnight after chloroform extraction in the experiments that follow.

The matrix effect on this spirolide detection assay was evaluated by comparing calibration curves of 13-desmethyl spirolide C obtained in buffer and in shellfish extract. The

Table 2

IC₅₀, dynamic range (IC₁₀–IC₉₀) and % cross-reactivity (%CR) values for 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C and 20-methyl spirolide G. The IC₁₀, IC₅₀ and IC₉₀ values were obtained from the experiments that generated Fig. 3 using a four parameter fit equation. For IC₅₀ calculation the concentration that corresponded to half of the response between maximum and minimum (background) binding was considered. The same criterion was followed for IC₁₀ and IC₉₀ (mean $\pm$ SEM, $n = 3$).

	13-DesMC	13,19-diDesMC	20-MetG
IC ₁₀ (nM)	11.0 $\pm$ 0.6	8.0 $\pm$ 1.1	16.1 $\pm$ 2.3
IC ₅₀ (nM)	37.7 $\pm$ 3.3	17.7 $\pm$ 4.5	55.4 $\pm$ 14.8
IC ₉₀ (nM)	150 $\pm$ 24	38.2 $\pm$ 16.1	134 $\pm$ 16
% CR	100	210	67

Table 3

Stability of spirolides at different points of the extraction procedure. Aliquots of acetone, water and chloroform extracts were spiked with 13-desmethyl spirolide C (13-DesMC), 13,19-didesmethyl spirolide C (13,19-diDesMC) and 20-methyl spirolide G (20-MetG), stored at 4 °C overnight and analyzed the next day by LC–MS/MS. Data are expressed as percent of toxin detected after storage versus the amount of toxin used for contamination.

	13-DesMC	13,19-diDesMC	20-MetG
Acetone	92.9 $\pm$ 7.1	94.3 $\pm$ 1.4	96.9 $\pm$ 4.1
H ₂ O before hexane	100.3 $\pm$ 11.3	100.7 $\pm$ 11.9	90.4 $\pm$ 4.5
H ₂ O after hexane	98.7 $\pm$ 3.5	97.6 $\pm$ 3.6	98.9 $\pm$ 3.6
Chloroform	98 $\pm$ 16.4	108.8 $\pm$ 11.6	95.4 $\pm$ 4

solvent of the standard stock solution was evaporated and the toxin was dissolved in scallop extract or in buffer (PBS-BT/EtOH). Serial dilutions of 13-desmethyl spirolide C at concentrations of 1, 5, 10, 50, 100, and 500 nM were prepared in buffer or extract, and assayed using the chemiluminescence inhibition assay. The calibration curves obtained in buffer and scallop extract were very similar (Fig. 2A), showing very close dynamic ranges and IC₅₀ values (Table 1). Considering the estimated LoD for 13-desmethyl spirolide C in the scallop extract calibration curve (Table 1) and the dilution during the extraction procedure, the estimated LoD in scallop meat was 50 μ g kg⁻¹. The maximum and minimum binding signals obtained in buffer and scallop extract with the chemiluminescence assay were not statistically different (Fig. 2B).

3.4. Recovery of 13-desmethyl spirolide C with chemiluminescence receptor-based assay

The recovery of 13-desmethyl spirolide C from spiked scallop homogenates using the extraction procedure described above and subsequent detection with the chemiluminescence assay was also tested. Aliquots of scallop tissue (1 g $\times$ 6) were fortified with 283 ng (283 μ g kg⁻¹, corresponding to a 50 nM concentration in the final extract assuming 100% recovery) of 13-desmethyl spirolide C, extracted and analyzed the following day simultaneously using the competition assay with a calibration curve in extract. The recovery of the extraction procedure was calculated using the following equation: % Recovery = 100 $\times$ (measured content/fortification level). The chemiluminescence assay detected an amount of 222.1 $\pm$ 7.0 μ g kg⁻¹ of 13-desmethyl spirolide C resulting in a recovery of 78.5 $\pm$ 2.5%. In order to analyze if calibration curves in buffer could be used for routine detection of spirolides using this technique, an experiment was performed including calibration curves in buffer and extract, and the extraction and analysis of 3 spiked samples (283 μ g kg⁻¹ 13-desmethyl spirolide C). The toxin content of the samples was calculated using the calibration curves in buffer and extract, yielding recoveries of 13-desmethyl spirolide C of 79.3 $\pm$ 3.1% and 81.2 $\pm$ 3.6% respectively.

3.5. Repeatability of the spirolide-detection assay

The intra-assay and inter-assay repeatabilities were determined for the detection of 13-desmethyl spirolide C

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considering the entire protocol which included extraction and inhibition assay. The intra-assay repeatability was evaluated by analyzing 6×1 g aliquots of scallop homogenates spiked with $283 \mu\text{g kg}^{-1}$ of 13-desmethyl spirolide C. The procedure was repeated in 3 different days for inter-assay variation. After the extraction procedure the spirolide content of each sample was calculated from the response value using a calibration curve in scallop extracts obtained in the same assay. The coefficient of variation (CV) within the same run (intra-assay CV) and the CV between runs (inter-assay CV) were 7.9% and 2.9% respectively.

3.6. Stability of 13-desmethyl spirolide C in buffer, extract and scallop homogenate

The stability of 13-desmethyl spirolide C in buffer and scallop extract in different storage conditions was also analyzed. Blank scallop samples were extracted and the final extracts were contaminated with a final concentration of 50 nM 13-desmethyl spirolide C. Similarly, a solution of 50 nM 13-desmethyl spirolide C was prepared in buffer (PBS-BT/EtOH). Both 13-desmethyl spirolide C solutions were divided in aliquots and stored over one or two weeks at $4-8^\circ\text{C}$ or -20°C . One set of 6 samples was analyzed using the receptor-based assay on the same day the aliquots were prepared. After one or two weeks the stored samples (6 per condition) were assayed with the competition assay and quantified with freshly prepared calibration curves in buffer or extract, as suitable. There was no degradation of 13-desmethyl spirolide C after one week of storage at both temperatures either in buffer or extract (Fig. 4A and B). However, longer storage of these samples generated interferences with the detection of 13-desmethyl spirolide C in some storage conditions (Fig. 4A and B). The stability of 13-desmethyl spirolide C in the scallop homogenate was also studied. For this purpose, aliquots of scallop homogenate (3×1 g) were spiked with 283 ng of 13-desmethyl spirolide C. Two samples were stored in the freezer for one or two weeks. Then the samples were thawed, extracted and assayed. The toxin content was compared with one sample of spiked scallop homogenate freshly analyzed on day 0 and no differences were observed among the spirolide content detected in frozen and fresh samples (Fig. 4C).

4. Discussion

In this study a previous solid-phase chemiluminescent assay for the detection of 13-desmethyl spirolide C was optimized for high-throughput screening. The performance of this assay was evaluated by a single-laboratory pre-validation. The scarcity of these toxins worldwide was the main limitation for a complete and thorough single laboratory validation. The guidelines of the European Decision 2002/657/EC (EC, 2002) were followed when possible. Instead of $\text{CC}\alpha$ (decision limit) and $\text{CC}\beta$ (detection capability) LoD and LoQ values are reported, because the determination of $\text{CC}\alpha$ and $\text{CC}\beta$ requires 20 blank samples. Twenty samples of different shellfish matrixes were tested for the presence of spirolides and all of them contained detectable amounts of these toxins, which made them not

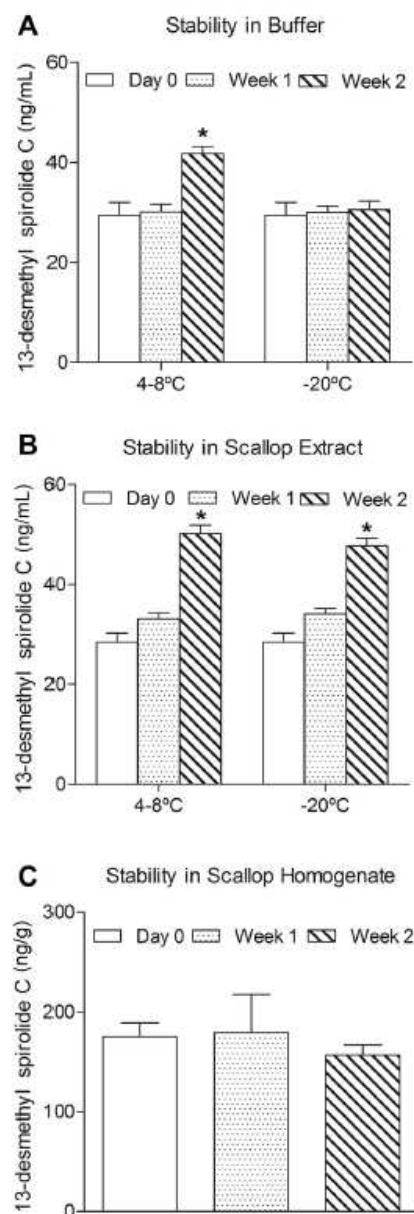


Fig. 4. Stability of the 13-desmethyl spirolide C in buffer, scallop extract, and scallop homogenate during storage. Stability of 13-desmethyl spirolide C in buffer (A) and scallop extract (B). Buffer (PBS-BT/EtOH) and scallop extract were spiked with 34 ng mL^{-1} of 13-desmethyl spirolide C and analyzed fresh, or stored over one or two weeks at $4-8^\circ\text{C}$ and -20°C . The samples (6 per storage condition) were assayed using freshly prepared calibrants (mean $\pm$ SEM, $n = 3$). (C) Stability of 13-desmethyl spirolide C in scallop homogenate at -20°C . Scallop homogenate was spiked with $283 \mu\text{g/kg}$ of 13-desmethyl spirolide C and stored in the freezer over one or two weeks. Finally, the samples were thawed and analyzed using the receptor-based assay. One sample was analyzed fresh (on the same day of the preparation).

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suitable to be used as blanks, except for the sample used in this study. The inhibition assay based on the competition of spirolides and α -BTX for binding to the nAChR was adapted to 384-well streptavidin-coated microplates for high-throughput screening. The 384-well microplate assay allows a reduction of the volume of reagents per sample to half the volumes used in the 96-well microplate assay and simultaneous testing of a maximum of 120 samples. The concentration of biotin- α -BTX was also reduced 10 times versus the previously published chemiluminescence assay. The nAChR membranes obtained from one Torpedo fish would allow the screening of thousands of samples. Overall, the 384-well plate assay allows the detection of 5 times more samples per plate than the previously published assay at a lower cost per sample.

In previous studies, the competition between α -BTX and several compounds of the cyclic imine group for binding to nAChRs has been demonstrated (Kharrat et al., 2008; Bourne et al., 2010). Actually, a recently published chemiluminescence assay, similar to the assay presented in this work, demonstrated the competition of α -BTX with 13-desmethyl spirolide C and gymnodimine for binding to nAChRs (Rodríguez et al., 2011). Interestingly, gymnodimine cross-reactivity was poor when the first incubation lasted for 30 min, although the sensitivity of the assay for this toxin increased with an incubation time of 2 h, conditions that yielded a sensitivity for gymnodimine about 10 times lower than for 13-desmethyl spirolide C (Rodríguez et al., 2011). The cross-reactivity of the chemiluminescence assay for other members of the cyclic imine group had not been tested previously. Actually, this work and a recently published receptor-based method for spirolide detection using a Luminex system are the first reports of the ability of the nAChR to bind 20-methyl spirolide G (Rodríguez et al., 2013). Our results demonstrate that this nAChR-based assay can detect the presence of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C or 20-methyl spirolide G. The assay seems to be more sensitive for 13,19-didesmethyl spirolide C, followed by 13-desmethyl spirolide C, as reported in a previous fluorescence polarization assay (Fonfría et al., 2010b), and finally it has a slightly lower sensitivity for 20-methyl spirolide G. Although this assay can detect several cyclic imines, representative toxins of other marine toxin groups, such as okadaic acid, saxitoxin, brevetoxin-2, yessotoxin and azaspiracid-2 are not detected by nAChR-based assays (Vilariño et al., 2009; Rodríguez et al., 2011). This method is specific for cyclic imines among marine toxins, but not selective among members of this toxin group. Ideally, a non-selective screening assay like this receptor-based assay should have a cross-reactivity profile for the target compounds that parallels their reported toxicity. This assay based on binding to $\alpha_1\beta\gamma\delta$ nAChR, which is a muscle type receptor, provides higher detection capabilities for spirolides versus gymnodimine, as it would be expected considering the affinities of representative toxins for the $\alpha_1\beta\gamma\delta$ Torpedo receptor (Bourne et al., 2010). The neuronal $\alpha_4\beta_2$ human receptor also displayed higher affinity for 13-desmethyl spirolide C than for gymnodimine. On the contrary, gymnodimine has higher affinity than the spirolide for $\alpha_4\beta_2$ human neuronal receptors and mollusk acetylcholine binding proteins

(Bourne et al., 2010). Cyclic imine toxicity data are scarce and in many occasions it is difficult to establish relative toxicity ratios because the data were obtained in different experimental conditions and because of the lack of certified standard solutions at the time the studies were done. In any case, gymnodimine seems to be much less toxic than the spirolides (500 $\mu\text{g kg}^{-1}$ oral LD₅₀ for 13-desmethyl spirolide C and >7500 $\mu\text{g kg}^{-1}$ oral LD₅₀ for gymnodimine) (Munday et al., 2004, 2012), and therefore the selection of the muscle $\alpha_1\beta\gamma\delta$ nAChR from Torpedo as detecting molecule provides an assay cross-reactivity for gymnodimine and 13-desmethyl spirolide C that resembles *in vivo* toxicity. In a recent work, the i.p. LD₅₀ for 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (in the same conditions) were 27.9 and 32.3 $\mu\text{g kg}^{-1}$ respectively (Otero et al., 2012). In another study the i.p. LD₅₀ values of 13-desmethyl spirolide C and 20-methyl spirolide G were 6.9 and 8 $\mu\text{g kg}^{-1}$ respectively, and their oral LD₅₀ was 500 $\mu\text{g kg}^{-1}$ for both compounds (Munday et al., 2012). The cross-reactivity of our assay for these 3 spirolides is close to the toxic potency derived from toxicity studies, with similar potencies for 13-desmethyl spirolide C and 20-methyl spirolide G. It should be pointed out that the cross-reactivity data of the 13,19-didesmethyl spirolide C presented in this study is an estimate, but not conclusive until a certified standard solution is available for adequate quantification.

