UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

DEPARTAMENTO DE
CIENCIAS CLÍNICAS 2016

TÓXICOS EN EL MEDIOAMBIENTE DE CANARIAS

ANÁLISIS DE LA SITUACIÓN EN FAUNA SILVESTRE Y DOMÉSTICA

NORBERTO RUIZ SUÁREZ

TESIS DOCTORAL EUROPEA

Anexo I

DON ALBERTO ARENCIBIA ESPINOSA, SECRETARIO DE LA FACULTAD DE VETERINARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que la Comisión de Asesoramiento Docente del programa de Doctorado Clínica e Investigación Terapéutica en su sesión de fecha ocho de abril de dos mil dieciséis, tomó el acuerdo de dar el consentimiento para su tramitación a la tesis doctoral titulada “Tóxicos en el medioambiente de Canarias: análisis de la situación en fauna silvestre y doméstica” presentada por el doctorando Don Norberto Ruiz Suárez y dirigida por los Doctores Don Octavio Pérez Luzardo y Don Luis Domínguez Boada.

Y para que así conste, y a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, defensa, tribunal y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a ocho de abril de dos mil dieciséis.-

Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Facultad de Veterinaria

Programa de Doctorado: Clínica Veterinaria e Investigación Terapéutica

Título de la Tesis

Tóxicos en el medioambiente de Canarias: análisis de la situación en fauna silvestre y doméstica

Tesis Doctoral presentada por D. Norberto Ruiz Suárez

Dirigida por el Dr. D. Octavio Pérez Luzardo

Codirigida por el Dr. D. Luis Domínguez Boada

El Director	**El Codirector**	**El Doctorando**
(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a _____de_____de 2016__

Islas Canarias, oasis y vergel, refugio de flora y fauna en el Atlántico, lugar donde tradición, dinamismo y sostenibilidad luchan por el equilibrio del desarrollo.

“Norberto Ruiz Suárez”

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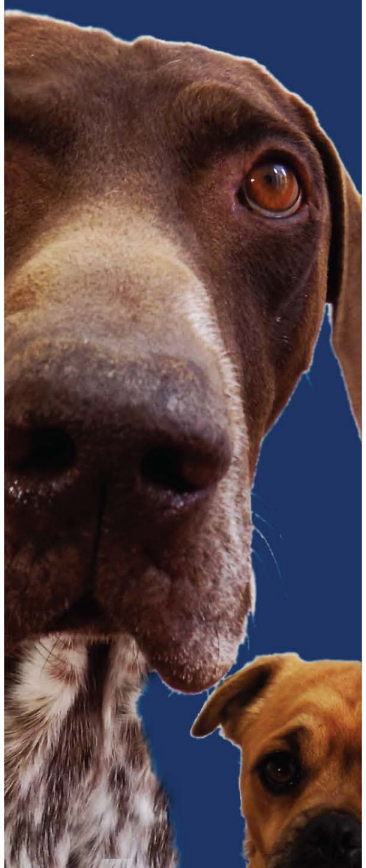
AGRADECIMIENTOS



AGRADECIMIENTOS

A mi familia: Que desde pequeño han inculcado en mí los valores que a día de hoy definen mi persona. La educación, el respeto, el compañerismo y el afán por superar cualquier obstáculo, sólo son pinceladas que con esfuerzo quisieron inculcar. No hay palabra ni gesto que exprese todo lo que significan para mí. No tengo la menor duda de que sin su sacrificio nada de esto hubiera sido posible. A mi hermano que más allá de las convicciones sociales siempre ha sido fiel a sus ideas, su tesón aún llevándole por los caminos menos fáciles, le ha brindado su recompensa. Agradecerle por supuesto todo el diseño de esta Tesis. Muchísimas gracias.

A Yessica: Apareciendo en el momento justo, no se me ocurren palabras para expresar toda la gratitud que tengo al haber estado estos 3 años aguantando mis nervios e indecisiones. A partir de ahora se abre una nueva etapa en la que no dudo que su presencia me aportará las fuerzas necesarias para seguir consiguiendo mis objetivos. Aunque nunca serán suficientes, gracias, mil gracias.

A Octavio: Que comenzó siendo el director de tesis que cualquier doctorando desearía, hasta convertirse hoy en día en un amigo, que creo que ha resuelto más dudas personales que aquellas relacionadas con la Tesis. Su energía y entusiasmo es contagioso. Sus consejos y afecto me han acompañado durante estos 5 años en los que hemos formado equipo en la lucha contra el veneno. Responsable de plantar la semilla de la investigación. Muchas gracias por tu confianza.

A Luis: El cual ha sabido a lo largo de los años en los que hemos trabajado, moldear la parte más importante de un equipo de investigación, las personas. Punto de referencia de todos y cada uno de nosotros, ha sabido combinar sus funciones de dirección sin dejar de ser compañero. Muchas gracias por todo.

A Manolo: Con su afán por el perfeccionismo y el buen hacer, trabajar a su lado supone un reto y una motivación. Con él siempre hay un paso más, algo que mejorar, algo sobre lo que reflexionar.

A mis compañeros: Son muchos con los que durante estos años he compartido despacho y trabajo. Si bien me gustaría comenzar con la primera persona con la que compartí laboratorio: Maira; es la persona que me inició y enseñó los primeros pasos en el laboratorio y con quién empecé a realizar lo que por aquel entonces no entendía bien y que se llamaban análisis cromatográficos, muchas gracias por tu paciencia y ganas de enseñar. Marta, que con su optimismo, alegría e ilusión me contagiaba todos los días sin importar cuanto trabajo tuviera que hacer. Gracias por escuchar. María, si alguien encarna la honradez y la fe ciega en sus convicciones, es ella. Muestra de integridad y compañerismo que hasta el último momento a mostrado su apoyo incondicional. Ángel, amigo y compañero de carrera y Tesis Doctoral, alguien en quien confiar y con el que ha sido un placer compartir vida laboral y social. Mayeya; trabajadora y compañera infatigable con la que a parte de compartir lugar, he compartido olores y algún que otro dolor de cabeza y arcadas. Luis; mente y diversión, el roquero del grupo, una de las personas más preparadas que conozco y capaz de ver el humor donde otros sólo ven rutina. Natalia; nuestra madrileña adoptada, cada vez que nos visita, su alegría

y buen humor inunda el despacho. No quisiera cerrar este grupo sin hacer mención a Yeray Brito, amigo desde hace muchos años, y culpable de que comenzara esta andadura en 2010. Muchas gracias por insistir, por tu apoyo y por tus ánimos. Gracias.

A los Centros de Recuperación de fauna silvestre, agentes de medio ambiente, agentes del SEPRONA, a SEO/Birdlife, AVAFES, a los Colegios Veterinarios de Las Palmas y Santa Cruz de Tenerife, al Gobierno de Canarias, al colectivo de cazadores y demás colectivos. Sin su ayuda, trabajo y esfuerzo diario, nada de esto se hubiera logrado. Muchísimas gracias a todos.

INTRODUCCIÓN

Introducción

El medioambiente, tanto su conocimiento y entendimiento, como su estudio y conservación, siempre ha sido de interés para la comunidad científica. Sin embargo, en las últimas décadas ha adquirido una dimensión mayor, incluso trascendental, ya que el ser humano ha entendido que nuestro desarrollo y supervivencia depende íntimamente del mismo y que el actual ritmo de vida no es compatible con un desarrollo sostenible. Este desarrollo sostenible según un informe titulado Nuestro Futuro Común de 1987 de la Comisión Mundial sobre el Medio Ambiente y el Desarrollo, de Naciones Unidas, debe entenderse como la satisfacción de las necesidades de la generación presente sin comprometer la capacidad de las generaciones futuras para satisfacer sus propias necesidades (1). Digamos que la salud y el bienestar de las personas van a estar íntimamente relacionados al estado del medioambiente (2). Según la Conferencia de las Naciones Unidas celebrada en Estocolmo en 1972, el medioambiente se define como el conjunto de componentes físicos, químicos, biológicos y sociales capaces de causar efectos directos o indirectos, en un corto o largo plazo, sobre los seres vivos y las actividades humanas. Una correcta conservación de los entornos naturales puede ofrecer múltiples beneficios al bienestar social. No obstante, cuando estos entornos por motivos de la contaminación tóxica del aire y las aguas, el ruido, la radiación, las sustancias químicas o los agentes biológicos, comienzan a degradarse, pueden repercutir negativamente en la salud (2).

El rol que representa la química en este panorama es crucial, ya que ésta se encuentra implícita en nuestra propia fisiología (procesos bioquímicos)

y además es básica en la obtención de medicamentos o alimentos (la enorme producción agrícola y ganadera actual se basa en el uso de plaguicidas o medicamentos que minimicen las pérdidas). En la actualidad podemos decir que se utilizan productos químicos sintéticos en todo lo que nos rodea, desde los plaguicidas a los cosméticos, desde los biberones a los ordenadores: la sociedad del siglo XXI depende de ellos. Es obvio que los productos químicos sostienen nuestro modo de vida, y que la mayor parte de ellos han tenido y tienen, efectos social y económicamente beneficiosos, pero no podemos olvidar que en muchas ocasiones una gran cantidad de sustancias comercializadas no han pasado ni una mínima evaluación de sus posibles efectos tóxicos a largo plazo para la fauna, el medioambiente o la salud de las personas (3).

El demostrar relaciones entre la presencia de tales sustancias y la aparición de un determinado efecto es complicado, debido en gran parte a que se liberan simultáneamente al medioambiente muchas sustancias diferentes (y sus respectivos productos de degradación) y por múltiples vías (de tal modo que los contaminantes tóxicos están presentes en aire y/o las aguas y/o los suelos) lo que impide establecer los correspondientes vínculos de asociación y causalidad (4).

Está demostrado que todos los seres vivos, población humana incluida, estamos expuestos diariamente a una innumerable cantidad de contaminantes tóxicos, y los habitantes de las Islas Canarias no son una excepción. De hecho, la población canaria ha sido extensamente estudiada en lo referente a su exposición a contaminantes tóxicos. En dicha población, se ha demostrado la presencia de residuos de contaminantes tóxicos persistentes (CTPs), entre los

que destacan los hidrocarburos aromáticos policíclicos (HAPs) (5), policlorobifenilos (PCBs) (6, 7), pesticidas organoclorados (POCs) (7-9) y metales pesados (Pb) (10). De igual manera, también se han evaluado los posibles efectos adversos que tal exposición puede causar, como por ejemplo patologías tumorales (11) y alteraciones hormonales (12, 13). Sin embargo, estudios destinados a evaluar la presencia de contaminantes tóxicos en el medioambiente de las Islas Canarias son menos numerosos. En este ámbito debemos destacar los artículos publicados por Ricardo Díaz Díaz en donde se ha demostrado la presencia de ciertos CTPs en el suelo (14, 15) y el publicado por Villa et al. quien confirma su presencia en las hojas de pino (16).

Como podemos deducir por anteriores estudios, los CTPs se encuentran distribuidos en nuestro medioambiente, pero la incógnita que nos planteábamos era la valoración de la biodisponiblidad real de estos compuestos para nuestra fauna. Por lo que el siguiente paso lógico suponía la medición directa de tales CTPs en la fauna. A excepción de artículos publicados por nuestro grupo de investigación sobre fauna marina (17-19), muy poco conocemos sobre los niveles de exposición de la fauna terrestre (20, 21), a pesar de que ésta comparte un ambiente más cercano al nuestro y además de estar expuesta a los contaminantes tóxicos ubicuos, puede verse expuesta de forma directa a otros productos tóxicos liberados por el ser humano directamente en su hábitat en el desarrollo de actividades agrícolas o sanitarias, tal es el caso de los plaguicidas.

Por todo lo expuesto anteriormente hemos planteado la presente Tesis Doctoral Europea en la que como objetivo principal se propone la evaluación

de la exposición de la fauna terrestre de las Islas Canarias a contaminantes tóxicos, ya sea de forma intencionada o no.

De forma genérica cuando queremos llevar a cabo una evaluación de la contaminación química, nos centramos en la detección, identificación y cuantificación de los CTPs de mayor interés en esa zona o población. En nuestro caso, dadas las peculiaridades de las Islas Canarias (en las que se usan o se han usado gran cantidad y variedad de plaguicidas) y teniendo en cuenta los estudios previos, decidimos incluir en este trabajo por un lado, la determinación de los contaminantes tóxicos ubicuos más evaluados en nuestro medio y cuya liberación en la actualidad no es intencionada, ejemplo de ello son los POCs, los PCBs, y los HAPs. Por otro lado, también evaluaremos aquellos compuestos que más recientemente son o han sido usados en sectores como la ganadería y la agricultura, entre ellos destacan los organofosforados, los carbamatos y los principales rodenticidas anticoagulantes.

Una vez planteado nuestro objetivo, el primer paso que tuvimos en cuenta fue el desarrollo de la metodología necesaria para abordar nuestros estudios. Ésta, en un laboratorio toxicológico, se erige como un pilar imprescindible y fundamental, ya que de ella parte cualquier línea de investigación o servicio de diagnóstico que queramos poner en funcionamiento. Dada la naturaleza biológica (y por tanto, compleja) de las muestras objeto de estudio, el análisis de las mismas representa un reto para cualquier laboratorio.

Durante el desarrollo de este trabajo hemos procesado muestras muy variadas. Ejemplo de ello es que hemos recibido muestras provenientes de

distintas especies, lo que implica distinta composición fisiológica. Pero no sólo eso, sino que en numerosas ocasiones, las muestras van a venir con distintos grados de descomposición, propios de los cambios postmortem o debido a una mala conservación de las mismas, lo que obliga al desarrollo de una metodología efectiva y robusta que garantice el éxito de los análisis.

Tradicionalmente, la detección de múltiples sustancias que pudieran estar involucradas en casos de intoxicación bajo una única metodología era bastante limitado, ya que la variedad en cuanto a naturaleza química de las moléculas que pueden estar implicadas es muy amplia, por lo que en la mayoría de las ocasiones se necesitaba realizar métodos complementarios entre si para poder abarcar el mayor espectro posible. Así pues, la aparición de nuevas tecnologías ha propiciado que se puedan desarrollar métodos de extracción y análisis multirresiduo que permiten el ahorro de recursos, no sólo económicos sino de tiempo. A la misma vez estos métodos ayudan a superar el escollo que supone en muchos casos la falta de información sobre el agente implicado.

En cuanto a extracción se refiere, poco a poco se ha ido imponiendo el uso de un método conocido como QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), que se corresponde con una extracción en fase sólida dispersiva. Es una herramienta de gran valor ya que supone un proceso de preparación de muestras sencillo e ideal para el análisis multirresiduo. Básicamente se fundamenta en la utilización de distintos componentes como lo puede ser el cloruro de sodio, el acetato de sodio anhidro, el sulfato de magnesio, amina primaria/secundaria (PSA), C18 o carbón grafitizado, cuya funciones varían desde la reducción del contenido en grasa o azúcar de la

muestra hasta la neutralización de ácidos orgánicos y pigmentos. El tipo de componentes que conforman el QuEChERS así como las cantidades son modificables, lo que facilita enormemente la adaptación de este sistema al tipo de muestra que queramos procesar.

En nuestro grupo de investigación hemos utilizado una adaptación de este método, lo que nos ha permitido la detección de varios contaminantes tóxicos ubicuos en matrices complejas. Este es el caso de los CTPs, estas sustancias siguen estando presentes en numerosos entornos y su estudio es primordial, ya que está demostrado que la presencia de tales sustancias en los organismos pueden causar efectos perjudiciales. La utilización del QuEChERS nos ha permitido la extracción de los CTPs más importantes en muestras tan significativas como lo son el calostro y la leche humana.

Aunque la tendencia en un futuro próximo es la utilización del QuEChERS, cuando hemos querido valorar la presencia de estos mismos compuestos en fauna al igual que otras sustancias como organofosforados y carbamatos nos hemos servido de una extracción sólido-líquido, extracción en fase sólida o la realización de un soxhlet.

De hecho gracias a la utilización de la extracción sólido-líquido, en la cual se ha utilizado una combinación de tres disolventes: diclorometano, etil éter y acetona, en relación 50/30/20, hemos sido capaces de realizar la detección de 117 sustancias de lo más variadas que, ya sea por su peligrosidad o por su frecuencia de utilización, se ven implicadas en los episodios de envenenamiento de fauna. Dicho método, robusto y fiable, tras ser validado para la matriz hígado, fue publicado y su utilidad ha quedado

demostrada tras su aplicabilidad en las muestras recibidas a lo largo de estos años en los que ha sido aplicado a otras matrices complejas como contenido gástrico de diferentes especies de animales, así como distintas clases de cebos.

Tomando como base este mismo método, nuestro grupo ha conseguido desarrollar una variación del mismo que ha sido utilizado con éxito en el análisis de muestras biológicas de muy variada procedencia, incluida sangre humana.

Tras el proceso de extracción, la técnica de la que nos servimos para analizar nuestras muestras es la cromatografía. Esta técnica nos permite a través de interacciones físico-químicas, entre otras, separar los distintos componentes dentro de una mezcla. Una vez separados los compuestos, la detección de los mismos, la realizaremos mediante la herramienta conocida como espectrometría de masas. Así pues, la combinación de la cromatografía (líquida o de gases) y la espectrometría de masas con sus múltiples variantes, es nuestra elección y la de muchos laboratorios para el análisis de este tipo de muestras complejas.

Aunque es una de las técnicas más sensibles y específicas del mercado, es bien sabido como dichos análisis van a estar sujetos a infinidad de variables. Ejemplo de ello son: la polaridad, distintos grupos químicos, tamaño de partícula, temperatura, distintas fases móviles y estacionarias, etc. De todas ellas queremos hacer hincapié en la que en nuestro caso particular destaca sobre las demás que es el efecto matriz. De todas las variables citadas anteriormente, es la única en la que en muchas ocasiones no vamos a poder

controlar. Los ejemplos de cómo puede afectar la matriz a nuestros análisis cromatográficos van desde aumentar la señal de nuestros analitos de interés, o incluso producir el efecto contrario como es la supresión de la señal; hasta propiciar el deterioro de nuestros equipos. Esto es fácilmente comprobable cuando comparamos cromatogramas en donde nuestros analitos están disueltos en un solvente frente a los mismos analitos disueltos en una matriz como puede ser carne o hígado. De esta situación se deriva que siempre que queramos desarrollar nuevos métodos, intentemos en la medida de lo posible, trabajar incluyendo la matriz sobre la cual vamos a recibir nuestras muestras.

Como podemos observar, el tiempo invertido en el desarrollo de una metodología eficaz nos brinda garantías de seguridad y de productividad científica; parámetros que un grupo de investigación siempre debe tener presente. De esta forma somos capaces de devolver el compromiso y el conocimiento que reclama la sociedad del siglo XXI de la que formamos parte.

Los resultados de esta primera parte metodológica se exponen en el Bloque A de esta Tesis Doctoral y han sido previamente publicados en: Analytical and Bioanalytical Chemistry “Multi-residue method for the determination of 57 Persistent Organic Pollutants in human milk and colostrum using a QuEChERS-based extraction procedure” (2013); Journal of Analytical Toxicology “Methodology for the identification of 117 pesticides commonly involved in the poisoning of wildlife using GC-MS-MS and LC-MS-MS” (2014); y en Science and Justice “Validated analytical methodology for the simultaneous determination of a wide range of pesticides in human blood using GC-MS/MS and LC-ESI/MS/MS and its application in two poisoning cases” (2015).

Una vez puesta a punto la metodología analítica necesaria, pasamos a evaluar la exposición de la fauna a los contaminantes tóxicos ubicuos. Es por ello que en el Bloque B se abordan los estudios tendentes a evaluar dicha exposición, valorando a su vez, si pueden ser de utilidad como bioindicadores de la contaminación del medioambiente y de la población humana.

El interés o la importancia de medir dichos contaminantes en fauna radica en que en muchas ocasiones el valorar el número de registros, autorizaciones y restricciones sobre los productos químicos sólo proporciona datos sobre las actividades emprendidas bajo los auspicios de las directivas de la UE. Tales observaciones no ofrecen información sobre la eficacia de estas medidas en el logro de sus objetivos. El control directo del aire, el suelo, el agua y los sedimentos pueden ser útiles para determinar el grado de contaminación en un área en particular, pero no proporciona una medida de la biodisponibilidad y la absorción resultante de la biota. Es sólo a través del biomonitoreo directo (el análisis de contaminantes en los tejidos de los organismos) a través del cual se puede determinar adecuadamente la exposición y la relación con los niveles en el entorno físico (22).

Desde épocas pasadas los animales han servido como alarmas frente a la exposición de los humanos frente a determinados tóxicos. Quizás el ejemplo más conocido de ello, sea la relación existente entre los canarios y los mineros. En el caso particular de los perros, al compartir el hábitat con los humanos y, sobre todo hoy en día donde la tendencia a considerar a nuestras mascotas como uno más de la familia se hace más evidente, ha hecho que cada vez más el tiempo que ambas especies compartimos en un mismo hábitat sea mayor. A

raíz de esta premisa, parece lógico pensar que los perros podrían servir como indicadores de la exposición de los humanos a ciertos tóxicos ubicuos liberados al medio de forma no intencionada. De hecho son varios los estudios que evidencian la presencia de sustancias de origen antropogénico en perros y gatos (23-26). Tal utilidad ha quedado demostrada en aquellos químicos cuya principal vía de entrada al organismo es la inhalatoria, pero ¿Qué pasa con aquellos cuya principal vía de absorción es la digestiva como en el caso de los CTPs? Este grupo posee una serie de características propias que los definen, tales como: la larga permanencia en el medio, elevada afinidad por las grasas y su capacidad de bioacumulación y biomagnificación a lo largo de la cadena alimentaria (27, 28). Son varios los estudios que muestran una baja concentración de algunos de estos contaminantes en los perros (29-32), lo que sugiere una metabolización más eficiente, por lo que podríamos llegar a pensar que para algunas de estas sustancias, los perros no serían adecuados como indicadores de la contaminación de estos compuestos. De ahí que en un principio tratáramos de valorar el grado de similitud del perfil de contaminación por CTPs (POCs, PCBs e HAPs) entre la población de las Islas y sus mascotas.

En este tipo de estudios, existe una variable que debemos tener en cuenta a la hora de comparar ambas especies, que es la exposición, y puesto que en humanos se ha demostrado que alrededor del 90% de la absorción de estas sustancias se debe al consumo de alimentos (33, 34), podríamos pensar que aunque perros y humanos compartamos el mismo hábitat, nuestro patrón alimentario es distinto y esto puede representar una importante diferencia. Por ello, en el segundo estudio que adjuntamos en este bloque, aparte de analizar

la presencia de ciertos CTPs en mascotas (perros y gatos), una muestra de varias marcas comerciales de comida húmeda y seca pertenecientes a distintas gamas, fueron analizadas con el objetivo de calcular la exposición a estos mismos CTPs a través de esta vía en las dos especies objeto de estudio. De esta manera podemos verificar de manera indirecta, la capacidad de metabolización de ambas especies y su potencial utilidad como posibles indicadoras de la exposición humana a este tipo de sustancias. Cabe resaltar que las comidas seleccionadas corresponden a marcas comerciales disponibles en todo el territorio español, lo que da un valor añadido a los datos de exposición ya que estos son extrapolables a otras zonas de nuestro país.

En la parte final de este Bloque B abordamos la evaluación de la presencia de contaminantes tóxicos ubicuos en fauna silvestre de las Islas.

Quizás el caso más conocido y ampliamente documentado en toxicología de fauna salvaje es el hecho de que ciertos contaminantes tóxicos ubicuos, en concreto los POCs, pueden reducir el espesor de la cáscara de huevo en las especies de rapaces. De hecho, durante los años 1940 y 1950, Derrick Ratcliff de la British Nature Conservancy señaló un descenso en las poblaciones de halcón peregrino (*Falco peregrinus*) en toda Europa. Poco después, el fracaso reproductivo en estos halcones, y en otras especies de aves, se asociaron a una disminución del grosor de la cáscara del huevo (35). La obra de Rachel Carson (1962), “Silent Spring” alimentó el debate entre los graves efectos de la liberación de contaminantes tóxicos al medioambiente. Rattner B.A. (36) realiza una amplia revisión de la historia de la toxicología en la fauna salvaje, describiendo intoxicaciones por plomo en faisanes (*Phasianus colchicus*) ya en el año 1876 (37) En 1979, debido a la

creciente necesidad de investigar en aspectos relacionados con la contaminación ambiental, se creó “The Society of Environmental Toxicology and Chemistry” (SETAC). Toxicólogos en fauna silvestre fueron los primeros miembros de esta sociedad, y hoy en día, sigue siendo una importante organización para la difusión de trabajos de investigación en este campo. El descubrimiento de masivas mortalidades de animales en el campo asociadas a la liberación de contaminantes tóxicos al medioambiente llevó a los toxicólogos de la época a iniciar experimentos en el laboratorio con el objetivo de establecer datos de referencia sobre la toxicidad aguda y crónica de los contaminantes tóxicos en fauna salvaje. Actualmente, la toxicidad de muchos de estos contaminantes ambientales, como el DDT, los PCBs, y varios metales, han sido ampliamente estudiados en la fauna salvaje. Sin embargo, y a pesar de que durante los últimos 30 años se ha hecho un enorme progreso, todavía queda mucho por aprender acerca de los efectos de estos contaminantes tóxicos por sí solos, y especialmente en las mezclas. Se ha de destacar que los estudios de monitorización de contaminantes tóxicos en fauna silvestre han ganado relevancia en los últimos años porque los datos derivados de los mismos se emplean en las evaluaciones de riesgo dirigidas a proteger la salud ambiental y humana, y en el establecimiento de los límites de “seguridad” para la población. Por lo tanto, estudios de monitorización en fauna silvestre como el desarrollado en esta Tesis, ofrecen la oportunidad de participar en procesos de regulación de enorme repercusión sanitaria.

En Canarias los estudios realizados en fauna son variados pero escasos. Es quizás en la fauna marina del archipiélago en donde más se ha estudiado la exposición a contaminantes tóxicos. Los datos que derivan de

tales investigaciones son especialmente interesantes ya que al estar dicha fauna (sobre todo mamíferos marinos) situada en la parte superior de la cadena trófica (como el ser humano), pueden sufrir una mayor exposición a algunos de estos contaminantes debido a su capacidad para ser almacenados en grandes cantidades en la grasa corporal a lo largo de su vida, siendo movilizados posteriormente durante ayunos prolongados, preñez, lactación o puesta de huevos.

En estudios de nuestro Grupo (Natalia Álvarez y cols.) se realiza la determinación de POCs, PCBs y PAHs en delfines mulares varados en las costas canarias (17), e incluso en delfines de vida libre, que fueron biopsiados dentro del periodo comprendido entre 2003 y 2011. Algunos de los contaminantes tóxicos de interés fueron detectados en el 100% de las muestras, como es el caso del p,p' DDE, p,p' DDT, PCB 180, PCB 153, PCB 138, endrín y fenantreno; presentando una tendencia al alza durante los últimos años de muestreo (18).

De forma similar, estudios de nuestro Grupo (María Camacho y cols.) en tortugas bobas (*Caretta caretta*), demostraron mayor presencia de POCs y PCBs en la población de Canarias en comparación con la población de Cabo Verde; se postula que debido al desarrollo de la industria. En este mismo estudio y al igual que ocurría con los delfines mulares, los PCBs que mayor porcentaje de detección mostraron fueron aquellos más pesados: PCB 138, PCB 153 y PCB 180. Cabe resaltar que se encontró una relación negativa en cuanto al estado de salud y las concentraciones del PCB 153, demostrando el posible efecto tóxico inducido por estos contaminantes. De igual manera que una correlación negativa entre el tamaño de las tortugas y la presencia de

determinados PCBs, sólo observable en la población de Canarias. En cuanto a los PAHs, el que mayor frecuencia de detección y concentración mostró fue el fenantreno. En este caso las poblaciones de Canarias y Cabo Verde mostraron similares concentraciones en cuanto al sumatorio de los PAHs (Σ PAHs). Si bien no podemos obviar que al igual que pasaba con otros contaminantes, las concentraciones de PAHs en las muestras de Canarias también han experimentado un aumento durante los últimos años (19).

Todos estos estudios vienen a corroborar un incremento en la contaminación del medio marino.

En cuanto a fauna terrestre se refiere, los estudios realizados son aún más escasos y es que bajo nuestro conocimiento el número de publicaciones realizadas se reduce a 2. La más reciente es la realizada por Gómara B. et al. en 2004 donde se analizó las concentraciones de 23 PCBs , p,p' DDT y dos de sus metabolitos, p,p' DDE y p,p'-TDE en guirres (*Neoprhon Pernocterus*). En este estudio la población diana fueron poblaciones de guirres presentes en 5 lugares distintos del territorio español. Tristemente la población que alcanzó los valores más elevados del ΣDDTs fue la población de la isla canaria de Fuerteventura, mostrando valores en suero de una media de 6,2 ng/ml (21). Estos resultados son importantes puesto que al ser la población de Guirres de Fuerteventura una población sedentaria, la podríamos considerar un fiel reflejo de la contaminación del entorno, aunque no podemos obviar una posible influencia debido a la cercanía de la isla al continente Africano, donde en determinadas zonas el uso del DDT se permite para el control de los vectores. En el caso de los PCBs los resultados obtenidos de este estudio son destacables ya que de los congéneres que mayor concentración mostraron,

tres (PCB 138, PCB 153 y PCB 180) coinciden con los que mayor concentración mostraron en las especies del medio marino (18, 19). La importancia radica en que estos PCBs se han relacionado con la contaminación industrial y al ser los más pesados con la contaminación local, y Fuerteventura no es de las islas más industrializadas del archipiélago, lo que impulsa una vez más la idea de monitorizar fauna terrestre y sobre todo en las islas más industrializadas.

El otro artículo del cual nos hacemos eco, es el estudio realizado por Rafael Mateo et al, en búsqueda del principal producto de degradación del DDT, que es el p,p'DDE, en los huevos de cernícalo (*Falco tinnunculus*). La concentración de este metabolito alcanzó una media de 4,9 µg/gr en peso húmedo (20). Teniendo en cuenta los resultados obtenidos, y publicaciones como la realizada por Newton (1979) en la que afirma que concentraciones que ronden lo los 4-5 µg/gr provocan una reducción del grosor de la cáscara de huevo en aves rapaces en torno a un 15% (39). Unido a que una disminución del grosor de la cáscara de huevo en torno al 18-20% o más durante varios años se ha postulado como causantes del declive de poblaciones (40). Activan la alarma para pensar que dicho tóxicos pueden ser los causantes del declive al cual muchas de las poblaciones de aves en Canarias se han visto abocadas.

A raíz de estos resultados, nos parecía lógico realizar nuevos estudios, sobre todo en la fauna terrestre, que por un lado fueran más recientes y que por otro nos sirvieran como indicadores de la biodisponibilidad de estos contaminantes en el medioambiente. La decisión de tomar como especies objeto de estudio las aves rapaces se basa en su reconocida valía como bioindicadores. Estas al tener una relativa larga vida y al estar en lo más alto

de la cadena alimentaria, hace que tengan la capacidad de almacenar estos compuestos a lo largo de su vida al igual que ocurría con delfines y tortugas en el medio marino. Por lo que un estudio en profundidad de los mismos nos puede servir para conocer la biodisponibilidad real de estos contaminantes en el medio para el resto de animales incluidos los humanos.

Los resultados de esta segunda parte se exponen en el Bloque B de esta Tesis y han sido previamente publicados en: Journal of Applied Animal Research “Are pet dogs good sentinels of human exposure to environmental polycyclic aromatic hydrocarbons, organochlorine pesticides and polychlorinated biphenyls? (2015); Science of the Total Environment “The assessment of daily dietary intake reveals the existence of a different pattern of bioaccumulation of chlorinated pollutants between domestic dogs and cats” (2015); y en Science of the Total Environment “Assessment of the exposure to organochlorine pesticides, PCBs and PAHs in six species of predatory birds of the Canary Islands, Spain” (2014).

El último bloque de la presente Tesis, el Bloque C, aborda una temática compleja en nuestro Archipiélago ya que trata de evaluar la liberación intencionada de productos tóxicos al medio. Ya sea con el fin de controlar plagas, como ocurre con los anticoagulantes empleados como rodenticidas, o cuando estas sustancias se emplean con fines delictivos como “veneno”.

Es bien conocido que ya sea de manera accidental o intencionada, el envenenamiento de la fauna es una práctica común en todo el mundo y representa un reto para toxicólogos y laboratorios forenses (41-43). Muchos de

los animales implicados en estos episodios, no sólo silvestres sino domésticos también, son encontrados por cazadores, senderistas, agentes de medio ambiente o cuerpos policiales. Si tuviéramos que establecer algún tipo de clasificación a estos episodios, utilizaríamos la clasificación que ha sido generalmente aceptada en la que en primer lugar, estarían aquellos en los que la sustancia involucrada está permitida y se ha utilizado correctamente (siguiendo las recomendaciones del fabricante); en segundo lugar, aquellos episodios en los que la sustancia es permitida, pero se ha hecho un mal uso de la misma; y en tercer lugar, aquellos en donde la sustancia empleada, legal o no, se utiliza deliberadamente para envenenar animales. En inglés esta clasificación se conoce como “approved use, misuse, or deliberate abuse.” respectivamente.

El número de envenenamientos en los animales es un aspecto de la protección de la naturaleza del que muy difícilmente llegaremos a tener datos precisos. Esto se debe en parte a que podemos suponer que las cifras comunicadas en este tipo de estudios sólo representan una aproximación de la incidencia real de la mortalidad de la fauna, ya que se ha estimado que menos del 10% de los casos de envenenamiento se detectan y se envían a un laboratorio forense (44, 45), cosa que en fauna silvestre ocurre con bastante frecuencia, y algo menos en fauna doméstica. También sucede que la muerte de estos animales es confundida con otros tipos de procesos como infecciosos o metabólicos entre otros. De ahí que siempre nos estemos moviendo sobre una línea difícil de definir.

El uso descontrolado o malintencionado de los pesticidas es una grave amenaza para la salud pública y la biodiversidad en Europa. En la Unión

Europea (UE), esta práctica ilegal representa uno de los mayores problemas para la conservación de algunas especies en peligro de extinción, a menudo convirtiéndose en la principal causa de muerte no natural.

Desde nuestro grupo queremos resaltar que la información que podemos obtener de los episodios de envenenamiento de la fauna doméstica, sobre todo en áreas rurales, no es nada desdeñable. Estos al ser objeto de mayor atención por parte de los propietarios, ofrecen información sobre la presencia y disponibilidad de los venenos en el medio rural. Llegados a este punto, la creación en un futuro cercano de una herramienta en la que los veterinarios que ejerzan su profesión en clínicas, pudieran comunicar sus casos, y de este modo recopilar todos los episodios de envenenamiento (puesto que en numerosas ocasiones, una intoxicación no tiene una resolución fatal, y la información que nos podría aportar se pierde), junto con puesta en marcha de una red a nivel nacional, donde los laboratorios dedicados a la lucha contra el veneno pudieran volcar datos y establecer un flujo de información, nos aproximaría un poco más a la realidad y nos dotaría de una importante información a la hora de registrar las tendencias y hábitos en el uso del veneno.

En 2008 publicado por WWF (World Wide Fund for Nature)/Adena, encontramos un documento en donde se plasma los episodios de envenenamiento acaecidos en España en el periodo comprendido entre 1990 y 2003. En el caso de Canarias, se registraron 33 episodios, afectando a un total de 165 animales, lo que hace una media de 5 animales afectados por caso (46). Estas cifras sólo vienen a ratificar que anteriormente a la creación del Servicio de diagnóstico que se presta desde la Unidad de Toxicología de la

Universidad de Las Palmas de Gran Canaria, el número de casos en los que se conseguía confirmar una intoxicación era muy inferior a los casos diagnosticados hoy en día.