The extraction procedure, based on acetone extraction followed by hexane clean-up and chloroform partition, had been tested previously with other matrixes and receptor-based assays. This method provides a final extract from scallops that displays no interferences with the chemiluminescence inhibition assay, as it had been previously demonstrated for cockles (Rodríguez et al., 2011). The recovery rate of 13-desmethyl spirolide C from spiked scallop samples was higher than the recoveries reported for cockles with a receptor-based chemiluminescence assay. Due to the lack of matrix interference the quantification of the toxin content of a sample with buffer or extract calibration curves was similar; suggesting that for routine analysis a calibration curve in buffer can be used for screening the presence of spirolides in shellfish extracts. Additionally, further analysis of the assay performance demonstrated adequate intra- and inter-assay repeatabilities. This extraction method has been shown to be compatible with nAChR-based methods designed for the detection of spirolides using both fluorescence polarization or chemiluminescence assays (Vilariño et al., 2009; Rodríguez et al., 2011), to provide extracts from mussel, scallop, cockle and clam suitable for nAChR-based methods (Vilariño et al., 2009; Fonfría et al., 2010a; Rodríguez et al., 2011) and to yield adequate recovery rates of 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C (Vilariño et al., 2009; Fonfría et al., 2010a, 2010b; Rodríguez et al., 2011). An important additional piece of information is the stability of spirolides after overnight interruption of the extraction procedure. This stability implies that the extraction protocol can be performed in two separate days, which provides the flexibility required by the schedules of many routine analysis laboratories. Moreover storage of samples, extracts or buffer containing 13-desmethyl spirolide C showed no significant degradation of the toxin

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after one week at 4 °C or –20 °C in buffer or extract, and after two weeks at –20 °C in scallop samples, which is also convenient for the organization of laboratory work and cost reduction. However, some interferences appear after longer storage of toxin solutions in buffer and extract, possibly due to alterations of the toxin or solution components during storage, that preclude the use of the assay in these conditions.

A safe level of spirolide toxin content in shellfish has been estimated to be approximately 400 µg kg⁻¹ by some authors (Munday, 2008), and this solid-phase chemiluminescence assay is capable of detecting 13-desmethyl spirolide C in the range of 50–1300 µg kg⁻¹ in shellfish samples. Recently, two new nAChR-based assays have been developed using colorimetric detection coupled to mass-spectrometry for toxin identification (Aráoz et al., 2012) or flow-fluorimetry in a Luminex system (Rodríguez et al., 2013). These two methods provide some advantages versus the current assay such as higher sensitivity, unequivocal identification of toxins in the case of the first assay, simpler extraction procedures or multi-detection capabilities for the second method, however laboratory instrumentation required for these methods is more expensive and less frequently available in routine detection laboratories.

5. Conclusion

In this work a pre-existing chemiluminescence nAChR-based assay for the detection of spirolides has been improved by increasing the number of samples and assay flexibility and reducing costs. This assay is sensitive enough to detect 13-desmethyl spirolide C in the range of 50–1300 µg kg⁻¹ in shellfish meat, with a good repeatability and can be used as a high-throughput screening assay with suitable flexibility for laboratory work organization. Additionally, this receptor-based assay is sensitive to the presence of other toxic members of the spirolide group, including 20-methyl spirolide G and 13,19-didesmethyl spirolide C, providing an overall estimation of the spirolide-related toxicity of a sample.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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**Bloque II. Diseño de métodos de detección de
iminas cíclicas y azaspirácidos mediante la
tecnología Luminex**





Artículo 3. Development of a Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines Using a Microsphere-Flow Cytometry System.

Laura P. Rodríguez, Natalia Vilariño, Jordi Molgó, Rómulo Aráoz, M. Carmen

Louzao, Palmer Taylor, Todd Talley, Luis M. Botana.

Development of a Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines Using a Microsphere-Flow Cytometry System

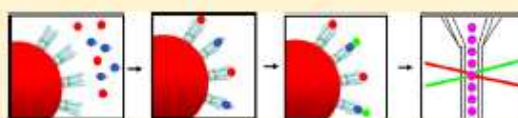
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ABSTRACT: Biologically active macrocycles containing a cyclic imine were isolated for the first time from aquaculture sites in Nova Scotia, Canada, during the 1990s. These compounds display a “fast-acting” toxicity in the traditional mouse bioassay for lipophilic marine toxins. Our work aimed at developing a receptor-based detection method for spirolides using a microsphere/flow cytometry Luminex system. For the assay, two alternatives were considered as binding proteins, the *Torpedo marmorata* nicotinic acetylcholine receptor (nAChR) and the *Lymnaea stagnalis* acetylcholine binding protein (Ls-AChBP). A receptor-based inhibition assay was developed using the immobilization of nAChR or Ls-AChBP on the surface of carboxylated microspheres and the competition of cyclic imines with biotin- α -bungarotoxin (α -BTX) for binding to these proteins. The amount of biotin- α -BTX bound to the surface of the microspheres was quantified using phycoerythrin (PE)-labeled streptavidin, and the fluorescence was analyzed in a Luminex 200 system. AChBP and nAChR bound to 13-desmethyl spirolide C efficiently; however, the cross-reactivity profile of the nAChR for spirolides and gymnodimine more closely matched the relative toxic potencies reported for these toxins. The nAChR was selected for further assay development. A simple sample preparation protocol consisting of an extraction with acetone yielded a final extract with no matrix interference on the nAChR/microsphere-based assay for mussels, scallops, and clams. This cyclic imine detection method allowed the detection of 13-desmethyl spirolide C in the range of 10–6000 μ g/kg of shellfish meat, displaying a higher sensitivity and wider dynamic range than other receptor-based assays previously published. This microsphere-based assay provides a rapid, sensitive, and easily performed screening method that could be multiplexed for the simultaneous detection of several marine toxins.



Shellfish constitute a worldwide food resource that may be contaminated with toxins produced by dinoflagellates, representing a potential threat to human health and having an important economic impact on the aquaculture industry. Spirolides and gymnodimines (Figure 1) are marine phycotoxins that belong to the group of macrocyclic imines^{1,2} due to the presence of a cyclic imine moiety in their macrocyclic chemical structure. Other marine toxins of the cyclic imine structural group are known as pinnatoxins, pteriatoxins, prorocentrolides, and spiro-procentrimine.^{3–7}

The spirolides were discovered during routine monitoring of the presence of the lipophilic diarrhetic shellfish poisoning (DSP) toxins in aquaculture sites along the South Eastern coast of Nova Scotia, Canada, in the early 1990s.⁸ The marine dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum* have been identified as the spirolide-producing microorganisms.^{9–11} Gymnodimines are produced by the planktonic dinoflagellate *Karenia selliformis* and were first isolated from New Zealand oysters in 1994.^{12,13} Although, spirolides and gymnodimines have not been linked to human intoxication so

far,¹⁴ these compounds displayed “fast-acting” toxicity in the traditional bioassay.^{14,15}

The mouse bioassay (MBA) has been the most commonly used method to detect marine toxins¹⁴ and it has been a basis for protecting human health for decades.¹⁶ However, such techniques have several drawbacks because of ethical concerns, sensitivity, and specificity.¹⁷ In the past few years, several techniques using mass spectrometry (MS) have also been developed for the detection of macrocyclic imines.^{1,18–24} These MS-based methods will only detect known compounds for which standards are available being blind to the presence of new toxic analogues or groups. Furthermore the use of MS-based techniques for routine detection of toxins requires expensive instrumentation and highly trained laboratory personnel. The evidence that spirolides and gymnodimine target muscular and neuronal nicotinic acetylcholine receptor

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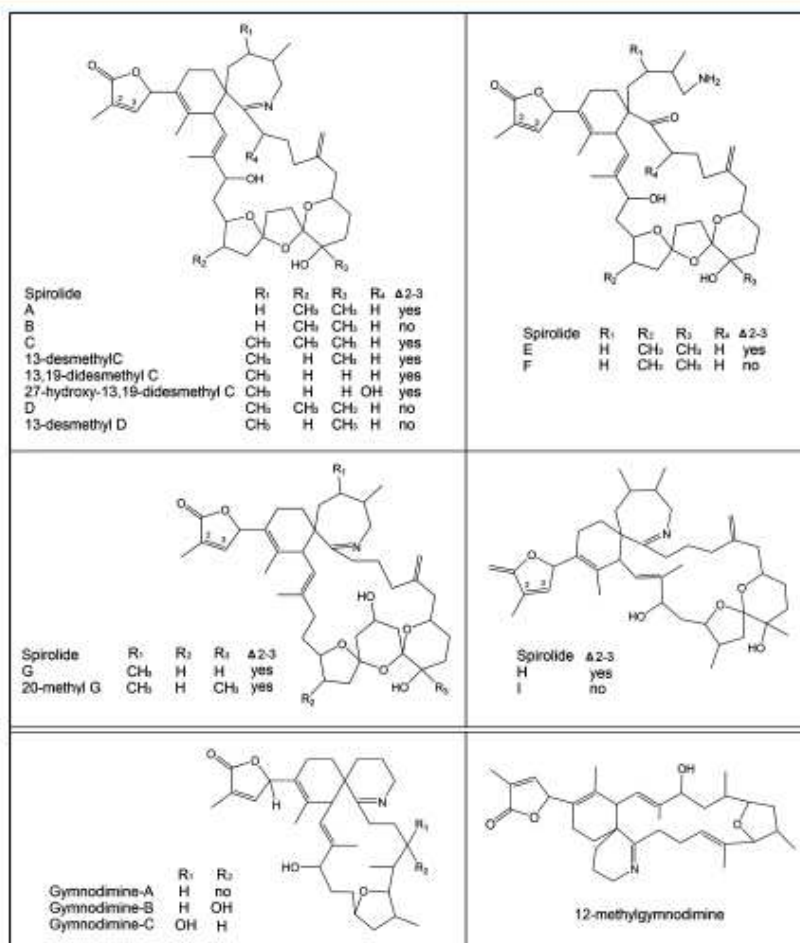


Figure 1. Chemical structures of spirolides and gymnodimines.

(nAChR) subtypes with high affinity^{25,26} has prompted the development of alternative techniques using a receptor-based approach for the detection of spirolides and gymnodimines by fluorescence polarization and chemiluminescence.^{27–29}

In recent years, multiplexed binding assays using microspheres have become a widely used tool for clinical diagnostics and research.³⁰ The Luminex technology is based on the use of polystyrene beads internally dyed with two spectrally distinct fluorochromes, resulting in 100 different microsphere populations. The surface carboxyl groups of the microspheres can be used to couple a binding molecule specific for the interaction with an analyte of interest. The binding molecule immobilized on the surface of a microsphere class confers specificity to each microsphere population for a certain analyte and the possibility of multidetection of several analytes by combining different microsphere classes in the same sample. The discrimination of microsphere classes and quantification of binding signal take place in a Luminex analyzer by detecting two different fluorescence signals per microsphere.

Macrocyclic imines, as mentioned above, have been shown to interact with nAChRs and to block their activity.^{25,26} Recently, a soluble acetylcholine binding protein (AChBP) from the water snail *Lymnaea stagnalis* has also been demonstrated to bind these toxins.²⁵ Actually, soluble AChBPs have been used as surrogates of nAChRs to study the interaction of different molecules with nAChRs by crystallographic and solution-based techniques due to the similarity of their ACh binding sites.^{25,31–33}

The aim of this study was to develop a microsphere-based assay for the detection of cyclic imines using binding proteins/receptors in a Luminex system and to characterize its advantages versus previously published receptor-based methods in terms of sensitivity, flexibility, simplicity, and assay duration.