Merece especial atención las sustancias clasificadas como rodenticidas anticoagulantes. Sabemos que a día de hoy y así ha sido durante muchos años, el uso de tales sustancias se ha convertido en el método preferido para el control de plagas de roedores. En el caso particular de las Islas Canarias, los raticidas han sido ampliamente utilizados en los últimos años debido a que la administración pública local ha proporcionado estos productos a los agricultores de forma gratuita (47). Aunque se ha demostrado que la exposición de aves rapaces a estas sustancias químicas puede condicionar la supervivencia de las poblaciones (48) y que por lo tanto podría ser una amenaza para las rapaces de estas islas, hasta la fecha no habían datos que documentaran tal situación en nuestra región. Debido a este vacío, se diseñó el estudio que presentamos en este bloque con el objetivo de evaluar la presencia y concentraciones a rodenticidas anticoagulantes, ya sean de primera o segunda generación en 104 hígados de aves pertenecientes a seis especies de aves rapaces en las Islas Canarias. De esta manera, podremos conocer el nivel de amenaza que representan tales sustancias para la conservación de las aves de nuestro territorio. Si bien somos consientes de que en muchas ocasiones, las aves rapaces o carroñeras lo que van a sufrir es una intoxicación secundaria tras el consumo de presas envenenadas, este artículo se integra dentro de este bloque debido a la intencionalidad que implica la colocación de estos raticidas en el medio, muchas veces realizado por personal no cualificado o debidamente formado.

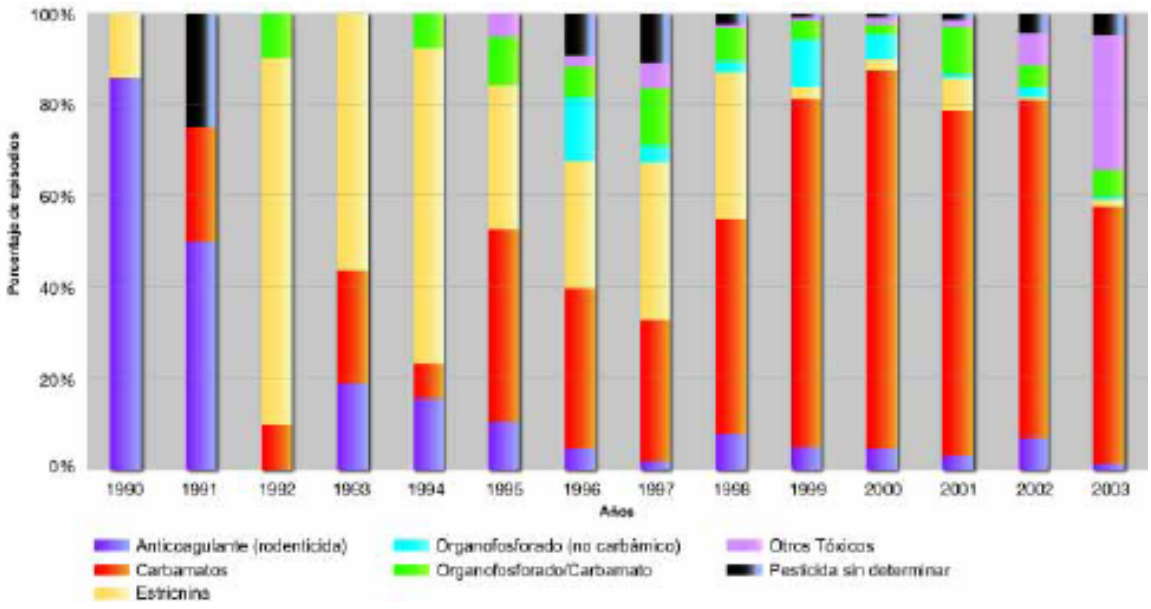
Gracias a la realización de este estudio y con motivo de la obtención del título de doctor con mención europea, durante el periodo de realización de la Tesis, se realizó una estancia en un centro dependiente del Gobierno Escocés llamado SASA (Science and Advice for Scottish Agriculture), específicamente en el Wildlife Incident Investigation Scheme. Durante esta estancia se planteó un estudio en el cual se evaluó la presencia y concentraciones de rodenticidas anticoagulantes en visones americanos (Neovison vison). Los visones americanos son una especie invasora distribuida por toda Escocia y debido a este motivo son objeto de captura. Sirviéndonos de tal proyecto, los hígados de los animales fueron procesados en búsqueda de dichas sustancias, corroborando finalmente que tal y como exponen numerosas publicaciones, el problema del uso de estas sustancias no está circunscrito a este archipiélago sino que es un problema a nivel mundial, que llega a afectar a distintas especies y que como podemos ver en el anexo II, estos depredadores al estar en lo más alto de la cadena trófica se convierten en unos buenos bioindicadores de la distribución de estas sustancias en el medio.

A través de la realización de estos dos estudios, queda confirmado como la distribución y colocación, así como las buenas prácticas de manejo, son esenciales para una correcta supervivencia de muchas especies, incluidas claro está las aves rapaces. Si bien como comentábamos anteriormente tales intoxicaciones se clasifican como un envenenamiento secundario, no podemos cerrar este capítulo sin hablar del ejemplo más claro en el envenenamiento intencionado de animales como lo es la colocación de cebos. En España, el uso del veneno como herramienta para dar muerte a los animales se encuentra arraigado en lo más profundo de nuestras costumbres y respaldado años atrás

por normativa que apoyaba esta práctica. Las primeras citas del uso del veneno las encontramos relacionadas con el sector de la ganadería. Sector afectado por el ataque que sufrían sus animales debido a los lobos principalmente y fue entonces cuando en 1542 Carlos I dio capacidad para legislar sobre esta materia y se aprobaron leyes que permitían el uso del veneno para dar caza a los lobos. Hasta entonces el uso del veneno se restringía a su empleo por ejemplo en lanzas, y de este modo estaríamos aún hablando de un método selectivo. Es en La Ley de Caza de 1879 en la que se hace mención al uso de los cebos envenenados y por tanto el uso del veneno ya pasa a ser el método masivo y poco selectivo que conocemos actualmente. Más específicamente en su artículo 41 dice: “Cuando las circunstancias lo exijan, los alcaldes, previa autorización del gobernador civil de la provincia, podrán disponer batidas generales para la destrucción de animales dañinos y el envenenamiento de estos. Tomarán las medidas necesarias para la seguridad y conservación de las personas y de las propiedades, el modo, la duración, el orden y la marcha de la operación, y todas las demás que sean necesarias para asegurar la regularidad y evitar peligros e inconvenientes”. Ya en 1983 se aprecian las últimas autorizaciones para este tipo de método, y en 1989 es cuando comienza a asentarse en nuestra legislación el rechazo al uso del veneno con la Ley 4/1989, de 27 de marzo, de Conservación de los Espacios Naturales y de la Flora y Fauna Silvestre, la cual prohíbe en su artículo 34 la tenencia, utilización y comercialización de todos los procedimientos masivos o no selectivos para la captura o muerte de animales y en particular los venenos. Su rechazo total viene de la mano del artículo 336 del código penal de 1995, el cual dice: “El que, sin estar legalmente autorizado,

emplee para la caza o pesca veneno, medios explosivos u otros instrumentos o artes de similar eficacia destructiva para la fauna, será castigado con la pena de prisión de seis meses a dos años o multa de ocho a veinticuatro meses. Si el daño causado fuera de notoria importancia se impondrá la pena de prisión antes mencionada en su mitad superior" (49) .

En el pasado, muchos de los casos de intoxicación se debieron a la estricnina (raticida), mientras que de 2000 a 2007, la mayoría de los casos de envenenamiento primario y secundario fueron causados por agentes anticolinesterásicos, principalmente aldicarb y carbofurano, y por rodenticidas anticoagulantes (45, 50-53). De hecho si rescatamos nuevamente el informe de WWF/Adena de 2008, podremos observar una grafica en la que claramente vemos esta tendencia cambiante en el uso de los venenos (46).



En la UE, la comercialización de productos que utilizaran como agente activo el aldicarb se retiró en 2003 (54), y los productos con carbofurano en 2007 (55). Por lo tanto, se debe esperar una reducción en los episodios de

intoxicación en los que se encuentren implicadas estas sustancias prohibidas. Sin embargo, en un estudio publicado por Martinez de Haro et al. Vemos como se concluye que en muchas ocasiones, las prohibiciones o restricciones en el uso de ciertas sustancias no impide su uso y es más, en ocasiones la DL50 se encuentra correlacionada con la frecuencia de uso, lo que denota cierto conocimiento del producto por parte del envenenador (56).

Volviendo de nuevo a nuestro archipiélago, en 2011, en un informe publicado también por la WWF/Adena en el cual se valoraba el estado actual de las distintas comunidades autónomas en cuanto a la lucha contra el veneno se refiere; y en el cual se valoraron los siguientes 8 factores:

- Existencia de un plan regional contra el veneno y su grado de desarrollo.
- Disponibilidad de recursos humanos y materiales.
- Existencia de planes de vigilancia y prevención.
- Especialización de agentes.
- Normativa propia y grado de desarrollo.
- Resoluciones ejemplarizantes.
- Sensibilización de grupos de riesgo.
- Trasparencia en el acceso a la información.

Y en donde las comunidades autónomas eran clasificadas dentro de las siguientes categorías dependiendo de la puntuación obtenida:

- Graves carencias (8-15 puntos).
- Muy insatisfactorio (16-19 puntos).
- Necesita mejorar (20-23 puntos).
- En el buen camino (24 o más puntos).

Canarias, sólo aprueba en el apartado 8, obteniendo una puntuación total de 13, categorizando su situación como con graves carencias (57)

Con la intención de cambiar dicha situación, y como punto de inflexión marcado por la celebración del I seminario contra el uso ilegal de sustancias tóxicas en el medio natural de Canarias, celebrado en 2010, diversos colectivos como la Universidad de Las Palmas de Gran Canaria a través de la Unidad de Toxicología y el Servicio de Toxicología Clínica y Analítica, la Administración Pública, los Colegios Oficiales de Veterinarios de ambas provincias, AVAFES (Asociación Veterinaria para la protección de Fauna Exótica y Silvestre) y SEO/Birdlife entre muchos otros; comenzaron a trabajar de manera integrada, y tras 4 años, en abril del 2014 llega a publicarse en el BOE la ORDEN de 28 de marzo de 2014, por la que se aprueba la Estrategia para la Erradicación del Uso Ilegal de Veneno en el Medio no Urbano de Canarias (Estrategia Canaria Contra el Veneno) (58). Esta estrategia viene a dar solución a un problema hasta el momento desconocido, aunque quizás el adjetivo que mejor lo califica es el de ignorado, puesto que hasta la puesta en marcha del laboratorio, la confirmación de un envenenamiento era una ardua tarea, ya que sabemos que cuando hablamos de veneno, si no se busca no se encuentra. A partir de entonces se abre una etapa de difícil gestión ya que se empieza a dar resolución a muchos casos de envenenamiento, y se hace evidente a la vista de los resultados, que Canarias tiene un serio problema. Las cifras arrojadas por el Servicio de Toxicología Clínica y Analítica (SERTOX) superan con creces las publicadas con anterioridad. La consecución de objetivos de esta estrategia es fundamental para cualquier plan de conservación, recuperación o reintroducción de fauna que se desee realizar en un futuro próximo. En el

Anexo II de esta Tesis Doctoral se presenta dicha estrategia con el objetivo de facilitar su consulta para todo aquel que quiera ahondar en el documento marco sobre el cual trabajará nuestra Comunidad Autónoma en la lucha contra el veneno.

Los resultados de esta tercera parte se exponen en el Bloque C de esta Tesis Doctoral y han sido previamente publicados en: Science of the Total Environment “Assessment of anticoagulant rodenticide exposure in six raptor species from the Canary Islands (Spain)” (2014); y en Science of the Total Environment “Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain)” (2015).

Sólo un desarrollo sostenible y responsable con el medioambiente es compatible con la protección de la salud de las personas y por ende de la fauna y flora perteneciente al mismo. El deterioro del medioambiente así como el uso irresponsable cuando no ilegal de pesticidas, tiene y tendrá un impacto de valor incalculable en la conservación de muchas especies, llegando a representar una seria amenaza y el camino hasta un punto de no retorno.

La presente Tesis Doctoral Europea brinda la oportunidad de abordar dicha problemática y conocer datos actualizados que a medio y largo plazo nos ofrezcan la posibilidad de concretar una solución viable que satisfaga a todas las partes involucradas y sea compatible con un desarrollo sostenible en las Islas Canarias.

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OBJETIVOS

Objetivos

1. Desarrollar una metodología de extracción robusta y fiable que permita la identificación y cuantificación de una amplia variedad de sustancias, no sólo de aquellas presentes en nuestro entorno de forma ubicua, sino de las involucradas comúnmente en los episodios de envenenamiento de fauna y humanos.

2. Evaluar la idoneidad de animales domésticos como centinelas de exposición a contaminantes tóxicos persistentes (CTPs) para los seres humanos.

3. Determinar la exposición de perros y gatos a determinados CTPs a través del consumo de dietas comerciales. Esto permitirá calcular la ingesta diaria de este grupo de contaminantes en perros y gatos según las indicaciones de consumo del fabricante. Además se evaluarán las potenciales diferencias de contaminación entre ambas especies teniendo en cuenta su exposición a través de la ingesta.

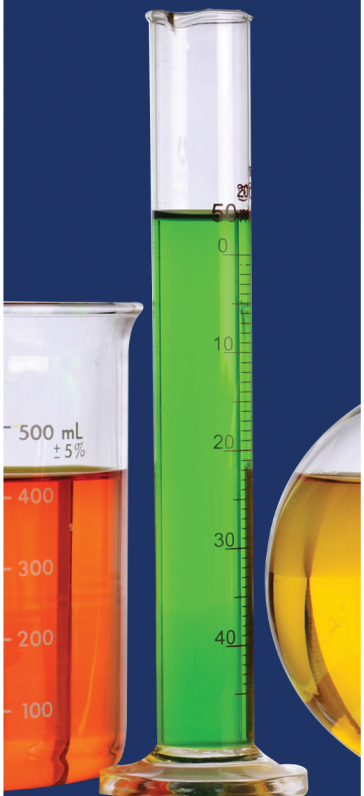
4. Monitorizar niveles de CTPs en aves silvestres del archipiélago canario, estudiando posibles diferencias entre especies y perfiles de contaminación.

5. Determinar niveles de exposición de aves rapaces del archipiélago canario a rodenticidas anticoagulantes de primera y segunda generación.

6. Ofrecer nuevos datos sobre la casuística de los envenenamientos de fauna silvestre y doméstica en Canarias, estudiando diferencias en las especies afectadas y las sustancias tóxicas más frecuentemente utilizadas.

BLOQUE A

DESARROLLO METODOLÓGICO



Resumen

En el presente bloque se exponen los artículos metodológicos desarrollados a lo largo del periodo en el que se ha elaborado la presente Tesis Doctoral Europea 2010-2015.

El primero de ellos contempla el desarrollo de un método para la detección y cuantificación de varios compuestos tóxicos en el calostro y la leche materna. En este caso nos servimos de una modificación del método conocido como QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), unido a la cromatografía de gases acoplada a espectrometría de masas de triple cuadrupolo, para la detección de 23 POCs, 18 PCBs y 16 PAHs.

El segundo de ellos tiene como objetivo la identificación y cuantificación de una gran variedad de pesticidas en muestras provenientes de especies silvestres y domesticas. El método presentado es una combinación de una extracción sólido-líquido seguida de otras fases de purificación de la muestra como es la cromatografía por permeación en gel (GPC) o mediante congelación y centrifugación. Para finalizar con la detección mediante el uso de 3 análisis complementarios de cromatografía líquida o gaseosa acoplada a espectrometría de masas. Dicho procedimiento nos permite la determinación de 117 pesticidas. La validez del método ha quedado demostrada tras su aplicación en 98 casos reales, en los cuales a parte de hígado se ha trabajado con diferentes matrices, algunas de ellas en avanzado estado de descomposición.

Basándonos en el método publicado anteriormente, se desarrolló otro el cual nos permite la identificación y cuantificación de 109 sustancias en sangre humana. En este caso la extracción primera se basa en una extracción liquido-líquido, seguida de una etapa de purificación y una detección utilizando cromatografía líquida y de gases acoplada a espectrometría de masas de triple cuadrupolo. El método fue completamente validado y su aplicabilidad demostrada tras la resolución de dos casos reales.

Anal Bioanal Chem (2013) 405:9523–9536
DOI 10.1007/s00216-013-7377-0

RESEARCH PAPER

Multi-residue method for the determination of 57 Persistent Organic Pollutants in human milk and colostrum using a QuEChERS-based extraction procedure

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Received: 11 August 2013 / Revised: 8 September 2013 / Accepted: 13 September 2013 / Published online: 27 October 2013

Abstract Human breast milk represents the best choice for the nutrition of infants. However, in addition to containing beneficial nutrients and antibodies, it can also be considered the best indicator of infant exposure to contaminants. We developed a multi-residue method using a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure and capillary gas chromatography–triple quadrupole mass spectrometry for the determination of 57 persistent organic pollutants, including 23 organochlorine pesticides, 18 polychlorinated biphenyl (PCB) congeners, and 16 polycyclic aromatic hydrocarbons in human milk and colostrum samples. We have used primary secondary amine in the clean-up step as it gave a more efficient separation of the analytes from fat and superior removal of the co-extracted substances compared with gel permeation chromatography. No significant matrix effect was observed for the tested pollutants, and therefore matrix-matched calibration was not necessary. The average recoveries from spiked samples were in the range of 74.8–113.0 %. The precision was satisfactory, with relative standard deviations below 16 %, while values of 0.1–0.4 μg L^{-1} were established as the limit of quantification for all the target analytes (0.05 and 100 μg L^{-1}). The method was successfully applied to the analysis of 18 human colostrum and 23 mature milk samples. All the samples tested were positive for at least nine different residues, with some samples containing up to 24 contaminants. Remarkably, the contaminants hexachlorobenzene, p,p'-DDE, PCB 138, PCB 180, phenanthrene, fluoranthene, and pyrene were present in 100 % of the colostrum and mature milk samples analyzed.

Keywords Organochlorine pesticides · Polychlorinated biphenyls · Polycyclic aromatic hydrocarbons · Breast milk · QuEChERS · GC-tandem MS

Introduction

Anthropogenic contaminants include many chemicals that are resistant to degradation in the environment and biota. Over the last 30 years, a number of these substances have been highlighted as a cause for concern [1] and have been the subject of extensive study and international regulation. Due to their stable structures and lipophilic properties, persistent organic pollutants (POPs), such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), often concentrate and magnify in the food chain, particularly in fat sources. Other contaminants, such as polycyclic aromatic hydrocarbons (PAHs), cannot be considered POPs sensu strictu because they are efficiently metabolised; however, due to their prevalence in the environment and their lipophillicity, they are frequently

Electronic supplementary material The online version of this article (doi:10.1007/s00216-013-7377-0) contains supplementary material, which is available to authorized users.

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categorised as and studied together with POPs. It is widely accepted that food consumption is the primary source of non-occupational human exposure to these contaminants, as opposed to alternative exposure routes such as inhalation and dermal contact. The ingestion of contaminated food constitutes over 90 % of total human exposure, and foodstuffs of animal origin are recognised as one of the main contributors [2–5].

OCPs were widely used prior to the recognition of their toxicity and persistence. Pesticide exposure has been associated with arthritis, various types of cancer and diabetes [6–9]. Organochlorine exposure has been associated with neurobehavioral and developmental changes and DNA hypomethylation [10]. Furthermore, PCBs were widely used in electrical systems and hydraulic fluids. Their production was banned worldwide in the 1970s. Nonetheless, PCBs are still detectable in wildlife and humans [11]. PCBs have been associated with adverse neurological development, including decreases in motor skills and cognitive development [12]. Prenatal exposure to PCBs and related chemicals, such as chlorinated dibenzofurans and dioxins, in highly exposed populations has been associated with altered pubertal timing and growth abnormalities, including decreased height and birth weight [13, 14]. Cancer and endocrine disruption have been associated with adult exposure [1, 15, 16]. Finally, PAHs are widespread chemical pollutants that are introduced into the environment from a number of different sources. They are mainly produced by pyrolysis but can also be of petrogenic origin from crude oils or refinery products. PAHs can enter the environment through atmospheric deposition, road run-off, industrial discharges and oil spills. More than 50 % of the PAH emitted into the atmosphere comes from car emissions, and 28 % results from residential and industrial combustion [17, 18]. PAHs have been identified in biological samples from wildlife [19] and humans [20]. Many PAHs are toxic, mutagenic and carcinogenic [21, 22].

Human milk not only contains nutrients and antibodies but can also be used as an indicator of the level of organic pollutants in human bodies [20, 23]. The presence of organochlorines and other contaminants is strongly correlated with maternal adipose tissue, plasma, cord blood and breast milk, demonstrating both placental and lactational transfer [24, 25]. A greater elimination of persistent organic pollutants from the maternal body occurs during breastfeeding [26, 27], contributing, along with the prenatal exposure of the foetus [28], to the newborn's bodily burden. Therefore, breast milk is a sensitive matrix for monitoring the maternal bodily burden of pesticides [23]. Furthermore, the sampling of milk is non-invasive and therefore generally accepted by mothers [29]. Within the lactation period, colostrum is secreted by women for 7 days following parturition. Because it contains a higher percentage of fat, it may contribute to the bioaccumulation of lipophilic contaminants in the newborn's body [23]. For this reason, it is important to develop rapid and sensitive analytical methods to identify and determine POP residues in human colostrum at trace levels.

Numerous methods for the extraction, purification and quantification of organic contaminants in fatty samples such as milk have been described in the literature. Since it was first developed, the quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction and clean-up method has become an important and widely used technique in the analysis of multiple chemical residues in a variety of matrices, including fatty tissues and foods such as milk [30–34]. Recently, the efficacy of this methodology has been proven for the extraction of pesticides, including some organochlorines [35]. Excellent recovery and repeatability has been obtained with this technique for a wide range of contaminants. Therefore, QuEChERS can be an effective, flexible and inexpensive choice for the multi-residue analysis of POPs in human milk and colostrum. In addition, tandem mass spectrometry (MS/MS) has been increasingly utilised in the final determining step of contaminant residue analysis and is considered a practical means of circumventing the challenges associated with the identification of target analytes in matrices containing excessive quantities of potentially interfering substances, such as the fat in human milk [36]. To our knowledge, the use of GC–MS/MS with an electron ionisation (EI)-QqQ analyser has not been applied to the analysis of POPs in human milk and colostrum, where the low levels of these residues necessitate a powerful technique with a low LOD and a confirmatory approach.

The aim of the present work was to develop and optimise a multi-residue method based on QuEChERS extraction and GC–MS/MS for the simultaneous determination of 23 OCPs, 18 PCBs and 16 PAHs in human colostrum and milk samples. The performance parameters were determined by the GC–MS/MS analysis of standard solutions, standard reference materials and spiked real samples. The proposed method was successfully applied to the characterisation of 41 human milk samples (18 of colostrum and 23 of mature milk) from volunteers recruited in the Insular Materno-Infantil University Hospital (Gran Canaria, Canary Islands, Spain; CHUIMI).

Materials and methods

Chemicals and reagents

Acetonitrile, hexane and cyclohexane were of the highest purity available (>99.9 %) and purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure water was produced from a Milli-Q Gradient A10 (Millipore, Molsheim, France). The OCPs (hexachlorobenzene, α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, δ-HCH, heptachlor, aldrin, endrin, dieldrin, dicofol, *o*,*p*′-DDT, *p*,*p*′-DDT, *o*,*p*′-DDE, *p*,*p*′-DDE, *o*,*p*′-DDD, *p*,*p*′-DDD, *cis*-chlordane, *trans*-chlordane, α-

endosulfan, β-endosulfan, endosulfan sulphate and metoxychlor and mirex), PCB congeners (IUPAC numbers 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180 and 189), surrogates (PCB 12, PCB 202, *p*,*p*′-DDE-d8 and acenaphtylene D8) and internal standards (tetrachloro-*m*-xylene, heptachloro epoxide *trans* and benzo[a]pyrene D12) were purchased from Dr. Ehrenstorfer Reference Materials (Augsburg, Germany). The PAHs (naphthalene, acenaphtylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene and benzo[ghi]perylene) were purchased from Absolute Standards, Inc. (Connecticut, USA). All standards were neat compounds (purity from 97 to 99.5 %). Stock solutions of each compound at 1 mg mL^{-1} were prepared in cyclohexane and stored at −20 °C. Diluted solutions ranging in concentration from 0.05 to 100 ng mL^{-1} were used to prepare the calibration curves. The QuEChERS bulk materials (anhydrous magnesium sulphate, sodium chloride, di-sodium hydrogen citrate 1.5 hydrate, tri-sodium citrate 2 hydrate and primary secondary amine (PSA)) were purchased from Panreac Ibérica (Barcelona, Spain). The standard reference material SRM 1953 (organic contaminants in human milk—non-fortified) was purchased from NIST (Gaithersburg, USA).

Extraction and clean-up procedure

A QuEChERS extraction procedure was developed for the milk and colostrum samples. A 5-mL volume of milk or colostrum was sampled in 50 mL polypropylene centrifuge tubes. A 5-mL volume of ultrapure water was then added and shaken. A 10-mL volume of acetonitrile saturated in *n*-hexane and 50 μL of the surrogates solution at 1 μg mL^{-1} were added, and the mixture was allowed to stand at room temperature for 30 min to allow the swelling of the matrix, vortexing it every 10 min. Subsequently, a mixture of 4 g of anhydrous magnesium sulphate, 1 g of sodium chloride, 0.5 g of di-sodium hydrogen citrate and 1 g of tri-sodium citrate salts was added directly to the tube; the mixture was immediately manually shaken for 1 min to prevent the agglomeration of salts. A centrifugation (5,000 rpm, 5 min, 20 °C) was performed, and the upper phase was removed and transferred to a glass tube. A second extraction was performed adding 5 mL of acetonitrile saturated in n-hexane to the remaining pellet, which was then vigorously shaken for 1 min and centrifuged a second time (5,000 rpm, 5 min, 20 °C) to separate the organic-upper layer, which was added to the previously obtained organic layer.

Finally, an additional clean-up step was performed. The acetonitrile phase was transferred to a 15 mL polypropylene centrifuge tube containing 0.9 g of anhydrous magnesium sulphate and 0.5 g of PSA. The mixture was vigorously shaken for 1 min and centrifuged (5,000 rpm, 5 min, 20 °C). The sample solution was filtered through a 0.2-μm PTFE filter. The filtered extract was evaporated to dryness under a gentle nitrogen stream. The residue was then reconstituted in 1 mL cyclohexane, 10 μL of the solution of internal standards at 1 μg mL^{-1} was added and the mixture was transferred to a GC vial that was used for the chromatographic analysis.

GC-QqQ-MS/MS analysis

The gas chromatography separations were performed on a Thermo Trace GC Ultra equipped with a TriPlus autosampler and a split/splitless injector with electronic pressure control (Thermo Fisher Scientific Inc., Waltham, MA, USA). A fused silica capillary column BPX5 (cross-linked 5 % phenyl methylpolysiloxane, SGE Inc., USA) with a length of 30 m, a 0.25 mm i.d. and a film thickness of 0.25 μm was used as the stationary phase. Helium (99.999 %) at a constant flow rate of 1.0 mL min^{-1} was used as the carrier gas. The temperatures were programmed as follows: the initial oven temperature of 60 °C was maintained for 1 min, ramped at 12 °C/min to 210 °C, then raised at 8 °C/min to 320 °C with a 6 min hold time. The total run time was 61 min. The injector and transfer line were set to 270 and 310 °C, respectively. The standards and samples were injected (1 μl) in the splitless mode.

The GC was interfaced with a TSQ Quantum Max QqQ mass spectrometer (mass range, *m*/*z*, from 10 to 1,050) for the detection of the contaminants included in this study. The instrument data system also contained an EI-MS/MS library, which was specially created for the target analytes under our experimental conditions. The mass spectrometer scale was calibrated weekly with perfluorotributylamine. ThermoFisher Xcalibur Software (Ver. 2.0.1) was used for the instrument control, data acquisition and data analysis.

After the retention times were determined in full scan mode (range *m*/*z* 45–650), a timed-selected reaction monitoring (SRM) method was developed to analyse the 57 target compounds plus four surrogates and three internal standards in one single run. A calibration curve was constructed from 0.05 to 100 ng mL^{-1} with all the compounds, with the exception of the surrogates and internal standards, contained in each calibration standard mixture. Argon (99.99 %) was used as the collision gas, and the collision cell pressure was set to 0.2 Pa. The QqQ mass spectrometer was operated under the following conditions: ionisation with electron impact at 70 eV in MRM with an emission current of 50 μA. The ionisation source temperature was set to 220 °C. A filament multiplier delay of 5 min was established to prevent instrument damage. The electron multiplier voltage was set to 1,500 V. The scan width was 0.15, and the scan time was 0.05 s. Peak widths of *m*/*z* 0.7 Da were set for both the first (Q1) and third quadrupole (Q3).

Validation

Eighteen samples of human colostrum and 23 samples of mature breast milk were collected as a sub-sample set from a cross-sectional study based on 103 women aged 18–40 years who gave birth at the CHUIMI in 2010. The samples selected were from primiparous mothers ranging from 17 to 37 years in age. The samples were stored at −20 °C until analysis. Standard reference material SRM 1953 was used for the validation experiments. The certified values of SRM 1953 were firstly assessed in our laboratory using a previously described method [37], and later with this QuEChERS-based method. The results with our method revealed a relative standard deviations (RSD) <17 %, which was considered acceptable (Electronic Supplementary Material, Table S1). We also used pooled colostrum samples, selected among those with the lowest contamination levels (e.g. <0.1 μg L^{-1}), in the validation experiments. To a 5-mL volume of the SRM 1953 or pooled colostrum, 100 μL of a 1 μg mL^{-1} or 50 ng mL^{-1} working standard solution in acetone was added to obtain concentrations of 20 and 1 μg L^{-1}, respectively. The samples were thoroughly mixed and allowed to stand at room temperature for 4 h to ensure that the analytes were homogenously distributed throughout the sample. The recoveries were determined in quintuplicate by comparing the obtained concentrations for the spiked SRM 1953 and the colostrum samples with the same concentrations of contaminants prepared in the solvent. The same experiments were also used to determine the intra-day and inter-day precision (five successive days).

To evaluate the possibility of a matrix effect, we also compared the spiked-extracted samples with the SRM 1953 samples spiked at the same concentration following the QuEChERS extraction.

The limit of quantification (LOQ) of the method was designated as the analyte concentration that produced a peak signal of ten times the background noise from the chromatogram. The quantification was based on peak area. Ten-point calibration curves were constructed using a least-squares linear regression from the injection of samples spiked with solutions to provide final concentrations ranging from 0.05 to 100 μg L^{-1}.

Quality control

In each batch of samples, two controls were included for every 12 samples, comprising a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of melted butter spiked at 20 ng g^{-1} with each of the analytes processed by the same

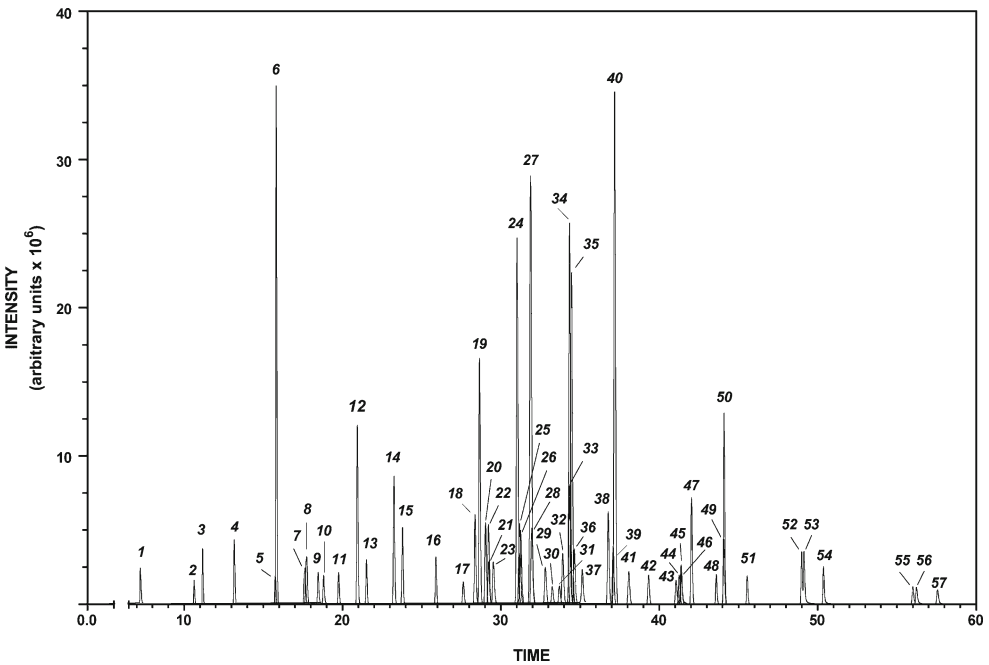

Fig. 1 Total ion chromatograms of the 57 MS/MS transition filters of a single mixture of contaminants prepared in cyclohexane (100 μg L^{-1}). *1* Naphthalene, *2* acenaphtylene, *3* acenaphtene, *4* fluorene, *5* α-HCH, *6* HCB, *7* β-HCH, *8* γ-HCH, *9* anthracene, *10* phenanthrene, *11* δ-HCH, *12* PCB 28, *13* heptachlor, *14* PCB 52, *15* aldrin, *16* dicofol, *17* fluoranthene, *18* chlordane (*trans*), *19* *o,p′*-DDE; *20* PCB 101, *21* α-endosulfan, *22* chlordane (*cis*), *23* pyrene, *24* *p,p′*-DDE, *25* PCB 81, *26* dieldrin, *27* *o,p′*-DDD, *28* PCB 77, *29* endrin, *30* PCB 123, *31* PCB 118, *32* β-endosulfan, *33* PCB 114, *34* *o,p′*-DDT, *35* *p,p′*-DDD, *36* PCB 153, *37* PCB 105, *38* endosulfan sulphate, *39* PCB 138, *40* *p,p′*-DDT, *41* PCB 126, *42* PCB 167, *43* benzo[*a*]anthracene, *44* PCB 156, *45* chrysene, *46* PCB 157, *47* PCB 180, *48* PCB 169, *49* metoxychlor, *50* mirex, *51* PCB 189, *52* benzo[*b*]fluoranthene, *53* benzo[*k*]fluoranthene, *54* benzo[*a*]pyrene, *55* indeno[*1,2,3-cd*]pyrene, *56* dibenzo[*a,h*]anthracene, *57* benzo[*ghi*]perylene

Table 1 Conditions of the optimised GC-MS/MS method

Name	t_R/min	precursor ion (m/z)	Product ions, m/z (collision energy/eV)	IPs
Polycyclic aromatic hydrocarbons (PAHs)				
Naphtalene	7.24	128	103 (15), 78 (15)	4
Acenaphtylene	10.63	152	151 (10), 126 (10)	4
Acenaphthene	11.16	154	153 (10), 152 (10)	4
Fluorene	13.18	166	165 (15), 163 (15)	4
Anthracene	18.46	178	176 (30), 152 (30)	4
Phenanthrene	18.81	178	176 (30), 152 (30)	4
Fluoranthene	27.62	202	201 (10), 200 (10)	4
Pyrene	29.52	202	201 (10), 200 (10)	4
Benzo[a]anthracene	41.05	228	226 (20), 202 (20)	4
Chrysene	41.37	228	226 (20), 202 (20)	4
Benzo[b]fluoranthene	48.66	252	250 (30), 226 (30)	4
Benzo[k]fluoranthene	48.82	252	250 (30), 226 (30)	4
Benzo[a]pyrene	50.36	252	250 (30), 226 (30)	4
Indeno[1,2,3-cd]pyrene	55.99	276	274 (35), 250 (35)	4
Dibenzo[a,h]anthracene	56.23	278	276 (35), 226 (35)	4
Benzo[ghi]perylene	57.56	276	274 (35), 250 (35)	4
Organochlorine pesticides (OCs)				
Hexachlorocyclohexane (alpha)	15.75	216	181 (15), 183 (15)	4
Hexachlorobenzene	15.81	284	214 (20), 249 (20)	4
Hexachlorocyclohexane (gamma)	17.50	217, 219	181 (15), 183 (15)	5
Hexaclorocyclohexane (beta)	17.65	217	181 (15), 183 (15)	4
Hexachlorocyclohexane (delta)	19.75	217	181 (15), 183 (15)	4
Heptachlor	21.51	258, 339	186 (22), 304 (15)	5
Aldrin	23.79	263	193 (32), 228 (26)	4
Dicofol	25.85	139, 251	111 (15), 139 (15)	5
Chlordane (*trans*)	28.36	373, 375	266 (15), 268 (17)	5
o,*p*′-DDE	28.65	318	246 (20), 248 (20)	4
Endosulfan (alpha)	29.23	196	159 (17), 161 (15)	4
Chlordane (*cis*)	29.72	373, 410	266 (18), 375 (5)	5
p,*p*′-DDE	31.02	318	246 (20), 248 (20)	4
Dieldrin	31.24	277	207 (20), 241 (10)	4
o,*p*′-DDD	31.72	235	165 (20), 199 (18)	4
Endrin	32.80	263	191 (25), 193 (27)	4
Endosulfan (beta)	33.91	196	159 (16), 160 (15)	4
o,*p*′-DDT	34.40	235	165 (20), 199 (18)	4
p,*p*′-DDD	34.57	235	165 (15), 199 (15)	4
Endosulfan sulphate	35.49	274	237 (10), 239 (15)	4
p,*p*′-DDT	37.15	235	165 (20), 199 (15)	4
Metoxychlor	44.08	274	239 (20), 259 (20)	4
Mirex	44.23	270, 272	235 (15), 237 (15)	5
Polychlorinated biphenyls (PCBs)				
PCB 28	20.93	256, 258	186 (22), 186 (42)	5
PCB 52	23.09	290, 292	220 (22), 220(20)	5
PCB 101	29.03	324, 326	254 (20), 256 (25)	5
PCB 81	31.16	290, 292	220 (22), 220 (20)	5
PCB 77	31.95	290, 292	220 (22), 220 (20)	5
PCB 123	33.33	324, 326	254 (20), 256 (25)	5

Table 1 (continued)

Name	t_R/min	precursor ion (m/z)	Product ions, m/z (collision energy/eV)	IPs
PCB 118	33.64	324, 326	254 (20), 256 (25)	5
PCB 114	34.33	324, 326	254 (20), 256 (25)	5
PCB 153	34.55	358, 360	288 (30), 290 (22)	5
PCB 105	35.12	324, 326	254 (20), 256 (25)	5
PCB 138	37.08	358, 360	288 (30), 290 (22)	5
PCB 126	38.07	324, 326	254 (20), 256 (25)	5
PCB 167	39.32	358, 360	288 (30), 290 (22)	5
PCB 156	41.19	358, 360	288 (30), 290 (22)	5
PCB 157	41.45	358, 360	288 (30), 290 (22)	5
PCB 180	42.03	392, 394	322 (30), 324 (20)	5
PCB 169	43.60	358, 360	288 (30), 290 (22)	5
PCB 189	45.54	392, 394	322 (30), 324 (20)	5

method used for the samples. The batch analyses were considered valid when the values of the analytes in the QC were within a 10 % of the deviation of the theoretical value.