MATERIALS AND METHODS

Materials. Biotin- α -bungarotoxin (α -bungarotoxin peptide sequence, IVCHTTATSPISAVTCTPPGENLCYRKMWCDFAF-CSSRGKVVVELGCAATCPSKKPYEEVTCSTDKCNP-HPKQRP; molecular formula, C₃₃₈H₅₂₉N₉₇O₁₀₅S₁₁) was

obtained from Molecular Probes (Eugene, OR). 13-Desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide G, okadaic acid, and yessotoxin standard solutions were purchased from Laboratorio Cifga (Lugo, Spain). Gymnodimine, saxitoxin, azaspiracid-1, and domoic acid standard solutions were obtained from the Institute for Marine Biosciences, National Research Council (Halifax, Canada). Bovine serum albumin (BSA), Tween-20, and N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich (Madrid, Spain), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Pierce (Rockford, IL). Acetone, absolute ethanol, sodium chloride, and sodium phosphates were obtained from Panreac (Barcelona, Spain). The nAChR-rich membranes from *Torpedo marmorata* were purified as previously described.²⁷ Toxin-free scallops (*Pecten maximus*), clams (*Ruditapes philippinarum*), and mussels (*Mytilus galloprovincialis*) were purchased from the market (Lugo, Spain). The acetylcholine binding protein from *Lymnaea stagnalis* (Ls-AChBP), the acetylcholine binding protein from *Aplysia californica* (Ac-AChBP), and the Y55W mutant of Ac-AChBP were purified and characterized as described previously.^{32,34} Calibration and performance verification kits for Luminex 200, carboxylated microspheres (LC10027-01 and LC10004-01), and sheath fluid were from Luminex Corporation (Austin, TX). PhycoLink Streptavidin-R-Phycoerythrin (PE) was purchased from Prozyme (Hayward, CA). Amicon Ultra-4, PLGC Ultracel-PL membrane, 10-kDa-molecular-weight-cutoff (MWCO) filters, 33 mm Millex filters with a 0.22 μm pore size, and Multiscreen 96-well filter plates (Dunapore membrane) were obtained from Millipore (Madrid, Spain). Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH_2PO_4 , 8.5 mM Na_2HPO_4 , pH 7.4. PBS-BT was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20, and PBS-x2BT was PBS supplemented with 0.2% w/v BSA and 0.2% v/v Tween-20. All solutions were filtered through a 0.22 μm pore size filter before use.

Coupling of Binding Proteins to Carboxylated Microspheres. Binding proteins were immobilized on the surface of microspheres following the protocol recommended by Luminex for protein coupling to carboxylated microspheres. The immobilization protocol was performed with LC10027-01 or LC10004-01 microspheres (4×10^5 to 1×10^6) in 1.5 mL microtubes. The carboxyl groups were activated by a mixture of 40 μL of EDC/NHS 1:1 (both reagents at concentrations of 50 mg/mL in water) and 160 μL of the activation buffer (0.1 M NaH_2PO_4 , pH 6.2). After 20 min of incubation, the microspheres were centrifuged and the supernatant was carefully discarded. The beads were then washed three times with 500 μL of PBS, and 200 μL of binding protein solution were added to preactivated microspheres and allowed to react for 2 h. Protein dilutions used for the immobilization were 1:40 for nAChR membrane stock (protein concentration of 1.5 mg/mL), 1:200 for Ls-AChBP stock (protein concentration of 6.4 mg/mL), and 1:10 and 1:100 for Ac-AChBP and Y55W Ac-AChBP stocks (protein concentrations of 6.5 mg/mL and 7 mg/mL, respectively). The protein solutions were sonicated for 10 s before being added to the microsphere suspension. Finally, the microspheres were washed with PBS-BT and stored in PBS-BT at 4 °C in the dark. Removal of reagents and washes were performed by centrifugation at 8000g for 2 min at room temperature, and all incubations were performed with constant shaking (200 rpm) at room temperature in the dark.

Microsphere-Based Assay Protocol for Detecting Cyclic Imines.

A microsphere-based assay was developed using the competition between α -bungarotoxin (α -BTX), which binds to the ACh binding site of the nAChR, and cyclic imines for the interaction with binding proteins attached to the microsphere surface. The assay was performed in a well of an opaque, 1.2 μm filter plate. For each well, 25 μL of the calibration solution or sample were added to 4×10^5 binding protein-coated microspheres (final volume 50 μL) and incubated for 30 min. Then, 50 μL of 50 nM biotin-labeled α -BTX were added to the same well and incubated for 30 min. The buffer was removed by filtration, and the microspheres were washed three times with PBS-BT. A volume of 100 μL of 4 $\mu\text{g/mL}$ PE-labeled streptavidin was added to the microspheres for 30 min. Finally, the microspheres were washed and suspended in 100 μL of PBS-BT by shaking immediately before detection. The reagents used in the assay were diluted to the required concentration in PBS-BT. Incubations were performed with constant shaking (700 rpm) at room temperature in the dark. All washes consisted of addition and subsequent removal of 200 μL of PBS-BT. For the removal of reagent and washing solutions, a vacuum manifold was used without exceeding 5 mmHg of vacuum pressure.

Quantification of the Fluorescence Signal. PE fluorescence bound to the microsphere surface was measured with a Luminex 200 analyzer (LuminexCorp, Austin, Texas). The Luminex system uses a 635 nm laser to discriminate between different microsphere classes based on their internal fluorescence. A second 532 nm laser is used to quantify PE-labeled streptavidin fluorescence bound to the microsphere surface. Default values of 7 500–13 500 were used for doublet discriminator gating of microspheres. The acquisition volume was 75 μL , and the minimum bead count was 100. Biotin- α -BTX binding response was calculated as follows: $\%R = \{(S_i - S_{\text{min}})/(S_{\text{max}} - S_{\text{min}})\} \times 100$, where $\%R$ is the percentage of α -BTX binding response, S_i is the signal obtained for a given condition, S_{max} the signal obtained in control wells containing protein-bound microspheres and biotin-labeled α -BTX, and S_{min} the signal obtained in control wells containing protein-bound microspheres and no biotin-labeled α -BTX.

Shellfish Extraction Procedure. Shellfish meat (whole body of mussel or clam and muscle plus gonad of scallop) was homogenized with a blender, divided into aliquots, and stored at -20 °C until use. The extraction procedure consisted of the addition of 4 mL of 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 3500g 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 20 mL of ethanol-PBS (4:6 v/v) and filtered through a 10 kDa MWCO filter (Millipore). Finally, an equal volume of PBS-2xBT was added. The mussel, clam, and scallop homogenates used for this study were previously analyzed by LC-MS/MS for the presence of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide C, and gymnodimine as described previously,²⁸ to be sure that the shellfish samples did not contain these compounds at levels at least 10 times lower than the limit of detection (LoD) of the Luminex assay.

Safety. Cyclic imines are fast-acting toxins with very high acute intraperitoneal (i.p.) toxicity in mice. Although no human intoxication has been reported, standard solutions should be

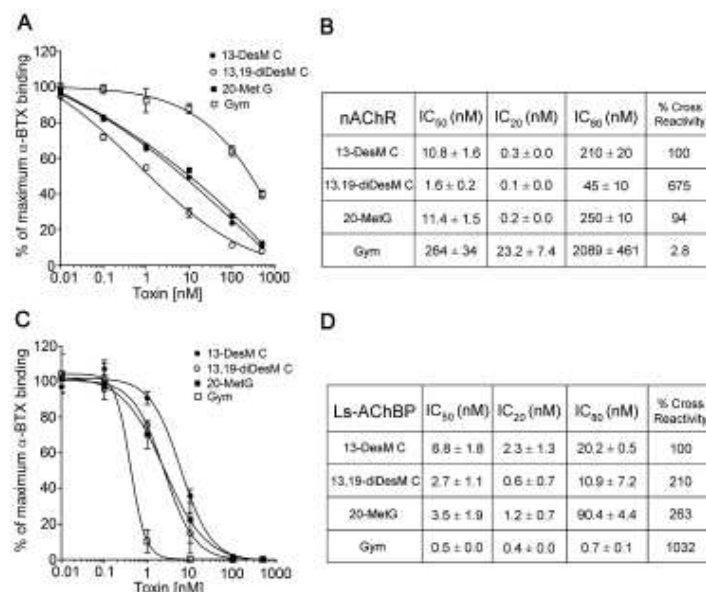


Figure 2. Cross-reactivity profiles for the detection of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide G, and gymnodimine with the nAChR- and Ls-AChBP-based inhibition assays. The data are expressed as percentage of biotin- α -BTX maximum binding response. The calibration curves were obtained with a four-parameter fit. (A) Calibration curves for the spirolides and gymnodimine with the nAChR inhibition assay. The four curves belong to nonmatched experiments (mean $\pm$ SEM; 13-desmethyl spirolide C, $n = 6$; 13,19-didesmethyl spirolide C, $n = 3$; 20-methyl spirolide G, $n = 3$; and gymnodimine, $n = 3$). (B) IC₅₀ dynamic range, and cross-reactivity (%) of the nAChR-microsphere-based assay were obtained from calibration curves in part A. (C) Calibration curves for the spirolides and gymnodimine with the Ls-AChBP inhibition assay. The four curves belong to nonmatched experiments (mean $\pm$ SEM; 13-desmethyl spirolide C, $n = 9$; 13,19-didesmethyl spirolide C, $n = 3$; 20-methyl spirolide G, $n = 4$; and gymnodimine, $n = 2$). (D) IC₅₀ dynamic range, and cross-reactivity (%) of the Ls-AChBP-microsphere based assay from calibration curves in part C.

handled with gloves, and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

Data Analysis. The results were expressed as mean $\pm$ standard error of the mean (SEM), and in every experiment each condition was tested in duplicate. The calibration curves were fitted using a four-parameter logistic equation obtained by a nonlinear regression fitting procedure (GraphPad Prism 5.0). The four-parameter fit equation was $y = \{(\text{Min} + (\text{Max} - \text{Min})) / (1 + 10^{\exp((\log \text{EC}_{50} - x) \cdot n_H)})\}$, where Min is the signal in the absence of ligand, Max is the maximum binding signal at saturating ligand, $\log \text{EC}_{50}$ is the concentration when the response is halfway between Min and Max, and n_H is the Hill slope. Statistical analysis for multiple comparisons was performed using ANOVA ($p < 0.05$). The LoD was calculated by subtracting 3 times the standard deviation (SD) of the raw binding signals of the highest point of the calibration curve from the maximum binding signal and interpolating this value in the calibration curve.

RESULTS

Selection of Binding Proteins for the Microsphere-Based Assay. The feasibility of using alternative binding proteins in a microsphere-based assay for the detection of cyclic imines using α -BTX competition was explored in order to select optimal binding characteristics. The proteins tested were nAChRs from the *Torpedo marmorata* electric organ, AChBP from the freshwater snail *Lymnaea stagnalis* (Ls-AChBP), AChBP from the saltwater mollusk *Aphysa californica* (Ac-

AChBP), and the Y55W mutant of Ac-AChBP. Ls-AChBP, nAChR, Ac-AChBP, and Y55W Ac-AChBP were immobilized on the microsphere surface, and binding of 13-desmethyl spirolide C was evaluated by inhibition of α -BTX binding to the protein-coated surface of the microspheres. The Ac-AChBP and Y55W-coated microspheres did not show a sufficient α -BTX binding signal under these conditions to perform a competition assay (data not shown), probably due to a much lower affinity for α -BTX than other AChBPs^{32,34} and therefore they were not suitable for this assay design. In contrast, Ls-AChBP- and nAChR-coated microspheres showed excellent α -BTX binding signals, with maximum binding signal to background ratios around 2000 and 500, respectively, under these assay conditions. Ls-AChBP and nAChR-immobilized microspheres were then used to obtain 13-desmethyl spirolide C calibration curves. The solvent of the 13-desmethyl spirolide C standard stock solution, methanol-TFA (trifluoroacetic acid), was evaporated, since methanol has been previously shown to interfere with binding properties of nAChR-rich membrane fragments,²⁷ and 13-desmethyl spirolide C was dissolved in PBS-BT containing 20% ethanol (PBS-BT/20% EtOH). Serial dilutions of 13-desmethyl spirolide C at concentrations of 0.01, 0.1, 1, 10, 100, and 500 nM were prepared in PBS-BT/20% EtOH (all assay wells contained the same final concentration of ethanol in order to ensure cyclic imine toxin solubility) and assayed using the nAChR and Ls-AChBP-based assays. 13-Desmethyl spirolide C inhibited binding of α -BTX to both proteins with similar IC₅₀ values (Figure 2). However, the

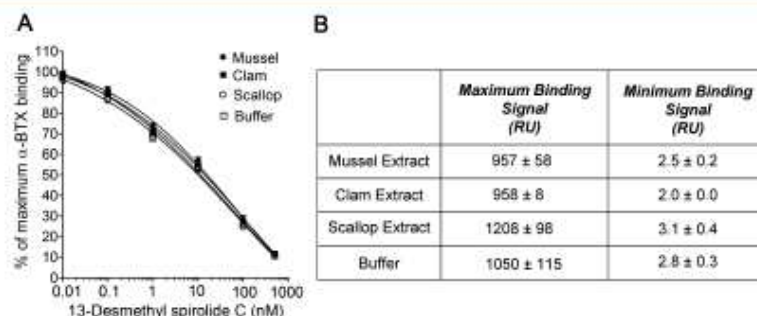


Figure 3. Effect of mussel, clam, and scallop matrices on the detection of 13-desmethyl spirolide C using the nAChR/microsphere-based assay. (A) Calibration curves of 13-desmethyl spirolide C in mussel, clam, and scallop extracts and in buffer using the nAChR inhibition assay. The data are expressed as percentage of biotin- α -BTX maximum binding response. The calibration curves were obtained with a four-parameter fit. The four curves belong to nonmatched experiments (mean $\pm$ SEM; mussel, $n = 6$; clam, $n = 2$; scallop, $n = 5$; and buffer, $n = 8$). (B) Maximum and minimum binding signals in shellfish extracts and buffer from the experiments in part A.

dynamic range ($IC_{20}-IC_{80}$) for 13-desmethyl spirolide C was considerably wider for the nAChR assay than for the Ls-AChBP assay (Figure 2B,D).

Cross Reactivity Profile of the nAChR and Ls-AChBP Assays. Cross-reactivities of the nAChR and Ls-AChBP assays were evaluated by comparing the inhibition of α -BTX binding by several members of the macrocyclic imine toxins. Calibration curves of 13-desmethyl spirolide C, 13,19-didesmethyl spirolide C, 20-methyl spirolide G, and gymnodimine were obtained using nAChR or Ls-AChBP-based competition assays in buffer (PBS-BT/20% EtOH). The concentrations of the calibration solutions for the four toxins ranged from 0.01 to 500 nM. All the phycotoxins tested inhibited binding of α -BTX to the surface of the two classes of protein-coated microspheres (Figure 2A,C). The IC_{50} of the calibration curves was used to evaluate the cross-reactivity of the assays for these compounds (Figure 2B,D). The cross-reactivity of every toxin tested was expressed related to 13-desmethyl spirolide C reactivity and calculated as follows: % cross-reactivity (% CR) = (IC_{50} of 13-desmethyl spirolide C/ IC_{50} of cyclic imine) $\times$ 100. The results showed that the cross-reactivity profile of cyclic imine binding to nAChR- and Ls-AChBP-coated microspheres differed considerably. The most remarkable difference was the efficient binding of gymnodimine relative to spirolides to Ls-AChBP-coated microspheres (Figure 2C,D), while gymnodimine binding in the nAChR assay was weaker than spirolide binding (Figure 2A,B). After the evaluation of the assay characteristics, the nAChR-based assay was selected for further development based on its dynamic range and cyclic imine cross-reactivity profile. The interference of representative toxins of the regulated toxin groups with the nAChR-based assay was tested using 10 μ M saxitoxin, 10 μ M okadaic acid, 10 μ M domoic acid, 5 μ M yessotoxin, and 1 μ M azaspiracid-1, and no effects on maximum and minimum binding signals were evidenced.

Additionally, the Luminex system offers the possibility of multidetection, and therefore nAChR- and Ls-AChBP-coated microspheres were combined in the same well to explore the performance of both assays simultaneously, in order to provide a higher sensitivity for gymnodimine if desirable. The combination of nAChR-coated microspheres (LC-10027-01) and Ls-AChBP-coated microspheres (LC-10004-01) in the same well yielded calibration curves, similar to the individual assays (data not shown).

Effect of Shellfish Matrices on the nAChR/Microsphere Assay.

The compatibility of this nAChR/microsphere-based assay with shellfish extracts was assessed using mussels, scallops, and clams. In order to evaluate the matrix effect on spirolide detection, calibration curves of 13-desmethyl spirolide C and maximum and minimum binding signals of the assay in buffer and in shellfish extracts were compared. The extracts were prepared as described in the Materials and Methods section, starting with an acetone extraction followed by evaporation and reconstitution in ethanol/buffer and filtration. The presence of 20% ethanol in the final extract did not affect the assay, since this concentration of ethanol was present in all assay conditions tested in this study, as reported in the previous sections. For 13-desmethyl spirolide C calibration curves, the calibration solutions were prepared as previously described in buffer or shellfish extract and assayed using the nAChR/microsphere-based assay. The calibration curves obtained in clam, mussel, and scallop extracts and in buffer were virtually identical (Figure 3A). The dynamic ranges, estimated LoDs, and IC_{50} values calculated from the calibration curves were very similar in buffer and shellfish extract (Table 1). Maximum and minimum binding signals of the nAChR/microsphere-based assay performed with mussel, scallop, or clam extracts did not

Table 1. IC_{50} , Dynamic Range, and Estimated LoD of the nAChR/Microsphere-Based Assay for 13-Desmethyl Spirolide C Detection in Shellfish Extracts and Buffer^a

	mussel	clam	scallop	buffer
IC_{50} (nM)	16.3 $\pm$ 1.5	14.9 $\pm$ 1.9	12.4 $\pm$ 2.2	10.8 $\pm$ 0.9
IC_{50} (ng/mL)	11.3 $\pm$ 1.1	10.3 $\pm$ 1.4	8.6 $\pm$ 1.5	7.4 $\pm$ 0.7
IC_{20} (nM)	0.6 $\pm$ 0.2	0.5 $\pm$ 0.01	0.5 $\pm$ 0.2	0.4 $\pm$ 0.07
IC_{20} (ng/mL)	0.4 $\pm$ 0.1	0.3 $\pm$ 0.01	0.4 $\pm$ 0.2	0.2 $\pm$ 0.04
IC_{80} (nM)	232 $\pm$ 11	231 $\pm$ 23	201 $\pm$ 9	1998 $\pm$ 8.0
IC_{80} (ng/mL)	160 $\pm$ 8	160 $\pm$ 6	139 $\pm$ 6.5	137 $\pm$ 5
LoD (nM)	0.1 $\pm$ 0.05	0.1 $\pm$ 0.08	0.05 $\pm$ 0.02	0.1 $\pm$ 0.03
LoD (ng/mL)	0.08 $\pm$ 0.03	0.07 $\pm$ 0.05	0.04 $\pm$ 0.02	0.08 $\pm$ 0.02

^aThe IC_{50} , dynamic range, and LoDs correspond to the experiments that generated Figure 3A (mean $\pm$ SEM; mussel, $n = 6$; clam, $n = 2$; scallop, $n = 5$; and buffer, $n = 8$).

differ significantly from those obtained with buffer (Figure 3B). The 13-desmethyl spirolide C IC_{50} values obtained in mussel, scallop, and clam extracts would correspond to levels of 451.3 ± 42.4 , 344.2 ± 60.3 , and 411.6 ± 54.8 $\mu\text{g}/\text{kg}$ of shellfish meat, assuming 100% recoveries. During the optimization of the extraction procedure, methanol was also tested as the extraction solvent instead of acetone, and the interference of the different shellfish extracts (mussels, scallops, or clams) obtained with this alternative extraction method was also very low in preliminary experiments (data not shown). However, the acetone extraction protocol was selected for future studies in order to shorten the duration of the assay, due to its rapid evaporation.

Recovery of 13-Desmethyl Spirolide C with the nAChR/Microsphere-Based Assay. The recovery of 13-desmethyl spirolide C using a simple acetone extraction protocol combined with detection by the nAChR/microsphere-based assay was evaluated in samples of different shellfish species. Blank samples of mussel, scallop and clam were spiked with 13-desmethyl spirolide C. Six 1 g aliquots of shellfish homogenate (6 per species) were contaminated with 280 ng (280 $\mu\text{g}/\text{kg}$) of 13-desmethyl spirolide C, extracted, and analyzed with the nAChR-based assay within the same day. The content of toxin was calculated using calibration curves in the extract. Recoveries are shown as a percentage: % recovery = $100(\text{measured content}/\text{fortification level})$. The amount of 13-desmethyl spirolide C detected with the assay was 209.5 ± 4.0 , 204.1 ± 0.7 , and 220.3 ± 13.3 $\mu\text{g}/\text{kg}$ in mussels, clams, and scallops resulting in recovery efficiencies of $74.8 \pm 1.4\%$, $72.9 \pm 0.3\%$, and $78.7 \pm 4.8\%$, respectively (mean $\pm$ SEM of six replicates).

Repeatability of the Spirolide-Detection Assay. The intra-assay and inter-assay repeatabilities of our method were determined for the detection of 13-desmethyl spirolide C in spiked shellfish samples. Intra-assay repeatability was evaluated for mussel, scallop, and clam and interassay repeatability was evaluated only for mussel. Aliquots of mussel, scallop, or clam homogenate (6×1 g) were spiked with 280 ng of 13-desmethyl spirolide C, extracted, and assayed. The procedure was repeated in 2 different days for mussels. Then the extracts were analyzed with the nAChR assay, and the amount of 13-desmethyl spirolide C was quantified using a calibration curve in shellfish extract. The coefficients of variation (CV) within the same run (intra-assay) were 11.3%, 20.5%, and 8.5% for mussel, scallop, and clam, respectively, and between runs (inter-assay) repeatability for mussels was 6.7%.

Stability of 13-Desmethyl Spirolide C in Buffer, Shellfish Extracts, and Mussel Homogenate. The stability of 13-desmethyl spirolide C was evaluated in buffer and shellfish extracts stored at $4-8$ $^{\circ}\text{C}$ or -20 $^{\circ}\text{C}$ for 2 or 4 weeks. The 13-desmethyl spirolide C was diluted in buffer (PBS-BT/20% EtOH) or extract (mussel and scallop) at a concentration of 7 ng/mL, divided in 30 aliquots and stored at $4-8$ $^{\circ}\text{C}$ (12 aliquots) or -20 $^{\circ}\text{C}$ (12 aliquots). The 6 remaining aliquots were analyzed on the same day. Half of the aliquots stored at $4-8$ $^{\circ}\text{C}$ or -20 $^{\circ}\text{C}$ were further analyzed after 2 weeks, and the other half after 4 weeks of storage. At the indicated times, the amount of 13-desmethyl spirolide C was detected with the nAChR/microsphere-based assay using freshly prepared calibration curves in buffer or shellfish extract (mussel or scallop) as required. These samples showed no significant degradation of 13-desmethyl spirolide C after 4 weeks of storage at -20 or 4 $^{\circ}\text{C}$ (Table 2).

Table 2. Stability of 13-Desmethyl Spirolide C in Buffer and Shellfish Extracts (Mussel and Scallop) for 4 Weeks at Different Storage Conditions^a

% recovery	buffer	mussel extract	scallop extract
$4-8$ $^{\circ}\text{C}$	109 ± 6	135 ± 23	92.0 ± 9.2
-20 $^{\circ}\text{C}$	98.1 ± 6.3	121 ± 16	85.5 ± 5.5

^aThe stability was expressed as a percentage of toxin detected in samples after storage versus freshly analyzed samples. Aliquots of buffer (PBS-BT/20% EtOH) and shellfish extracts were spiked with 7 ng/mL of 13-desmethyl spirolide C and analyzed fresh or stored over 4 weeks at 4 or -20 $^{\circ}\text{C}$. After this time period, the stored samples were assayed using freshly prepared calibrants (mean $\pm$ SEM, $n = 6$).

The stability of 13-desmethyl spirolide C in mussel homogenate was also analyzed. For this purpose, three aliquots of 1 g of mussel homogenate were spiked with 13-desmethyl spirolide C, one aliquot was analyzed fresh and the other two were stored at -20 $^{\circ}\text{C}$ for 1 and 2 weeks. After these two periods, the mussel homogenate was thawed and extracted to measure the amount of toxin using the nAChR/microsphere-based assay. The calibration curve was freshly prepared in mussel extract. After 2 weeks of storage in the freezer, $105.7 \pm 5.3\%$ of 13-desmethyl spirolide C was recovered.