Results and discussion

Optimisation of the instrumental method

Gas chromatography coupled with a triple quadrupole mass spectrometer was demonstrated to provide low detection limits and successfully enabled the identification and confirmation of the peak identities. Combining the SRM transitions with their retention times allowed us to positively confirm the contaminant identity. Occasionally, a chromatographic separation is not critical to the development of a multi-residue method with QqQ analysers because the high QqQ acquisition speed permits the monitoring of co-eluted compounds with a high number of transitions simultaneously in the SRM mode [38]. However, to achieve a good separation, various alterations to the temperature program were assayed. The selected GC operating conditions and optimised oven temperature programs are explained fully in the "Materials and methods" section. A chromatogram of the mixture of 57 contaminants with good analyte separation was successfully obtained under these optimised conditions in 61 min (Fig. 1).

To optimise the triple quadrupole MS/MS conditions, relevant considerations included the choices of precursor ions and product ions as well as the optimisation of the collision energies to achieve the best response from each target compound. After obtaining full scan spectra, the precursor ion for every analyte was selected and subjected to collision energy voltages (the potential on the second quadrupole) to generate MS/MS product ions; in this work, collision energies from 5 to 35 eV were evaluated (Table 1). The final aim was to develop a timed SRM method with two reactions or transitions per compound. Moreover, the peak shapes were highly related to the scan time, dwell time, scan rate and the number of monitored transitions [39, 40]. To obtain low detection limits and well-shaped chromatographic peaks, the dwell time was adjusted to allow 10 cycles per second throughout the chromatographic run, providing a sufficient number of chromatographic points for all the compounds. The final MS/MS conditions used in this study are detailed in Table 1.

According to the European Commission Decision 2002/657/EC [41], which introduced the concept of identification points (IPs) for the confirmation stage, the confirmation of the analytes included in this work involved monitoring two product ions from the same precursor ion, which resulted in four IPs, or two product ions derived from two different precursor ions, which resulted in five IPs. Therefore, the timed-SRM method used in this work met the requirements of the aforementioned regulation. The resulting number of IPs for each analyte is outlined in the Table 1.

Optimisation of the sample extraction and clean-up

In the present study, the QuEChERS [42] principle was adopted for the extraction of contaminants from human milk and colostrum. The classic QuEChERS method, initially developed for vegetables and fruits, involves an extraction with acetonitrile due to the ability of this solvent to separate from water upon the addition of an appropriate mixture of salts. Nevertheless, the QuEChERS approach allows considerable flexibility and can be modified to accommodate the specific analyte properties, matrix composition, equipment and analytical techniques available in the laboratory [43]. After a careful review of the bibliography in this study, we chose to use acetonitrile and citrate salts because several authors have demonstrated that this combination is the best choice for fatty

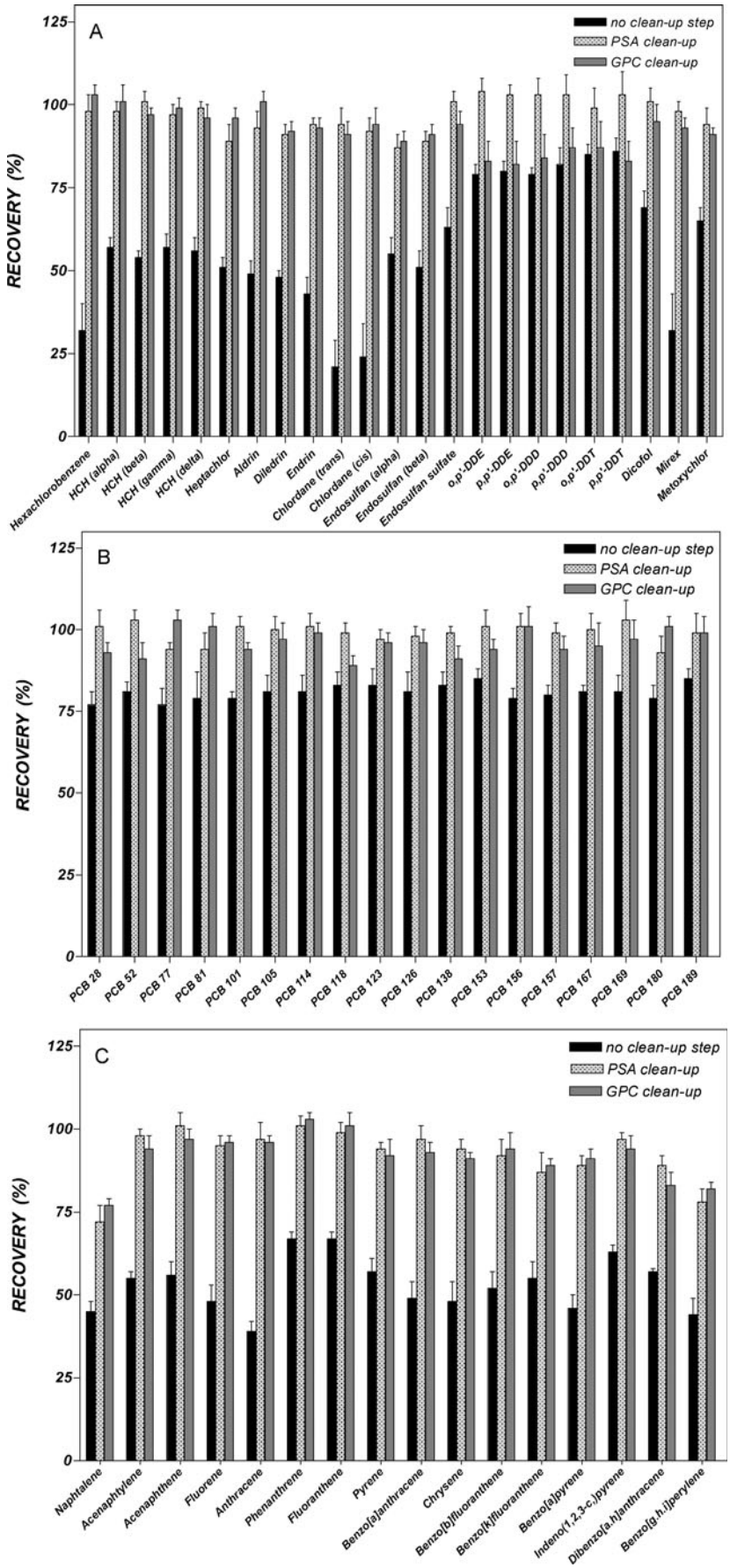

Fig. 2 Effect of the clean-up step by PSA or GPC on the extraction efficiency of the compounds from human colostrum spiked with 57 contaminants (20 μg L^{-1}). **A**. Organochlorine pesticides; **B**. Polychlorinated byphenils; **C**. Polycylic aromatic hydrocarbons

Table 2 Validation parameters ($n=5$) obtained for the 57 contaminants at two concentration levels in colostrum

Compound	Average recovery, % (RSD[a,b])		LOQ (μg L^{-1})	RSD for retention time, %	R^2
	1 μg L^{-1}	20 μg L^{-1}			
Organochlorine pesticides (OCs)					
Hexachlorobenzene	98 (6, 7)	98 (8, 10)	0.1	0.009	0.9980
α-HCH	98 (10, 13)	97 (6, 9)	0.5	0.004	0.9962
β-HCH	101 (9, 11)	99 (7, 11)	0.2	0.011	0.9977
γ-HCH	97(12, 15)	96 (11, 14)	0.2	0.009	0.9963
δ-HCH	99 (11, 9)	97 (8, 12)	0.3	0.018	0.9977
Heptachlor	89 (5, 8)	93 (8, 10)	0.4	0.021	0.9837
Aldrin	93 (14, 11)	99 (8, 7)	0.4	0.019	0.9985
Dieldrin	91 (13, 13)	98 (9, 11)	0.05	0.024	0.9935
Endrin	94 (12, 9)	94 (4, 7)	0.5	0.022	0.9972
Chlordane (*trans*)	94 (7, 11)	101 (9, 13)	0.4	0.033	0.9954
Chlordane (*cis*)	92 (8, 5)	88 (4, 8)	0.4	0.035	0.9995
α-Endosulfan	87 (16, 12)	96 (8, 11)	0.2	0.021	0.9826
β-Endosulfan	89 (11, 10)	87 (11, 9)	0.2	0.018	0.9813
Endosulfan sulphate	76 (13, 16)	82 (9, 14)	0.3	0.011	0.9971
o,p′-DDE	104 (11, 8)	95 (12, 7)	0.05	0.009	0.9995
p,p′-DDE	103 (6, 12)	93 (14, 11)	0.05	0.011	0.9981
o,p′-DDD	103 (5, 8)	99 (5, 9)	0.1	0.020	0.9985
p,p′-DDD	105 (9, 5)	101 (3, 6)	0.1	0.014	0.9987
o,p′-DDT	99 (11, 13)	94 (6, 11)	0.1	0.014	0.9996
p,p′-DDT	102 (9, 9)	97 (7, 11)	0.1	0.009	0.9997
Dicofol	95 (12, 9)	89 (11, 8)	0.1	0.023	0.9960
Mirex	101 (5, 7)	92 (11, 14)	0.1	0.021	0.9985
Metoxychlor	94 (12, 8)	97 (7, 6)	0.4	0.008	0.9915
Polychlorinated biphenyls (PCBs)					
PCB 28	101 (10, 8)	93 (4, 11)	0.2	0.009	0.9985
PCB 52	103 (12, 8)	91 (14, 10)	0.2	0.013	0.9824
PCB 77	94 (5, 9)	103 (4, 8)	0.2	0.017	0.9959
PCB 81	94 (11, 12)	101 (5, 7)	0.2	0.016	0.9816
PCB 101	101 (4, 7)	94 (9, 13)	0.1	0.017	0.9904
PCB 105	100 (9, 6)	97 (6, 9)	0.1	0.009	0.9934
PCB 114	101 (6, 9)	99 (9, 6)	0.1	0.013	0.9813
PCB 118	99 (11, 8)	89 (12, 9)	0.1	0014	0.9890
PCB 123	97 (8, 12)	96 (6, 9)	0.1	0.018	0.9992
PCB 126	98 (9, 8)	91 (11, 11)	0.1	0.019	0.9899
PCB 138	99 (5, 8)	94 (7, 10)	0.1	0.021	0.9972
PCB 153	101 (5, 9)	101 (3, 7)	0.05	0.009	0.9948
PCB 156	101 (4, 8)	94 (8, 9)	0.05	0.021	0.9982
PCB 157	99 (7, 11)	101 (5, 9)	0.05	0.023	0.9929
PCB 167	100 (4, 6)	99 (6, 4)	0.05	0.025	0.9994
PCB 169	103 (5, 9)	97 (9, 4)	0.05	0.027	0.9856
PCB 180	93 (10, 7)	99 (6, 8)	0.05	0.025	0.9908
PCB 189	99 (4, 8)	93 (8, 11)	0.05	0.028	0.9873
Polycyclic aromatic hydrocarbons (PAHs)					
Naphtalene	77 (8, 10)	77 (6, 9)	0.2	0.019	0.9878
Acenaphtylene	98 (11, 11)	94 (8, 12)	0.1	0.008	0.9877
Acenaphthene	101 (7, 9)	97 (6, 8)	0.1	0.015	0.9887

Table 2 (continued)

Compound	Average recovery, % (RSD[a,b])		LOQ (μg L^{-1})	RSD for retention time, %	R^2
	1 μg L^{-1}	20 μg L^{-1}			
Fluorene	95 (11, 9)	96 (10, 11)	0.1	0.009	0.9964
Anthracene	97 (14, 12)	96 (9, 11)	0.09	0.021	0.9987
Phenanthrene	101 (10, 14)	97 (7, 7)	0.05	0.017	0.9945
Fluoranthene	113 (9, 12)	99 (8, 11)	0.09	0.008	0.9976
Pyrene	103 (14, 11)	94 (18, 12)	0.07	0.011	0.9995
Benzo[a]anthracene	98 (6, 8)	93 (12, 8)	0.09	0.013	0.9883
Chrysene	100 (8, 9)	94 (12, 14)	0.07	0.017	0.9978
Benzo[b]fluoranthene	94 (12, 10)	92 (11, 13)	0.1	0.019	0.9889
Benzo[k]fluoranthene	96 (8, 11)	87 (12, 8)	0.1	0.026	0.9887
Benzo[a]pyrene	95 (6, 8)	89 (7, 11)	0.1	0.023	0.9856
Indeno[1,2,3-cd]pyrene	99 (11, 14)	87 (9, 13)	0.2	0.019	0.9819
Dibenzo[a,h]anthracene	101 (9, 12)	89 (11, 13)	0.2	0.019	0.9881
Benzo[ghi]perylene	98 (7, 9)	78 (9, 12)	0.2	0.023	0.9883
Surrogates					
PCB 12	97 (4, 6)	94 (6, 11)	0.2	0.005	0.9948
PCB 202	95 (8, 7)	96 (4, 9)	0.05	0.022	0.9932
p,*p*′-DDE-d8	101 (5, 9)	99 (6, 11)	0.05	0.018	0.9921
Acenaphtylene-d8	96 (6, 10)	94 (7, 5)	0.1	0.011	0.9904

[a] Intra-day

[b] Inter-day

matrices, minimising the co-extraction of lipids due to the low solubility of the lipids in this solvent [30, 44, 45].

Nevertheless, as human milk and colostrum in particular are matrices with a relatively high fat content, a clean-up step was presumed necessary to eliminate lipids that could reduce signal or cause column damage. In the QuEChERS methodology, the separation of the acetonitrile and aqueous phases upon the addition of sodium chloride, anhydrous $MgSO_4$ and citrate salts is usually followed by a dispersive solid-phase extraction clean-up step using a small quantity of SPE sorbents (PSA, GCB and/or C18). Because apolar compounds can be adsorbed on solid phases such as C18 and GCB, we decided to use PSA in this work and compare it with the traditional approach for lipid removal by gel permeation chromatography (GPC). Thus, we compared the chromatographic signals of the extracts of 57-contaminant-spiked (20 ng mL^{-1}) human milk and colostrum samples obtained either without an additional clean-up step or with a clean-up using 500 mg PSA or GPC. For each spiked concentration, the results were expressed as the percentage of the signal of the contaminant prepared in solvent. As displayed in Fig. 2, the chromatographic results revealed that a clean-up step was necessary for the human colostrum samples, which displayed weaker signals when a clean-up step was not performed. Figure 2 also demonstrates that the clean-up using 500 mg d-SPE produced very similar results (or even cleaner extracts, as in the case of DDTs, see Fig. 2) to those obtained by the GPC method. We performed a statistical comparison of the mean result values for all the analytes in each of the three experiments (no clean-up, PSA clean-up and GPC clean-up). For the PAHs and PCBs, there were statistically significant differences between "no clean-up" and "clean-up" as well as between the two types of clean-up. In all instances, the p value was below 0.001. For the OCPs, we also found statistically significant differences ($p < 0.001$) between performing and not performing the clean-up step, but not between the two types of clean-up evaluated. Similar results were obtained for the human milk samples (data not shown). Because d-SPE is a much less solvent- and time-consuming method, we concluded that using d-SPE with PSA was the better clean-up procedure for removing co-extracted material from human milk and colostrum samples.

Analytical performance

After optimising the clean-up procedure and analysis program, the confirmation criteria, precision, linearity, LOQs and repeatability were studied in order to evaluate the usefulness of the method for the quantitative determination of contaminants in milk and colostrum.

Compounds were identified as target analytes only when their chromatographic peaks satisfied all of the following criteria: (1) the retention time (tR) of the candidate was within three standard deviations (SD) of the average tR (tR±3SD) obtained when six blank samples spiked at the second level of calibration were injected; (2) the ion ratios matched those of the standard with a tolerance of ±30 % in absolute ion abundances, and (3) the S/N ratio of the target analytes was >10 for the sample extract.

To assess the extraction efficiency of the proposed method, recovery studies were conducted by spiking human milk and colostrum with the mixture of 57 analytes included in this work. As previously described, we used SRM 1953 and a pool of previously screened colostrum samples that exhibited the lowest numbers and concentrations of contaminants. The recoveries were determined from five replicates at two spiking concentrations (1.0 and 20.0 μg L^{-1}). The recovery values and the relative standard deviations were calculated for each concentration by comparing the areas of the analytes in the extracted, spiked samples with those of the same concentrations in a solvent (Table 2). The results ranged from 72.0 to 113.0 %, with the majority of the recoveries greater than 90 % at both concentrations. The precision was satisfactory, with the majority of unfavourable RSDs below 16 %. We also evaluated the recoveries and precision over five consecutive days (inter-day measurement), and the RSD was again observed to be below 16 %. Table 2 demonstrates that all the results were within the acceptable range and indicates the adequate precision of the method, with RSD values of 4–16 % for all the analytes.

To determine the presence of a matrix effect, the same comparison was performed using SRM 1953 or a colostrum "blank" spiked after the QuEChERS extraction; no significant differences were observed. Thus, we concluded that there were no significant matrix effects, and the remainder of the studies and calculations were therefore performed against the calibration curves of standards prepared in solvent.

The quantifications of the measurements were performed over ten different concentrations in solvent, ranging from 0.05 to 100 μg L^{-1} (three replicates for each concentration were analysed). Calculations were performed using the peak areas. The calibration curves were constructed without including the origin point. The calibration graphs obtained by plotting the concentration versus the average peak area are summarised in Table 2. The calibration curves were found to exhibit good linearity, with correlation coefficients (r^2) of more than 0.9813 for all the analyses (Table 2). The residual analyses

Table 3 Levels of organochlorine pesticides levels detected in samples of human colostrum (n=18) and mature milk (n=23)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
Hexachlorobenzene	0.75	100	0.22–0.54	0.76	100	0.18–1.76
α-HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-HCH	2.24	72.22	1.05–3.18	0.81	86.96	0.20–2.87
γ-HCH	2.49	77.78	0.67–5.25	0.76	60.87	0.57–2.49
δ-HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aldrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dieldrin	2.58	50.00	1.75–3.58	1.21	73.91	0.74–3.78
Endrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlordane (*trans*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlordane (*cis*)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α-Endosulfan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-Endosulfan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endosulfan sulphate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
o,p′-DDE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDE	8.84	100	2.90–110.34	9.14	100	2.64–72.78
o,p′-DDD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDD	0.55	22.22	0.12–0.88	0.28	43.48	0.1–0.47
o,p′-DDT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p′-DDT	0.19	11.11	0.11–0.23	0.12	13.04	0.12–0.14
Dicofol	0.35	27.78	0.12–0.59	n.d.	n.d.	n.d.
Mirex	0.13	61.11	0.1–0.29	0.19	43.48	0.1–0.44
Metoxychlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 4 Levels of polychlorinated biphenyls detected in samples of human colostrum ($n=18$) and mature milk ($n=23$)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
PCB 28	0.49	61.11	0.22–0.60	0.23	43.48	0.13–0.58
PCB 52	0.15	44.44	0.10–0.20	0.12	21.74	0.10–0.32
PCB 77	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 81	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 101	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 105	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 114	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 118	0.16	61.11	0.10–0.32	0.14	91.30	0.10–0.31
PCB 123	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 126	0.15	66.67	0.10–0.42	0.13	52.17	0.10–0.21
PCB 138	1.19	100	0.39–6.89	0.62	100	0.15–3.34
PCB 153	0.98	100	0.39–5.68	0.53	82.61	0.18–2.77
PCB 156	0.21	83.33	0.11–0.40	0.13	82.61	0.10–0.22
PCB 157	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 167	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 169	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 180	1.31	100	0.45–7.35	0.56	100	0.10–3.27
PCB 189	0.11	72.22	0.10–0.37	0.00	30.43	0.10–0.16

revealed values within the −9.588 to 7.765 range, indicating that the linear regression method can be used to accurately calculate the concentrations of the analytes included in this study within the concentration range investigated.

Application to real samples

The validated method was applied to the routine contaminant analysis of real samples. Eighteen human colostrum and 23

Table 5 Levels of polycyclic aromatic hydrocarbons detected in samples of human colostrum ($n=18$) and mature milk ($n=23$)

Compound	Colostrum			Mature milk		
	Median (μg/L)	Frequency (%)	Detection range (μg/L)	Median (μg/L)	Frequency (%)	Detection range (μg/L)
Naphtalene	2.33	77.78	1.27–9.82	1.04	82.61	0.45–2.96
Acenaphtylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acenaphthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluorene	1.12	83.33	0.60–2.76	1.07	100	0.24–1.22
Anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenanthrene	11.38	100	7.15–13.51	5.33	100	4.21–14.44
Fluoranthene	0.79	100	0.49–0.89	0.41	100	0.34–0.80
Pyrene	2.15	100	1.22–2.66	1.06	100	0.81–3.71
Benzo[a]anthracene	0.61	11.11	0.36–0.86	0.30	13.04	0.27–0.32
Chrysene	0.34	61.11	0.1–0.62	0.17	30.43	0.13–0.21
Benzo[b]fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[k]fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[a]pyrene	0.19	16.67	0.16–0.19	n.d.	n.d.	n.d.
Indeno[1,2,3-cd]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[a,h]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[ghi]perylene	0.11	5.56	0.11–0.11	n.d.	n.d.	n.d.

mature milk samples were analysed using the developed method. Each batch of samples was processed in duplicate together with an SRM 1953 aliquot that was subjected to the entire procedure. Each field sample and QC sample was spiked with the surrogates at 50 μg L^{-1} and internal standards at 10 μg L^{-1}.

As observed in Table 3, of the 23 OCPs analysed, we identified nine residues in both colostrum and mature milk samples. The remaining OCPs were either absent or present at much lower levels that were below the detection limits of the method. Hexachlorobenzene and *p,p′*-DDE were present in 100 % of the analysed samples, with median concentrations of 0.75 and 8.84 μg L^{-1} in colostrum and 0.76 and 9.14 μg L^{-1} in milk, respectively. The contaminant present at the highest concentrations was *p,p′*-DDE (ranging from 2.64 to 110.34 μg L^{-1}), followed by dieldrin, β-HCH, γ-HCH and hexachlorobenzene. As displayed in Table 3, the pattern of contamination was similar for both types of samples, although the colostrum samples exhibited higher contaminant levels overall than the mature milk samples. This result is likely due to the higher level of fat in colostrum as well as to a progressive "purging" of contaminants from the mothers over the course of the lactation period.

Detailed data for the PCB residues detected in the colostrum and milk samples are displayed in Table 4. The results revealed that the most frequently detected congeners were the highly chlorinated marker-PCBs nos. 138 and 180, which were identified in 100 % of both types of samples, and no. 153, which was present in 100 % of the colostrum samples and in 82.61 % of the milk samples. In total, we identified nine of the 18 congeners analysed, with six of them being marker-PCBs (nos. 28, 52, 118, 138, 153 and 180); marker-PCB no. 101 was not detected in any of the analysed samples. Remarkably, the dioxin-like and highly toxic PCB no. 126 was found in 66.67 % of the colostrum samples and in 52.17 % of the milk samples. The concentrations of the PCBs were typically low (with the average concentrations of the individual congeners ranging from 0.11 to 1.94 μg L^{-1}), although some colostrum samples exhibited concentrations as high as 7.35 μg L^{-1} for some of the congeners (Table 4).

Finally, Table 5 displays the analytical figures for the PAH residue levels in the analysed samples. The results indicate the presence of naphthalene, fluorene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene and chrysene in both types of samples. In colostrum, we also identified benzo[a]pyrene and benzo[ghi]perylene in 16.67 and 5.56 % of the samples, respectively. A frequency of detection of 100 % was found for phenanthrene, fluoranthene and pyrene. Phenanthene was also the compound present at the highest concentrations (Table 5).

Conclusions

The applicability of a QuEChERS-based extraction procedure combined with triple quadrupole GC-MS/MS for the simultaneous detection and quantification of 23 OCPs, 18 PCBs and 16 PAHs was demonstrated. The results revealed satisfactory validation parameters. The studied contaminants were detected at very low concentrations, and highly linear calibration curves were developed within the investigated calibration range (0.05–100 μg L^{-1}, with r^2>0.98). The LOQ values of the instrument varied from 0.05 to 0.4 μg L^{-1}. The recovery rates were between 72 to 113 % with very good precision (RSD<16 %). The proposed method is recommended for routine monitoring applications due to its simplicity, sensitivity and utility. The applicability of the optimised method was demonstrated by monitoring contaminant residues in human milk samples (colostrum and mature milk) collected from women who gave birth at the CHUIMI in 2010. The results indicated a high presence of contaminant residues in this important newborn food supply, with a minimum of nine out of 57 contaminants detected. These results demonstrate the necessity of regularly monitoring persistent pollutants in human milk over extended time periods.

Acknowledgments The authors would like to thank the human milk and colostrum sample providers, Dr. Lluis Serra Majem and Dr. Adriana Ortiz Andrelluchi from the Nutrition Research Group of the University of Las Palmas de Gran Canaria and Mrs. María de los Reyes Suárez Hanna for her for her technical assistance.

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Journal of Analytical Toxicology 2014;38:155–163
doi:10.1093/jat/bku009 Advance Access publication February 10, 2014

Article

Methodology for the Identification of 117 Pesticides Commonly Involved in the Poisoning of Wildlife Using GC–MS-MS and LC–MS-MS

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Poisoning with agricultural chemicals is a major threat for wildlife all over the world. We have developed and validated an analytical scheme aimed to the identification and quantification of a wide range of pesticides in fresh liver from wildlife specimens that are routinely delivered to the forensic laboratories for toxicological investigation. The proposed method is comprised of a general solid–liquid extraction followed by purification steps and three complementary liquid or gas chromatographic analyses with triple quadrupole mass spectrometry detection. The developed methodology allows for the determination of 117 highly toxic pesticides in a variety of samples from wildlife poisoning incidents. The validity of the method has been demonstrated in samples from 98 real cases submitted to our laboratory between 2010 and 2012. This method allowed the identification and quantification of poison in 78 of 94 fresh liver samples from wild animals and was successfully used for the identification of pesticides in 35 of 46 non-liver samples. Therefore, the extraction and cleanup method with minor modifications and the potency of triple quadrupole mass spectrometry allow this method to be used to simultaneously detect and quantify or semi-quantify a majority of the most toxic pesticides in a variety of complex and degraded matrices.

Introduction

Accidental or malicious poisoning episodes in wildlife are very common all over the world and represent a major research challenge for toxicologists and forensic laboratories (1). Poisoned animals are usually found dead by hunters, hikers or wildlife protection agents. The investigation of an incident that involves the death of wildlife specimens generally consists of a field inquiry, a postmortem examination and, when necessary, chemical analysis to determine whether a poison might be responsible (2). In addition to biological samples from wildlife specimens, any bait or recipients found at the scene are also usually sent to the laboratory for chemical identification. The variety and complexity of biological samples in different states of decomposition and the wide range of chemicals that can be responsible for a lethal poisoning in wildlife are major sources of difficulty in these toxicological investigations. The development of powerful, sensitive multiresidue identification methodologies is necessary to identify unknown toxicants in this context.

Multistage mass spectrometry (MS-MS) is considered a very useful tool to detect low levels of analyte when coupled with chromatographic techniques. The use of triple quadrupole mass spectrometry (QqQ) analyzers operated in the selective reaction monitoring (SRM) mode significantly improves both the sensitivity and selectivity of the analytical determination, when compared with single-stage mass spectrometry (MS). This is mainly due to the elimination of isobaric interferences and a substantial reduction of background noise (3). Currently, the use of this technique in forensic toxicology laboratories represents a practical way to overcome the complexity that represents the identification of the target analytes in difficult matrices (4). Moreover, the high speed of the electronics of the QqQ analyzers permits the simultaneous acquisition of several transitions and thus the monitoring of co-eluted compounds. This allows the development of multiclass, multiresidue methods that can include dozens or even hundreds of compounds that can be analyzed simultaneously (5).

Among all the chemicals that threaten wildlife, pesticides are particularly important. It has been described that the great majority of all the pesticides used in the agriculture is dispersed into the environment never reaching their target organisms (6). In addition, the deliberate poisoning of prey species that compete with hunters and poachers usually involves bait material that has been laced with pesticides due to their high toxicity (2). It has been estimated that the illegal use of pesticides can be involved in as much as 68% of all suspected poisoning cases (7). Epidemiological studies have revealed that pesticides account for ~52.5% of bird poisonings, and that pesticides are also major agents in wild mammal deaths by poisoning (1). Not all pesticides have the same relevance in wildlife poisonings, with most primary and secondary cases being caused by anticholinesterase agents and anticoagulant rodenticides (7–10).

It is noteworthy that, in general, it is difficult to carry out thorough analytical investigations in the usually decayed samples that are sent to the laboratory, where information on the toxic substance involved is lacking in most cases. Many authors have published methods for the analysis of pesticides in wildlife samples, but most of them have been designed for the analysis of a few pesticides belonging to the same chemical group, making it necessary to use several of these methods in a complementary manner (2, 11–13). For this reason, sensitive and specific multiresidue techniques that cover a wide spectrum of highly toxic substances can substantially contribute to minimizing the costs and maximizing the chance of identifying the toxicant involved. Thus, we have developed a highly sensitive methodology for the extraction, detection and quantification of 117 pesticides that have been selected based on either their high toxicity for wildlife (7, 14), or their frequent use (14). As far as we know to the present, no work has been published that cover such a wide range of highly toxic pesticides in a single analytical scheme and that have been specifically oriented to wildlife forensic samples.

Materials and methods

Ethical statement

The chicken liver samples used in the validation experiments were purchased from a butcher and came from poultry that had been slaughtered in accordance with European legislation. The rest of the samples analyzed came from poisoned animals found dead in the countryside or that died during their stay in the Wildlife Recovery Centers of the Canary Islands, Spain. No animal was killed for the purposes of this study, and no experiments on living animals or with samples coming from them were performed.

Chemicals and reagents

All the solvents (>99.9%) were purchased from Fisher Scientific (Leicestershire, UK). The quality of solvents was: Optima™ LC/MS for acetonitrile and methanol, and Pesticide grade for the rest. Diatomaceous earth Celite® 503 and Bio-Beads SX3 were purchased from Sigma-Aldrich (St. Louis, USA) and BioRad Laboratories (Hercules, USA), respectively. About 0.20-μm polyester syringe filters were from Macherey-Nagel (Düren, Germany). Fifty- and 15-mL polypropylene conical centrifuge tubes were from VWR International (Radnor, PA, USA). Neat standards (97–99.5%) of the analytes included in this method were purchased from Dr. Ehrenstorfer Reference Materials (Augsburg, Germany). Surrogate standards were used to monitor the entire analytical process, especially the step of sample preparation (extraction and clean-up), but also the instrument performance. We used aldicarb-d_3, carbofuran-d_3, chlorfenvinphos-d_{10} and chlorpyriphos-d_{10} for this purpose. We also used a mixture of (±)-warfarin-d_5, thiobencarb, chloropropham, diazinon-d_{10} and heptachloro epoxide, *cis* as internal standards (ISs), since we used the IS method of quantification. Surrogates and ISs were also purchased from Dr. Ehrenstorfer Reference Materials. Diluted working solutions of each compound (0.5 to 500 ng/mL) either in acetonitrile or cyclohexane were used for the calibration curves. Two mixtures of all the standards were prepared in acetone at 10 μg/L or 500 ng/L for the fortification experiments.

Extraction and cleanup procedure

For the extraction, 5 mL of ultrapure water were added to the 2 g of the sample (animal tissue, meat, plant or insects), and homogenization was performed using a disperser at 10,000 rpm (Ultra-turrax T 25, IKA Laboratory Equipment, China). About 25 μL of the mixture of surrogates (40 μg/mL in acetone) were added to give a final concentration of 500 ng/g sample. Diatomaceous earth (10 g) was added to absorb the moisture in the sample, and 10 mL of dichloromethane/ethyl acetate/acetone (50/30/20) were added. The samples were placed in an orbital shaker (Cel-Gro Tissue Culture Rotator, Thermo Fisher Scientific, CA, USA) for 10 min and then sonicated for 5 min. The whole extraction procedure is summarized in Figure 1.

Owing to the usually high content of interfering substances in the extracts one or two additional cleanup steps were needed, depending on the matrix and its degradation status. The fastest

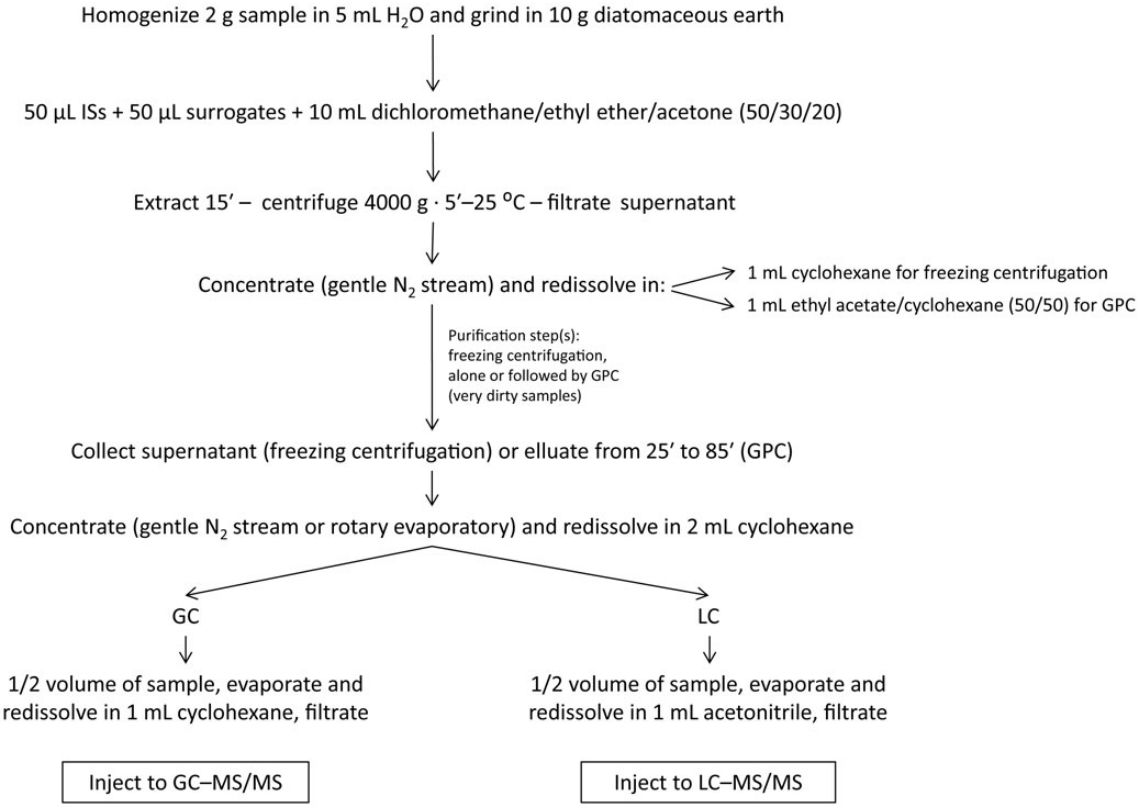

Figure 1. Scheme of the extraction and cleanup method.

and most economical purification method was freezing centrifugation in which the concentrated extract was redissolved in 2 mL of cyclohexane in an Eppendorf tube and placed in an −82°C freezer for 20 min. After this period, the sample was centrifuged (4,000 g, 5 min, −10°C), and the supernatant was carefully removed and separated from the frozen lipids in the bottom of the tube. This purification step was repeated three times, and the resulting supernatant was divided into two aliquots, which were evaporated under a gentle nitrogen stream. The one of them was redissolved in 1 mL of cyclohexane and used for GC–MS-MS and the other, redissolved in acetonitrile and used for LC–MS-MS. This method was applied as the unique cleanup step for extracts from fresh animal tissues, plants or blood. For highly degraded matrices or insect homogenates, which yielded very dirty extracts, freezing centrifugation was also used as a pre-cleaning step, but in these cases, the pre-cleaned extracts were evaporated to dryness, redissolved in 1 mL of ethyl acetate/cyclohexane (50/50) and subjected to an additional purification step by gel permeation chromatography (GPC). We used a 500 × 25 mm column (Omnifit, New York, USA) packed with 30 g Bio-beads S-X3 as the stationary phase. The eluent used was ethyl acetate/cyclohexane (50/50) at a continuous flow of 2 mL/min. Larger molecules (>600 Da) were discarded with the first 25 min of elution (50 mL). The following 120 mL were collected (from min 25 to 85) and concentrated in a rotary evaporator (Heidolph, Schwabach, Germany) to a volume of 10 mL. This concentrate was split into two 5-mL aliquots that were filtrated and evaporated under a gentle nitrogen stream. One of the aliquots was redissolved in cyclohexane and used for GC–MS-MS, and the other was redissolved in acetonitrile and used for LC–MS-MS. Previous to the chromatographic analysis, 10 μL of the mixture of ISs (50 μg/mL in acetone) were added to each vial to reach a final concentration of 500 ng/mL. The efficacy of this purification method for fatty samples has been demonstrated previously in our laboratory (15, 16).

GC-QqQ-MS-MS analysis

We used a Thermo Trace GC Ultra with split/splitless injector for the chromatographic analyses (Thermo Fisher Scientific, Inc., USA). We used as the stationary phase a column of 30 m × 0.25 mm, 0.25 μm film thickness (BPX5, SGE, Inc., USA). As the carrier gas we used helium (99.999%) that was set at constant flow (1.0 mL/min). The 61-min oven temperature program was: 60°C held for 1 min, ramped to 210°C at 12°C/min and then to 320°C at 8°C/min with a 6-min hold time. The injector temperature was set at 270°C, and the transfer line was heated to 310°C. All the injections (1 μL) were done in the splitless mode.

The 91 pesticides that were separated by GC were detected with a triple quadrupole TSQ XLS mass spectrometer (QqQ, Thermo Fisher Scientific, Inc., USA). We first determined their retention times (RTs) in the full scan mode (range: m/z 45–500), and then a timed-SRM method was constructed to analyze all the target compounds, surrogates and ISs in a single run. The calibration curve ranged from 0.5 to 500 ng/mL and included all the compounds in each calibration standard level. The surrogates and ISs were excluded from the calibration mix. As the collision gas in the collision cell we used argon (99.99%) that was set at 0.2 Pa. The operating conditions of the mass spectrometer were: electron impact ionization at 70 eV in SRM (emission current of 50 μA); ionization source temperature 220°C; electron multiplier voltage 1,500 V; scan width 0.15; scan time 0.05 s; peak widths m/z 0.7 Da (first and third quadrupole).

LC-QqQ-MS-MS analysis

We performed two chromatographic methods using a Thermo LC Accela Ultra instrument (Thermo Fisher Scientific, Inc., USA).

For LC–MS-MS method 1 (Table I), we used an analytic Accucore C18 column (2.6 μm, 150 × 3 mm; Thermo Fisher Scientific, Inc., USA) as the stationary phase. The mobile phases were (A) ultrapure water as the aqueous phase and (B) methanol (HPLC–MS grade) as the organic phase. The flow was set at 800 μL/min. The injection volume was 25 μL, and the total run time was 5 min. The gradient program was programmed as follows: 0–1 min: 50% A; 1–1.5 min: 50% A → 5% A; 1.5–3.5 min: 5% A; 3.5–3.7 min: 5% A → 50% A; 3.7–5 min: 50% A.

For LC–MS-MS method 2 (Table I), an analytic Synergi Hydro-RP column (4.0 μm, 150 × 4.6 mm; Phenomenex, Torrance, USA) was used as the stationary phase. The mobile phases were (A) 7.5 mM ammonium formate in ultrapure water as the aqueous phase, (B) methanol (HPLC–MS grade) as the organic phase and (C) 2% formic acid. The solvent flow was 1,000 μL/min. The injection volume was 25 μL, and the total run time was 26 min. During the entire run, solvent C was set at 2.5%. The infusion of the other two mobile phases was programmed as a gradient as follows: 0–12 min: 87.5% A → 7.5% A; 12–16 min: 7.5% A; 16.0–16.2 min: 7.5% A → 87.5% A; 16.2–25.0 min: 87.5% A.

The 26 most polar pesticides that were separated by LC were detected using a TSQ Quantum Max QqQ mass spectrometer equipped with the H-ESI II heated electrospray ionization source (Thermo Fisher Scientific, Inc., USA). For LC–MS-MS methods 1 and 2, the mass spectrometer and the ionization source were programmed according to the following parameters, respectively: skimmer offset (4 and 10 V), sheat gas pressure (10 and 15 arbitrary units, a.u.), ion sweep gas pressure (8 and 0 a.u.), capillary temperature (250°C in both cases), spray voltage (3,500 and 3,000 V) and vaporization temperature (200 and 180°C). The spectrometer was programmed in the negative ionization mode for method 1 and the positive mode for method 2.

We first determined their RTs in the full scan mode (range: m/z 45–500), and then we constructed a timed-SRM method by directly infusing pure standard methanolic solutions into the source to analyze 26 target compounds as well as surrogates and ISs in two separate runs. The calibration curve included 10 levels that ranged from 0.5 to 500 ng/mL for all compounds, excluding the surrogates and ISs. The gas in the collision cell was argon (99.99%) at a pressure of 0.25 Pa.

Validation

Chicken liver samples that were purchased from a butcher were used for the validation experiments. We added 40 μL of a 10-μg/mL or 500-ng/mL working standard solution in acetone (containing all the pesticides included in the method) to 2 g of liver tissue homogenized in 5 mL of ultrapure water to obtain concentrations of 200 and 10 ng/g, respectively. We determined the recoveries from spiked liver tissue in quintuplicate experiments by comparing the obtained concentrations with the same concentrations of the pesticides prepared in the dissolvent. In the same experiments, we also determined the intra- and inter-day precision

Table I

Toxicities of the pesticides detected by LC–MS-MS, method settings and results from recovery experiments

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings							Validation parameters			
		Birds	Mammals	RT (min)	CV (V)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)	IPs	LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
LC–MS-MS method 1														
1	Coumatetralyl	38.3	42.5	1.57	65	291.1 → 140.9	28	291.1 → 247.0	22	4	0.01	0.03	89.2 (13.6)	1
2	Warfarin	942.0	6.5	1.71	56	307.1 → 116.9	39	307.1 → 250.0	24	4	0.005	0.02	92.7 (8.3)	1
3	Chlorophacinone	430.0	7.5	1.76	123	373.1 → 116.0	50	373.1 → 200.9	25	4	0.01	0.03	87.9 (12.4)	1
4	Difenacoum	50.0	50.0	1.83	90	443.2 → 134.9	36	443.2 → 293.0	33	4	0.005	0.01	91.3 (11.7)	1
5	Brodifacoum	4.5	2.5	1.88	108	521.1 → 135.0	44	521.1 → 186.9	39	4	0.005	0.01	97.4 (5.8)	1
6	Bromadiolone	138	16.5	2.02	96	525.1 → 180.9	37	525.1 → 249.9	37	4	0.005	0.01	94.3 (8.9)	1
7	Difethialone	0.9	4.0	2.08	100	537.1 → 150.9	45	537.1 → 370.9	36	4	0.01	0.03	86.9 (13.4)	1
LC–MS-MS method 2														
8	Metamidofos	14.3	18.5	2.26	148	142.1 → 94.0	14	142.1 → 125.0	16	4	0.005	0.02	62.8 (14.6)	2
9	Oxamyl	4.2	30.0	2.78	85	237.2 → 163.0	14	237.2 → 196.0	18	4	0.01	0.05	63.2 (11.6)	2
10	Phoxim	5.6	250	4.03	117	300.1 → 129.3	18	300.1 → 283.0	10	4	0.005	0.02	61.9 (15.5)	2
11	Acephate	125.0	321.0	4.51	100	184.1 → 125.0	16	184.1 → 143.0	5	4	0.01	0.05	89.7 (12.4)	2
12	Omethoate	125.0	50.0	5.03	106	214.0 → 155.0	19	214.0 → 183.0	13	4	0.01	0.05	93.3 (13.7)	2
13	Metomil	20.5	24.9	6.71	98	163.1 → 88.1	11	163.1 → 106.0	12	4	0.005	0.02	74.6 (9.8)	2
14	Imidacloprid	152.0	98.0	8.05	99	256.1 → 175.0	18	256.1 → 209.0	16	4	0.005	0.02	97.3 (4.3)	2
15	Dimethoate	45.6	220.0	8.64	150	230.0 → 125.0	23	230.0 → 199.0	11	4	0.01	0.05	88.2 (9.7)	2
16	Aldicarb	3.8	1.9	9.98	115	208.0 → 89.2	19	208.0 → 116.2	10	4	0.005	0.02	67.2 (12.4)	2
17	Carbofuran	22.4	10.2	10.34	147	222.0 → 123.1	25	222.0 → 137.5	24	4	0.005	0.03	98.4 (3.1)	2
18	Propoxur	19.9	51.2	10.76	123	210.0 → 111.2	18	210.0 → 168.0	11	4	0.005	0.02	70.1 (10.0)	2
19	Carbaryl	56.0	150.0	11.20	145	202.1 → 127.0	33	202.1 → 145.1	13	4	0.005	0.02	64.1 (12.9)	2
20	Pirimicarb	45.5	100.0	11.33	92	239.1 → 72.3	27	239.1 → 182.1	16	4	0.01	0.05	84.3 (10.7)	2
21	Carboxin	42.2	430.0	11.49	92	236.1 → 93.2	33	236.1 → 143.0	15	4	0.01	0.05	65.2 (12.7)	2
22	Bromoxynil	50.0	78.0	12.01	95	275.9 → 79.2	29	275.9 → 81.1	33	4	0.01	0.05	85.7 (9.1)	2
23	Methiocarb	2.4	16.0	12.97	135	226.0 → 121.0	19	226.0 → 169.0	8	4	0.01	0.05	62.8 (14.3)	2
24	Cyproconazole	150.0	352.0	13.46	107	292.1 → 70.3	17	292.1 → 125.1	34	4	0.01	0.02	58.4 (11.1)	2
25	Benfuracarb	92.0	102.0	15.02	111	411.2 → 190.1	13	411.2 →252.3	15	4	0.01	0.05	66.2 (16.5)	2
26	Profenofos	1.9	116.0	15.52	122	373.0 →302.8	18	373.0 →344.8	13	4	0.01	0.03	69.9 (13.4)	2
Surrogates														
S1	Aldicarb-d_3			9.98	115	211.0 → 89.2	19	211.0 → 119.2	10	4	–	–	–	
S2	Carbofuran-d_3			10.34	147	225.0 → 123.1	25	225.0 → 140.5	25	4	–	–	–	
Internal standards														
IS1	(±)-Warfarin-d_5			1.71	56	312.1 → 116.9	39	312.1 → 250.0	24	4	–	–	–	
IS2	Thiobencarb			8.65	132	258.1 → 89.1	35	258.1 → 125.0	19	4	–	–	–	

RT: retention time; CV: cone voltage; CE: collision energy; IPs: identification points; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.

[a]Average data from different species. These data have been taken from Mineau *et al.* (14) and the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

(5 successive days). The matrix effect was evaluated by comparing spiked samples after the extraction of liver tissue with the spiked-extracted samples at the same concentrations.

Those concentrations of analytes, which produced a signal peak of 10-fold the background noise of the chromatogram, were set as the limit of quantification (LOQ) of the method. We quantified on the basis of the peak areas. Least squares linear regressions were constructed from the areas of each of the 10 calibration levels (0.5–500 μg/kg).

Quality control

In each batch of samples, two controls were included: a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of melted butter spiked at 20 μg/kg of each of the analytes processed using the same method as the samples. The batch analyses were considered valid when the values of the analytes in the QC were within a 10% of deviation of the theoretical value.

Results and discussion

Optimization of the instrumental method

In this study, 91 apolar pesticides suitable for gas chromatography (Table II) were investigated under the same optimized temperature program and analysis time to obtain the most efficient quantitative results with maximum separation. Twenty-six polar pesticides (Table I) were separated by liquid chromatography in two different chromatographic runs because the conditions that yielded the optimal ionization of the parent compounds for MS were different. The complete chromatographic separation of all the analytes is not always necessary when using QqQ analyzers, since the high-speed acquisition of these spectrometers (high number of simultaneous SRM transitions) allows the identification of co-eluted compounds (17). Nevertheless, to achieve a good separation, various changes in the temperature program were assayed in the gas chromatography method, and various gradient programs were assayed in the liquid chromatography methods. The chosen GC and LC operating conditions were described in the Material and Methods section.

For the optimization of the conditions of the triple quadrupole MS-MS, the precursor ion for each of the 91 GC analytes was first selected after analyzing the pesticides separately to obtain the full scan spectra. The product ions from the precursor ion were selected from another set of experiments at different collision energy (CE) voltages. The precursor ions of each of the 26 LC analytes (usually $M-H^+$ or $M-H^-$) were selected from the bibliography, and the CEs and MS-MS product ions were chosen

Table II

Toxicities of the pesticides detected by GC–MS-MS, method settings and results from recovery experiments

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings						Validation parameters			
		Birds	Mammals	RT (min)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)	IPs	LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
27	Dimefox	1.7	3.5	5.33	154.1 → 58.0	10	154.1 → 111.1	10	4	0.001	0.01	62.5 (13.3)	3
28	Dichlorphos	8.8	61.0	7.61	185.0 → 109.0	15	185.0 → 127.0	12	4	0.001	0.01	78.7 (9.5)	3
29	Metamidophos	8.0	18.0	9.32	141.0 → 80.0	10	141.0 → 95.0	10	4	0.001	0.01	93.2 (7.6)	3
30	Mevinphos	1.4	4.0	9.72	192.0 → 127.0	12	192.0 → 164.0	10	4	0.001	0.02	68.7 (11.4)	4
31	Chlormephos	65.0	12.5	9.94	154.0 → 121.0	5	154.0 → 121.0	14	4	0.001	0.02	82.3 (9.9)	4
32	Metolcarb	100.0	109.0	10.56	108.1 → 79.0	10	108.1 → 107.1	10	4	0.001	0.01	62.4 (15.7)	4
33	Heptenophos	17.0	117.0	12.26	250.0 → 124.0	10	250.0 → 215.0	4	4	0.001	0.05	76.5 (9.3)	4
34	Thionazin	2.4	5.0	12.96	192.0 → 96.0	10	248.0 → 140.0	10	5	0.001	0.01	79.2 (8.2)	4
35	TEPP	1.3	2.3	13.07	263.1 → 179.1	15	263.1 → 235.1	5	4	0.001	0.02	97.6 (7.5)	3
36	Propachlor	91.0	392.0	13.25	176.1 → 120.0	10	196.1 → 120.0	10	5	0.001	0.01	78.3 (11.2)	3
37	Etoprophos	4.2	34.0	13.70	158.0 → 114.0	10	158.0 → 130.0	10	4	0.001	0.01	63.3 (11.5)	4
38	Sulfotep	25.0	22.0	14.50	322.0 → 202.0	15	322.0 → 294.0	10	4	0.001	0.01	81.5 (10.7)	3
49	Dicrotophos	1.2	11.0	14.61	127.0 → 95.0	10	127.0 → 109.0	10	4	0.001	0.05	92.4 (9.3)	4
40	Bendiocarb	21.0	35.0	14.79	166.1 → 151.1	15	223.1 → 166.1	15	5	0.001	0.01	94.5 (4.5)	3
41	Cadusafos	16.0	71.4	14.93	159.1 → 97.0	20	159.1 → 131.0	10	4	0.001	0.01	96.7 (8.9)	4
42	Phorate	1.0	20.0	15.24	260.0 → 75.0	5	260.0 → 231.0	8	4	0.001	0.02	88.3 (11.8)	4
43	Diallate	167.0	395.0	15.30	236.0 → 152.0	20	236.0 → 194.0	15	4	0.001	0.01	99.3 (10.6)	4
44	Monocrotophos	0.8	15.0	15.80	127.0 → 95.0	20	127.0 → 109.3	25	4	0.001	0.01	88.5 (14.3)	3
45	Thiometon	100.0	37.0	15.85	88.0 → 60.0	15	248.0 → 88.0	15	5	0.001	0.05	91.2 (11.0)	4
46	Dazomet	424.0	415.0	16.56	89.0 → 75.0	20	162.0 → 89.0	8	5	0.001	0.01	87.6 (6.8)	4
47	Dioxathion	200.0	10.0	17.13	125.0 → 97.0	15	197.0 → 141.0	15	5	0.0005	0.005	97.6 (8.2)	4
48	Lindane	127.0	25.0	17.16	216.9 → 180.9	15	218.9 → 182.9	15	5	0.001	0.01	97.7 (13.2)	5
49	Propetamphos	49.0	130.0	17.35	236.1 → 166.1	15	236.1 → 194.1	5	5	0.001	0.01	94.7 (6.4)	4
50	Terbufos	15.0	3.5	17.37	231.0 → 175.0	15	231.0 → 203.0	10	4	0.001	0.01	99.1 (3.4)	4
51	Diazinon	2.0	76.0	17.57	179.1 → 127.0	15	179.1 → 137.1	15	4	0.001	0.01	92.1 (9.9)	5
52	Chlorfenvinphos	13.0	20.0	17.60	267.0 → 159.0	15	323.0 → 269.0	10	5	0.001	0.01	97.9 (11.6)	4
53	Cyanophos	3.0	215.0	17.60	243.0 → 109.0	12	243.0 → 127.0	15	4	0.001	0.01	89.7 (12.2)	4
54	Fonofos	10.0	3.0	17.69	137.0 → 109.0	10	246.0 → 137.0	10	5	0.001	0.01	93.3 (7.9)	4
55	Disulfoton	2.4	5.0	18.32	274.0 → 88.0	10	274.0 → 245.0	10	4	0.001	0.02	99.7 (5.8)	4
56	Tefluthrin	267.0	22.0	18.40	197.0 → 141.0	15	197.0 → 161.0	10	4	0.001	0.01	79.3 (12.1)	3
57	Isazophos	244.0	27.0	18.40	257.0 → 119.0	15	257.0 → 162.0	15	4	0.001	0.02	95.2 (7.7)	4
58	Dichlone	–	160.0	18.58	191.0 → 135.0	15	226.0 → 191.0	10	5	0.001	0.01	88.6 (12.0)	3
59	Formothion	630.0	175.0	19.94	224.0 → 125.0	15	224.0 → 196.0	10	4	0.001	0.01	97.2 (3.7)	4
60	Phosphamidon	1.8	6.0	20.08	264.0 → 127.0	15	264.0 → 127.0	15	4	0.001	0.01	98.6 (6.8)	4
61	Chlorpyriphos methyl	13.0	2000.0	20.57	285.9 → 93.0	25	285.9 → 272.9	13	4	0.001	0.01	93.2 (12.4)	4
62	Parathion methyl	5.0	57.0	21.10	263.0 → 109.0	15	263.0 → 127.0	15	4	0.0005	0.005	95.4 (12.1)	4
63	Heptachlor	125.0	50.0	21.36	338.8 → 267.9	15	338.8 → 303.8	15	4	0.001	0.01	85.6 (9.7)	5
64	Fenitrothion	11.0	142.0	22.74	277.0 → 109.0	20	277.0 → 260.0	15	4	0.001	0.01	96.7 (7.6)	4
65	Pirimiphos methyl	30.0	1150.0	23.19	290.1 → 125.0	15	290.1 → 233.1	10	4	0.001	0.01	96.1 (8.4)	4
66	Malathion	400.0	53.0	23.27	173.0 → 127.0	10	173.0 → 145.0	5	4	0.001	0.01	92.2 (6.3)	4
67	Chlorpyrifos	5.2	60.0	23.60	197.0 → 169.0	15	199.0 → 171.0	15	5	0.0005	0.003	95.3 (11.2)	4
68	Aldrin	7.2	65.0	23.60	262.9 → 192.9	32	262.9 → 227.9	26	4	0.001	0.01	97.3 (13.7)	5
69	Fenthion	1.4	46.2	24.08	278.0 → 169.0	20	278.0 → 245.0	15	4	0.001	0.01	91.5 (10.0)	4
70	Parathion ethyl	1.3	0.9	24.26	291.0 → 109.0	15	291.0 → 263.0	10	4	0.005	0.02	96.3 (7.8)	4
71	Isobenzan	1.0	5.0	24.41	310.8 → 274.8	10	312.8 → 276.8	10	5	0.005	0.05	76.4 (8.9)	4
72	Cyanazine	400.0	141.0	24.59	225.1 → 189.1	10	225.1 → 198.1	10	4	0.005	0.04	78.3 (9.1)	4
73	Trichloronat	1.6	10.0	24.70	296.9 → 268.9	15	299.9 → 271.9	15	5	0.001	0.01	63.9 (13.2)	4
74	Pirimiphos ethyl	3.0	25.0	26.08	333.1 → 288.1	20	333.1 → 318.1	15	4	0.001	0.01	92.1 (11.6)	4
75	Isofenphos	3.0	91.5	26.44	255.1 → 185.1	10	255.1 → 213.1	10	4	0.001	0.02	95.2 (6.7)	4
76	Allethrin	2030.0	370.0	26.93	123.1 → 81.1	10	136.1 → 93.1	10	4	0.001	0.05	78.9 (8.8)	4
77	Phenthoate	58.6	138.0	27.10	274.0 → 125.0	7	274.0 → 246.0	10	4	0.001	0.03	76.5 (9.6)	4
78	Quinalphos	20.0	75.0	27.17	146.0 → 91.0	15	146.0 → 118.0	15	4	0.001	0.03	92.1 (13.2)	4
79	Mephospholan	2.8	11.0	27.60	196.0 → 140.0	15	196.0 → 168.0	10	4	0.0005	0.005	98.3 (3.6)	4
80	Chlordane, *trans*	220.0	50.0	28.04	372.8 → 265.9	15	374.8 → 267.9	16	5	0.001	0.01	92.1 (7.9)	5
81	Bromophos ethyl	20.5	125.0	28.07	358.9 → 302.9	20	358.9 → 330.9	10	4	0.001	0.01	94.4 (9.5)	4
82	Methidathion	80.0	25.0	28.23	145.0 → 58.0	15	145.0 → 85.0	10	4	0.001	0.01	98.7 (9.3)	4
83	Propaphos	2.5	61	28.58	220.1 → 140.0	15	304.1 → 220.1	15	5	0.001	0.01	92.2 (10.9)	4
84	Tetrachlorvinphos	100.0	4200.0	28.64	330.9 → 109.0	22	330.9 → 316.0	22	4	0.001	0.01	97.7 (2.5)	4
85	Endosulfan, alpha	35.0	26.0	28.88	195.9 → 158.9	16	195.9 → 159.9	15	4	0.0005	0.005	93.6 (9.9)	5
86	Chlordane, *cis*	220.0	50.0	28.90	372.8 → 265.9	18	409.8 → 374.8	5	5	0.001	0.01	95.9 (10.0)	5
87	Fenamiphos	2.4	10.0	29.98	303.1 →260.1	15	303.1 → 288.1	15	4	0.0005	0.005	96.6 (10.0)	4
88	Dieldrin	13.3	65	30.87	276.9 → 206.9	20	276.9 → 240.9	10	4	0.0005	0.005	93.3 (9.3)	5
89	Endrin	1.7	3.0	32.42	262.9 → 190.9	25	262.9 → 192.9	26	4	0.001	0.01	95.8 (9.5)	5
90	Isoxathion	21.6	112.0	32.47	177.0 → 130.0	15	313.0 → 177.0	15	5	0.001	0.01	95.7 (6.7)	4
91	Endosulfan, beta	35.0	26.0	33.50	195.9 → 158.9	16	195.9 → 159.9	15	4	0.001	0.01	93.9 (8.9)	5
92	Fensulfothion	0.3	2.2	33.84	293.0 → 97.0	16	293.0 → 125.0	0	4	0.001	0.01	99.1 (9.1)	4
93	Ethion	45.0	13.0	33.96	231.0 → 175.0	15	231.0 → 203.0	15	4	0.001	0.01	89.9 (11.7)	4
94	Chlorthiophos	45.0	20.0	34.26	325.0 → 269.0	15	325.0 → 297.0	10	4	0.001	0.01	94.3 (6.6)	4
95	Sulprofos	65.0	70.0	35.31	322.0 → 139.0	15	322.0 → 156.0	15	4	0.001	0.01	94.1 (7.9)	4
96	Triazophos	4.2	57.0	35.55	161.0 → 105.0	13	161.0 → 134.0	10	4	0.001	0.01	96.4 (7.3)	4
97	Famphur	1.8	59.0	35.87	218.0 → 109.0	10	218.0 → 127.0	10	4	0.001	0.01	95.2 (7.9)	4

(*continued*)

Table II Continued

No.	Compound	Toxicity (LD_{50}, mg/kg)[a]		Mass spectrometry settings					IPs	Validation parameters			
		Birds	Mammals	RT (min)	First transition ($m/z \rightarrow m/z$)	CE (V)	Second transition ($m/z \rightarrow m/z$)	CE (V)		LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
98	Carbophenothion	5.8	14.0	36.02	342.0 → 157.0	10	342.0 → 296.0	5	4	0.001	0.01	95.8 (11.0)	4
99	Ediphenphos	350.0	100.0	36.23	173.0 → 109.0	15	310.0 → 173.0	10	4	0.001	0.01	98.3 (9.3)	4
100	Endosulfan sulphate	52.4	18.0	36.38	273.9 → 236.9	10	273.9 → 239.0	15	4	0.0005	0.005	89.7 (8.7)	5
101	DDT	1135.0	200.0	36.77	234.9 → 165.0	20	234.9 → 198.9	15	4	0.001	0.05	93.3 (7.8)	5
102	Nuarimol	200.0	2450.0	37.69	235.1 → 139.0	15	314.1 → 139.0	15	5	0.001	0.05	68.9 (5.6)	4
103	Resmethrin	75.0	250.0	39.00	171.1 → 128.0	9	171.1 → 143.0	9	4	0.001	0.01	79.4 (11.3)	4
104	Carbosulfan	120.0	115.0	39.80	163.1 → 107.1	15	163.1 → 135.1	10	4	0.001	0.01	79.1 (5.4)	4
105	Phosmet	18.0	40.0	40.66	160.0 → 104.0	20	160.0 → 133.0	15	4	0.0005	0.005	96.7 (9.1)	4
106	EPN	2.4	20.0	40.75	169.0 → 77.0	16	169.0 → 141.0	10	4	0.003	0.04	98.2 (9.9)	4
107	Bifenthrin	1975.0	54.5	40.81	181.0 → 153.0	6	181.0 → 166.0	15	4	0.003	0.04	75.9 (10.2)	4
108	Tebufenpyrad	2000.0	210.0	41.87	333.1 → 171.1	20	333.1 → 276.1	10	4	0.001	0.01	76.9 (17.9)	4
109	Leptophos	268.8	65.0	42.96	374.9 → 359.9	26	376.9 → 361.9	26	5	0.001	0.02	96.8 (4.5)	4
110	Phosalone	–	112.0	43.10	182.0 → 111.0	15	182.0 → 138.0	10	4	0.001	0.01	91.0 (9.2)	4
111	Azinphos methyl	8.5	10.0	43.57	132.0 → 77.0	15	160.0 → 104.0	10	5	0.003	0.05	97.3 (9.4)	4
112	Amitraz	–	100.0	44.30	293.2 → 147.1	15	293.2 → 162.1	10	4	0.001	0.01	85.9 (9.2)	4
113	Pyrazophos	118.0	184.0	44.92	221 .0→ 177.0	15	221.0 → 193.0	10	4	0.001	0.01	98.2 (4.3)	4
114	Azinphos ethyl	34.4	12.0	45.36	160.0 → 104.0	10	160.0 → 132.0	5	4	0.005	0.05	91.7 (11.5)	4
115	Cifluthrin	250.0	300.0	49.00	163.0 → 91.0	12	163.0 → 127.0	10	4	0.005	0.05	78.5 (13.2)	4
116	Flucythrinate	2708.0	76.0	50.00	199.1 → 107.0	22	199.1 → 157.0	10	4	0.005	0.05	77.0 (5.4)	4
117	Deltamethrin	1000.0	22.0	53.01	253.0 → 93.0	18	253.0 → 192.0	30	4	0.005	0.05	81.4 (9.4)	4
Surrogates													
S3	Chlorfenvinphos-d_{10}			17.60	263.0 → 159.0	15	369.0 → 101.0	30	5	–	–	–	–
S4	Chlorpyrifos-d_{10}			23.60	197.0 → 169.0	15	362.0 → 131.0	20	5	–	–	–	–
Internal standards													
IS3	Chloropropham			11.32	213.0 → 127.0	15	213.0 → 171.0	10	4	–	–	–	–
IS4	Diazinon-d_{10}			17.81	179.1 → 137.1	15	315.0 → 170.0	20	5	–	–	–	–
IS5	Heptachloro epoxide, *cis*			26.30	352.8 → 262.9	15	352.8 → 288.9	15	4	–	–	–	–

RT: retention time; CE: collision energy; IPs: identification points; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.

[a]Average data from different species. These data have been taken from Mineau *et al.* (14) and the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

by the direct infusion of a 1-μg/mL of methanolic solution of each pesticide into the ionization source. All the selected CEs of these complementary methods ranged from 5 to 39 eV (Tables I and II). The final goal was to develop three timed-SRM methods with two reactions or transitions per compound. The dwell time was adjusted to 10 cycles per second throughout the chromatogram to obtain low detection limits and well-shaped chromatographic peaks. The peak shapes were satisfactory and were highly related to the number of monitored transitions, the scan and dwell times, and the scan rate (18, 19). According to the European Commission Decision 2002/657/EC (20), which introduced the concept of identification points (IPs) for the confirmation stage, the confirmation of the analytes included in this study involved the monitoring of two product ions from the same precursor ion, which resulted in four IPs, or two product ions derived from two different precursor ions, which resulted in five IPs. Therefore, the timed-SRM methods used in this study meet the requirements of the aforementioned regulation (20). The resulting number of IPs and also the final MS-MS conditions used in this study for each analyte are summarized in Tables I and II.

Optimization of sample extraction and cleanup

In the present study, solid–liquid principles were adopted for the extraction of contaminants from samples coming from wildlife poisoning episodes. When considering the simultaneous extraction of analytes belonging to different chemical classes, selecting the appropriate solvents and extraction methods is critical to achieving a satisfactory recovery from the matrix. Many organic solvents are applied in the literature for the extraction of pesticides (21). We selected mixtures of solvents that have been applied to the extraction of pesticides of each chemical group in the literature due to the wide range of polarities of the pesticides included in this method. Thus, we assayed the extraction efficacy of various mixtures of solvents of different polarities, and dichloromethane/ethyl acetate/acetone (50/30/20) provided the best combination of extract recovery and purity and was therefore chosen for the extraction. The use of sonication has also been described in the literature, as it may improve the extraction efficiency. In our case, we observed a slight improvement in the recoveries of certain key pesticides, such as carbofuran. Therefore, a 5-min sonication step was added to the extraction protocol.

The samples sent to the laboratory for poison identification are usually matrices with a relatively high content of fat and other interfering substances derived from degradation processes, a cleanup step was included to eliminate substances that could reduce the signal or cause column damage. Many strategies can be used for lipid removal, such as freezing centrifugation, partitioning lipid extraction, adsorption chromatography, GPC and sulfuric acid treatment. We chose GPC for those extracts from complex matrices (e.g., animal tissues, insects and laced baits) because its efficacy had been proven previously in our laboratory, yielding residual lipid concentrations of $<3\%$ of the initial amount (15, 16). Nevertheless, GPC is a solvent- and time-consuming method; thus, in extracts from such samples as blood or fresh animal tissue, we preferred to assay the efficacy of freezing centrifugation, where pesticides remain dissolved in

the solvent while frozen lipids can be removed by centrifugation due to their lower melting points relative to the solvent. Our experiments showed that freezing centrifugation alone was an adequate cleanup method for these samples, because it gave suitable extracts for chromatographic analysis with MS detection.