DISCUSSION

In this study, a solid-phase receptor-based assay was developed and prevalidated for the detection of cyclic imines using the xMap Luminex technology. This assay is based on the competition between cyclic imines and α -BTX for the interaction with a binding protein immobilized on the surface of microspheres. Two different binding proteins known to interact with spirolides, the *Torpedo marmorata* nAChR, and the *Lymnaea stagnalis* AChBP^{2,25} were considered suitable for assay development. While both proteins bound efficiently to 13-desmethyl spirolide C, the nAChR provided a much wider dynamic range for this toxin and a cross-reactivity profile that matched better the relative toxicity of 13-desmethyl spirolide C and gymnodimine. The broader dynamic range of the nAChR assay is probably a consequence of the nonidentical binding sites at the $\alpha\gamma$ and $\alpha\delta$ subunit interfaces of the *Torpedo marmorata* electric organ nAChR (heteropentamer $\alpha_7\beta\gamma\delta$) for these competing ligands, similar to what has been previously reported for other muscle nAChR antagonists with apparent Hill coefficients less than 1.0.³⁵⁻³⁸ The AChBP, instead, is a homomeric pentamer with identical subunit interfaces and therefore binding sites.³² Although, AChBP characteristics provide some advantages such as sharp concentration dependence for accurate measurements of affinity and a greater homogeneity and stability of the binding protein preparation, the wide dynamic range of the nAChR assay is a valuable feature for screening the presence of toxins.

Regarding cross-reactivity, in our hands the Ls-AChBP assay is 14-fold more efficient for gymnodimine than 13-desmethyl spirolide C. On the contrary, the nAChR assay detects 13-desmethyl spirolide C 24 times more efficiently than gymnodimine. Actually, since AChBP is considered a structural homologue of the extracellular domain of the $\alpha 7$ nAChR subtype,³⁹ the higher sensitivity of our AChBP assay for gymnodimine is consistent with previous reports of higher affinity of gymnodimine for the chimeric protein $\alpha 7$ -SHT3 than for the *T. marmorata* nAChR.²⁶ The LD_{50} of gymnodimine and 13-desmethyl spirolide C fed to fasted mice has been reported

as $>7500 \mu\text{g/kg}$ and $500 \mu\text{g/kg}$, respectively,^{40,41} which means that gymnodimine would be >15 times less toxic than the spirolide by this route of administration. The cross-reactivity data for 13,19-didesmethyl spirolide C should be considered as preliminary estimations, since to date there are no certified standard solutions available for this compound worldwide. A certified standard solution of 20-methyl spirolide G has been recently released to the market and has been used for cross-reactivity profiling in this study. The 95% cross-reactivity obtained for 20-methyl spirolide G agrees with previous toxicity studies performed in mice with 13-desmethyl spirolide C and 20-methyl spirolide G, with very similar oral LD_{50} values of $160 \mu\text{g/kg}$ by gavage and $500 \mu\text{g/kg}$ when administered with food for both toxins.⁴¹ However, some of these toxicity studies were performed before certified standards of these toxins were produced, and therefore, the correlation between assay cross-reactivity and relative toxicity of these compounds should be reviewed when more toxicological data and standard solutions are available. So far, our results clearly demonstrate that the nAChR assay can detect the three spirolides and gymnodimine with an acceptable correlation with toxicity data currently available. Therefore the nAChR was chosen as the binding protein for subsequent assay development and prevalidation taking into consideration assay cross-reactivity and dynamic range.

Additionally, in the Luminex system the different cross-reactivities of nAChR and Ls-AChBP assays can be used to generate a multiplexed assay with additional higher sensitivity toward gymnodimine than the single plex nAChR assay. The multiplexed alternative does not seem necessary at the moment since macrocyclic imines have not been regulated yet. However, some investigators in the marine toxin field regard the spirolides as potentially more toxic, while gymnodimine is generally considered a lesser threat. In case the presence of spirolides in seafood, and not the gymnodimines, become regulated, a multiplexed assay would be useful to discriminate between them.

The characterization of the nAChR/Luminex microsphere assay performance was conducted following the recommendations for method validation of the European Decision 2002/657/EC⁴² as far as the availability of toxins and blank shellfish samples would allow. The nAChR/Luminex microsphere-based assay provides higher sensitivity for the 13-desmethyl spirolide C ($\text{IC}_{50} = 10 \text{ nM}$) than previously published competition assays based also on interactions with the nAChR, such as the chemiluminescence assay ($\text{IC}_{50} = 35 \text{ nM}$) and the fluorescence polarization assay ($\text{IC}_{50} = 100 \text{ nM}$).^{27,28} Another characteristic of the Luminex assay is a wider dynamic range than other receptor-based assays.^{27–29} The increased sensitivity of the Luminex-based method for spirolide detection has allowed an important improvement versus other receptor-based competition assays for cyclic imines previously published: the elimination of matrix interferences by higher dilutions, allowing the use of a simple and fast extraction method. The extraction method optimized for this assay consists of an extraction with acetone, reconstitution in ethanol/PBS, and filtration, which implies the elimination of several solvent partitions needed to clean extracts for other assays.^{27,28} The new extraction method is much shorter and less laborious, permitting the simultaneous extraction of a higher number of samples. The recovery rates of the new method also appear improved, probably due to the reduction of the number of extraction steps.

The nAChR microsphere-based assay in combination with this sample preparation protocol allows the detection of the 13-desmethyl spirolide C in three different shellfish extracts, mussels, clams, and scallops, in the range of $10\text{--}6000 \mu\text{g/kg}$ of shellfish meat, a wider detection range than the previous chemiluminescent assay ($40\text{--}1000 \mu\text{g/kg}$),²⁸ and a LoD of $3 \mu\text{g/kg}$. The LoD has been used to report sensitivity instead of $\text{CC}\alpha$ and $\text{CC}\beta$ due to the impossibility to obtain enough blank shellfish matrixes and purified toxin to perform the experiments required for $\text{CC}\alpha$ and $\text{CC}\beta$ determination. The assay performance for the detection of 13-desmethyl spirolide C in spiked shellfish samples was deemed adequate in terms of recovery, with values around 75%, and repeatability, with intra- and inter-assay CVs lower than 12%, except for the scallop matrix. Although the toxicity of spirolides to humans is still unknown and no regulatory limits have been defined for these toxins, a safe level of spirolide content in shellfish has been estimated by some investigators as $400 \mu\text{g/kg}$,⁴³ a value within the range of our assay. The stability of spirolides in buffer and shellfish extracts as well as in mussel homogenates stored at -20 or 4°C during 4 weeks makes it feasible to include in a single assay samples received in the lab at different times.

The nAChR/Luminex-based assay can be used as a semiquantitative method to screen shellfish samples for the presence of cyclic imines, yielding an overall estimation of cyclic imine-related sample toxicity based on binding to the nAChR. Moreover, representative toxins of other groups, saxitoxin, okadaic acid, yessotoxin, domoic acid, and azaspiracid-1 do not interfere with the Luminex nAChR-based assay. In previous studies, nAChR based assays have not shown any interferences with saxitoxin, okadaic acid, brevetoxin-2, yessotoxin, and azaspiracid-2.^{27,28}

The Luminex system has been developed as a multiplex detection technology that would allow simultaneous detection of several classes of analytes in the same sample. This cyclic imine-detection assay could be included in the future in a multidetection assay for several groups of marine toxins. In this regard, the compatibility with methanol extraction in this nAChR assay is useful information, since acetone is not a commonly used solvent to extract other marine toxin groups. However, recoveries of cyclic imines with methanol extraction would have to be tested in the Luminex assay and the multiplexed assay optimized due to the possibility of unexpected interactions appearing when combining several competition assays.⁴⁴

In summary, this nAChR/Luminex-based assay provides a suitable system to screen shellfish samples for the presence of cyclic imines. This new method is capable of detecting concentrations of 13-desmethyl spirolide C above $3 \mu\text{g/kg}$. This offers a screening method that is more sensitive, faster, and easier to perform than previously published receptor-based assays. Moreover, this technology affords the possibility of including spirolide detection in future toxin multidetection assays.

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Notes

The authors declare no competing financial interest.

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* **ANEXO al artículo 3.** Ensayo basado en unión a receptor para la detección de ANTX mediante la tecnología xMAP de Luminex.

Debido a la afinidad de ciertas toxinas, como la anatoxina-a, por los receptores colinérgicos nicotínicos, el método basado en la interacción de las toxinas con estas proteínas de membrana, se optimizó también para la detección de la cianotoxina usando el sistema Luminex. Igualmente se probaron como proteínas de unión para la anatoxina-a, los receptores nicotínicos de acetilcolina (nAChRs) procedentes de las membranas del *Torpedo marmorata* y la proteína de unión a acetilcolina (L-AChBP) procedente del gasterópodo *Lymnaea stagnalis*.

La L-AChBP proporcionó un método con mayor sensibilidad para la anatoxina-a que los el nAChR de las membranas del *Torpedo marmorata* (Figura 20). Este ensayo se ha incluido en un futuro método de multidetección para detectar la presencia de anatoxina-a, microcistinas, cilindrospermopsinas, saxitoxina y ácido domoico en extractos de muestras naturales de agua dulce.

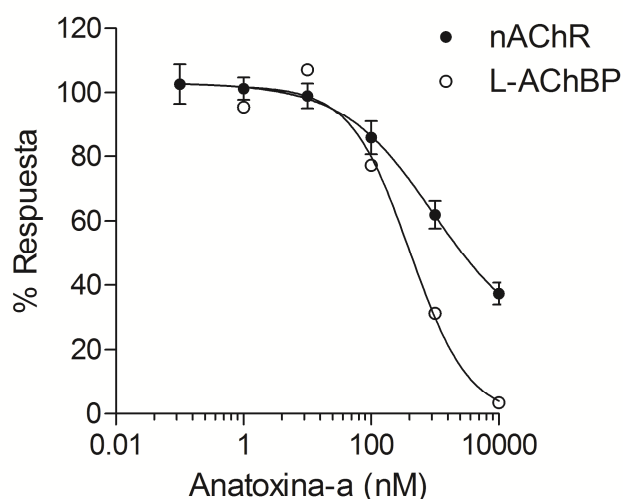


Figura 20. Curva de calibrado de anatoxina-a en el tampón de ensayo usando el ensayo de inhibición basado en la unión a los receptores nicotínicos (nAChR) (media $\pm$ SEM, $n = 3$) y a la proteína de unión a acetilcolina (L-AChBP) ($n = 1$). Los datos se expresan como porcentaje de unión máxima de la α -bungarotoxina. Las curvas de calibrado se obtuvieron con una ecuación de cuatro parámetros.



Artículo 4. *Microsphere-Based Immunoassay for the Detection of Azaspiracids.*

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Microsphere-Based Immunoassay for the Detection of Azaspiracids

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Abstract

Azaspiracids (AZAs) are a group of lipophilic toxins discovered in mussels from Ireland in 1995 following a human poisoning incident. Nowadays the regulatory limit for AZAs in many countries is set at 160 µg of azaspiracid equivalents per kg of shellfish meat. In this work a microsphere-based immunoassay has been developed for the detection of AZAs using a Luminex system. This method is based on the competition between AZA-2 immobilized onto the surface of microspheres and free AZAs for the interaction with a monoclonal anti-azaspiracid antibody (mAb 8F4). In this inhibition immunoassay the amount of mAb 8F4 bound to AZA-2-microspheres was quantified using a phycoerythrin-labeled anti-mouse antibody, and the fluorescence was measured with a Luminex analyzer. Simple acetate/methanol or methanol extractions yielded final extracts with no matrix interferences and adequate recovery rates of 86.5% and 75.8%, respectively. In summary, this work presents, for the first time, a rapid and easily performed screening method capable of detecting AZAs at concentrations below the range of the European regulatory limit using a microsphere/flow cytometry system.

Keywords

Azaspiracid poisoning, anti-azaspiracid antibody, flow fluorimetry system

Introduction

Azaspiracids (AZAs) are a class of polyether toxins discovered in 1995 due to a human intoxication episode reported in The Netherlands after the consumption of contaminated mussels (*Mytilus edulis*) harvested from Killary Harbour, Ireland [1,2]. These toxins are produced by the photosynthetic thecate dinoflagellate *Azadinium spinosum* [3].

The main toxin found in contaminated samples, azaspiracid-1 (AZA-1) was isolated in 1998 [2], and its structure was later fully elucidated through synthetic studies [4–10]. To date, more than 30 analogues have been described [11], and AZA toxins have been reported from many locations around the world [2,12–17]. The symptoms observed during azaspiracid poisoning (AZP) in humans are similar to signs of diarrhetic shellfish poisoning (DSP), including nausea, vomiting, severe diarrhea and stomach cramps [1]. These toxins have been responsible for several incidents of shellfish poisoning in Europe [18], and, as a consequence, a maximum level of 160 µg of AZA equivalents per kg of shellfish meat destined for human consumption has been established in many countries for AZA-1, azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) [19] (Fig. 1), the better-known compounds of the group.

Azaspiracids have been described to induce multiple system damage in rodents and cause, occasionally, the appearance of lung tumors [20]. Several *in vitro* studies have demonstrated the high cytotoxicity of AZA-1, affecting different cell lines [21]; however, the mechanism of action of these toxins remains to be determined.