Analytical performance

To evaluate the usefulness of this methodology for the quantitative determination of pesticides in fresh liver samples, the confirmation criteria, precision, linearity and method limits of detection (LODs) and quantification (LOQs) were studied.

The obtained chromatographic peaks were identified as target analytes only if satisfied all of the following criteria: (i) the RT of the unknown peak coincided with that obtained from six replicates of the second level of calibration (RT $\pm$ 3 SD); (ii) there was a match with the ion ratios of the standard. We applied the tolerances of absolute ion abundances that are specified in the 2002/657/EC Directive [$\pm$20% tolerance for >50% relative intensity (% of base peak); $\pm$25% for > 20–50%; $\pm$30% for >10–20% and $\pm$50% for <10%] and (iii) we obtained a S/N ratio higher than 10 for a sample extract.

The extraction efficiency of the proposed methodology was evaluated by spiking five chicken liver samples with a mixture of the 117 pesticides at two concentrations (10.0 and 200.0 μg/kg). We calculated the recovery values and their relative standard deviations (RSDs) for each level by comparing the areas of the analytes in the extracted spiked samples with those of the same concentrations in the dissolvent (Tables I and II). The results ranged from 61.0 to 99.7%, with most of the recoveries being >90% at both concentrations. The precision was satisfactory, with the most unfavorable RSD being <18%. We also evaluated the recoveries and precision during 5 consecutive days (inter-day measurement), which yielded an RSD that was also <18% (Tables I and II).

To evaluate the possible existence of matrix effects, the same comparison was performed with chicken liver samples that were extracted without having being spiked, and where the spiking was performed after the extraction to evaluate if the extracted components of the matrix had an enhancement/suppression effect on the signal of the target pesticides. We did not found significant differences. Thus, we could conclude that there were no significant matrix effects, and the rest of the studies and calculations were performed against calibration curves of standards (0.5 and 500 μg/kg). We used the peak areas for performing the calculations. The origin point was not included when constructing the calibration curves. A good linearity was found and we concluded that it is possible to use the linear regression method to calculate the concentrations of these analytes since the correlation coefficients (r^2) were >0.9804 for all the analyses and the residual analysis showed values within the range of −11.32 to 9.42%.

Application to real samples

The validated methodology has been applied to the routine analysis of 140 real samples from 98 wildlife poisoning incidents that were submitted between 2010 and 2012 to the Clinical and Analytical Toxicology Service of the University of Las Palmas de Gran Canaria (SERTOX, Canary Islands, Spain).

Table III presents the results of the analysis of well-preserved fresh liver samples from suspected wild animal poisonings. We positively identified a pesticide in 78 of 94 liver samples, and their quantified levels were compatible with death by poisoning. The most frequently detected pesticides belonged to the group of anticoagulant rodenticides (brodifacoum, bromadiolone and

Table III

Summary of the positive detection of pesticides in fresh liver samples from wildlife poisoning episodes (2010–2012) with the validated methodology, and application to the identification and semi-quantification of pesticides in degraded liver tissue, laced baits and other samples from the poisoning scenarios

Liver	No. of animals	Principal toxicant(s)				
		Anticoagulants	Carbamates	OPs[a]	Pyrethroids	Others
Western Canaries Lizard (*Gallotia galloti*)	12		12			
Barn Owl (*Tyto alba*)	12	12				
Common Kestrel (*Falco tinnunculus*)	11	8		2	1	
Long-eared Owl (*Asio otus*)	8	8				
Common Buzzard (*Buteo buteo*)[a]	7		6	1		1
European hedgehog (*Erinaceus europaeus*)	6		6			
Common Raven (*Corvus corax*)	5		4		1	
European Turtle Dove (*Streptopelia turtur*)	5	5				
Barbary Falcon (*Falco pelegrinoides*)	4	2	2			
European Sparrowhawk (*Accipiter nisus*)	4	2	2			
Long-eared Bat (*Plecotus teneriffae*)	3					3
Cory's Shearwater (*Calonectris diomedea*)	1			1		
Other samples	No. of samples					
Degraded liver tissue (different species)	9	3	3	1	1	1
Meat	4		3	1		1
Containers and plastic[b]	4		3	1	1	
Gastrointestinal content	4		3	1		
Chicken carcasses	3		3			
Feed	3		2	1		
Insects	3		2	1		
Soil	3		1	1	1	
Bones and flesh	2		2			

[a]In one animal, two pesticides were found (carbofuran and aldicarb).

[b]In one plastic container, two pesticides were found (carbofuran and methomyl).

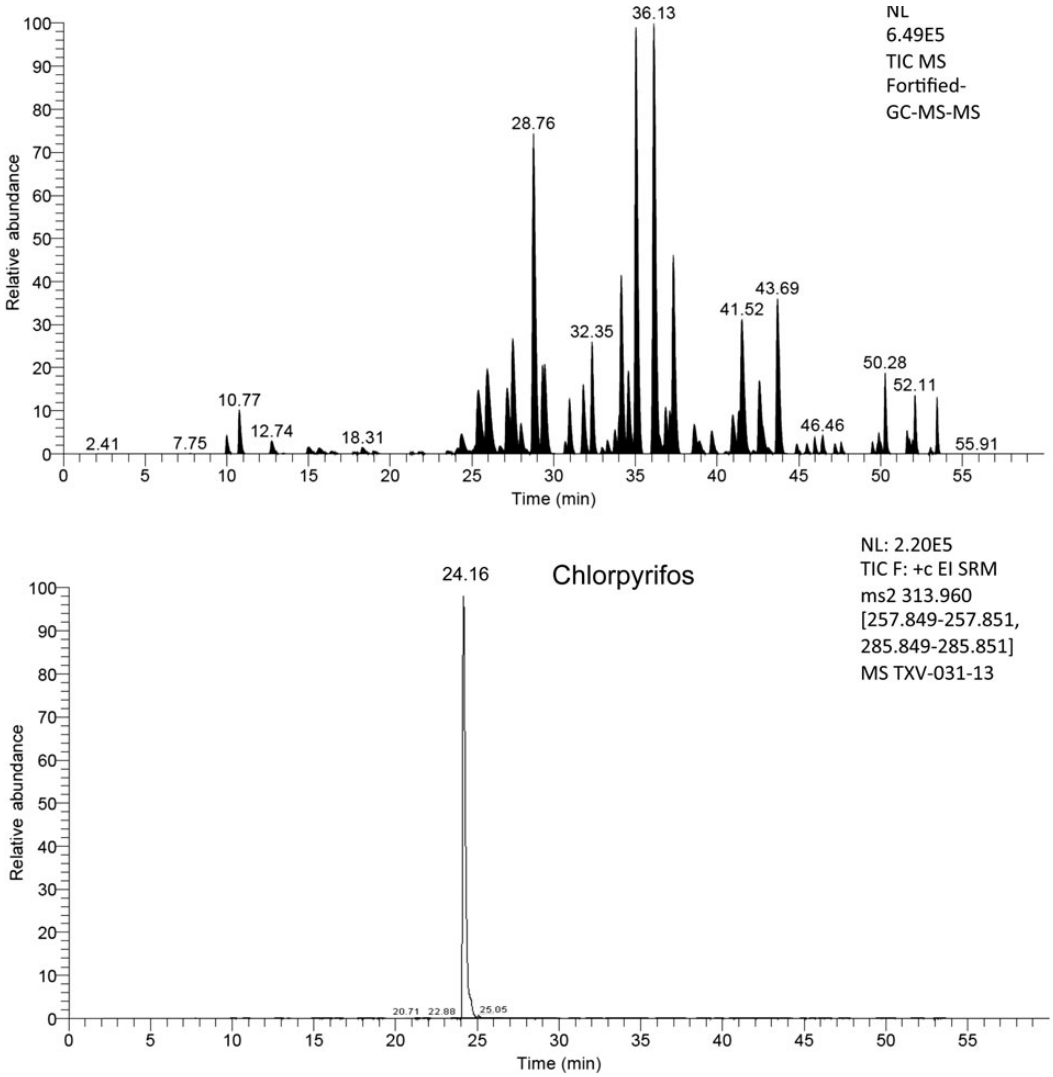

Figure 2. Representative GC–MS-MS chromatogram (A) sample spiked with the 91 apolar pesticides analyzed by gas chromatography; (B) SRM chromatogram of chlorpyrifos from a real sample. This SRM chromatogram results from the product ions spectra (m/z 169.0 and 171.0) from the precursor ion at m/z 197.0 for chlorpyrifos.

difenacoum), followed by carbamate insecticides (mainly carbofuran but also aldicarb and methomyl) and organophosphorus pesticides (chlorpyrifos, chlorpyrifos-methyl and diazinon). In some of the livers, multiple pesticides were detected and quantified. A representative chromatogram is shown in Figure 2.

The proposed methodology was also applied to degraded liver, other animal tissues, materials suspected of being bait and other samples that were submitted to our laboratory. We applied minor modifications to the extraction and cleanup procedures depending on the moisture content, adding enough water in the homogenization step to obtain a homogenate of similar physical consistency than that obtained from liver, and also depending on the state of conservation of the samples and the dirtiness of the extracts (necessity of GPC or not). As summarized in Table III, its application allowed the detection of a pesticide in 35 of 46 samples of very different natures, from biological samples to plastic materials.

We must note that, although the results of the validation experiments were satisfactory for chicken liver, given the wide range and conservation status of the samples from cases of wildlife poisoning, the usual rigorous method validation standards cannot be applied to all the real samples. Thus, many measurements of pesticides using this method must be regarded as only semi-quantitative in samples other than fresh liver (2).

Conclusions

This study reports the application of a solid–liquid extraction procedure in combination with triple quadrupole GC– and LC–MS-MS for the simultaneous detection and quantification or semi-quantification of 117 pesticides in a variety of samples from wildlife poisoning. The results show satisfactory validation parameters in liver tissue. All the pesticides could be detected at very low concentrations, with a good linearity of the calibration curves within the investigated calibration range (0.5–500 μg/kg, with $r^2 > 0.98$). The recovery rates were between 62 and 99%, with very good precision (RSD <18%). The proposed method can be recommended for routine application in environmental

forensic studies because it is simple, sensitive and very useful. Our results show that our methodology represents a valuable tool in the task of identifying unknown toxicants, as at least one pesticide was identified in 113 samples at levels compatible with death by poisoning.

Acknowledgments

The authors thank to Mrs María de los Reyes Suárez Hanna for her technical assistance.

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Science and Justice 55 (2015) 307–315

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Science and Justice

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Technical note

Validated analytical methodology for the simultaneous determination of a wide range of pesticides in human blood using GC–MS/MS and LC–ESI/MS/MS and its application in two poisoning cases

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ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form 13 April 2015
Accepted 15 April 2015

Keywords:
Forensic science
Pesticide poisoning
Forensic toxicology
Multiresidue
GC–MS/MS
LC–MS/MS

ABSTRACT

Pesticides are frequently responsible for human poisoning and often the information on the involved substance is lacking. The great variety of pesticides that could be responsible for intoxication makes necessary the development of powerful and versatile analytical methodologies, which allows the identification of the unknown toxic substance. Here we developed a methodology for simultaneous identification and quantification in human blood of 109 highly toxic pesticides. The application of this analytical scheme would help in minimizing the cost of this type of chemical identification, maximizing the chances of identifying the pesticide involved. In the methodology that we present here, we use a liquid–liquid extraction, followed by one single purification step, and quantitation of analytes by a combination of liquid and gas chromatography, both coupled to triple quadrupole mass spectrometry, which is operated in the mode of multiple reaction monitoring. The methodology has been fully validated, and its applicability has been demonstrated in two recent cases involving one self-poisoning fatality and one non-fatal homicidal attempt.

1. Introduction

The large group of pesticides, which are widely used throughout the world primarily to control pests affecting crops, is often implicated in human poisoning [1]. Morbidity and mortality attributable to these substances vary from country to country, depending on many variables such as the level of socioeconomic development, accessibility to these chemicals, and the importance of the agricultural sector. Fatalities involving pesticides are a consequence of accidents, self-injury or more rarely homicides, and range from less than 1% of deaths from poisoning in EU countries [1,2] to up to 71% of all violent deaths in the Western Pacific and Southeast Asia [3–5]. In all these cases chemical analysis to investigate the poison involved is mandatory, and the forensic laboratory is facing a challenge because in most cases there is no information on what the substance involved was. The main difficulties of this type of toxicological identifications are the wide variety of biological matrices that are sent to the laboratory, often in advanced state of decomposition, along with the wide range of pesticides to which the poisoning could be attributed.

In recent years the use of chromatographic techniques (HPLC or GC) combined with detection of analytes by mass spectrometry (MS/MS) has been considered as a very useful tool in forensic toxicology laboratories, since it enables high selectivity along with a very low detection limits. Triple quadrupole mass spectrometers (QqQ) allow operating in the mode of selective multiple reaction monitoring (SRM). This allows the monitoring of parent ions fragmenting into product ions. This mode of operation improves selectivity and sensitivity of the determination, in comparison with one-stage mass spectrometry. With this technique, the virtual elimination of isobaric interferences is allowed, as well as a significant decrease in chemical noise from the matrix [6]. The use of any of these analytical techniques is currently seen as a practical way to overcome the difficulties posed by complex biological matrices, which may contain an excessive amount of potentially interfering substances, such as fat, protein, sugars, and chemicals [7]. In addition, high acquisition speed in the MRM mode allows the development of methods for the simultaneous analysis of tens or even hundreds of compounds belonging to different chemical classes [8–12].

It needs to be emphasized that when the information of the pesticide involved is lacking it is generally difficult to conduct thorough analytical investigations in complex biological matrices such as ante- or post-mortem blood, and usually several complementary analyses are needed. That is why all the techniques of high sensitivity and specificity that allow the simultaneous analysis of a wide series of chemicals of high

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http://dx.doi.org/10.1016/j.scijus.2015.04.007
1355-0306/

toxicity may contribute to reducing the costs associated with this type of analytical, as well as to increase the chances of identifying the "unknown toxic substance". In this work we have developed an analytical method for the detection and quantification of 109 pesticides in human blood. The pesticides have been selected on the basis of both, their high toxicity to humans [13], and the frequency with which they are involved in cases of poisoning [14,15]. This methodology is based on a liquid–liquid extraction, clean-up, chromatographic separation, and detection by QqQ operated in the MRM mode, and has been successfully applied to the detection of the toxicant involved in 2 recent cases of poisoning by an unknown pesticide that were submitted to our laboratory. Besides, this methodology has been also applied in our laboratory to the identification of the pesticides in matrices other than blood [16].

2. Materials and methods

2.1. Chemicals and reagents

Acetone, acetonitrile, cyclohexane, dichloromethane, ethyl acetate, and methanol (>99.9%) were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure (UP) water was obtained from a Milli-Q Gradient A10 (Millipore, Molsheim, France). Blank blood was purchased from Medichem (Medidrug® Basis Line, Medichem, Germany). All the pesticide standards (purity from 97% to 99.5%), as well as the internal standards (ISs, aldicarb-D3, carbofuran-D3, chlorfenvinphos-D10, chloropropham, chlorpyriphos-D10, diazinon-D10, heptachloro epoxide cis, and thiobencarb), were purchased from Dr Ehrenstorfer Reference Materials (Augsburg, Germany). We prepared stock solutions of target compounds (0.1 and 1 mg/mL) in cyclohexane or acetonitrile. Stock solutions were stored at −20 °C. From these stock solutions matrix-matched calibration curves were prepared (0.5 ng/mL to 500 ng/mL) using blank blood. For the fortification experiments we used mixtures of all the standards in acetone (10 μg/mL and 500 ng/mL).

2.2. Pesticide selection

A wide variety of pesticides belonging to different chemical classes are currently used in agricultural practices but, because of their high toxicity, several restrictions have been applied and most of the most toxic compounds are nowadays banned. Nevertheless, it has been shown that legal and commercial restrictions have not influenced the intentional illegal use of some pesticides as poisons [16,17]. For this reason for the selection of the 109 pesticides included in this multiresidue method we have mainly taken into account their known toxicities for either humans and other mammals (Table 1), and also according to the available data, the frequency with which these compounds have been implicated in human poisonings [14,15], regardless of whether their use is currently allowed or not.

2.3. Extraction and cleanup procedure

A liquid–liquid extraction procedure was developed for human whole blood. For the extraction, 2 mL of the sample (pH = 7.0) was placed in 50-mL polypropylene centrifuge tubes. Next, 5 mL of ultrapure water and 50 μL of the ISs solution at 1 μg/mL were added and thoroughly vortexed. 10 mL of a mixture of dichloromethane/ethyl acetate/acetone (50/30/20) were added to the tubes. The tubes were then placed in an orbital shaker for 10 min. The tubes were then sonicated for 5 min. The samples were centrifuged at 5000 rpm, 5 min, 20 °C, and the supernatant collected. The samples were then placed under a gentle nitrogen stream to evaporate the solvent. The concentrated extracts were re-dissolved in 3 mL of cyclohexane in Eppendorf tubes.

After the extraction an additional cleanup step by freezing centrifugation was performed to minimize the content of interfering substances (mainly lipids). The tubes were placed in a −82 °C freezer for 20 min, and then centrifuged at 5000 rpm, 5 min, −10 °C. The frozen lipids remained in the bottom of the tube and thus separated from the pesticides dissolved in the supernatant, which was carefully removed. This procedure was performed three times, and the resulting supernatant was divided into two 1-mL aliquots. One aliquot was directly used for GC–MS/MS. The other was evaporated and re-dissolved in acetonitrile for LC–MS/MS analysis.

2.4. GC–QqQ-MS/MS analysis

For the GC–MS–MS detection of the pesticides included in this study we used a Trace GC Ultra tandem coupled with a TSQ XLS triple quadrupole (QqQ) mass spectrometer instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). As the stationary phase a 30 m × 0.25-mm i.d., 0.25 μm film thickness column was used (BPX5, SGE Inc., Austin, TX, USA). Helium (99.999%) was used as the carrier gas at a constant flow of 1 mL/min. The 61-min oven temperature program was: 60 °C held for 1 min, ramped to 160 °C at 15 °C/min, then to 230 °C at 2.3 °C/min, and finally to 290 °C at 5 °C/min and held for 8.9 min. The injector temperature was set al 270 °C and the transfer line was heated to 310 °C. The injection volume was 1 μl in the splitless mode.

The GC was tandem-coupled to a TSQ XLS QqQ mass spectrometer, which was used for the detection and quantification of the 90 most apolar pesticides. An electron ionization (EI)–MS/MS library was specially created for the target analytes under our experimental conditions. We calibrated the mass spectrometer scale with perfluorotributylamine on a weekly basis to ensure an optimal response over time and proper mass assignments. The instrument control, data acquisition and data analysis was performed using the Thermo Fisher Xcalibur software (Ver. 2.0.1).

We constructed a timed MRM method for the simultaneous analysis of 90 pesticides plus ISs in a single run. Matrix-matched calibration curves contained all of the target compounds except for the ISs at each level (0.5 to 500 ng/mL). The operation conditions of the mass spectrometer were: electron impact ionization (70 eV) in MRM; emission current, 50 μA; ionization source temperature, 220 °C; electron multiplier voltage, 1500 V; scan width, 0.15; scan time, 0.05 s; and peak width, *m/z* 0.7 Da. Argon (99.99%) was used as the collision gas at 0.2 Pa.

2.5. LC–MS–MS analysis

Because some of the most relevant pesticides causing poisoning in humans, such as carbofuran and aldicarb, can only be analyzed by liquid chromatography due to their high polarity (unless prior derivatization is performed), we developed a complementary method by LC–MS–MS. In this second method we included 19 compounds. Some of them can be analyzed both, by liquid chromatography and by gas chromatography (such as metamidofos, dimethoate, or pirimicarb), but we chose the technique with which a higher sensitivity for each one of them is achieved. However, we have avoided duplicating them in both methods to minimize the number of transitions and to gain sensitivity.

For the LC–MS–MS detection, we used an Accela LC tandem coupled to a TSQ Quantum Max QqQ mass spectrometer instrument equipped with an H-ESI II electrospray ionization source (Thermo Fisher Scientific Inc.). As the stationary phase we used an analytic Synergi Hydro-RP column (4.0 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). We used the following mobile phases for LC: (A) 7.5 mM ammonium formate in ultrapure water; (B) methanol (HPLC–MS grade); and (C) 2% formic acid. The solvent flow was 1000 μl/min. The injection volume was 25 μl. During the entire run (26 min), solvent C was set at 2.5%. The infusion of the other two mobile phases was programmed as a gradient as follows: 0–12 min: 87.5% A → 7.5% A; 12–16 min: 7.5% A; 16.0–16.2 min: 7.5% A → 87.5% A; and 16.2–25.0 min: 87.5% A.

Table 1
Acute toxicity values (mg/kg) and toxic and lethal blood concentrations (μg/mL) of the pesticides included in the methodology.

Compound	Toxicity			Compound	Toxicity			Compound	Toxicity		
	LD_{50}[a]	[Toxic][b]	[Lethal][b]		LD_{50}	[Toxic][b]	[Lethal][b]		LD_{50}	[Toxic][b]	[Lethal][b]
Acephate	321.0	>200	–	Dieldrin	65	0.15	0.5	Mevinphos	4.0	–	–
Aldicarb	1.9	1.5	6.1	Dimefox	3.5	–	–	Monocrotophos	15.0	–	12
Aldrin	65.0	0.005	0.7	Dimethoate	220.0	–	4	Nuarimol	2450.0	–	–
Allethrin	370.0	–	–	Dioxathion	10.0	0.2	–	Omethoate	50.0	–	3.2
Amitraz	100.0	0.6	–	Disulfoton	5.0	–	–	Oxamyl	30.0	0.23	–
Azinphos ethyl	12.0	–	0.9	Ediphenphos	100.0	–	1.4	Parathion ethyl	0.9	–	0.5
Azinphos methyl	10.0	–	–	Endosulfan sulfate	18.0	–	–	Parathion methyl	57.0	–	–
Bendiocarb	35.0	1	40	Endosulfan, alpha	26.0	0.5	2.8	Phenthoate	138.0	–	–
Benfuracarb	102.0	–	–	Endosulfan, beta	26.0	0.5	2.8	Phorate	20.0	–	0.83
Bifenthrin	54.5	–	–	Endrin	3.0	0.01	–	Phosalone	112.0	–	–
Bromophos ethyl	125.0	–	1.6	EPN	20.0	–	0.8	Phosmet	40.0	–	–
Bromoxynil	78.0	20	–	Ethion	13.0	–	–	Phosphamidon	6.0	–	–
Cadusafos	71.4	5	6	Etoprophos	34.0	–	–	Phoxim	250	–	–
Carbaryl	150.0	–	2	Famphur	59.0	–	–	Pirimicarb	100.0	3.7	32.8
Carbofuran	10.2	0.06	0.4	Fenamiphos	10.0	–	–	Pirimiphos ethyl	25.0	–	–
Carbophenothion	14.0	–	–	Fenitrothion	142.0	1	–	Pirimiphos methyl	1150.0	–	–
Carbosulfan	115.0	–	–	Fensulfothion	2.2	–	–	Profenofos	116.0	–	1.2
Carboxin	430.0	–	–	Fenthion	46.2	1	–	Propachlor	392.0	–	–
Chlordane, cis	50.0	0.005	2	Flucythrinate	76.0	–	–	Propaphos	61	–	–
Chlordane, trans	50.0	0.005	2	Fonofos	3.0	–	–	Propetamphos	130.0	–	–
Chlorfenvinphos	20.0	–	0.1	Formothion	175.0	–	–	Propoxur	51.2	0.12	–
Chlormephos	12.5	–	–	Heptachlor	50.0	–	–	Pyrazophos	184.0	–	–
Chlorpyrifos	60.0	0.2	1.6	Heptenophos	117.0	–	–	Quinalphos	75.0	–	4.5
Chlorpyriphos methyl	2000.0	–	–	Imidacloprid	98.0	–	2.1	Resmethrin	250.0	–	–
Chlorthiophos	20.0	–	–	Isazophos	27.0	–	–	Sulfotep	22.0	0.08	–
Cifluthrin	300.0	3.1	37.4	Isobenzan	5.0	0.03	–	Sulprofos	70.0	–	–
Cyanazine	141.0	–	–	Isofenphos	91.5	–	–	Tebufenpyrad	210.0	–	–
Cyanophos	215.0	–	–	Isoxathion	112.0	–	–	Tefluthrin	22.0	0.9	7.1
Cyproconazole	352.0	–	–	Leptophos	65.0	–	–	TEPP	2.3	–	2.7
Dazomet	415.0	–	–	Lindane	25.0	0.3	1.3	Terbufos	3.5	–	–
DDT	200.0	1	–	Malathion	53.0	0.5	1	Tetrachlorvinphos	420.0	–	–
Deltamethrin	22.0	0.2	9.3	Mephospholan	11.0	–	–	Thiometon	37.0	–	–
Diallate	395.0	–	–	Metamidofos	18.5	–	13.5	Thionazin	5.0	–	0.8
Diazinon	76.0	0.05	0.97	Methidathion	25.0	0.1	–	Triazophos	57.0	–	–
Dichlone	160.0	–	–	Methiocarb	16.0	–	–	Trichloronat	10.0	0.15	–
Dichlorphos	61.0	–	29	Metolcarb	109.0	–	–				
Dicrotophos	11.0	0.3	2.8	Methomyl	24.9	–	0.45				

[a] Average data from different species. These data have been taken from Mineau et al. (2001) and the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

[b] These data are values from non-fatal or fatal case reports of human poisonings when available in the literature and have been obtained from the National Library of Medicine Resource Toxicology Data Network (http://toxnet.nlm.nih.gov/index.html) and from the database of the Spanish National Institute of Toxicology and Forensic Sciences, available in http://busca-tox.com/.

The LC was tandem-coupled to a TSQ Quantum Max QqQ mass spectrometer, which was used for the detection and quantification of the 19 pesticides. As the ionization source we used a heated electrospray H-ESI II (Thermo Fisher Scientific Inc., Waltham, USA). The mass spectrometer was programmed according the following parameters: skimmer offset (10 V), sheath gas pressure (15 arbitrary units, a.u.), capillary temperature (250 °C), spray voltage (3000 V), and vaporization temperature (180 °C). The spectrometer was programed in the positive ionization mode. Argon (99.99%) was used as the collision gas at 0.25 Pa.

The MRM method was constructed for these pesticides plus ISs by means of the infusion of pure standard solutions in methanol into the ionization source. A matrix-matched calibration curve was constructed for all compounds (except for the ISs) from 0.5 to 500 ng/mL.

2.6. Validation

Blank whole blood (Medidrug Basis-Line VB, Medichem, Germany) was used for the validation experiments. For the fortification experiments 40 μL of a 10 μg/L or 500 ng/L of a mixture of all of the pesticides in acetone were added to 2 mL of whole blood to obtain concentrations of 200 μg/kg and 10 μg/kg, respectively. The samples were thoroughly mixed and allowed to stand at room temperature for 4 h to ensure that the analytes were homogenously distributed throughout the sample. The matrix effect was determined in quintuplicate by comparing the obtained concentrations between the spiked blood and the same concentrations of the pesticides prepared in the dissolvent. The recovery was calculated as the quotient of signals obtained from the spiked samples and the matrix matched standards. The intra- and inter-day precisions (five successive days) were also determined from the same experiments.

The analyte concentration that produced a peak signal of ten times the background noise from the chromatogram was set as the method limit of quantification (LOQ). Quantifications were based on the peak areas. Calibration curves were constructed using a least-squares linear regression from the injection of samples spiked with solutions to give final concentrations ranging from 0.5 to 500 μg/kg

2.7. Quality control

All the measurements were performed in triplicate, and the values used for calculations were the mean of the three values. In each batch of samples, two controls were included every 12 samples: a reagent blank consisting of a vial containing only cyclohexane and an internal laboratory quality control (QC) consisting of blank whole blood spiked with a mixture of all of the pesticides (20 μg/kg), and processed with the same method as the samples. The results were considered acceptable when the quantification of the analytes in the QC was within 15% of the deviation of the theoretical value.

Table 2
Method settings and validation parameters (n = 5) obtained for the 109 pesticides at the concentration of 0.2 μg/mL in human blood.

N°	Compound	Mass spectrometry settings						Validation parameters		
		RT	First transition *m/z* → *m/z*	CE (V)	Second transition *m/z* → *m/z*	CE (V)	IPs	LOQ (μg/mL)	Linearity (R^2)	Average recovery (RSD[a,b]) (%)
LC–MS/MS method										
1	Metamidofos	2.26	142.1 → 94.0	14	142.1 → 125.0	16	4	0.01	0.9985	78 (14, 11)
2	Oxamyl	2.78	237.2 → 163.0	14	237.2 → 196.0	18	4	0.03	0.9824	93 (8, 10)
3	Phoxim	4.03	300.1 → 129.3	18	300.1 → 283.0	10	4	0.01	0.9959	97 (6, 9)
4	Acephate	4.51	184.1 → 125.0	16	184.1 → 143.0	5	4	0.03	0.9816	95 (7, 11)
5	Omethoate	5.03	214.0 → 155.0	19	214.0 → 183.0	13	4	0.05	0.9904	96 (11, 14)
6	Methomyl	6.71	163.1 → 88.1	11	163.1 → 106.0	12	4	0.02	0.9934	97 (8, 12)
7	Imidacloprid	8.05	256.1 → 175.0	18	256.1 → 209.0	16	4	0.01	0.9813	88 (8, 10)
8	Dimethoate	8.64	230.0 → 125.0	23	230.0 → 199.0	11	4	0.05	0.9890	92 (8, 7)
9	Aldicarb	9.38	208.0 → 89.2	19	208.0 → 116.2	10	4	0.01	0.9906	94 (9, 11)
10	Carbofuran	10.34	222.0 → 123.1	25	222.0 → 137.5	24	4	0.01	0.9899	97 (4, 7)
11	Propoxur	10.76	210.0 → 111.2	18	210.0 → 168.0	11	4	0.01	0.9972	101 (9, 13)
12	Carbaryl	11.20	202.1 → 127.0	33	202.1 → 145.1	13	4	0.01	0.9948	82 (4, 8)
13	Pirimicarb	11.33	239.1 → 72.3	27	239.1 → 182.1	16	4	0.03	0.9982	96 (8, 11)
14	Carboxin	11.49	236.1 → 93.2	33	236.1 → 143.0	15	4	0.03	0.9929	71 (11, 9)
15	Bromoxynil	12.01	275.9 → 79.2	29	275.9 → 81.1	33	4	0.05	0.9994	92 (9, 11)
16	Methiocarb	12.97	226.0 → 121.0	19	226.0 → 169.0	8	4	0.05	0.9856	97 (12, 15)
17	Cyproconazole	13.46	292.1 → 70.3	17	292.1 → 125.1	34	4	0.01	0.9908	99 (11, 9)
18	Benfuracarb	15.02	411.2 → 190.1	13	411.2 → 252.3	15	4	0.05	0.9873	85 (5, 8)
19	Profenofos	15.52	373.0 → 302.8	18	373.0 → 344.8	13	4	0.03	0.9814	93 (14, 11)
GC–MS/MS method										
20	Dimefox	5.33	154.1 → 58.0	10	154.1 → 111.1	10	4	0.01	0.9834	91 (11, 9)
21	Dichlorphos	7.61	185.0 → 109.0	15	185.0 → 127.0	12	4	0.01	0.9921	90 (14, 12)
22	Mevinphos	9.72	192.0 → 127.0	12	192.0 → 164.0	10	4	0.01	0.9953	101 (10, 14)
23	Chlormephos	9.94	154.0 → 121.0	5	154.0 → 121.0	14	4	0.02	0.9878	105 (9,12)
24	Metolcarb	10.56	108.1 → 79.0	10	108.1 → 107.1	10	4	0.02	0.9877	103 (14, 11)
25	Heptenophos	12.26	250.0 → 124.0	10	250.0 → 215.0	4	4	0.01	0.9887	93 (6, 8)
26	Thionazin	12.96	192.0 → 96.0	10	248.0 → 140.0	10	5	0.05	0.9964	100 (8, 9)
27	TEPP	13.07	263.1 → 179.1	15	263.1 → 235.1	5	4	0.01	0.9987	94 (12, 10)
28	Propachlor	13.25	176.1 → 120.0	10	196.1 → 120.0	10	5	0.02	0.9913	96 (8, 11)
29	Etoprophos	13.70	158.0 → 114.0	10	158.0 → 130.0	10	4	0.01	0.9978	95 (6, 8)
30	Sulfotep	14.50	322.0 → 202.0	15	322.0 → 294.0	10	4	0.01	0.9889	92 (11, 14)
31	Dicrotophos	14.61	127.0 → 95.0	10	127.0 → 109.0	10	4	0.01	0.9887	101 (9, 12)
32	Bendiocarb	14.79	166.1 → 151.1	15	223.1 → 166.1	15	5	0.05	0.9856	93 (7, 9)
33	Cadusafos	14.93	159.1 → 97.0	20	159.1 → 131.0	10	4	0.01	0.9948	941 (8, 12)
34	Phorate	15.24	260.0 → 75.0	5	260.0 → 231.0	8	4	0.01	0.9932	93 (6, 8)
35	Diallate	15.30	236.0 → 152.0	20	236.0 → 194.0	15	4	0.02	0.9921	95 (10, 11)
36	Monocrotophos	15.80	127.0 → 95.0	20	127.0 → 109.3	25	4	0.01	0.9904	92 (9, 11)
37	Thiometon	15.85	88.0 → 60.0	15	248.0 → 88.0	15	5	0.01	0.9878	87 (7, 7)
38	Dazomet	16.56	89.0 → 75.0	20	162.0 → 89.0	8	5	0.05	0.9877	89 (8, 11)
39	Dioxathion	17.13	125.0 → 97.0	15	197.0 → 141.0	15	5	0.01	0.9887	101 (7, 9)
40	Lindane	17.16	216.9 → 180.9	15	218.9 → 182.9	15	5	0.005	0.9964	92 (11, 9)
41	Propetamphos	17.35	236.1 → 166.1	15	236.1 → 194.1	5	5	0.01	0.9987	94 (14, 12)
42	Terbufos	17.37	231.0 → 175.0	15	231.0 → 203.0	10	4	0.01	0.9945	101 (10, 14)
43	Chlorfenvinfos	17.60	267.0 → 159.0	15	323.0 → 269.0	10	5	0.01	0.9995	103 (14, 11)
44	Cyanofos	17.60	243.0 → 109.0	12	243.0 → 127.0	15	4	0.01	0.9883	94 (6,11)
45	Fonofos	17.69	137.0 → 109.0	10	246.0 → 137.0	10	5	0.01	0.9978	92 (4, 9)
46	Diazinon	17.81	179.1 → 127.0	15	179.1 → 137.1	15	4	0.01	0.9907	92 (9, 6)
47	Disulfoton	18.32	274.0 → 88.0	10	274.0 → 245.0	10	4	0.01	0.9889	94 (6,11)
48	Tefluthrin	18.40	197.0 → 141.0	15	197.0 → 161.0	10	4	0.02	0.9887	91 (7,5)
49	Isazophos	18.40	257.0 → 119.0	15	257.0 → 162.0	15	4	0.01	0.9856	84 (4,6)
50	Dichlone	18.58	191.0 → 135.0	15	226.0 → 191.0	10	5	0.02	0.9819	89 (8,7)
51	Formothion	19.94	224.0 → 125.0	15	224.0 → 196.0	10	4	0.01	0.9881	94 (5,9)
52	Phosphamidon	20.08	264.0 → 127.0	15	264.0 → 127.0	15	4	0.01	0.9883	91 (6,10)
53	Chlorpyrifos methyl	20.57	285.9 → 93.0	25	285.9 → 272.9	13	4	0.01	0.9890	88 (8, 11)
54	Parathion methyl	21.10	263.0 → 109.0	15	263.0 → 127.0	15	4	0.01	0.9992	89 (6, 8)
55	Heptachlor	21.36	338.8 → 267.9	15	338.8 → 303.8	15	4	0.005	0.9899	94 (8, 6)
56	Fenitrothion	22.74	277.0 → 109.0	20	277.0 → 260.0	15	4	0.01	0.9972	90 (15, 12)
57	Pirimifos methyl	23.19	290.1 → 125.0	15	290.1 → 233.1	10	4	0.01	0.9948	93 (12, 8)
58	Malathion	23.27	173.0 → 127.0	10	173.0 → 145.0	5	4	0.01	0.9982	94 (12, 14)
59	Chlorpyrifos	23.60	197.0 → 169.0	15	199.0 → 171.0	15	5	0.01	0.9929	92 (11, 13)
60	Aldrin	23.60	262.9 → 192.9	32	262.9 → 227.9	26	4	0.003	0.9994	78 (12, 8)
61	Fenthion	24.08	278.0 → 169.0	20	278.0 → 245.0	15	4	0.01	0.9856	81 (7, 11)
62	Parathion ethyl	24.26	291.0 → 109.0	15	291.0 → 263.0	10	4	0.01	0.9908	87 (9, 13)
63	Isobenzan	24.41	310.8 → 274.8	10	312.8 → 276.8	10	5	0.02	0.9873	82 (11, 13)
64	Cyanazine	24.59	225.1 → 189.1	10	225.1 → 198.1	10	4	0.05	0.9948	75 (9, 12)
65	Trichloronat	24.70	296.9 → 268.9	15	299.9 → 271.9	15	5	0.04	0.9932	93 (6, 8)
66	Pirimifos ethyl	26.08	333.1 → 288.1	20	333.1 → 318.1	15	4	0.01	0.9921	95 (8, 9)
67	Isofenphos	26.44	255.1 → 185.1	10	255.1 → 213.1	10	4	0.01	0.9904	94 (12, 10)
68	Allethrin	26.93	123.1 → 81.1	10	136.1 → 93.1	10	4	0.02	0.9987	92 (11, 14)
69	Phenthoate	27.10	274.0 → 125.0	7	274.0 → 246.0	10	4	0.05	0.9945	91 (8, 12)
70	Quinalphos	27.17	146.0 → 91.0	15	146.0 → 118.0	15	4	0.03	0.9976	90 (8, 10)

Table 2 (*continued*)

N°	Compound	Mass spectrometry settings						Validation parameters		
		RT	First transition *m/z* → *m/z*	CE (V)	Second transition *m/z* → *m/z*	CE (V)	IPs	LOQ (μg/mL)	Linearity (R^2)	Average recovery (RSD[a,b]) (%)
GC–MS/MS method										
71	Mephospholan	27.60	196.0 → 140.0	15	196.0 → 168.0	10	4	0.03	0.9995	94 (8, 7)
72	Chlordane, trans	28.04	372.8 → 265.9	15	374.8 → 267.9	16	5	0.005	0.9878	93 (9, 11)
73	Bromophos ethyl	28.07	358.9 → 302.9	20	358.9 → 330.9	10	4	0.01	0.9877	92 (4, 7)
74	Methidathion	28.23	145.0 → 58.0	15	145.0 → 85.0	10	4	0.01	0.9887	92 (9, 13)
75	Propafos	28.58	220.1 → 140.0	15	304.1 → 220.1	15	5	0.01	0.9964	77 (4, 8)
76	Tetrachlorvinphos	28.64	330.9 → 109.0	22	330.9 → 316.0	22	4	0.01	0.9819	94 (10, 8)
77	Endosulfan, alpha	28.88	195.9 → 158.9	16	195.9 → 159.9	15	4	0.01	0.9881	91 (12, 8)
78	Chlordane, cis	28.90	372.8 → 265.9	18	409.8 → 374.8	5	5	0.005	0.9883	94 (5, 9)
79	Fenamiphos	29.98	303.1 → 260.1	15	303.1 → 288.1	15	4	0.01	0.9883	94 (11, 12)
80	Dieldrin	30.87	276.9 → 206.9	20	276.9 → 240.9	10	4	0.001	0.9978	92 (4, 7)
81	Endrin	32.42	262.9 → 190.9	25	262.9 → 192.9	26	4	0.001	0.9889	93 (9, 6)
82	Isoxathion	32.47	177.0 → 130.0	15	313.0 → 177.0	15	5	0.01	0.9887	101 (6, 9)
83	Endosulfan, beta	33.50	195.9 → 158.9	16	195.9 → 159.9	15	4	0.01	0.9856	89 (11, 8)
84	Fensulfothion	33.84	293.0 → 97.0	16	293.0 → 125.0	0	4	0.01	0.9985	93 (4,11)
85	Ethion	33.96	231.0 → 175.0	15	231.0 → 203.0	15	4	0.01	0.9824	91 (14, 10)
86	Chlorthiophos	34.26	325.0 → 269.0	15	325.0 → 297.0	10	4	0.01	0.9959	101 (4, 8)
87	Sulprofos	35.31	322.0 → 139.0	15	322.0 → 156.0	15	4	0.01	0.9816	93 (5, 7)
88	Triazofos	35.55	161.0 → 105.0	13	161.0 → 134.0	10	4	0.01	0.9904	94 (9, 13)
89	Famphur	35.87	218.0 → 109.0	10	218.0 → 127.0	10	4	0.01	0.9934	97 (6, 9)
90	Carbophenothion	36.02	342.0 → 157.0	10	342.0 → 296.0	5	4	0.01	0.9813	89 (9, 6)
91	Ediphenphos	36.23	173.0 → 109.0	15	310.0 → 173.0	10	4	0.01	0.9890	88 (12, 9)
92	Endosulfan sulfate	36.38	273.9 → 236.9	10	273.9 → 239.0	15	4	0.01	0.9992	92 (6, 9)
93	DDT	36.77	234.9 → 165.0	20	234.9 → 198.9	15	4	0.002	0.9899	91 (7, 11)
94	Nuarimol	37.69	235.1 → 139.0	15	314.1 → 139.0	15	5	0.05	0.9803	90 (4, 6)
95	Resmethrin	39.00	171.1 → 128.0	9	171.1 → 143.0	9	4	0.05	0.9815	93 (5, 9)
96	Carbosulfan	39.80	163.1 → 107.1	15	163.1 → 135.1	10	4	0.01	0.9907	87 (10, 7)
97	Phosmet	40.66	160.0 → 104.0	20	160.0 → 133.0	15	4	0.01	0.9928	89 (4, 8)
98	EPN	40.75	169.0 → 77.0	16	169.0 → 141.0	10	4	0.005	0.9994	86 (6, 9)
99	Bifenthrin	40.81	181.0 → 153.0	6	181.0 → 166.0	15	4	0.04	0.9856	91 (11, 11)
100	Tebufenpyrad	41.87	333.1 → 171.1	20	333.1 → 276.1	10	4	0.04	0.9908	94 (7, 10)
101	Leptophos	42.96	374.9 → 359.9	26	376.9 → 361.9	26	5	0.01	0.9873	101 (3, 7)
102	Phosalone	43.10	182.0 → 111.0	15	182.0 → 138.0	10	4	0.02	0.9972	94 (8, 9)
103	Azinphos methyl	43.57	132.0 → 77.0	15	160.0 → 104.0	10	5	0.01	0.9948	91 (5, 9)
104	Amitraz	44.30	293.2 → 147.1	15	293.2 → 162.1	10	4	0.05	0.9982	92 (6, 4)
105	Pyrazophos	44.92	221.0 → 177.0	15	221.0 → 193.0	10	4	0.01	0.9929	94 (9, 4)
106	Azinphos ethyl	45.36	160.0 → 104.0	10	160.0 → 132.0	5	4	0.01	0.9992	91 (14, 10)
107	Cifluthrin	49.00	163.0 → 91.0	12	163.0 → 127.0	10	4	0.05	0.9836	77 (4, 8)
108	Flucythrinate	50.00	199.1 → 107.0	22	199.1 → 157.0	10	4	0.05	0.9812	79 (5, 7)
109	Deltamethrin	53.01	253.0 → 93.0	18	253.0 → 192.0	30	4	0.05	0.9801	83 (9, 13)
Internal standards										
IS1	Aldicarb-D3 (LC)	9.38	211.0 → 89.2	19	211.0 → 119.2	10	4	–	–	–
IS2	Carbofuran-D3 (LC)	10.34	225.0 → 123.1	25	225.0 → 140.5	25	4	–	–	–
IS3	Chlorfenvinphos-D10 (GC)	17.60	263.0 → 159.0	15	369.0 → 101.0	30	5	–	–	–
IS4	Chloropropham (GC)	11.32	213.0 → 127.0	15	213.0 → 171.0	10	4	–	–	–
IS5	Chlorpyrifos-D10 (GC)	23.60	197.0 → 169.0	15	362.0 → 131.0	20	5	–	–	–
IS6	Diazinon-D10 (GC)	17.81	179.1 → 137.1	15	315.0 → 170.0	20	5	–	–	–
IS7	Heptachloro epoxide *cis* (GC)	26.30	352.8 → 262.9	15	352.8 → 288.9	15	4	–	–	–
IS8	Thiobencarb (LC)	258.1	258.1 → 89.1	35	258.1 → 125.0	19	4	–	–	–

[a] Intra-day.
[b] Inter-day.

3. Results and discussion

3.1. Optimization of the instrumental method

LC–MS/MS and GC–MS/MS provided very low detection limits and could be applied to the identification and confirmation of the peak identities. Two transitions were selected for each analyte included in this study (Table 2). The combination of the transitions and their retention times allowed the pesticide identity to be confirmed.

In this work, we simultaneously investigated 90 pesticides suitable for GC (Table 2) to obtain the most efficient quantitative results with maximum separation. Additionally, we investigated 19 polar pesticides, which were separated by LC (Table 2). Although last generation QqQ analyzers permits the monitoring of co-eluted compounds with a high number of transitions simultaneously in MRM mode [18], and thus, the chromatographic separation is not always a critical stage in the development of a multi-residue method, we assayed several temperature programs (GC method), as well as various gradient programs (LC method), to achieve a good separation of the analytes. The chosen GC and LC operating conditions were those described above (Material and Methods section).

To optimize the triple quadrupole MS/MS conditions, the relevant considerations included the choices of the precursor and product ions and the optimization of the collision energies for the best response of each target compound. For the GC method, we firstly analyzed all the pesticides separately, with the aim of obtaining the full scan spectra and to select the parent ions. After that, another set of analyses was conducted at different collision energy voltages (potential on the second quadrupole) to generate the MS/MS product ions. Similarly, the parent ions described in the bibliography for each one of the 19 LC analytes (usually $M–H^+$ or $M–H^-$) were confirmed by analyzing the pesticides in separate runs to obtain their full scan spectra. Then, we selected the collision energies and MS/MS product ions after the direct infusion of a solution of each pesticide in methanol (1 μg/mL)

into the ionization source with the aid of Thermo Fisher Scientific Tune software. The collision energies (5 to 39 eV) are detailed in Table 2.

At the end of this procedure we developed two timed-MRM methods (GC–MS/MS and LC–MS/MS) with two transitions per compound (Table 2). The dwell time was adjusted so that the number of >cycles per second was 10 throughout the chromatographic run to obtain well-shaped chromatographic peaks, low detection limits, and to provide a sufficient number of chromatographic points for all compounds (>15). The peak shapes of all of the analytes in these methods were highly related to the scan time, dwell time, scan rate and number of monitored transitions [19,20]. The final MS/MS conditions used in this study are detailed in Table 2.

The concept of identification points (IPs) for the confirmation stage of mass spectrometry analysis was introduced by the European Commission Decision 2002/657/EC [21]. Meeting the requirements of this regulation, the confirmation of the pesticides included in the present protocol resulted in 4 IPs (two product ions from the same parent ion), or in 5 IPs (two product ions derived from two different parent ions). The resulting number of IPs for each pesticide is also shown in Table 2.

3.2. Optimization of sample extraction and cleanup

The liquid–liquid extraction procedure consists of shaking the samples several times in selected organic solvents to extract the pesticide residues from the bulk of the sample matrix. We considered that this method could be convenient for the extraction of pesticides from blood samples. Considering that the pesticides that can be involved in a poisoning episode can belong to different chemical classes, it is critical to select the appropriate solvents to achieve a satisfactory recovery of all of the analytes from the matrix of interest. Organic solvents such as ethanol, methanol, ethyl acetate, hexane and petroleum ether, and their mixtures, including ethanol/ethyl acetate, acetone/hexane, ethyl acetate/acetone/methanol and hexane/dichloromethane have been described for the efficient extraction of pesticides [22]. From the literature we chose various solvent mixtures that would be appropriate for pesticides included in this study, considering their range of polarities. Thus, we assayed the extraction efficacy of mixtures of acetone/hexane (50/50), ethyl acetate/acetone/acetonitrile (40/30/30) and dichloromethane/ethyl acetate/acetone (50/30/20) for all of the pesticides from fortified blood samples. The best combination of purity and recovery was obtained with the dichloromethane/ethyl acetate/acetone (50/30/20) mixture. Therefore we chose this mixture for the extraction.

According to the literature the use of sonication may improve the liquid–liquid extraction efficiency, so we assayed different times of sonication of the samples (5 to 30 min). A slight improvement in the recoveries of certain key pesticides (i.e. aldicarb, carbofuran, diazinon and methomyl) was observed with 5 min of sonication, and therefore this step was added to the extraction protocol.

We also included a cleanup step, since the liquid–liquid extraction is a non-selective method and many potentially interfering substances, such as lipids, sugars or pigments, can be co-extracted. Especially lipids should be eliminated to prevent column damage and signal reduction. There are many strategies that can be used for lipid removal: freezing centrifugation, adsorption chromatography, gel permeation chromatography, or sulfuric acid treatment, among others. We chose freezing centrifugation because blood samples yielded relatively clean extracts. Lipids possess a lower melting point than the solvent, and thus with this cleanup method, the lipids can be removed by centrifugation while pesticides remain dissolved in the solvent. According to our experiments, freezing centrifugation was an adequate single-stage cleanup method for blood samples, as it yielded extracts that were suitable for both, LC–MS/MS and GC–MS/MS analyses.

3.3. Analytical performance

After the optimization of the analytical methodology we studied the confirmation criteria, precision, linearity, method limits of quantification (LOQs) and repeatability, to evaluate its usefulness for the quantitative determination of pesticides in blood samples.

We only identified the compounds as target analytes if the chromatographic peaks satisfied all of the following criteria: 1) the retention time (tR) of the candidate was the same as that averaged plus or minus three standard deviations (SD) of the tR (tR ± 3SD) obtained when six blank samples spiked at the second level of calibration were injected, 2) there was a match with the ion ratios of the standard with a tolerance of ±30% of absolute ion abundances and 3) the S/N ratio of the target analyte was >10 for a sample extract.

To check whether there were matrix effects we carried out experiments in which blank blood samples were spiked with a mixture of the 109 analytes included in this work at two concentrations: 10.0 and 200.0 μg kg^{-1} (five replicates each). By means of the comparison between the quantifications of the recovered pesticides with those of the same concentrations of pesticides in dissolvent we calculated the recovery percentages (Table 2). The results ranged from 68% to 105%, with most of the recoveries being greater than 85% at both concentrations. As the most unfavorable RSD was below 15%, we also found that the precision was satisfactory. The inter-day measurement (recoveries and precision during five consecutive days) also yielded an RSD that was below 15%. Table 2 shows that all results were within the acceptable range and the methods were precise, with RSD values of 3.0–18.0% for all pesticides.

The quantifications were done against matrix-matched calibration curves, ranging between 0.5 and 500 μg kg^{-1} (three replicates for each level were analyzed). Calculations were performed using the peak areas. The calibration curves were constructed without including the origin point and were found to have good linearity based on the correlation coefficients (r^2), which were greater than 0.9801 for all analyses. After performing the residual analysis (values within the range of −10.618 to 11.337) we concluded that the linear regression method may be used for quantifications within the range investigated.

3.4. Application to real samples

The validated method was applied to the analysis of real samples from two recent cases (May and June of 2013) of pesticide poisoning that were received in the toxicology service of the Institute of Legal Medicine of Las Palmas (Canary Islands, Spain).

3.4.1. Case 1

A 79-year-old man was found dead by a friend who went to his house, as the man did not answer the phone. Upon entering he detected a strong odor of "chemical" and found the man on the couch with a belt tied around his neck. According to statements by the sister of the deceased, he lived alone and was being treated for prostate disease and depression. She also reports that he had attempted suicide twice, and for this reason he was being treated in the Mental Health Unit of his area. At autopsy, the remarkable features were: edematous and emphysematous lungs; bloody fluid from the parenchymal cut; presence of yellowish white mucus in the bronchial tubes and trachea; the liver appeared congested; the stomach contained a clear liquid with strong solvent odor, and walls with signs of erosive gastritis; and erosions were also observed in the esophageal mucosa. Gastric content and blood samples were submitted to our laboratory for toxicological analysis. The results of abuse of drugs and alcohol were negative. When we applied the protocol described in this paper to the blood sample in the GC–MS/MS analysis we found the organophosphate insecticide diazinon at a concentration of 6.48 μg/mL (Fig. 1A), which is more than six times higher than the value described by Repetto and Repetto (2007) in a previous fatality [15]. The proposed methodology was also applied to the gastric content sample. We first homogenized the sample and diluted it with ultrapure water (1:10), and we also performed an additional centrifugation in the clean-up step. In Fig. 1B

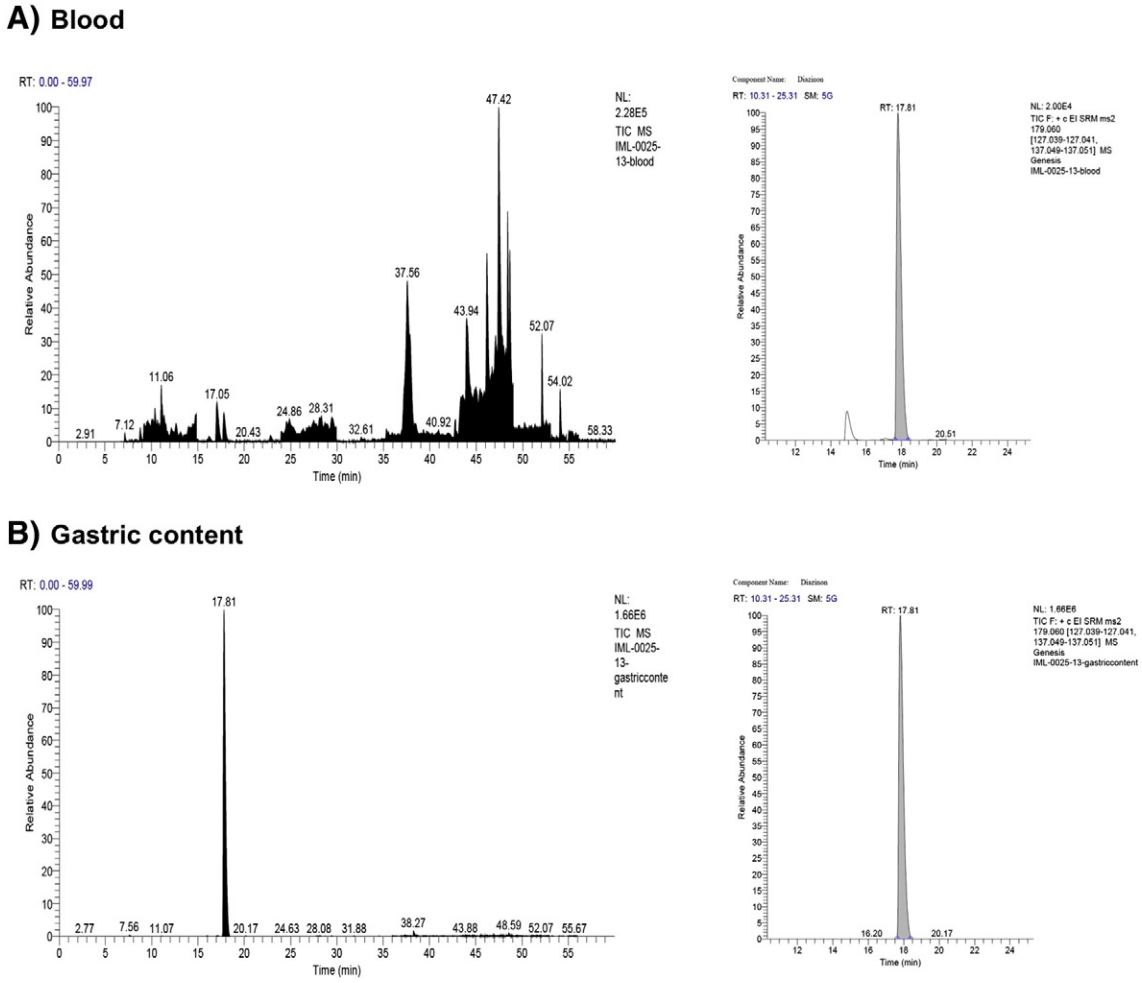

Fig. 1. (A) *Left*, GC–MS/MS total ion current chromatogram (TIC) of blood sample from case 1; *right*. Extracted chromatogram of this sample. Diazinon concentration was 6.48 μg/mL. (B) *Left*, TIC of gastric content sample from case 1. *Right*, extracted MRM chromatogram of this sample, showing the identification of diazinon.

we show the raw and the filter-extracted chromatograms that we obtained from this sample, in which we clearly identified the diazinon.

3.4.2. Case 2

A 63 year old woman was taken to the hospital after eating three tablespoons of vegetable soup that her husband had prepared for her. She did not eat more because "the soup tasted like dirt". According to her own statement, she soon vomited at home and felt strong nausea and abdominal pain. Her son took her to the hospital, and on admission to ICU she displayed marked cholinergic symptoms and was semiconscious. The patient was given pralidoxime and atropine. Her condition gradually improved on days 2 and 3 and she was discharged at 87 h after admission. A blood sample that was taken on admission was submitted to the laboratory and the described methodology was applied. In LC–ESI–MS/MS analysis the carbamate insecticide aldicarb was detected at a concentration of 2.32 μg/mL (Fig. 2). Days later the police brought to our laboratory a plastic container containing the remains of the soup, which had been located by the son in a dumpster quite out of the marital home. According to the police report, the son suspected of an attempted homicide by her father because of his strange behavior and bad relationship and frequent quarrels they had. By visual examination the soup showed abundant black colored granules (Fig. 3). One gram of this material was diluted in 10 mL of ultrapure water and subjected to the same method of extraction and chromatographic analysis and the presence of aldicarb was confirmed.

3.5. Limitations of the methodology

The proposed methodology has been successfully applied to the identification of pesticides in samples from real human poisoning episodes, allowing their quantification in the case of blood samples. Nevertheless, in spite of being quick, easy and very useful, it should be noted that this methodology has several limitations, such as the use of large amounts of expensive and hazardous organic solvents; the necessity of evaporation of solvents, which is a source of environmental contamination; the multiple steps that suppose a risk of analyte losses; and that very relevant pesticides could not be included (i.e. strychnine, paraquat, alpha-chloralose), since they are chemically very different from the rest, so that additional analysis should be specifically targeted to the identification of these compounds in particular. Besides, the whole procedure is time consuming, and because of the nature of the samples and the extraction and cleanup the methodology cannot be automated and therefore costs remain high.

4. Conclusions

We have shown in this paper the applicability of a methodology based on a liquid–liquid extraction followed by a combination of two chromatographic methods (LC and GC) with mass spectrometry detection for the identification and quantification of 109 toxic pesticides in blood samples from human pesticide poisoning episodes. The validation parameters were satisfactory. For all the pesticides we found good

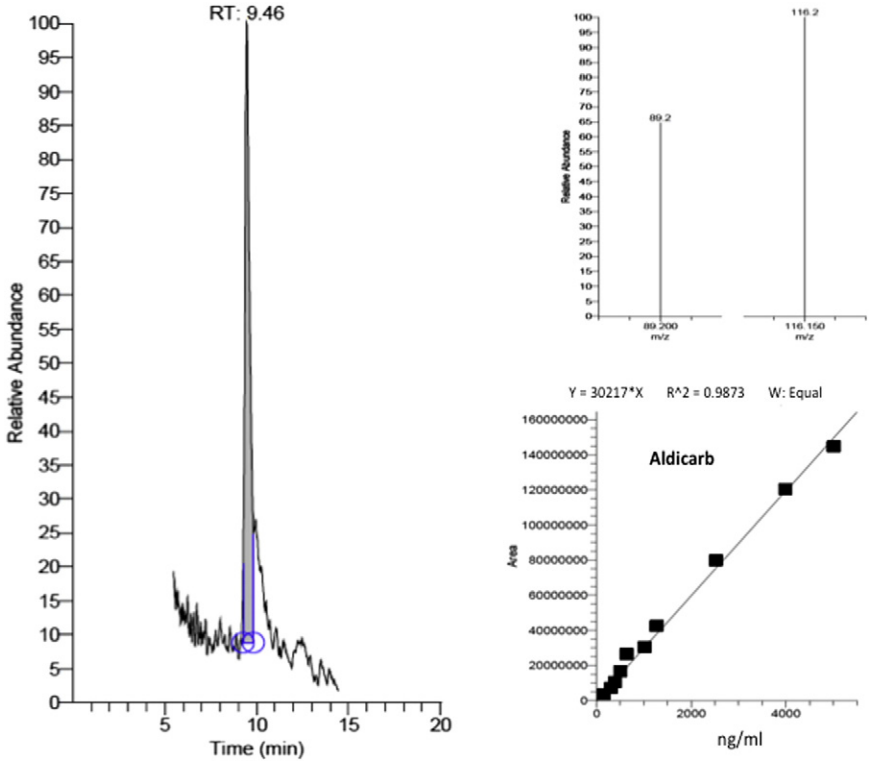

Fig. 2. *Left*, LC–MS/MS extracted MRM chromatogram of the blood sample from case 2; *right*, ion ratio, and calibration plot of aldicarb. Concentration was 2.32 μg/mL.

linearity (0.5–500 μg/mL, with $r^2 > 0.98$) and low detectability. The recoveries (68 to 105%) were good, and the precision (RSD < 15%) was acceptable. Thus we conclude that this methodology, which is simple, sensitive, and very reliable, may be recommended for its routine application in forensic toxicology laboratories. The applicability of the optimized method was proven in the analysis of samples from two recent poisoning cases. Our results also showed that this methodology is robust enough to be applied to samples different from blood, because the involved pesticide was also identified in gastric contents and food samples.

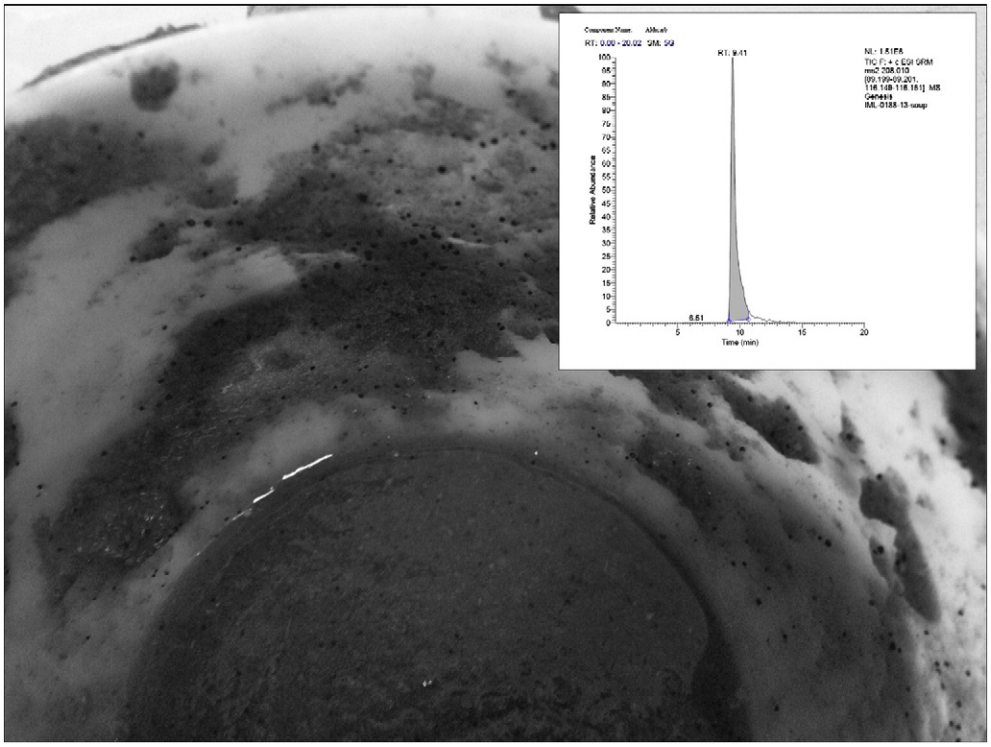

Fig. 3. Vegetable soup showing abundant black colored granules, which were positively identified as aldicarb.

Conflict of interest

There are no financial or other relations that could lead to a conflict of interest.

Acknowledgments

The authors would like to thank to Mrs. María de los Reyes Suárez Hanna and to Dr. Esmeralda Romero-Covo for their technical assistance.

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BLOQUE B

CONTAMINACIÓN UBICUA

Resumen

En este bloque se presentan aquellos trabajos destinados a la evaluación de varios contaminantes tóxicos ubicuos en fauna doméstica y silvestre.

El primero de ellos trata de verificar la hipótesis de si los perros son buenos indicadores de la exposición humana a COPs a través de la medición de 11 POCs, 18 PCBs y 27 PAHs en el plasma de 87 perros y en el plasma de 100 personas. Los resultados advierten diferencias entre ambas especies en lo que se refiere a frecuencias de detección y concentraciones, en especial en el grupo de POCs y PCBs. Por lo que aunque humanos y perros compartan el mismo hábitat, tanto la exposición como metabolización pueden ser lo suficientemente diferentes para que estos no sirvan como centinelas de la exposición humana a este tipo de sustancias.

A la vista de los anteriores resultados, el siguiente paso a dar era comprobar la exposición a través del consumo de alimento que sufrían no sólo perros sino gatos también. Para ello se determinó la presencia de 19 POCs, 18 PCBs y 16 PAHs, en varias marcas comerciales de comida de ambas especies. De este modo se pudo calcular la ingesta de dichos contaminantes. Cabe resaltar que en el caso de la comida para perros los resultados doblaban a los obtenidos para la comida de gatos: ΣPOCs: 233.1 vs. 83 (ng/kg pv/día), ΣPCBs: 101.8 vs. 43.8 (ng/kg pv/día) y ΣPAHs: 274.8 vs. 141.8 (ng/kg pv/día). De forma paralela, estos mismos compuestos fueron analizados en el plasma de 42 perros y 35 gatos. El plasma de perro mostró mayor concentración de

PAHs que el de los gatos, resultado esperable al ver los datos de exposición, pero inferiores en cuanto a POCs y PCBs, lo que denota una menor capacidad de bioacumulación o una mayor capacidad de metabolización.

El último de los estudios presente en este bloque, trata de comprobar la presencia y concentraciones de 23 POCs, 18 PCBs y 16 PAHs en los hígados de aves rapaces. Y de si estas pueden actuar como bioindicadores para el monitoreo de la contaminación del medioambiente en la región. De todas las especies estudiadas fueron el gavilán (*Accipiter nisus*), el halcón (*Falco pelegrinoides*) y el cernícalo (*Falco tinnunculus*) las que mayor contaminación presentaron. En el caso de lo POCs, los patrones de contaminación fueron bastantes similares entre especies, siendo el DDT y sus metabolitos los más abundantes. La contaminación por dichos compuestos al igual que por dieldrín, se considera alta y consecuentes con las halladas por nuestro grupo de investigación en otras muestras provenientes de humana, alimentos u otros animales. Por el contrario, la contaminación por PCBs y PAHs fue baja.

Journal of Applied Animal Research, 2016
Vol. 44, No. 1, 135–145, http://dx.doi.org/10.1080/09712119.2015.1021808

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Are pet dogs good sentinels of human exposure to environmental polycyclic aromatic hydrocarbons, organochlorine pesticides and polychlorinated biphenyls?

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(*Received 26 August 2014; accepted 13 February 2015*)

Several studies have shown that pet dogs and cats, since they share the habitat with people, could be good sentinels for human exposure to environmental pollutants. However, some publications have suggested that dogs could efficiently metabolize and eliminate some persistent organic pollutants (POPs), which are accumulated by humans throughout life. For this reason, the role of domestic dogs as sentinels could not be appropriate, at least for certain contaminants. To test this hypothesis, we designed this study in which we determined plasma levels of 56 POPs (11 organochlorine pesticides [OCPs], 18 polychlorinated biphenyls [PCBs] and 27 polycyclic aromatic hydrocarbons) in the plasma of 87 dogs and 100 people from the same area (the Canary Islands, Spain). We detected most of these contaminants in the plasma of both species, although the frequencies of detection, concentrations and profiles were very different from each other, especially for ∑OCPs and ∑PCBs. In light of these results, we can conclude that, although they share the environment, sources of exposure to these pollutants and/or the metabolic capabilities do not seem to be comparable between the two species, so that the dog does not seem to be a good sentinel for human exposure to these contaminants.

Keywords: organochlorine pesticides; polychlorinated biphenyls; polycyclic aromatic hydrocarbons; dogs; sentinels

1. Introduction

Since the iconic 'canary in the cage' began to be used to detect the presence of toxic gases in the coal mines, the potential for all kinds of animals to act as sentinels for human exposure to toxic substances has been explored in many scientific papers (Reif 2011). The pets, particularly the dogs and cats, are of particular interest, given that they share the habitat with humans and they respond to toxic assaults similar to their owners (Backer et al. 2001). Thus, many studies have been designed to reveal the shared exposure of humans and dog/cats to different environmental toxicants of anthropogenic origin, such as asbestos (Glickman et al. 1983; Backer et al. 2001), tobacco smoke (Ka et al. 2014), industrial pollutants (Maciejewski et al. 2008; Bischoff et al. 2010), pesticides (Knapp et al. 2013) or smog (Heyder & Takenaka 1996; Calderon-Garciduenas et al. 2001), among others. Most of these studies have found associations between exposure and elevation of the selected biomarkers, as well as correlations between the incidence of diseases related to exposure to such pollutants between pets and humans. It is noteworthy, however, that the majority of these studies have focused on pollutants whose primary route of exposure is by inhalation. However, it has been widely reported that the main route of exposure of many other environmental chemicals, such as persistent organic pollutants (POPs), is the ingestion of contaminated food, in which these contaminants are particularly associated with the fat fraction (El-Shahawi et al. 2010). This is relevant since pets, while sharing the habitat with their owners, currently can be said that mostly do not share the dietary pattern with humans, because most domestic dogs and cats are fed specially formulated feed (Rumbeiha & Morrison 2011).

This group of food-associated contaminants – the POPs – is constituted by a high variety of synthetic, lipophilic, persistent and cumulative substances (Safe 1990). Many POPs come from the production and use of organochlorine compounds, such as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants containing two or more fused benzene rings that are produced during the incomplete combustion of organic matter and during human or industrial activities (Guo et al. 2012). Because of their efficient metabolization, strictly speaking, PAHs cannot be considered as POPs, but due to their high prevalence in the environment and their lipophilicity, PAHs are usually considered as POPs (Lammel et al. 2013). Numerous studies have revealed that POPs, individually and in combination, may contribute to the development of severe health problems such as immune suppression, genotoxic effects or cancer and are of

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public health concern worldwide (WHO 2003; Kortenkamp 2007; Kortenkamp et al. 2007, 2011; Valeron et al. 2009; Lauby-Secretan et al. 2013; Jarvis et al. 2014). As far as we know, there is no research that has assessed exposure to PAHs in dogs to date, and very few scientific studies have evaluated organochlorine contamination of dogs and cats (Kunisue et al. 2005; Storelli et al. 2009; Ali et al. 2013; Dirtu et al. 2013). These studies have indicated that dogs, as occurs with other canines (Georgii et al. 1994; Shore et al. 2001), accumulated minor amounts of some of these compounds (particularly DDT and its metabolites, and the highly chlorinated biphenyls) than other mammalian species. Therefore, it has been suggested that the canines may have greater metabolic capacity and elimination of these compounds than other mammalian species (Georgii et al. 1994; Kunisue et al. 2005).