Mouse and rat bioassays have been used for the detection of AZAs [1]. Nevertheless, mammalian bioassays have several disadvantages due to their lack of sensitivity and specificity and ethical considerations. Analytical methods using mass spectrometry (MS) have been developed for the detection of marine toxins, in order to reduce the number of laboratory animals sacrificed every year for this purpose. Recently, LC-MS/MS has been established as the official

reference method to detect AZAs in Europe [22]. MS-based methods have some drawbacks due to the use of large amounts of solvents as well as requiring highly qualified personnel and expensive instrumentation. Moreover, these methods need certified standards of all analogues of a toxin group for accurate estimation of toxicity [23], and the presence of new possibly toxic compounds would be missed with these techniques. The development of alternative assays suitable for screening high numbers of samples for marine biotoxins is needed to reduce the number of analyses by more expensive and time consuming methods.

In the last few years, multiplexed assays that enable the rapid analyses of a large number of samples by combining the use of microspheres with flow fluorimetry have been described for the analysis of several contaminants in food [24,25]. The Luminex system is based on laboratory multi-analyte profiling (LabMAP™) technology that combines the use of fluorescently dyed 5.6 micron polystyrene microspheres with a Luminex analyzer. One hundred classes of microspheres are differentiated by their internal fluorescence. The surface carboxyl groups of each microsphere are used for coating with an analyte specific detection molecule, and a reporter molecule labeled with phycoerythrin (PE) enables quantification of analyte-related signal. Therefore, Luminex technology allows the simultaneous detection of multiple analytes within a single sample by combining different analyte-specific microsphere classes.

The aim of this work was to develop a flow fluorimetry-based immunoassay for AZAs detection using a specific monoclonal antibody, known as mAb 8F4, and a Luminex system.

Materials and methods

Materials

Certified reference standard materials of azaspiracid-1 (AZA-1), 8-methylazaspiracid (AZA-2) and 22-demethylazaspiracid (AZA-3) were obtained

from the Institute for Marine Biosciences, National Research Council (Halifax, Canada). AZA-2, used for immobilization on the microsphere surface, was synthesized by Nicolaou and co-workers as previously described [5,7,9,26,27]. *N*-hydroxysuccinimide (NHS), boric acid, bovine serum albumin (BSA), Tween-20, dimethyl sulfoxide (DMSO) and sodium tetraborate decahydrate were obtained from Sigma-Aldrich (Madrid, Spain), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, Illinois). Ethylenediamine and sodium azide were obtained from Fluka (Steinheim, Germany). Phycoerythrin (PE) Goat Anti-Mouse IgG was purchased from Invitrogen (Eugene, Oregon), and the anti-azaspiracid monoclonal antibody (mAb) 8F4 was produced as previously described [28]. Sodium chloride, acetic acid, methanol, sodium acetate anhydrous and sodium phosphates were obtained from Panreac (Barcelona, Spain). Ethanolamine was supplied by BiaCore AB (Uppsala, Sweden). Mussels (*Mytilus galloprovincialis*), scallops (*Pecten maximus*), clams (*Ruditapes philippinarum*) and cockles (*Cerastoderma edule*) were purchased from the market (Lugo, Spain). Calibration and performance verification kits for Luminex 200, carboxylated microspheres (LC10077-01) and sheath fluid were purchased from Luminex Corporation (Austin, Texas). Multiscreen 96-well filter plates (Durapore membrane), 33 mm Millex filter with 0.22 μm pore size and 0.45 μm pore size Ultrafree-MC centrifugal filters (Durapore membrane) were purchased from Millipore (Madrid, Spain).

Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH_2PO_4 , and 8.5 mM Na_2HPO_4 , pH 7.4. PBS-BT solution was PBS supplemented with 0.1% w/v BSA and 0.1% v/v Tween-20. Buffer solutions were filtered through a 0.22 μm pore size filter before use.

Coupling of azaspiracid to carboxylated microspheres

AZA-2 was immobilized on the surface of carboxylated microspheres using EDC/NHS. The coupling protocol was performed in an opaque 1.2 μm filter microplate where 150 μL of a mixture of equal volumes of EDC and NHS, at concentrations of 75 mg/mL and 11.5 mg/mL, respectively (both reagents dissolved in water), were added to a well-microplate containing 1.2×10^6 prewashed microspheres (LC10077-01). After 30 min of incubation the EDC/NHS mixture was removed by filtration and then 150 μL of 1 M ethylenediamine (dissolved in 50 mM borate buffer solution, pH 8.5) were added to the well and let to react for 1h. Then ethylenediamine was removed and 150 μL of 1 M ethanolamine were added. The ethanolamine was filtered off after 20 min. Finally, 50 μg of AZA-2 dissolved in 10 μL of DMSO, 30 μL of acetate buffer solution pH 4.5 and 10 μL of a mixture of equal volumes of EDC (54 mg/mL) and NHS (24 mg/mL) were added to the preactivated microspheres and allowed to couple for 4h. Once the coupling reaction period was completed, the microspheres were washed three times with 200 μL of PBS-BT and stored in PBS-BT supplemented with 0.01% w/v sodium azide at 4 °C in the dark until use. All the incubations were performed with constant shaking (700 rpm) at room temperature in the dark. All washes consisted of the addition of 200 μL PBS-BT, and solutions were removed using a vacuum manifold without exceeding 5 mm Hg of pressure.

Microsphere-based immunoassay for the detection of AZAs

An inhibition immunoassay for the detection of AZAs was designed using the competition between AZA-2 immobilized on the microsphere surface and free AZAs for the interaction with a monoclonal anti-AZA antibody (mAb 8F4). The first step was the incubation of 60 μL of sample or calibration solution with 60 μL of a 1:50000 dilution of the mAb 8F4 (2.8 mg/mL, stock solution) in 1.5 mL microtubes for 30 min. Then 100 μL of this mixture were added to a well of an

opaque, 1.2 μm filter plate containing 2×10^3 prewashed AZA-2-coupled-microspheres. The AZA-2-microspheres were incubated with the sample-mAb 8F4 mixture overnight at 4 °C with constant shaking. After three washing steps with PBS-BT, 100 μL of a 1:2000 dilution of PE-labeled anti-mouse antibody (1 mg/mL, stock solution) were added to the microspheres for 30 min, followed by three additional washes. Finally, the microspheres were resuspended in 100 μL of PBS-BT.

Quantification of the binding signal

PE fluorescence intensity bound to the AZA-2-microspheres was measured with a Luminex 200 analyzer (LuminexCorp, Austin, Texas). The Luminex analyzer detects individual beads by a flow fluorimetry system using a 635 nm laser to classify microspheres. PE (analyte-related) fluorescence is quantified after excitation with a 532 nm green laser. Default values of 7500–13500 were used for doublet discriminator gating of microspheres. The acquisition volume was 75 μL and minimum bead count was 100.

The mAb 8F4 binding response for each condition was expressed as percent of maximum binding and calculated as follows: $\% R = \{(S_i - S_{\min}) / (S_{\max} - S_{\min})\} \times 100$, where % R is the percentage of mAb 8F4 binding response, S_i is the signal obtained for a given condition, $S_{\max}$ is the signal obtained in control wells containing AZA-2-microspheres and mAb 8F4, and $S_{\min}$ is the signal obtained in control wells containing AZA-2-microspheres and no mAb 8F4.

Shellfish extraction procedure

Shellfish meat (whole body of mussel, cockle or clam, and muscle plus gonad of scallop) was homogenized with a blender, divided into aliquots and stored at $-20\text{ }^{\circ}\text{C}$ until use. The extraction procedure consisted of the addition of 5 mL of the extraction solution (70% methanol in 0.2 M sodium acetate buffer, pH 5.0,

MeOH/NaOAc) to 1 g of sample homogenate. The mixture was vortexed for 3 min and then centrifuged at 3600 g during 8 min at room temperature. The supernatant was saved and the pellet was re-extracted with 3 mL of the extraction solution, as described. The supernatants were combined and the volume was made up to 10 mL with MeOH/NaOAc. This extract was then diluted 1:10 (v/v) in PBS-BT and filtered through a 0.45 µm filter. Mussels, scallops, clams and cockles used in this work did not contain detectable amounts of AZAs when tested by LC-MS/MS [29].

Safety

Azaspiracids are toxins with high, acute intraperitoneal toxicity in mice. Although no human intoxication has been reported, standard solutions should be handled with gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

Data analysis

The results are expressed as mean ± SEM (standard error of the mean). For every experiment all conditions were tested in duplicate. The calibration curves were fitted using a four-parameter logistic equation obtained by a nonlinear regression fitting procedure (GraphPad Prism 5.0). The four-parameter fit equation was: $y = \{ \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{\exp((\log EC_{50} - x) n_H)}) \}$, where Min is the bottom or the response at infinite concentration, Max is the top or the response at 0 concentration, $\log EC_{50}$ is the concentration when the response is halfway between Min and Max and n_H is the Hill Slope. The limit of detection (LoD) was calculated using three times the standard deviation (SD) of raw binding signals of the highest point of the calibration curve, corresponding to the lower toxin concentration, and interpolating this value in the calibration curve.

Results and Discussion

This study presents for the first time a flow fluorimetry-based competition immunoassay to detect AZAs using a specific antibody. The assay design consist of the competition of free AZA toxins and AZA-2 immobilized on the surface of microspheres for binding to monoclonal antibody mAb 8F4. Finally, the mAb 8F4 antibody bound to AZA-2-microspheres is quantified using a PE-labeled anti-mouse antibody.

The assay was optimized for maximum sensitivity by varying antibody dilutions and incubation times. In order to evaluate sensitivity, AZA-1 calibration curves were obtained for every condition. Serial dilutions of AZA-1 at several concentrations, ranging from 0.1 to 100 nM or 0.01 to 100 nM, depending on expected sensitivity, were prepared in buffer (PBS-BT/10% MeOH) and assayed with the microsphere-based detection method. At first, 1:2000 and 1:5000 dilutions of the mAb 8F4 stock (2.8 µg/mL) were tested with short incubation times (30 min for Ab + sample incubation, and 30 min for Ab + sample + microsphere incubation). These conditions provided calibration curves with IC_{50} values for AZA-1 of 7.4 ± 1.3 nM and 3.6 ± 0.1 nM, for 1:2000 and 1:5000 dilutions, respectively (Fig. 2, Table 1). In order to increase the sensitivity of the method, a 1:50000 dilution of the mAb 8F4 stock was also tested in combination with overnight incubation. Therefore the anti-AZA antibody was added to the calibration solution or sample for 30 min, and then this mixture was incubated with AZA-2-microspheres overnight at 4 °C with constant shaking. These conditions provided calibration curves with an average IC_{50} value of 1.4 ± 0.2 nM (Table 1). The overnight assay also showed a wider dynamic range (Fig. 2) and a lower LoD than shorter protocols (Table 1). Moreover, this inhibition immunoassay has a high sensitivity compared to the sensitivity of previously published immunoassays for AZAs or AZA fragments [28,30].

The cross-reactivity of this AZA-detection method was also studied by comparing IC_{50} values of the calibration curves obtained for AZA-1, AZA-2 and AZA-3 within

the same assay. Dilutions of 0.01, 0.1, 1, 3, 10, 30 and 100 nM of the three toxins were prepared in buffer and assayed using the overnight protocol. The IC_{50} values obtained in these matched experiments were 1.7 ± 0.4 , 4.2 ± 0.5 and 1.4 ± 0.5 nM for AZA-1, AZA-2 and AZA-3, respectively (mean $\pm$ SEM; $n = 3$) (Figs. 3A and 3B). The % cross-reactivity (% CR) was calculated as follows (AZA-1 was considered the reference toxin with 100% cross-reactivity): % CR = $(IC_{50} \text{ of AZA-1} / IC_{50} \text{ of AZA}) \times 100$. These results demonstrate that the microsphere-based immunoassay is capable of detecting AZA-1, AZA-2 and AZA-3. The assay seems to be more sensitive for AZA-1 and AZA-3 and less efficient for AZA-2 (Figs. 3A and 3B). The cross-reactivity of this immunoassay did not show a good correlation with the reported relative toxicity of these molecules (LD_{50} values of 200 $\mu\text{g/kg}$, 110 $\mu\text{g/kg}$ and 140 $\mu\text{g/kg}$, for AZA-1, AZA-2 and AZA-3, respectively, intraperitoneal, mouse). [2,31]. However, the studies of AZA-1, 2 and 3 toxicity were performed before certified standards of these toxins were produced, and therefore the correlation of assay cross-reactivity with relative toxicity should be reevaluated when more toxicity data are available.

The performance of the microsphere-based assay with shellfish extracts was evaluated using two extraction solvents and different shellfish matrixes. Mussel, clam, cockle and scallop extracts were prepared using a simple extraction with MeOH/NaOAc, and analyzed in duplicate following the overnight assay protocol. The extraction procedure, was also carried out using methanol instead of MeOH/NaOAc, and mussels as shellfish extracts. Maximum and minimum binding signals in buffer and in shellfish extracts were compared in order to analyze the matrix effect; and no statistically significant differences were observed between the data obtained in buffer and shellfish extracts in any condition (Table 2).