In light of the above, the domestic dog does not seem to be a good sentinel for human exposure to PCBs and some OCPs. To test this hypothesis, and also to assess the role of the dog as monitor of human exposure to PAHs, we designed the present study. We have determined the plasmatic levels of 56 POPs (11 OCPs and metabolites, 18 PCBs, and 27 PAHs and metabolites) in dog (n = 87) and human (n = 100) plasma samples. Sampling was performed in the Canary Islands (Spain), a well-studied area regarding the levels of these pollutants (Zumbado et al. 2005; Luzardo et al. 2009, 2014; Henríquez-Hernández et al. 2011; Almeida-Gonzalez et al. 2012; Boada et al. 2012; Luzardo, Rodriguez-Hernandez, et al. 2013; Burillo-Putze et al. 2014; Rodríguez-Hernández et al. 2014;), and all the samples were analysed under the same instrumental conditions. The normalized results of both species were statistically analysed and compared with each other.

2. Materials and methods

2.1. Sampling and ethics statement

Dog blood samples were collected between November 2013 and March 2014 through cephalic vein puncture from 87 pet dogs (46 males and 41 females, 0.5–13 years old) visiting the Veterinary Hospital of the Veterinary Faculty of the University of Las Palmas de Gran Canaria (Canary Islands, Spain) for routine care. Only clinically normal animals (negative stool sample, negative result on a heart-worm test and no overt disease) were included in the study, after owners' consent. Human blood samples from 60 males and 40 females (19–34 years old) were collected from the Spanish Red Cross blood bank from October to December 2013. All participants were residents in Gran Canaria (Canary Island, Spain) and determined to be eligible as blood donors based on the screening by nurses before recruitment. All participants provided their written informed consent to participate in this study. The study was approved by the Research and Ethics Committee of our Institution (University of Las Palmas de Gran Canaria, Canary Islands, Spain). Samples of blood were collected in heparinized tubes, maintained at 4°C, and centrifuged at 1000 g for 15 min to separate the plasma and kept at − 20°C until chemical analysis. Although for logistical reasons we could not get blood from the owners of the dogs included in this study, it has been estimated that there are about six million domestic dogs in Spain and that more than 40% of Spanish homes have at least one dog, so we can assume that a high percentage of these blood donors share habitat with some dog.

2.2. Analytical chemical methods

We measured the serum levels of 11 OCPs: p,p′-Dichlorodiphenyltrichloroethane (p,p′ -DDT), p,p′-Dichlorodiphenyldichloroethylene (p,p′ -DDE), p,p′-Dichlorodiphenyldichloroethane (p,p′ -DDD), hexachlorobenzene (HCB), hexachlorocyclohexane (α-, β-, γ- and δ-HCH), aldrin, endrin and dieldrin. We also determined 18 PCB congeners, including marker-PCBs (M-PCBs) and dioxin-like PCBs (DL-PCBs): IUPAC numbers # 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180 and 189. We also included 21 PAHs listed in the Toxics Release Inventory Program of the United States and the Environmental Protection Agency's (EPA) Priority Chemical list (EPA 2001): benzo(a)anthracene, benzo(a)phenanthrene (chrysene), benzo(a)pyrene, benzo(b)fluoranthene, benzo(j) fluoranthene, benzo(k)fluoranthene, benzo(j,k)fluorene (fluoranthene), dibenzo(a,h)anthracene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,l)pyrene, indeno(1,2,3-cd) pyrene, 5-methylchrysene, acenaphthene, acenaphthylene, anthracene, benzo(g,h,i)perylene, fluorene, phenanthrene, pyrene and naphthalene. Finally, we also determined the levels of six common metabolites of PAHs: 1-naphthol, 2-naphthol, 2-OH-fluorene, 1-OH-phenanthrene, 7-OH-benzo (c)fluorene, and 1-OH-pyrene.

The details of sample extraction, validated chromatographic method and quality control have been previously reported (Camacho, Boada, et al. 2013; Camacho, Luzardo, et al. 2013; Luzardo, Ruiz-Suarez, et al. 2013; Camacho et al. 2014). Briefly, samples were subjected to solid-phase extraction using Chromabond® C18ec columns (Macherey-Nagel, Germany) that yielded recoveries in the range of 79%–107%. Without further purification steps, the samples were subjected to chromatographic analysis on a Trace GC Ultra coupled with a Quantum Max triple quadrupole mass spectrometer (Thermo Fisher Scientific, Palo Alto, CA, USA) for the quantification of 56 analytes in addition to internal standards and surrogates. The limit of quantification (LOQ) was set at 5 pg/mL for all the compounds. A zero value was assigned to all the compounds below the limit of detection, and those compounds below the LOQ were assigned

half of the LOQ. The total cholesterol and triglyceride concentrations were used for the lipid adjustment of results of organochlorine compounds, as recommended (Bernert et al. 2007). Therefore, the results have been expressed in ng/g lipid weight (lw).

2.3. Statistical analysis

We used PASW Statistics v 19.0 (SPSS Inc., Chicago, IL, USA) to manage the database of the study and to perform statistical analyses. Normality was examined using the Kolmogorov–Smirnov test. The POPs distributions lacked normality and homoscedasticity; therefore, we used non-parametric tests (the Mann–Whitney and Kruskal–Wallis tests). We used the chi-square test to examine the relationships between the categorical variables. The results were reported as medians and ranges (or interquartile ranges). Probability levels of less than 0.05 (two-tailed) were considered statistically significant.

3. Results and discussion

3.1. Concentrations of PAHs in dog and human plasma

All PAHs included in this study were detected in this series of samples (humans and dogs), with the exception of benzo(a)pyrene, which was not detected in none of the dog plasma samples. The compounds most frequently detected in both species were phenanthrene, fluorene and fluoranthene, and 2-naphthol, which were present in nearly 100% of the samples. The frequencies of detection of the rest of compounds of this chemical group were highly variable and different between the two species, as can be seen in Table 1.

We also show in Table 1 the fat-normalized median values, ranges and means plus standard deviations (SDs) of the 21 PAH and 6 PAH metabolites in dog and human plasma, respectively. The median values of ∑PAH21 were 747.6 and 1404.1 ng/g lw (0.87 and 1.71 ng/g lw), for dogs and humans, respectively. When we considered only those PAHs that are classified as probable human carcinogens by the US EPA (PAH7, benzo(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, benzo (k)fluoranthene, dibenzo(a,h)anthracene, and indeno (1,2,3-c,d)pyrene) the median (and mean ± SD) values were 0 (6.8 ± 17.2) ng/g lw in dog plasma and 10.4 (21.9 ± 43.5) ng/g lw in human plasma. We consider that it is also interesting to highlight the results of the sum of PAH metabolites in plasma samples, whose median values were 144.8 and 93.7 ng/g lw in dogs and humans, respectively. As far as we know, there is no study that has reported plasma levels of PAHs in dogs, and we can only compare the results with other studies in humans. Thus, the levels found in human plasma are comparable to those found in cord blood in Hong Kong in 2005 (1158 ng/g lw) (Tsang et al. 2011) and in China in 2011 (1370 ng/g lw) (Yu et al. 2011), but much lower than those found in maternal and cord blood in Texas, USA (5.5 and 12.8 ng/g ww) (Sexton et al. 2011). In any case, it should be noted that these comparisons are not totally reliable since in these papers a different number of compounds were included, with 15 analytes in the works of Hong Kong and China and 50 individual PAHs in the work of Texas. However, if we compare only the values of the most toxic PAHs (PAH7) with those reported in the literature, we found that levels of this study were much lower than those described in China (Yu et al. 2011; Guo et al. 2012; Song et al. 2013) and Hong Kong (Tsang et al. 2011).

However, our main objective in this study was the comparison of the levels and detection frequencies of pollutants between the two species, and in this sense, we have found many significant differences. So, we found remarkable differences for both PAH21 (Figure 1a) and for PAH7 (Figure 1a, inset) levels, which were detected at much higher levels in humans than in dogs ($p < .0001$). These results suggest that exposure of both species to this contaminant group could be different. However, it also seems quite remarkable that for metabolites of PAHs, the relationship was the opposite, plasma levels of these chemicals being significantly higher in dogs than in humans (Figure 1b), which could also indicate that dogs possess superior ability to metabolize these compounds than humans. Obviously, to confirm this point additional research is needed, but our results allow us to hypothesize that the lower levels of PAHs detected in the plasma of dogs could be due to a higher rate of biotransformation and elimination thereof.

Furthermore, as shown in Figure 1c, the profiles of PAH contamination were not similar between dogs and humans, with a clear predominance of the four ring compounds in human and three ring compounds in dogs. In fact, as seen in Table 1, it is noteworthy that some compounds such as pyrene, which were detected in almost 100% of the human samples, were barely detectable in 17% of samples from dogs, and yet, others such as acenaphthylene or 1-naphthol were much more frequently detected in dog plasma than in human plasma. Thus, in the light of the above discussion, we can conclude that the dog does not seem to be a good sentinel for human exposure to PAHs.

3.2. Concentrations of OCPs in dog and human plasma

In this study we included only a selection of 11 OCPs, among the most frequently detected in environmental samples, and as it was expected, all of them were detected in plasma samples of both dog and humans. However, for many of them, we found significant differences in both the frequency of detection and, above all, in concentrations. In Table 2 and in Figure 2, we summarize the results of this group of contaminants. It is

Table 1. Individual PAHs, PAH metabolites, ∑PAH7 and ∑PAH21 concentrations (ng/g lw) in dog (n = 87) and human (n = 100) plasma samples from the Canary Islands, Spain.

	Dog plasma			Human plasma			
	Mean ± SD	Median (range)	Frequency (%)	Mean ± SD	Median (range)	Frequency (%)	Mann–Whitney test (p)
Carcinogenic PAHs (PAH7, EPA, 2001)							
Benzo(a)anthracene	4.2 ± 5.1	0 (0–17.2)	12.6	6.0 ± 17.9	0 (0–127.6)	12.0	.9728
Benzo(a)phenanthrene (chrysene)	5.4 ± 7.3	0 (0–51.6)	10.3	4.4 ± 17.1	0 (0–110.9)	13.0	.7437
Benzo(a)pyrene	n.d.	n.d.	0.0	4.4 ± 8.5	0 (0–51.1)	8.0	–
Benzo(b)fluoranthene	4.6 ± 5.4	0 (0–42.5)	6.9	4.7 ± 9.2	0 (0–68.2)	10.0	.6995
Benzo(k)fluoranthene	4.3 ± 6.9	0 (0–35.7)	11.9	**17.9 ± 34.1**	**0 (0–246.9)**	**36.0**	.0015**
Dibenzo(a,h)anthracene	4.9 ± 6.1	0 (0–10.4)	2.3	4.43 ± 7.8	0 (0–51.1)	6.0	.5999
Indeno(1,2,3-cd)pyrene	5.2 ± 7.4	0 (0–12.3)	2.3	5.2 ± 4.4	0 (0–34.8)	3.0	.8955
∑PAH7	6.8 ± 17.2	0 (0–78.2)	53.2	**21.9 ± 43.5**	**10.4 (0–25.3)**	**78.0**	.01**
Other priority PAHs (EPA, 2001)							
Benzo(j)fluoranthene	4.5 ± 5.6	0 (0–27.2)	7.9	**11.7 ± 3.7**	**0 (0–101.8)**	**14.0**	.0056**
Benzo(j,k)fluorene (fluoranthene)	6.6 ± 4.3	35.1 (0–195.5)	97.7	**77.5 ± 26.4**	**70.1 (0–197.5)**	**99.0**	<.0001***
Dibenzo(a,e)pyrene	5.2 ± 5.6	0 (0–10.5)	1.2	4.3 ± 5.3	0 (0–17.8)	5.0	.6046
Dibenzo(a,h)pyrene	6.3 ± 7.4	0 (0–17.5)	2.3	4.5 ± 5.8	0 (0–17.1)	6.0	.7145
Dibenzo(a,l)pyrene	4.4 ± 6.1	0 (0–13.5)	1.2	6.2 ± 5.9	0 (0–18.3)	5.0	.5099
5-Methylchrysene	6.3 ± 6.1	0 (0–34.2)	12.6	7.6 ± 17.9	0 (0–127.1)	14.0	.7855
Acenaphthene	7.6 ± 25.5	0 (0–104.7)	13.8	8.7 ± 19. 2	0 (0–69.2)	16.0	.7911
Acenaphthylene	**51.2 ± 34.4**	**52.1 (0–152.9)**	**75.8**	12.5 ± 6.3	0 (0–16.8)	6.0	<.0001***
Anthracene	4.7 ± 26.2	0 (0–178.7)	4.6	6.8 ± 34.1	0 (0–35.5)	10.0	.4671
Benzo(g,h,i)perylene	n.d.	n.d.	0.0	4.5 ± 5.2	0 (0–42.1)	3.0	–
Fluorene	**76.9 ± 42.5**	**68.1 (0–212.8)**	**98.8**	42.5 ± 17.2	33.7 (0–144.7)	98.0	<.0001***
Phenanthrene	**382.5 ± 0.21**	**332.7 (153.6–1175.8)**	**100.0**	313.3 ± 137.5	307.5 (0–1007.7)	100.0	<.0001***
Pyrene	7.6 ± 17.2	0 (0–85.7)	17.2	**43.7 ± 25.9**	**48.9 (0–213.5)**	**94.0**	<.0001***
Naphthalene	34.1 ± 51.6	0 (0–220.1)	28.7	34.1 ± 59.6	0 (0–161.1)	41.0	.3100
PAH metabolites (m-PAHs)							
1-Naphthol	**76.9 ± 69.8**	**42.3 (0–288.7)**	**79.3**	5.2 ± 17.2	0 (0–165.4)	8.0	<.0001***
2-Naphthol	**95.1 ± 52.6**	**86.6 (0–356.7)**	**96.6**	67.2 ± 23.5	59.4 (0–306.3)	98.0	<.0001***
2-OH-fluorene	5.8 ± 8.5	0 (0–85.7)	11.5	7.9 ± 12.3	0 (0–213.9)	21.0	.1623
1-OH-phenanthrene	**17.4 ± 35.6**	**0 (0–177.7)**	**23.5**	4.2 ± 6.6	0 (0–43.2)	10.0	.0459*
7-OH-benzo(c)fluorene	**51.4 ± 8.5**	**0 (0–34.2)**	**36.8**	4.5 ± 8.5	0 (0–51.4)	12.0	.0089**
1-OH-pyrene	4.5 ± 17.2	0 (0–136.1)	14.9	17.0 ± 43.5	0 (0–93.5)	6.0	.4185
∑PAH21	782.2 ± 323.8	747.6 (272.1–2069.1)	100.0	**1623.5 ± 799.2**	**1404.1 (0–5454.9)**	**100.0**	<.0001***
∑m-PAH	**198.1 ± 110.5**	**144.8 (10.2–468.2)**	**100.0**	131.6 ± 148.5	93.7 (0–973. 4)	100.0	<.0001***

Bold values indicate those values which are significantly higher.
Note: ∑PAH7: sum of carcinogenic PAHs . ∑PAH21: sum of 21 priority PAHs (EPA, 2001). n.d., not determined.

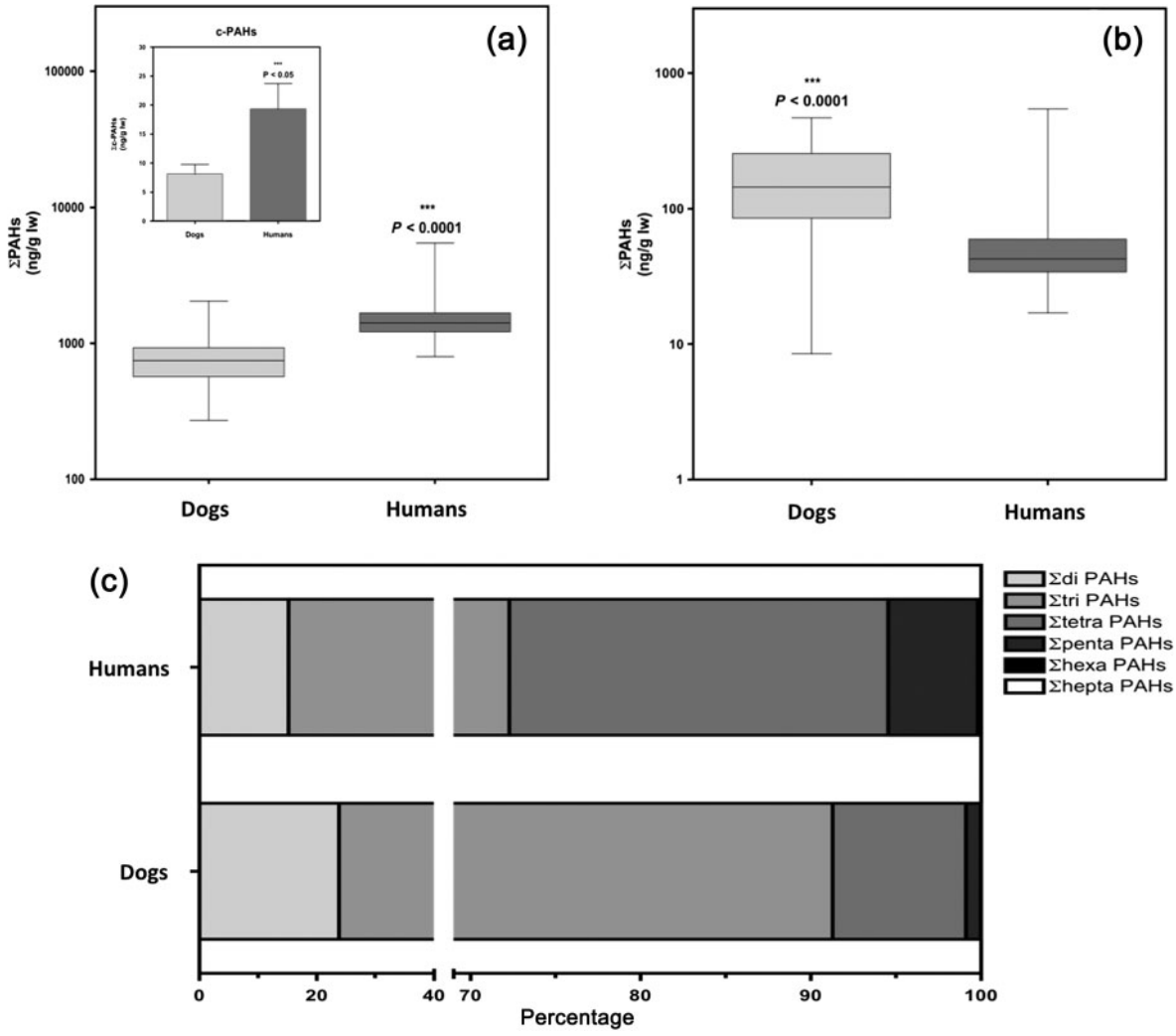

Figure 1. Levels of PAHs in plasma samples. (a) Main body: Box plots of ∑PAH21 in dogs and humans. Inset: Bar graph of ∑PAH7 (carcinogenic PAHs, median and interquartile range) in dogs and humans. (b) Box plots of ∑PAH metabolites in dog and humans. (c) Profile of distribution of PAHs in dogs and humans. The line inside the boxes represents the median, the bottom and top of the boxes are the first and third quartiles of the distribution and the lines extending vertically from the boxes indicate the variability outside the upper and lower quartiles.

noteworthy that the levels of the sum of OCPs was an order of magnitude higher in humans than in dogs (Figure 2a), although the differences were not homogeneous among the different pollutants in this group.

Thus, the OCP that was detected more frequently and at higher concentrations in canine plasma was HCB, as was detected in 96.5%. This detection rate was similar to that found in human plasma (98%), but the levels were higher in the latter (median 0.2 vs. 10.1 ng/g lw, $p < .01$). It is also noteworthy that the concentrations of this pollutant in dogs accounted for over 60% of all pollutants in this group (Figure 2b), whereas in humans represented only 2.5% of ∑OCPs. There are very few references that have reported the levels of this pollutant in dogs, and, as far as we know, none in plasma. However, the results reported here are comparable in concentration to those described in the livers from Russian wolves, although the detection rate was considerably lower in those animals (3.5% vs. 96.5%, respectively, in wolves and dogs) (Shore et al. 2001). Our results, however, were much higher than those described in gonadal tissues of domestic dogs in Japan in 2005 (Kunisue et al. 2005), although this comparison may not be appropriate, since it has been reported that HCB in dogs is distributed effectively firstly to the lungs, and later to other visceral organs (mainly liver, kidneys and spleen), and afterwards, much more slowly, to fatty tissues and other target tissues, where this pollutant reaches low concentrations (Sundlof et al. 1982). On the other hand, the plasma levels of HCB in humans of this

Table 2. OCP concentrations (ng/g lw) in dog (n = 87) and human (n = 100) plasma samples from the Canary Islands, Spain.

	Dog plasma			Human plasma			
	Mean ± SD	Median (range)	Frequency (%)	Mean ± SD	Median (range)	Frequency (%)	Mann–Whitney test (p)
HCB	21.8 ± 34.9	0.2 (0–204.1)	96.5	**14.8 ± 10.7**	**10.1 (0–138.2)**	**98.0**	<.01***
∑HCH	11.8 ± 40.8	0 (0–238.0)	27.6	**27.6 ± 79.6**	**6.7 (0–722.5)**	**79.0**	<.0001***
α-HH	0.2 ± 1.3	0 (0–8.5)	2.3	0.6 ± 2.5	0 (0–17.2)	6.0	.6415
β-HCH	10.3 ± 40.7	0 (0–238.1)	13.8	**20.7 ± 68.8**	**0 (0–620.5)**	**60.0**	<.0001***
γ-HCH	3.2 ± 12.1	0 (0–74.3)	5.9	**6.2 ± 21.9**	**0 (0–192.7)**	**48.0**	<.0001***
δ-HCH	0.5 ± 2.1	0 (0–7.9)	5.3	4.8 ± 17.1	0 (0–119.1)	21.0	.0564
∑DDT	1.8 ± 4.7	0 (0–25.6)	17.2	**559.6 ± 642.5**	**361.3 (23.1 ± 4420.7)**	**100.0**	<.0001***
p,p′ -DDT	0.7 ± 2.3	0 (0–6.9)	8.0	**9.8 ± 24.4**	**8.4 (0–212.5)**	**52.0**	<.0001***
p,p′ -DDE	0.2 ± 1.3	0 (0–8.4)	2.3	**542.3 ± 635.2**	**352.8 (23.1 ± 4412.2)**	**100.0**	<.0001***
p,p′ -DDD	0.4 ± 1.8	0 (0–6.7)	4.6	**6.9 ± 19.7**	**0 (0–136.1)**	**34.0**	<.0001***
∑Cyclodienes	14.8 ± 21.0	8.2 (0–153.2)	80.5	**48.3 ± 41.7**	**38.2 (0–170.3)**	**93.0**	<.0001***
Aldrin	0.8 ± 2.8	0 (0–17.1)	8.0	1.3 ± 4.0	0 (0–25.5)	11.0	.7032
Dieldrin	13.2 ± 21.3	8.2 (0–153.2)	71.3	**46.2 ± 40.4**	**34.1 (0–161.5)**	**91.0**	<.0001***
Endrin	0.5 ± 2.0	0 (0–9.3)	2.3	4.8 ± 17.2	0 (0–119.2)	7.0	.0564
∑OCPs	75.6 ± 52.7	59.5 (6.7–246.5)	100.0	**724 ± 499.6**	**486.5 (23.1–5540.5)**	**100.0**	<.0001***

Bold values indicate those values which are significantly higher.

study were fully comparable with those described recently in other European countries (Koppen et al. 2009; Mari et al. 2013).

With regard to plasma concentrations of OCPs in dogs, the most important group was that of cyclodienes, mainly dieldrin (median 8.2 ng/g lw), representing 20.1% of total OCPs detected in this species (Figure 2b). However, both the frequency and the plasma levels of this pollutant were significantly lower than those detected in human plasma (91%, 34.1 ng/g lw, $p < .0001$). The other two cyclodienes included in this study, aldrin and endrin, were also detected in both types of samples, albeit at very low concentrations, without significant differences, neither in their concentrations nor in their detection frequencies between the two species. The values and the frequency of detection of dieldrin described for dogs of this study are very similar to those described in livers of Russian wolves in 2001 (67.2%, 13.1 ng/g lw) (Shore et al. 2001). We do not have further information of other works that have been published the levels of these cyclodienes in dogs or other canines. With respect to the values detected in humans in this study, we found that the profile was somewhat different to that previously described in a population sample taken in this same region in 1998, as now we have found higher levels of dieldrin and lower levels of aldrin and endrin in people from the Canary Islands (Luzardo et al. 2006).

The second set of most relevant pollutants in terms of concentrations in canine plasma was formed by the isomers of HCH, which represented 16% of total OCPs in this species (Figure 2b), whereas in humans only represents 1.7%. The four isomers were detected in both species, but at very low concentrations (the median value was 0 ng/g lw in all cases). The mean values of this group of pollutants in dogs were higher than those reported in Pakistan in 2013 (Ali et al. 2013), but the median values were much lower, indicating that there was a high dispersion of the concentrations. However, it should be noted that the sample sizes of both studies were very different, being much higher in this study (n = 87 vs. 16), so probably the median value of this study more accurately reflects the actual level of contamination in this species. Still, because the detection frequencies of the beta and gamma isomers were much higher in the samples of human plasma, we found that the values were significantly higher in humans than in dogs ($p <$ .0001 for both chemicals).

Finally, we have to remark that the most striking difference between the two species is the relative to the group of DDTs, as can be seen in the inset of Figure 2a. The concentrations of these contaminants in humans, in particular p,p′ -DDE, were very high (median 352.8 ng/g lw) as has been described in numerous publications for this and other Western populations (Zumbado et al.

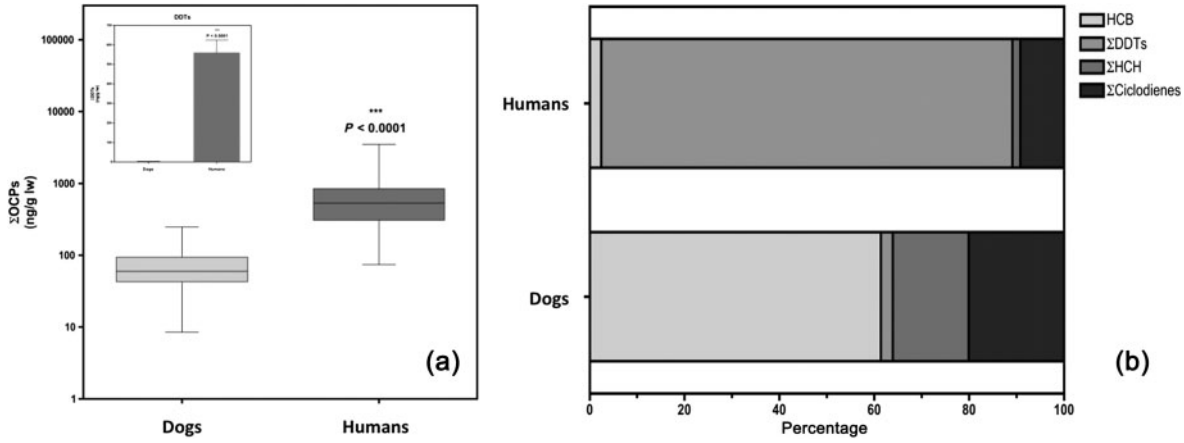

Figure 2. Levels of OCPs in plasma samples. (a) Main body: Box plots of ∑OCPs in dogs and humans. The line inside the boxes represents the median, the bottom and top of the boxes are the first and third quartiles of the distribution and the lines extending vertically from the boxes indicate the variability outside the upper and lower quartiles. Inset: Bar graph of ∑DDTs (median and interquartile range) in dogs and humans. (b) Profile of distribution of OCPs in dogs and humans.

2005). Indeed, in human plasma samples, the p,p′ -DDE was detected in 100% of the samples, and its parent compound (p,p′ -DDT) in 52% samples. In fact, the concentration of these compounds represented 86.7% of ∑OCPs in human samples. Reversely, in samples of canine plasma the situation was radically different, as the median value was 0 ng/g lw, and detection frequency did not exceed 8% for any of these analytes. This had already been described in dogs of Pakistan and Japan (Kunisue et al. 2005; Ali et al. 2013), and also in Russian wolves (Shore et al. 2001), so it has been suggested that canines, unlike many other mammalian species, do not accumulate these pollutants throughout their life, probably because they are equipped with efficient mechanisms of biotransformation and elimination (Kunisue et al. 2005).

All the evidences set out above, in which the values of most of the contaminants in this group were much higher in humans than in dogs, especially so different pollution profiles between the two species, indicate that the dog is not a good indicator of human exposure to OCPs.

3.3. Concentrations of PCBs in dog and human plasma

With respect to contaminants in this group, we also found highly significant differences for both the group (∑PCBs) and for each one of the individual congeners between both species. With respect to the summed values, the medians were almost 20 times higher in humans than in dogs (276.3 vs. 16.7 ng/g lw) (Table 3 and Figure 3a). The levels reported in this work in humans are comparable to those of any other Western populations (Koppen et al. 2009; Mari et al. 2013), and the profile of contamination was very similar in both species, as shown in Figure 3b, with a predominance the M-PCBs, which accounted for about 75% of total plasma PCB concentrations in both canine and human plasma. Indeed seven congeners of this group were detected in both the samples of both species. However, the significant difference levels remained for all of the congeners, whose concentrations were much higher in human plasma, where they also reach percentages of detection very close to 100% (except PCB 118) (Table 3).

Regarding the DL-PCBs, all congeners (except the PCB 126) were detected in human plasma, whereas in the canine plasma congeners #77, 81, 105, 114, 123 and 126 were not detected in any sample, and the median values for the remaining congeners were 0 ng/g lw in this species. In fact, as shown in the insert of Figure 3a, the differences between species became more evident in this subgroup of PCBs.

As it was described for the previously discussed groups of pollutants that were included in this study, it is possible that the source of exposure to contaminants (in this case PCBs) is different between the two species, given that these are food-associated pollutants and the eating pattern is generally also different between humans and dogs. In any case, it has also been suggested that the canines could have the ability to efficiently metabolize PCBs, or at least some congeners (Kunisue et al. 2005), since the few studies that have investigated the levels of these pollutants in dogs have also reported a very low level thereof as compared to those commonly found in mammalian species (Shore et al. 2001; Kunisue et al. 2005; Ali et al. 2013).

So, as we have concluded for the other groups of contaminants, in the light of the above discussion, it does not appear that the dogs can be used as indicators or sentinels for human exposure to PCBs.

Table 3. PCB concentrations (ng/g lw) in dog (n = 87) and human (n = 100) plasma samples from the Canary Islands, Spain.

	Dog plasma			Human plasma			
	Mean ± SD	Median (range)	Frequency (%)	Mean ± SD	Median (range)	Frequency (%)	Mann–Whitney test (p)
∑M-PCBs	17.6 ± 17.3	8.5 (0–85.6)	89.6	**283.6 ± 199.2**	**238.0 (7.3–1666.2)**	**100.0**	<.0001***
PCB 28	5.5 ± 5.2	0 (0–17.1)	47.1	**56.9 ± 42.1**	**46.8 (0–289.1)**	**98.0**	<.0001***
PCB 52	0.6 ± 2.2	0 (0–8.1)	6.9	**27.2 ± 19.4**	**25.5 (0–93.5)**	**91.0**	<.0001***
PCB 101	0.7 ± 2.3	0 (0–7.2)	8.0	**47.9 ± 36.5**	**42.5 (0–187.0)**	**95.0**	<.0001***
PCB 118	0.3 ± 1.6	0 (0–9.5)	3.5	**10.6 ± 13.1**	**8.3 (0–102.3)**	**70.0**	<.0001***
PCB 138	0.9 ± 2.7	0 (0–7.7)	11.5	**35.6 ± 36.5**	**25.5 (0–289.7)**	**95.0**	<.0001***
PCB 153	1.8 ± 4.3	0 (0–17.2)	16.1	**63.2 ± 58.7**	**51.4 (0–425.6)**	**100.0**	<.0001***
PCB 180	4.5 ± 10.6	0 (0–59.5)	26.4	**42.4 ± 53.4**	**34.2 (7.3–467.5)**	**94.0**	<.0001***
∑DL-PCBs	5.7 ± 10.9	0 (0–-50.9)	32.2	**81.4 ± 155.5**	**42.5 (0–1207.5)**	**100.0**	<.0001***
PCB 77	n.d.	n.d.	–	**3.3 ± 9.9**	**0 (0–119.3)**	**33.0**	–
PCB 81	n.d.	n.d.	–	**1.9 ± 6.0**	**0 (0–42.5)**	**14.0**	–
PCB 105	n.d.	n.d.	–	**47.9 ± 36.5**	**42.5 (0–187.3)**	**52.0**	–
PCB 114	n.d.	n.d.	–	**3.3 ± 11.8**	**0 (0–84.9)**	**17.0**	–
PCB 118	0.3 ± 1.6	0 (0–9.5)	3.5	**10.6 ± 13.1**	**8.3 (0–102.3)**	**70.0**	<.0001***
PCB 123	n.d.	n.d.	–	**3.2 ± 13.7**	**0 (0–118.7)**	**16.0**	–
PCB 126	n.d.	n.d.	–	n.d.	n.d.	–	–
PCB 156	0.9 ± 2.6	0 (0–9.5)	10.3	**8.1 ± 13.8**	**0 (0–76.5)**	**47.0**	<.0001***
PCB 157	0.7 ± 2.7	0 (0–16.7)	6.9	**8.6 ± 14.5**	**0 (0–110.4)**	**54.0**	<.0001***
PCB 167	0.3 ± 1.6	0 (0–9.2)	3.5	**5.7 ± 20.5**	**0 (0–168.9)**	**24.0**	<.0001***
PCB 169	0.6 ± 2.2	0 (0–6.4)	6.9	**11.9 ± 30.5**	**0 (0–253.9)**	**54.0**	<.0001***
PCB 189	0.7 ± 2.3	0 (0–7.1)	8.0	**42.4 ± 53.5**	**34.1 (0–467.5)**	**52.0**	<.0001***
∑PCBs	24.3 ± 26.0	16.7 (0–127.6)	96.5	**364.7 ± 333.2**	**276.3 (7.3–2873.6)**	**100.0**	<.0001***

Bold values indicate those values which are significantly higher.
Note: n.d., not determined.