Moreover, the matrix effect on the AZA-detection method was evaluated by comparing calibration curves of AZA-1 in buffer and in mussel extract using the MeOH/NaOAc extraction procedure. Serial dilutions of AZA-1 at concentrations of 0.01, 0.1, 1, 3, 10, 30 and 100 nM were prepared in buffer or extract, and

assayed following the overnight microsphere-based inhibition assay. Both calibration curves were very similar (Fig. 4A). The dynamic range (IC_{10} – IC_{90}), estimated LoD and IC_{50} values for AZA-1 obtained in shellfish extract were very close to the values obtained in buffer (Fig. 4B). The lack of matrix interference suggests that the quantification of the AZA content in natural samples could be performed using a calibration curve in buffer.

Considering sample dilution during the MeOH/NaOAc extraction protocol, the dynamic range of the assay in extract and the LoD (Fig. 4B), the microsphere-based immunoassay is able to detect AZA-1 in the range of 10–230 $\mu\text{g/kg}$ in shellfish meat. The EU has set a regulatory limit for AZAs of 160 $\mu\text{g/kg}$ [19], which is within the range of our assay.

The recovery of AZA-1 with this sample extraction protocol was also evaluated using methanol or MeOH/NaOAc as extraction solutions. For this purpose, an aliquot (1 g) of blank mussel homogenate was spiked with 84 ng of AZA-1. The spiked blank materials were extracted following the procedure described above and analyzed with the overnight microsphere-based immunoassay. The toxin content was quantified using calibration curves in MeOH/NaOAc or methanol extract depending on the solvent used for sample extraction, and the recovery rate was calculated as follows: % Recovery = (measured content / fortification level) $\times$ 100. The amount of AZA-1 detected by the microsphere-based assay in 1 g of mussel was 72.7 ng in the MeOH/NaOAc extract and 63.7 ng in the methanol extract, resulting in recovery efficiencies of 86.5% and 75.8%, respectively. These results indicate that both methanol and MeOH/NaOAc extracts are adequate in terms of recovery and matrix effects for shellfish sample screening. The extraction with MeOH is used for AZA detection by LC-MS/MS [29], however, for a multiplexed assay using Luminex technology with the capability of multidetection of hydrophilic and lipophilic toxins, we have been testing a combination of MeOH and acetate buffer to favor extraction of some hydrophilic toxins. The inclusion of acetate buffer in the extraction solution

seems to improve AZA-1 recovery; and, additionally, it will facilitate inclusion of this single assay in a multiplexed marine toxin assay.

In summary, this paper reports, for the first time, a new, rapid and sensitive screening assay to detect AZAs in the range of the European regulatory limit using a microsphere/flow fluorimetry-based immunoassay. This detection method could be included in the future in a multiplexed assay to allow the simultaneous detection of several groups of toxins in the same sample.



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TABLES

Table 1. IC_{50} , dynamic range (IC_{10} – IC_{90}), limit of detection (LoD) and maximum and minimum binding signal of the microsphere-based assay for AZA-1 in buffer with several assay conditions. The IC_{50} , IC_{10} , IC_{90} , LoD and binding signals values were obtained from the experiments that generated Fig. 2 using a four-parameter fit equation (mean $\pm$ SEM, $n = 4$ for 1:2000 mAb 8F4 dilution, $n = 3$ for 1:5000 mAb 8F4 dilution and $n = 7$ for 1:50000 mAb 8F4 dilution).

Incubation time	30 min		Overnight
mAb 8F4 dilution	1:2000	1:5000	1:50000
IC_{50} (nM) IC_{50} (ng/mL)	7.4 ± 1.3 6.2 ± 1.1	3.6 ± 0.1 3.0 ± 0.1	1.4 ± 0.2 1.2 ± 0.2
IC_{10} (nM) IC_{10} (ng/mL)	2.3 ± 0.5 1.9 ± 0.4	1.4 ± 0.0 1.2 ± 0.0	0.3 ± 0.1 0.3 ± 0.1
IC_{90} (nM) IC_{90} (ng/mL)	23.7 ± 3.3 19.9 ± 2.8	10.4 ± 0.5 8.8 ± 0.4	10.2 ± 3.4 8.6 ± 2.9
LoD (nM) LoD (ng/mL)	2.6 ± 0.4 2.2 ± 0.3	1.5 ± 0.1 1.3 ± 0.1	1.0 ± 0.6 0.8 ± 0.5
Maximum Binding Signal (RU)	2109 ± 180	1052 ± 78	617 ± 159
Minimum Binding Signal (RU)	2.6 ± 0.2	2.3 ± 0.2	3.2 ± 0.1

Table 2. Matrix effect of MeOH/NaOAc or methanol shellfish extracts on maximum and minimum binding signals using the overnight microsphere-based inhibition assay (mean $\pm$ SEM; n = 3).

MeOH/NaOAc		
	<i>Maximum Binding Signal (RU)</i>	<i>Minimum Binding Signal (RU)</i>
Mussel	1519 $\pm$ 292	1.8 $\pm$ 0.2
Scallop	1613 $\pm$ 193	1.7 $\pm$ 0.2
Clam	1637 $\pm$ 139	2.4 $\pm$ 0.1
Cockle	1680 $\pm$ 207	2.0 $\pm$ 0.3
Buffer	1644 $\pm$ 398	2.0 $\pm$ 0.0
Methanol		
	<i>Maximum Binding Signal (RU)</i>	<i>Minimum Binding Signal (RU)</i>
Mussel	971 $\pm$ 313	3.8 $\pm$ 1.3
Buffer	1109 $\pm$ 409	3.7 $\pm$ 0.9

FIGURES

Figure 1

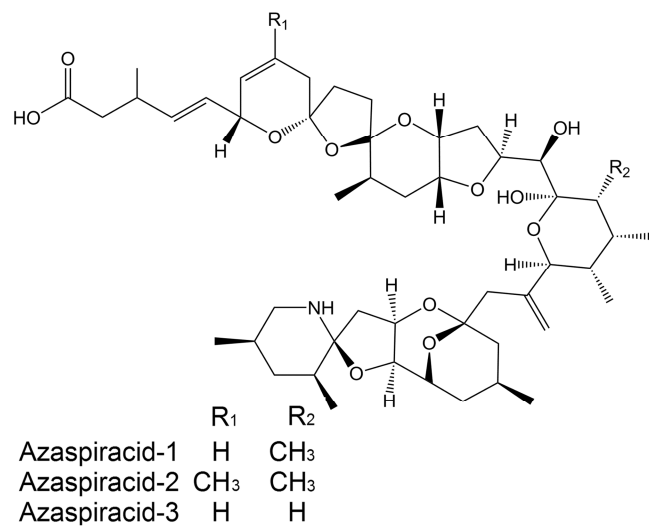


Figure 1. Chemical structure of AZA-1, AZA-2 and AZA-3.

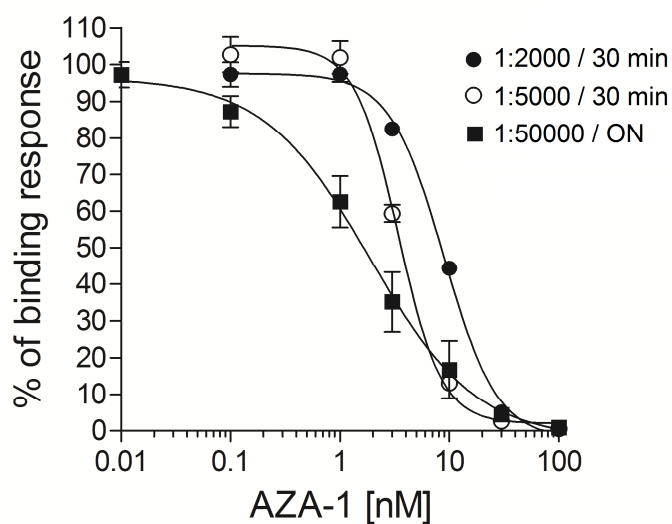
Figure 2

Figure 2. Calibration curves of AZA-1 in buffer using the microsphere-based inhibition assay with several assay conditions. Calibration curves were obtained using the inhibition assay with mAb 8F4 dilutions of 1:2000 and 1:5000 combined with 30 min incubations, and 1:50000 combined with an overnight incubation with AZA-2-coated microspheres. Data are expressed as percentage of maximum mAb 8F4 binding. The three calibration curves belong to non-matched experiments (mean $\pm$ SEM; $n = 4$ for 1:2000 dilution, $n = 3$ for 1:5000 dilution and $n = 7$ for 1:50000 dilution).

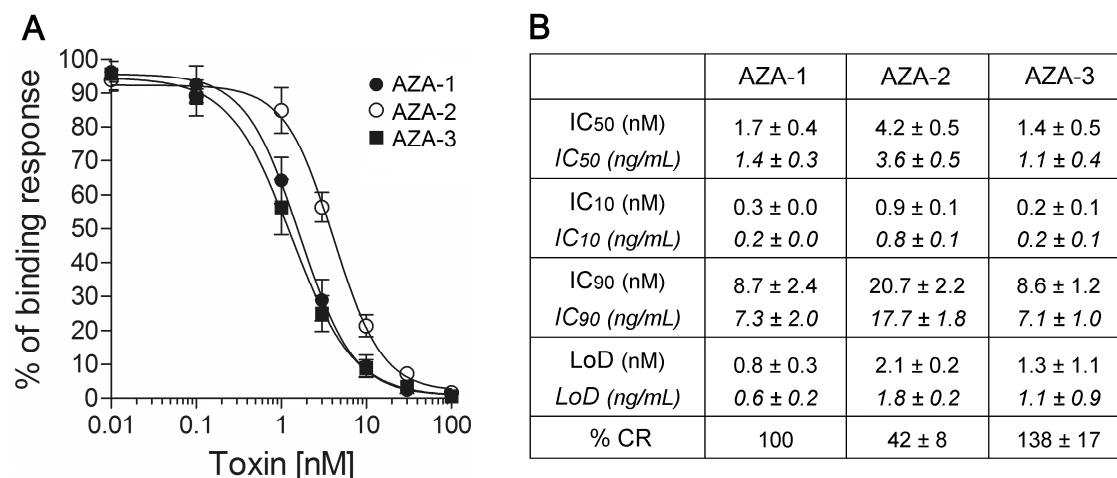
Figure 3

Figure 3. Cross-reactivity profile of AZA-1, AZA-2 and AZA-3 using the overnight microsphere-based inhibition assay. (A) Calibration curves of AZA-1, AZA-2 and AZA-3. Calibration solutions were prepared at concentrations ranging from 0.01 to 100 nM in buffer for the three toxins and assayed using the inhibition assay. The data are expressed as a percentage of maximum mAb 8F4 binding response. The calibration curves were obtained with a four-parameter fit. (B) IC₅₀, dynamic range, LoD and % cross-reactivity (% CR) values for AZA-1, AZA-2 and AZA-3. Data obtained from the curves in (A) (mean ± SEM; n = 3).

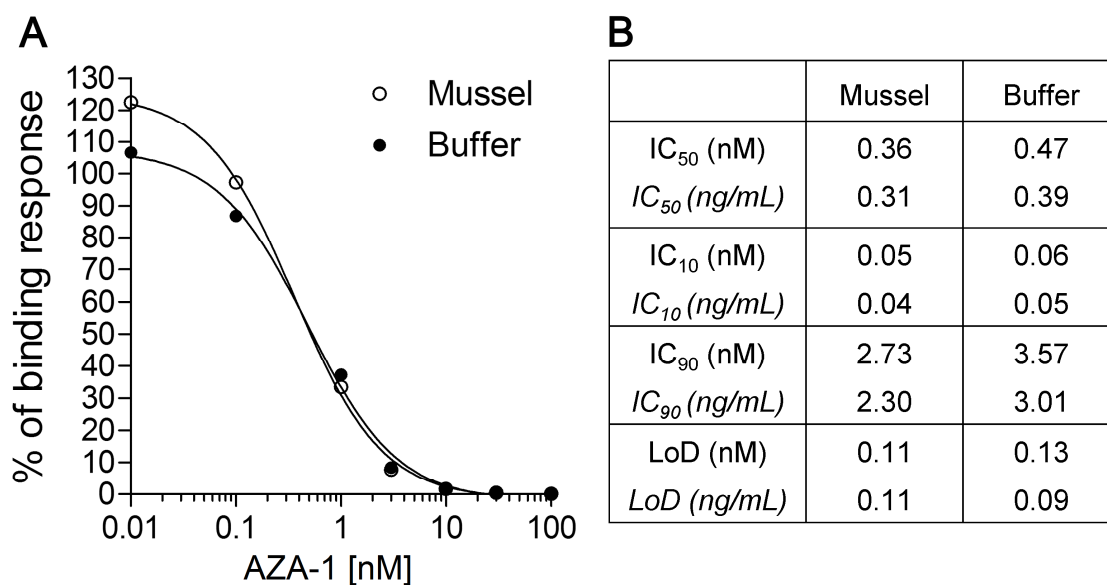
Figure 4

Figure 4. Effect of mussel matrix on the detection of AZA-1 using the microsphere-based Luminex system. (A) Calibration curves of AZA-1 in buffer and shellfish extract using the overnight microsphere-based immunoassay and MeOH/NaOAc as extraction solvent. AZA-1 standard was diluted at concentrations ranging from 0.01–100 nM in buffer and mussel extract. The data are expressed as a percentage of maximum mAb 8F4 binding response. The calibration curves were obtained with a four-parameter fit ($n = 1$). (B) IC_{50} , dynamic range ($IC_{10} - IC_{90}$) and LoD values obtained from the curves in (A).



DISCUSIÓN

4. DISCUSIÓN

Los episodios tóxicos de origen algal son cada vez más frecuentes en nuestro planeta, por lo que, en los últimos años se han establecido nuevas regulaciones y programas de vigilancia. La continua aparición de nuevos compuestos tóxicos, hace cada vez más necesario el desarrollo de nuevos métodos de detección rápidos, sensibles y económicos.

En general, los métodos desarrollados durante la presente tesis doctoral, poseen ciertas ventajas frente a los métodos oficiales existentes hasta ahora para la detección de ficotoxinas. Estas técnicas, no requieren el sacrificio continuo de gran número de animales y resultan más específicas que los bioensayos, ya que emplean la interacción de las toxinas con su molécula diana o con un anticuerpo específico. Por otra parte, con respecto a los métodos analíticos, como el LC-MS/MS, las técnicas presentadas, resultan más sencillas y menos costosas, ya que no requieren personal especializado, los equipos utilizados son más económicos y no usan grandes cantidades de disolventes (se emplean pequeños volúmenes durante los procesos de extracción).

De forma individual, el ensayo de unión a receptor para la detección de iminas cíclicas en microplaca mediante técnicas luminométricas, ofrece tres modalidades de detección, lo cual resulta muy ventajoso para los laboratorios de análisis rutinario. De las tres modalidades de detección, la quimioluminiscencia resultó ser la que mejor relación señal/ruido proporcionaba, y tras ser optimizado, este método ofrece la posibilidad de analizar más muestras por placa (hasta 120).

Uno de los inconvenientes de la quimioluminiscencia es la rápida disminución de la señal luminiscente, lo que provoca cierta variabilidad en los resultados. Una vez solventado este problema mediante la adición simultánea del sustrato

luminiscente a los pocillos de la placa, este ensayo detecta el 13-desmetil espirólido C con mayor sensibilidad que otros métodos publicados anteriormente usando el mismo procedimiento de extracción con acetona/hexano/cloroformo [271].

Por otro lado, usando una plataforma de multidetección, el sistema Luminex, se desarrolló un segundo método para la detección de iminas cíclicas. Esta técnica ofrece mayor sensibilidad por el 13-desmetil espirólido C con respecto al método de quimioluminiscencia presentado en esta tesis y a otros ensayos basados en unión a receptor desarrollados previamente para la detección de estas toxinas [271,337]. Esta mayor sensibilidad permite diluir más los extractos, eliminando las interferencias de la matriz con un procedimiento de extracción sencillo, sin necesidad de realizar particiones en distintos disolventes durante la preparación de la muestra. La sencillez del proceso de extracción permite la preparación simultánea de un mayor número de muestras.

Actualmente ninguna de las iminas cíclicas está regulada. Sin embargo, debido a la elevada toxicidad que los espirólidos y la gymnodimina presentan durante los bioensayos, algunos expertos sugieren la posible necesidad de establecer ciertos límites (400 µg/kg para los espirólidos y 18 mg/kg para la gymnodimina) para salvaguardar la salud de los consumidores [38,53]. En caso de que en un futuro, estas toxinas fueran reguladas, la reactividad cruzada de los ensayos individuales de nAChR y L-AChBP podría usarse en un ensayo de multidetección en el que se combinen dos clases de microesferas marcadas, cada una, con una proteína detectora para poder discriminar entre ambos grupos de compuestos.

Los métodos desarrollados durante esta tesis para la detección de iminas cíclicas, se basan en la interacción de las toxinas con sus dianas biológicas, por lo que estas técnicas serían útiles a la hora de detectar cualquier otro compuesto que comparta esta misma diana. Debido a esto, el ensayo de unión a receptor

para la detección de espirólidos y gymnodimina mediante el sistema Luminex, es capaz de detectar la anatoxina-a, un potente agonista de los receptores colinérgicos nicotínicos. Por el contrario, otras toxinas como el ácido okadaico, ácido domoico, saxitoxina o la yessotoxina, cuyo mecanismo de acción no está relacionado con los receptores nicotínicos, no producen ningún tipo de interferencia en los métodos. Además, el hecho de usar la competición con α -bungarotoxina, un antagonista que se une de forma específica a la acetilcolina, contribuye a la especificidad de ambos métodos.

Se han publicado diversos ensayos de unión a receptor para la detección tanto de iminas cíclicas como de anatoxina-a que proporcionan mayor sensibilidad que los expuestos anteriormente [323-325]. Sin embargo, el uso de compuestos radioactivos de alguno de ellos o el acoplamiento a equipos de espectrometría de masas en otro, dificulta su uso como métodos de criba de alto rendimiento. Por otra parte, la tecnología Luminex, aporta una gran ventaja frente a las técnicas anteriores, ya que ofrece la posibilidad de incluir estos ensayos en un método de multidetección. El ensayo puesto a punto durante esta tesis para la detección de la anatoxina-a, basado en la interacción de esta cianotoxina con la proteína de unión a acetilcolina (L-AChBP), se ha incluido en un método en desarrollo para la detección simultánea de anatoxina-a, microcistinas, cilindrospermopsina, saxitoxina y ácido domoico. Además, esta plataforma de multidetección proporciona resultados similares a los obtenidos en otros ensayos similares para la detección de toxinas marinas, en términos de sensibilidad, rango dinámico y reactividad cruzada, como se ha comprobado en inmunoensayos desarrollados recientemente para la detección de toxinas PSP, ASP y DSP mediante el sistema Luminex [318,319].

En la actualidad existen más de 15 grupos de toxinas, y es probable que este número se incremente con el paso del tiempo. A pesar de que los métodos

analíticos como el LC-MS, son capaces de detectar gran cantidad de compuestos simultáneamente, estas técnicas resultan costosas y necesitan estándares certificados de cada una de las toxinas a analizar, por lo que el desarrollo de métodos rápidos, sencillos y económicos para la detección simultánea de distintas toxinas dentro de una misma muestra, sería una herramienta muy útil para emplearse en los laboratorios de análisis rutinario.

Aunque por el momento no existan métodos para el análisis simultáneo de tantos analitos, la tecnología Luminex es capaz de distinguir hasta 100 clases de microesferas, ofreciendo la posibilidad de analizar un gran número de muestras simultáneamente para varios grupos de toxinas. Sin embargo la detección de distintos compuestos tóxicos dentro de una misma muestra, requiere la optimización de un protocolo de ensayo y de extracción adecuado para todos ellos, lo cual podría resultar complicado debido a las diferencias entre las propiedades químicas de los distintos grupos de toxinas.

Además de las ventajas técnicas de los métodos presentados para la detección de iminas cíclicas, la estabilidad de los espirólidos en el tampón de ensayo, en los extractos, o en la propia carne de los moluscos, tanto en el ensayo quimioluminiscente como en el realizado mediante el sistema Luminex, permite el almacenamiento de muestras para el posterior análisis de todas ellas, abaratando los costes que supondría su procesamiento de forma independiente.

Para que este tipo de técnicas se puedan usar en los laboratorios de análisis rutinario, es necesario garantizar la estabilidad y disponibilidad de los reactivos. En nuestro caso, los receptores, el anticuerpo y la proteína de unión a acetilcolina utilizados durante los ensayos proporcionaron resultados satisfactorios tras largos periodos de almacenamiento, sin embargo las membranas que contienen a los receptores nicotínicos no se encuentran disponibles en el mercado, lo cual resulta una desventaja.

En el caso de los AZAs, el anticuerpo anti-azaspirácido 8F4 utilizado en la presente tesis permitió la detección de AZA-1, AZA-2 y AZA-3 en el rango nanomolar a concentraciones inferiores al límite regulador establecido en Europa para estas toxinas (160 µg/kg), usando un sencillo protocolo de extracción. Las dos soluciones empleadas (metanol y metanol/acetato), resultaron adecuadas en términos de recuperación y efectos de matriz, sin embargo, debido a las diferencias entre las propiedades químicas de las distintas toxinas (hidrofílicas, lipofílicas...). La incorporación del tampón acetato a la solución de extracción facilitaría la inclusión de este ensayo en un futuro método para la detección simultánea de distintos grupos de toxinas, como se ha comprobado recientemente un ensayo desarrollado para la detección de toxinas PSP, ASP y DSP, en el que extrayendo simultáneamente toxinas hidrofílicas (ácido okadaico) y lipofílicas (saxitoxina y ácido domoico), se han obtenido resultados satisfactorios [319].

Aunque los datos de reactividad cruzada obtenidos durante esta tesis para el AZA-1, AZA-2 y AZA-3 no se correlacionan exactamente con los estudios de toxicidad realizados previamente [66,75], son lo suficientemente próximos como para garantizar la detección de las tres toxinas. Por otro lado, la variabilidad inherente a los bioensayos (cepa del ratón, sexo, estado del animal...), o el hecho de que las toxinas administradas a los animales no fueran estándares certificados, son factores que pueden influir en los valores de toxicidad relativa descritos hasta el momento para estos análogos. Por lo tanto, para realizar una buena comparación de ambos métodos (bioensayos e inmunoensayo) deberían de estandarizarse todos estos parámetros y emplearse estándares certificados de las toxinas objeto de estudio. La falta de estándares certificados o la gran cantidad de toxina que se requiere para realizar la validación completa de un método, ha dificultado la inclusión de este tipo de técnicas como métodos de análisis. Por otra parte, la continua aparición de nuevos grupos de toxinas o

nuevos análogos, junto con el hecho de depender de floraciones tóxicas naturales e impredecibles para la purificación de algunos de estos compuestos como las ostreocinas, las ciguatoxinas u otros análogos de estos grupos [338], contribuyen a la escasez, a nivel mundial, de estándares certificados.

De forma general, los métodos presentados en esta tesis son específicos, no usan animales ni moléculas radiactivas, no requieren personal especializado y ofrecen la posibilidad de detectar moléculas de estos grupos que aún no están descritas sin necesidad de utilizar estándares certificados de todas las toxinas pertenecientes a un grupo, por lo que proporcionarían una capacidad de detección adicional. Además, estas técnicas proporcionan resultados rápidos que permiten la detección de uniones moleculares. Aunque ninguno de estos métodos podría sustituir por completo a los bioensayos *in vivo* o a los métodos analíticos, estas técnicas podrían usarse como métodos de criba en combinación con los anteriores, disminuyendo el número de muestras procesadas mediante métodos más costosos o éticamente más cuestionables, siendo una herramienta útil para poder hacer frente a la carga de trabajo en los laboratorios de análisis rutinario que se incrementa con los años debido a la intensificación de los programas de monitorización, a la aparición de nuevas toxinas y al aparente aumento de los episodios tóxicos.



CONCLUSIONES

5. CONCLUSIONES

1. El ensayo de inhibición desarrollado para la detección de iminas cíclicas basado en la competición de estas toxinas con la α -bungarotoxina por la unión a los receptores nicotínicos de acetilcolina, es capaz de detectar tanto espirólidos como gymnodimina en la carne de molusco ofreciendo distintas modalidades de detección. Además la optimización del ensayo quimioluminiscente, permite usar esta técnica como método de criba de alto rendimiento para analizar un mayor número de muestras por placa, disminuyendo los costes e incrementando la flexibilidad para la organización del trabajo de laboratorio.
2. La inhibición de la unión entre la α -bungarotoxina y las proteínas de unión a acetilcolina (nAChRs y L-AChBP) por parte de espirólidos y gymnodimina, puede usarse para detectar la presencia de estas toxinas en moluscos usando la plataforma de multidetección Luminex. Esta técnica, más sensible que las anteriores, emplea un sencillo protocolo de extracción con acetona que permite la preparación simultánea de gran número de muestras y la posibilidad de incluir a estas toxinas en un método de multidetección.
3. El método de detección de azaspirácidos basado en el uso de la plataforma de multidetección Luminex, puede utilizarse como método de criba usando una extracción con metanol/acetato, ya que detecta concentraciones inferiores al límite regulador establecido en Europa para estas toxinas. Este método presenta características adecuadas para ser incluido en el futuro en un método de multidetección.



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