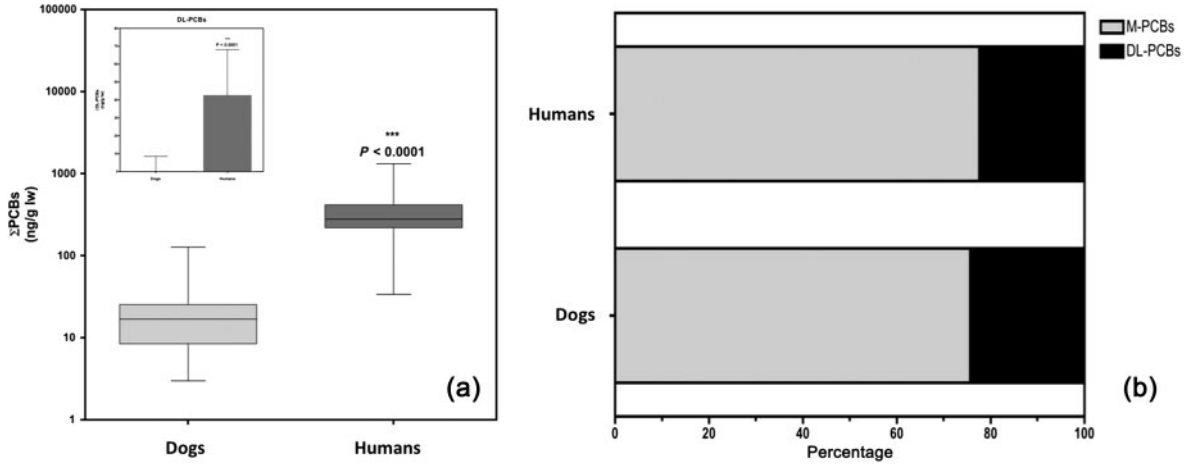

Figure 3. Levels of PCBs in plasma samples. (a) Main body: Box plots of ∑PCBs in dogs and humans. The line inside the boxes represents the median, the bottom and top of the boxes are the first and third quartiles of the distribution and the lines extending vertically from the boxes indicate the variability outside the upper and lower quartiles. Inset: Bar graph of ∑DL-PCBs (median and interquartile range) in dogs and humans. (b) Profile of distribution of PCBs in dogs and humans.

4. Conclusions

In this paper, we compared the plasma levels of a broad panel of contaminants commonly associated with food (PAHs, PCBs and OCPs) in two species that closely share the habitat, such as dogs and humans. The main purpose of this study was to test whether domestic dogs are equally exposed than humans to these pollutants, and thus whether they could serve as sentinels for human exposure to them. In accordance with the results found in this study, the levels of most pollutants investigated were much higher in humans than in dogs, and the profiles of contamination were completely different, indicating that the sources of exposure between the two species are probably different. Furthermore, different evidences such as significantly higher levels of PAH metabolites in dogs, or a null rate of bioaccumulation of contaminants so common in humans such as DDTs and PCBs, suggest that the dog could be provided with different enzymatic capabilities to the human, which would allow them to effectively remove these pollutants. Therefore, we can conclude that, although the domestic dog has been shown as an excellent indicator of human exposure to contaminants by inhalation, for the pollutants studied in this work this species does not behave as a good indicator of human exposure. It will be extremely interesting to carry out further research to elucidate the differential biotransformation routes between canines and humans that may explain these results.

Acknowledgements

The authors thank Mrs María de los Reyes Suárez Hanna for her technical assistance. The authors also thank the veterinarians Dr Elena Carretón and Dr Alberto Montoya for providing dog blood samples.

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Science of the Total Environment 530–531 (2015) 45–52

Contents lists available at ScienceDirect

Science of the Total Environment

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The assessment of daily dietary intake reveals the existence of a different pattern of bioaccumulation of chlorinated pollutants between domestic dogs and cats

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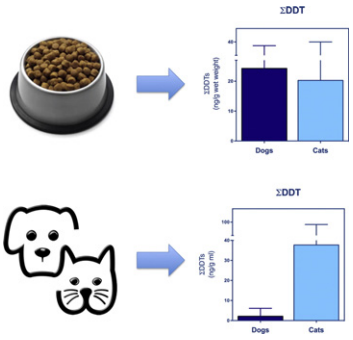
HIGHLIGHTS

- First assessment of the dietary intake of POPs in pet animals.
- Intake levels of pollutants are more than double in dogs than in cats.
- Proportionality between intake of PAHs and their plasma levels in both species.
- Lower levels of organochlorines in dog plasma, although their intake was higher.
- Dogs seem to be able of eliminating certain recalcitrant contaminants.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 14 May 2015
Accepted 15 May 2015
Available online 27 May 2015

Editor: D. Barcelo

Keywords:
Daily intake
Pets
Sentinels
Polycyclic aromatic hydrocarbons
Polychlorinated biphenyls
Organochlorine pesticides
POPs
Pet food

ABSTRACT

Pet dogs and cats have been proposed as sentinel species to assess environmental contamination and human exposure to a variety of pollutants, including POPs. However, some authors have reported that dogs but not cats exhibit intriguingly low levels of some of the most commonly detected POPs, such as DDT and its metabolites. This research was designed to explore these differences between dogs and cats. Thus, we first determined the concentrations of 53 persistent and semi-persistent pollutants (16 polycyclic aromatic hydrocarbons (PAHs), 18 polychlorinated biphenyls (PCBs) and 19 organochlorine pesticides (OCPs)) in samples of the most consumed brands of commercial feed for dogs and cats, and we calculated the daily dietary intake of these pollutants in both species. Higher levels of pollutants were found in dog food and our results showed that the median values of intake were about twice higher in dogs than in cats for all the three groups of pollutants (ΣPAHs: 274.8 vs. 141.8; ΣOCPs: 233.1 vs. 83; ΣPCBs: 101.8 vs. 43.8 (ng/kg bw/day); respectively). Additionally, we determined the plasma levels of the same pollutants in 42 and 35 pet dogs and cats, respectively. All these animals lived indoors and were fed on the commercial brands of feed analyzed. As expected (considering the intake), the plasma levels of PAHs were higher in dogs than in cats. However, for organochlorines (OCPs and PCBs) the plasma levels were much higher in cats than in dogs (as much as 23 times higher for DDTs), in spite of the higher intake in dogs. This reveals a lower capacity of bioaccumulation of some pollutants in dogs, which is probably related with higher metabolizing capabilities in this species.

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http://dx.doi.org/10.1016/j.scitotenv.2015.05.070
0048-9697/

1. Introduction

Certain environmental contaminants, including organochlorine pesticides (OCPs), and industrial products such as polychlorinated biphenyls (PCBs), are known for their toxicity and their resistance to degradation in the environment and biota. For these reasons they are included within the group of chemicals known as persistent organic pollutants (POPs) (El-Shahawi et al., 2010). Other compounds, such as polycyclic aromatic hydrocarbons (PAHs), strictly speaking cannot be considered as POPs because of their efficient metabolization. However, due to their high prevalence in the environment and their toxicity, they are frequently considered as such, and therefore are studied together (Lammel et al., 2013). It has been established that the ingestion of contaminated food contributes more than 90% to the total exposure to these compounds, and foodstuffs of animal origin are recognized as one of the main contributors (Almeida-González et al., 2012; Boada et al., 2014; Formigaro et al., 2014; Luzardo et al., 2012, 2013a; Malisch and Kotz, 2014; Rodríguez-Hernández et al., 2014; Schwarz et al., 2014; Zhou et al., 2012). As all these compounds are highly soluble in fat, their ingestion usually leads to bioaccumulation throughout the life and to biomagnification in the food chain (El-Shahawi et al., 2010; Safe, 1995). Numerous studies have revealed that many POPs, individually and in combination, may contribute to the development of severe health problems such as immune suppression, genotoxic effects, cancer, or endocrine disruption (Bergman et al., 2012; Boada et al., 2012; Kortenkamp et al., 2011; Lauby-Secretan et al., 2013; Valerón et al., 2009). For these reasons the majority of POPs have been banned or severely restricted (El-Shahawi et al., 2010).

Despite the time that has elapsed since the ban of many of these chemicals, today still relevant concentrations of many of them are detected, as witnessed by very recent studies (Boada et al., 2015; Henríquez-Hernández et al., 2011; Luzardo et al., 2014b; Storelli and Zizzo, 2014). Indeed, in some regions of the planet it has been reported that the levels of some compounds, such as PCBs, are even increasing (Garcia-Alvarez et al., 2014; Luzardo et al., 2014a). So, the monitoring of their levels in the environment remains a priority, especially, as regards to exposure of human populations (Diamond et al., 2015). This assessment of exposure to POPs can be done by directly measuring levels in biological samples donated by human volunteers. However, the assessment can be also performed by indirect estimates. Among these, calculations of the intake of pollutants in a given population to assess the exposure, or the employment of sentinel species are usually considered. Firstly, dietary intake estimations are made by combining food consumption data with the concentrations of contaminants found in food samples (Kesse-Guyot et al., 2013; Llobet et al., 2003; Luzardo et al., 2012; Zhou et al., 2012). These are studies that are usually linked to surveillance systems of human diseases in order to obtain quick and reliable information on the prevalence and occurrence of foodborne diseases and risks associated to food (Riviere et al., 2014; Veyrand et al., 2013). Additionally, this methodology has been also used to assess the exposure of animal species to pollutants (Formigaro et al., 2014). Secondly, all kinds of animals, which are convenient to sample, have been used to act as sentinels that allow the assessment of the environmental contamination status, and the estimation of the exposure of other species, including humans (Reif, 2011).

It seems obvious that the more suitable species to act as sentinels of human exposure are the pets, because they closely share the habitat with their owners. So, there are numerous authors that have explored the potential of dogs and cats in this sense (Andrade et al., 2010; Baker et al., 2005; Calderón-Garciduenas et al., 2001; Heyder and Takenaka, 1996; Rabinowitz et al., 2008; Reif, 2011). However, in the case of exposure to POPs the results have been variable, because although some authors have suggested that cats seem to be adequate sentinels of human exposure to these contaminants (Ali et al., 2013; Dirtu et al., 2013; Guo et al., 2012), the role of dogs as such does not seem so clear (Ruiz-Suárez et al., 2015; Sévère et al., 2015). One reason is that several authors have reported that, intriguingly, dogs and other canines exhibit extremely low levels of some of the more abundant POPs in most mammals (including cats and humans), such as DDE and DDT, which suggests a higher metabolic capacity of these animals (Georgii et al., 1994; Kunisue et al., 2005; Ruiz-Suárez et al., 2015; Sévère et al., 2015; Shore et al., 2001; Storelli et al., 2009). This is what led us to design the present investigation, to explore these differences between dogs and cats.

In light of the above, the objectives of the present study were the following: (1) To determine the levels of selected POPs (OCPs, PCBs, and PAHs) in commercial feed for dogs and cats; (2) to estimate the daily dietary intake of these POPs by dogs and cats on the basis of the recommended consumption of these feeds; (3) To analyze the plasma samples collected from two groups of domestic dogs and cats fed on these commercial feeds; and (4) to evaluate the potential differences in contaminant levels between both species in relation with their respective intakes.

2. Material and methods

2.1. Sampling

Blood samples of pet dogs (n = 42, 24 females and 18 males) and cats (n = 35, 19 females and 16 males) were collected during 2013–2014 through cephalic vein puncture. All samples were collected in the Veterinary Hospital of the University of Las Palmas de Gran Canaria (ULPGC, Canary Islands, Spain) during a routine care. Only clinically normal animals were included in the study after owner's consent. All the dogs and cats were adults. The mean age of dogs was 5.2 y.o. (range = 2–14), and the mean age of cats was 4.8 y.o. (range = 2–11). No statistically significant differences in age were observed between males and females. All the animals included in this study were healthy, lived inside the houses with their owners, and were fed with commercial feed. Samples of blood were collected in heparinized tubes and maintained at 4 °C. Plasma was separated after centrifugation and kept frozen at −20 °C in the Laboratory of Toxicology of the ULPGC until sample preparation for chemical analysis.

In addition, we made a random purchase of different brands of commercial feed for dogs and cats in supermarkets and specialty stores from Gran Canaria (Canary Islands, Spain). The feed brands were chosen having into account their composition, and were matched between species according to raw materials they contain. Samples were acquired in triplicate, and chosen among the top selling brands (7 brands of dog feed, and 9 brands of cat feed). All the samples were individually processed as described below, and we used the mean values of the triplicate samples of each brand in the calculations of dietary intake.

2.2. Chemicals, reagents and analytes of interest

All the organic solvents (dichlorometane, hexane, ethyl acetate, and cyclohexane) were of mass spectrometry grade (VWR International, PA, USA). Ultrapure (UP) water was produced in the laboratory using a Milli-Q Gradient A10 apparatus (Millipore, Molsheim, France). The inert desiccant (Celite ® 545) was purchased from Sigma-Aldrich (St. Louis, USA). Bio-Beads SX-3 were purchased from BioRad Laboratories (Hercules, USA). Standards of OCPs, PCB congeners, and internal standards (ISs, PCB 202, tetrachloro-m-xylene, p,p′-DDE-d8, heptachloro epoxide cis, and phenanthrene-d10), were purchased from Dr Ehrenstorfer, Reference Materials (Augsburg, Germany). Standards of PAHs were purchased from Absolute Standards, Inc. (Connecticut, USA). All standards were neat compounds. Stock solutions of each compound at 1 mg/ml were prepared in cyclohexane and stored at −20 °C. Diluted solutions from 0.05 ng/ml to 40 ng/ml were used for calibration curves (9 points).

We determined the levels of 53 organic compounds in plasma samples and commercial feed for dogs and cats: (a) 19 OCPs: methoxychlor;

dicofol; four isomers of hexachlorocyclohexane (α-, β-, γ-, and δ-HCH); p,p′-dichloro-diphenyl-trichloroethane (p,p′-DDT) and its metabolites (p,p′-DDE, and p,p′-DDD); hexachlorobenzene (HCB); aldrin; dieldrin; endrin; chlordane (cis- and trans-isomers); mirex; endosulfan (α- and β-isomers) and endosulfan-sulfate; (b) we also determined the levels of 6 marker (M-PCBs) and 12 dioxin-like PCBs (DL-PCBs) which were numbered according to the International Union of Pure and Applied Chemistry (IUPAC): IUPAC numbers # 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180, 189; and (c) the 16 most environmentally relevant PAHs listed by the United States Environmental Protection Agency (US-EPA): naphthalene, acenaphthylene, acenaphtene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo [b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno [1,2,3,-cd]pyrene, dibenzo[a,h]anthracene, and benzo[ghi]perylene.

2.3. Extraction and clean-up procedure

Plasma samples were subjected to solid-phase extraction using Chromabond® C18ec cartridges (Macherey-Nagel, Germany) that yielded recoveries in the range of 89–107% for the analytes studied. Before application of the plasma samples, the cartridges were cleaned and conditioned with three 1-ml volumes of methanol followed by four 1-ml volumes of ultrapure water. The samples were then passed through the cartridge by gravity flow. The plasma tubes were rinsed with four 1-ml aliquots of ultrapure water, and each aliquot was applied to the corresponding cartridge by gravity flow. The cartridges were rinsed with three 1-ml portions of ultrapure water and dried for 20 min under gentle vacuum (~15 mm Hg). The adsorbed analytes were eluted with 1 ml of methylene chloride by gravity flow. Gentle vacuum was then used to elute the residual methylene chloride from the cartridges. Solvent of the extracts was then evaporated under gentle nitrogen stream, and the extracted analytes were solubilized in 200 μl of cyclohexane that was transferred to 1.8-ml GC vials with 250 μl inserts (Chromatographic Research Supplies, Inc., USA). No additional purification was necessary for the plasma samples that were subjected to chromatographic analysis.

Because the contaminants included in this study are totally lipid-soluble and therefore found bound to the lipid fraction, when we extracted contaminants from the fish, we first extracted the fat of the fish. The mean fat content was 17.8% for Pacific herring, 0.7% for whiting and 17.9% for capelin. A total of 10 g of the homogenated fish were spiked with the 10-ppm surrogate's mix in acetone to yield a final concentration of 100 ppb and mixed with 30 g of diatomaceous earth to absorb all the humidity. The method of extraction and purification followed that recommended by the European Standard for the determination of pesticides and PCBs in fatty food (EN, 1996a,b), whose validity has been previously proven in our laboratory for fatty foods (Almeida-González et al., 2012; Hernández et al., 2015; Luzardo et al., 2012, 2013a). This method combines an automated Soxhlet extraction method (FOSS Soxtec Avanti 2055) with a purification step using gel permeation chromatography (GPC). This method gives acceptable recoveries that ranged between 74.5% and 104.7%. No additional purification steps were required, and the 1-ml extracts in cyclohexane obtained at the end of the GPC were used for the gas chromatography/triple quadrupole mass spectrometry (GC–MS/MS) analysis.

2.4. Procedure of chemical analysis

Gas chromatography analyses of 53 contaminants plus ISs were performed in a single run on a Thermo Trace GC Ultra equipped with a TriPlus Autosampler and coupled to a Triple Quadrupole Mass Spectrometer Quantum XLS (Thermo Fisher Scientific Inc., Waltham, MA, USA), as previously described (Luzardo et al., 2013b), and identifications were done using an electron ionization (EI)-MS/MS based on the retention time and the relative ion transition ratios of each of the analytes. Quantifications were performed against calibration curves as mentioned above. The limit of quantification was set at 0.01 ng/ml for all of the analytes.

2.5. Quality of analyses and quality control (QA/QC)

All of the measurements were performed in triplicate, and we used the means for the calculations. In each batch of samples, four controls were included for every 18 vials (6 samples): a reagent blank consisting of a vial containing only cyclohexane; a vial containing 2 ng/ml of each of the pollutants in cyclohexane; and two internal laboratory quality control samples (QCs) consisting of: a) blank serum (lyophilized human serum, Medidrug Basis Line, Medichem, Germany), and b) melted meat fat, both spiked at 20 ng/ml of each of the analytes. These QCs were processed using the same method as the plasma and feed samples, respectively. The results were considered to be acceptable when the concentration of the analytes determined in the QC sample was within 15% of the deviation of the theoretical value.

2.6. Dietary intake estimates and calculations

The exposure assessment of dogs and cats through feed consumption was calculated by multiplying the respective concentrations of the contaminants in the extracted feed fat (mean values) by the amount of fat contained in the average daily feed consumption, as recommended by the manufacturer for either dogs or cats, and divided by the body weight (bw) of each animal. Thus we obtained the consumption of each one of the pollutants expressed in ng/kg bw/day. The exposures were assessed for all of the contaminants, both individually considered and also grouped in different forms. For the calculations, when the concentration of a given contaminant was below the limit of quantification (LOQ) but above the limit of detection (LOD) of the technique, the value was assumed to be ½ LOQ. Otherwise the value was considered to be 0.

In this work, we expressed the total value of the OCP residues (ΣOCPs) as the sum of the 19 OCPs and metabolites measured; the total value of the DDTs (ΣDDT) was expressed as the sum of the measured values of p,p′-DDT, p,p′-DDE and p,p′-DDD; the total value of the HCH residues (ΣHCH) was expressed as the sum of the 4 HCH isomers measured (α-, β-, δ- and γ-HCH); and the total value of the cyclodiene residues (Σcyclodienes) was expressed as the sum of aldrin, dieldrin, endrin, cis-chlordane, trans-chlordane and heptachlor. PCB congeners: 6 (M-PCB = PCBs #28, 52, 101, 138, 153 and 180) and 12 dioxin-like PCBs (DL-PCB = PCBs #77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189). Finally, we considered the total content of the PAHs (ΣPAHs) as the sum of the values of the 16 US-EPA compounds included in this study.

2.7. Statistical analysis

Database management and statistical analysis were performed with PASW Statistics v 19.0 (SPSS Inc., Chicago, IL, USA). To determine whether the data were normally distributed, we used the Kolmogorov–Smirnov test. Because the levels of organic pollutants did not follow a normal distribution, we used a non-parametric test. The differences between two independent groups were tested with the Mann–Whitney *U*-test. The results were reported as medians and ranges (or interquartile ranges). Probability levels of less than 0.05 (two tailed) were considered statistically significant.

3. Results and discussion

As expected, our results showed that many of the studied pollutants were present in all of the commercial feed samples, as well as in cat and dog plasma samples, indicating that dogs and cats are continuously exposed to POPs through their diets. Following the objectives of this research we present the levels of the studied pollutants (OCPs, PCBs,

and PAHs) in feed in Table 1; the estimated intake of these contaminants in Table 2; and the plasma levels found in dog and cat plasma in Table 3. Moreover, in all the three tables we show the results of the statistical comparison and, additionally we present a graphical representation of the most relevant differences in Figs. 1 to 3. There are very few references in the literature that have examined the levels of PCBs, OCPs and PAHs in the blood and tissues of pets (Ali et al., 2013; Kunisue et al., 2005; Ruiz-Suárez et al., 2015; Storelli et al., 2009), and even less in commercial feed for these species (Kunisue et al., 2005). This paper studies a wide range of these compounds in two groups of domestic dogs and cats, and in their food, comparing between them. It also presents two characteristics that confer its originality: as far as we know, a) this is the first time that dietary intake of contaminants in these species is evaluated, b) is also the first time that levels of PAHs are reported in cats. In the following sections we present and discuss the results in detail.

Table 1
Concentrations of POPs in commercial feed for dogs and cats. All the results are presented as median, percentiles 25th–75th, and frequency of detection, and expressed in ng/g (fresh product weight).

Name	Commercial feed for dogs			Commercial feed for cats			*p*
	Median	P25–P75	Freq.	Median	P25–P75	Freq.	
	(ng/g w.w.)		(%)	(ng/g w.w.)		(%)	
Polycyclic aromatic hydrocarbons (PAHs)							
Naphtalene	0.50	0.42–1.24	100.0	0.22	0.13–0.55	100.0	n.s.
Acenaphthene	0.31	0.09–0.37	100.0	0.14	0.12–1.12	100.0	n.s.
Acenaphthylene	0.38	0.0–0.69	57.1	0.0	0.0–0.20	22.2	n.s.
Fluorene	0.18*	0.08–0.50	100.0	0.03	0.0–0.10	55.5	0.011*
Phenanthrene	12.1*	6.33–14.53	100.0	3.75	2.61–6.79	100.0	0.010*
Anthracene	–	–	n.d.	–	–	n.d.	–
Fluoranthene	3.75**	3.40–4.22	100.0	2.15	1.15–2.97	100.0	0.003**
Pyrene	3.84	3.71–5.21	100.0	2.44	2.10–4.53	100.0	n.s.
Benzo[a]anthracene	0.0	0.0–0.0	14.3	0.0	0.0–0.07	33.3	n.s.
Chrysene	0.0	0.0–0.27	42.8	0.0	0.0–0.08	22.2	n.s.
Benzo[b]fluoranthene	0.0	0.0–0.0	14.3	0.0	0.0–0.15	22.2	n.s.
Benzo[k]fluoranthene	0.0	0.0–0.16	28.6	0.0	0.0–0.07	33.3	n.s
Benzo[a]pyrene	–	–	n.d.	–	–	n.d.	–
Dibenzo[a,h]anthracene	–	–	n.d.	–	–	n.d.	–
Benzo[g,h,i]perylene	–	–	n.d.	–	–	n.d.	–
Indeno[1,2,3-c,d]pyrene	–	–	n.d.	–	–	n.d.	–
Organochlorine pesticides (OCs)							
Hexachlorocyclohexane (alpha)	0.02	0.0–0.10	71.4	0.0	0.0–0.1	77.7	n.s.
Hexachlorocyclohexane (beta)	0.33	0.02–0.66	85.7	0.06	0.03–0.29	100.0	n.s.
Hexaclorocyclohexane (gamma)	0.27	0.01–0.57	71.4	0.12	0.05–0.023	88.8	n.s.
Hexachlorocyclohexane (delta)	0.01	0.0–0.09	14.3	0.0	0.0–0.1	22.2	n.s.
Heptachlor	–	–	n.d.	–	–	n.d.	–
Aldrin	0.04	0.0–0.21	57.1	0.01	0.0–0.03	66.6	n.s.
Dieldrin	13.22*	6.14–17.25	100.0	4.68	3.05–9.18	100.0	0.0229*
Endrin	–	–	n.d.	–	–	n.d.	–
Chlordane (cis)	–	–	n.d.	–	–	n.d.	–
Chlordane (trans)	–	–	n.d.	–	–	n.d.	–
Endosulfan (alpha)	–	–	n.d.	–	–	n.d.	–
Endosulfan (beta)	–	–	n.d.	–	–	n.d.	–
Endosulfan sulfate	–	–	n.d.	–	–	n.d.	–
p,p′-DDT	0.67	0.53–0.81	71.4	0.53	0.33–0.89	88.8	n.s.
p,p′-DDE	0.43	0.29–1.31	100.0	0.32	0.24–1.76	100.0	n.s.
p,p′-DDD	0.05	0.02–0.08	14.3	0.04	0.0–0.13	22.2	n.s.
Dicofol	–	–	n.d.	–	–	n.d.	–
Metoxychlor	–	–	n.d.	–	–	n.d.	–
Mirex	–	–	n.d.	–	–	n.d.	–
Polychlorinated biphenyls (PCBs)							
PCB 28	5.54*	2.38–5.79	100.0	1.82	0.69–2.63	100.0	0.0079*
PCB 52	1.81**	0.61–1.90	100.0	0.41	0.26–0.92	100.0	0.0115**
PCB 77	0.03	0.01–0.04	85.7	0.01	0.0–0.04	66.6	n.s.
PCB 81	0.01	0.0–0.02	71.4	0.01	0.0–0.02	66.6	n.s.
PCB 101	0.56**	0.28–0.59	100.0	0.19	0.11–0.29	100.0	0.0079**
PCB 105	0.03	0.02–0.04	100.0	0.02	0.01–0.03	88.8	n.s.
PCB 114	0.0	0.0–0.01	28.6	0.0	0.0–0.0	33.3	n.s.
PCB 118	0.15	0.08–0.16	100.0	0.06	0.04–0.18	100.0	n.s.
PCB 123	0.0	0.0–0.01	14.3	0.0	0.0–0.01	33.3	n.s.
PCB 126	–	–	n.d.	–	–	n.d.	–
PCB 138	0.05	0.04–0.06	100.0	0.06	0.03–0.09	100.0	n.s.
PCB 153	0.07	0.05–0.17	100.0	0.09	0.03–0.14	100.0	n.s.
PCB 156	–	–	n.d.	–	–	n.d.	–
PCB 157	–	–	n.d.	–	–	n.d.	–
PCB 167	–	–	n.d.	–	–	n.d.	–
PCB 169	–	–	n.d.	–	–	n.d.	–
PCB 180	0.02	0.0–0.07	100.0	0.02	0.0–0.05	88.8	n.s.
PCB 189	–	–	n.d.	–	–	n.d.	–

n.d. non detected.
n.s. not significant.
* $P < 0.05$ ** $P < 0.01$.

Table 2
Values of daily dietary intakes of POPs (ng/kg b.w./day) in domestic dogs and cats which are feed on commercial feed. All the results are presented as mean ± SD, median, and range.

	Daily intake (dogs)			Daily intake (cats)			
Name	Mean ± SD	Median	Range	Mean ± SD	Median	Range	*p*
Polycyclic aromatic hydrocarbons (PAHs)							
∑ c-PAHs[a]	8.5 ± 16.4	0.0	0.0–44.3	6.0 ± 12.7	1.23	0.0–39.5	n.s.
∑ PAHs	**323.6 ± 102.1**	**274.8**	**244.4–511.8**	183.6 ± 104.9	141.8	71.6–409.2	0.023*
Organochlorine pesticides (OCs)							
∑ HCH	**10.5 ± 8.9**	**8.0**	**1.0–27.3**	2.8 ± 2.9	1.5	0.3–9.7	0.031*
∑ DDTs	24.3 ± 14.3	17.4	13.6–53.1	20.3 ± 19.7	15.6	3.4–71.6	n.s.
∑ cyclodienes	**179.3 ± 59.3**	**207.0**	**84.2–255.0**	99.8 ± 73.3	68.4	24.3–265.3	0.031*
∑ OCPs	**214.0 ± 60.5**	**233.1**	**119.0–299.7**	122.9 ± 92.7	83.0	29.6–340.6	0.030*
Polychlorinated biphenyls (PCBs)							
∑ M-PCBS	**99.9 ± 28.0**	**98.5**	**56.0–132.8**	48.0 ± 29.3	42.7	22.2–106.8	0.008**
∑ DL-PCBS	3.3 ± 0.5	3.2	2.5–4.2	3.3 ± 3.5	1.9	0.9–12.2	n.s.
∑ $TEQ_{DL\text{-}PCBs}$[b]	0.4 ± 0.1	0.4	0.24–0.5	0.3 ± 0.3	0.3	0.1–1.1	n.s.
∑ PCBs	**103.2 ± 27.7**	**101.8**	**59.4–136.3**	41.4 ± 32.1	43.8	24.1–119.0	0.012*

Figures in bold indicate the group of samples having a significantly higher value.
n.d. non detected.
n.s. not significant.
* $P < 0.05$ ** $P < 0.01$.
[a] Carcinogenic PAHs = the sum of the 8 compounds for which there are evidences of carcinogenicity: benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene; dibenz[a,h]anthracene; and indeno[1,2,3-cd]pyrene.
[b] Expressed in pg/g wet weight.

3.1. Levels of PAHs, OCPs and PCBs in feed for dogs and cats, and estimation of their daily intake through diet

3.1.1. PAHs

Eleven out of the sixteen priority USEPA PAHs were detected in the commercial feed samples analyzed in this work. The compounds detected were the same in feed for dogs than in feed for cats. Only anthracene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[*ghi*]perylene and indeno[1,2,3,-*cd*]pyrene were not detected in any of the samples. On the contrary, naphthalene, acenaphthene, phenanthrene, fluoranthene, and pyrene were detected in 100% of the samples of feed for both species, and in addition fluorene was also detected in 100% of the samples of feed for dogs (Table 1). When we considered the concentrations of these contaminants (and not the frequencies), we found that the levels were in general higher in feed for dogs than in feed for cats, and for fluorene, phenanthrene, and fluoranthene these differences reached statistical significance (Table 1). Thus, the ΣPAHs was also significantly higher in dog food (median = 21.86 vs. 7.58 ng/g w.w., in feed for dogs and cats, respectively; $P < 0.05$). According to the levels found in the food, it was expected that the daily intake levels of these contaminants were also higher in dogs than in cats, as indeed our estimates demonstrated. So, intake of ΣPAHs was 274.8 ng/kg bw/day in dogs and 141.8 ng/kg bw in cats ($p < 0.05$) (Table 2 and Fig. 1, left).

3.1.2. OCPs

Within this group of organochlorine compounds we detected nine out of the nineteen compounds analyzed (Table 1) in the analyzed feed samples, and the compounds detected were the same in both types of feed. Heptachlor, endrin, cis-chlordane, trans-chlordane, α-endosulfan, β-endosulfan, endosulfan-sulfate, dicofol, metoxychlor, mirex were not detected in any of the samples. The frequencies of detection of each of the OCPs were pretty similar between the samples of feed for dogs and feed for cats. However, the median concentrations were found to be higher for all of them in the group of feeds for dogs (although only statistically significant for dieldrin). Therefore, the median levels of these compounds reached statistical significance when summed (median ΣOCPs = 14.84 vs. 6.24 ng/g in feed for dogs and cats, respectively, $p < 0.05$). Subsequently, as also occurred with PAHs, our estimations showed that the daily intake of OCPs were also higher in dogs than in cats (Table 2). As observed in this table, dogs consume statistically significant higher levels of ΣHCHs, Σcyclodienes, and ΣOCPs ($p < 0.05$ in all cases), whereas the intake of ΣDDTs can be considered almost the same in both species.

As far as we know, only one previous study has analyzed the levels of organochlorine contaminants (OCPs and PCBs) in food for domestic animals (canned food for dogs and cats). In this study the residues detected were similar in both types of food, either in frequency and in concentration (Kunisue et al., 2005). Although no estimation of intake was performed in that work, given the results it is probable that the dietary exposure would be also the same in both species. Other authors have studied the levels of pollutants in pet food, but these other studies have focused in food contaminants as the cause of toxic outbreaks (Dobson et al., 2008; Rumbeiha and Morrison, 2011), and therefore do not have considered the food as a source of exposure to environmental pollutants.

3.1.3. PCBs

All the M-PCBs and 6 out of the 12 DL-PCBs were detected in both types of feed samples. The congeners #126, 156, 157, 167, 169 and 189 were not detected in any of the samples. As occurred with the other two groups of chemicals, the frequencies of detection of PCBs were similar between samples of feed for dogs and samples of feed for cats, but the concentrations were higher in samples of feed for dogs. Indeed, the levels detected were more than three times higher, when we considered all the congeners as a group (8.49 vs. 2.37 ng/g, $p < 0.05$), and also individually for PCB 28 ($p < 0.01$), PCB 52 ($p < 0.05$), and PCB 101 ($p < 0.01$) (Table 1). It is remarkable that all of the M-PCBs were detected in 100% of the samples analyzed, and that this group of congeners accounted for >95% of ΣPCBs in both types of feed. Thus, also similar to that described above, the estimated dietary exposure to these pollutants is much higher in dogs than in cats (median ΣPCBs intake = 101.8 vs. 43.8 ng/kg bw/day, $p < 0.05$) (Table 2). Probably due to the enormous difference of concentration between ΣM-PCBs and ΣDL-PCBs in feed, the higher exposure in dogs seems to be only attributable to the intake of M-PCBs (no statistically significant differences in the intake of ΣDL-PCBs nor of $\Sigma TEQs_{DL\text{-}PCBs}$ (Van den Berg et al., 2006) were found).

3.2. Plasma levels of PAHs, OCPs, and PCBs in dogs and cats

3.2.1. PAHs

As shown in Table 3, and consistently with the levels found in feed and the estimation of daily intake, the plasma levels of ΣPAHs were significantly higher in dogs than in cats (422.5 vs. 253, respectively;

Table 3
Concentrations of POPs (mean ± SD, median and range) in plasma samples of dogs and cats, which are feed on commercial feed. Results are expressed in ng/g lw.

	Plasma levels (dogs)			Plasma levels (cats)			
Name	Mean ± SD	Median	Range	Mean ± SD	Median	Range	*p*
Polycyclic aromatic hydrocarbons (PAHs)							
∑ c-PAHs[a]	1.8 ± 2.5	1.0	0.0–9.1	4.6 ± 20.2	0.0	0.0–120.0	n.s.
∑ PAHs	**447.3 ± 189.9**	**422.5**	**230.0–995.0**	346.3 ± 211.4	253.0	52.0–962.0	0.025*
Organochlorine pesticides (OCs)							
∑ HCH	2.7 ± 7.9	0.0	0.0–25.1	**3.1 ± 3.7**	**2.0**	**0.0–14.0**	<**0.001****
∑ DDTs	2.1 ± 3.9	0.0	0.0–15.0	**49.2 ± 90.4**	**12.0**	**1.0–441.0**	<**0.001****
∑ cyclodienes	26.8 ± 25.4	25.0	0.0–125.4	40.5 ± 43.3	26.3	0.0–174.2	n.s.
∑ OCPs	31.6 ± 29.9	25.1	0.0–152.3	**92.7 ± 122.7**	**47.9**	**3.1–618.0**	**0.022***
Polychlorinated biphenyls (PCBs)							
∑ M-PCBS	67.1 ± 61.6	50.1	0.0–225.7	**87.7 ± 43.5**	**87.0**	**24.1–216.4**	0.040*
∑ DL-PCBS	6.1 ± 8.3	2.5	0.0–30.9	3.3 ± 3.7	2.0	0.0–21.2	n.s.
∑ $TEQ_{DL-PCBs}$[b]	3.9 ± 6.7	0.4	0.0–25.9	1.8 ± 5.5	0.4	0.1–30.7	n.s.
∑ PCBs	73.1 ± 69.2	50.8	0.0–255.1	**90.9 ± 45.7**	**89.2**	**22.3–221.6**	0.039*

Figures in bold indicate the group of samples having a significantly higher value.
n.d. non detected.
n.s. not significant.
[a] Carcinogenic PAHs = the sum of the 8 compounds for which there are evidences of carcinogenicity: benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene; dibenz[a,h]anthracene; and indeno[1,2,3-cd]pyrene.
[b] Expressed in pg g^{-1} wet weight.

$P < 0.05$). On the contrary, when we considered only those PAHs that are classified as probable carcinogens for animals and humans by the USEPA (benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and indeno[1,2,3,-cd]pyrene), no differences were observed between dog and cat plasma samples, but this was also consistent with the intake levels in both species (Table 2). As shown in Fig. 1, proportionality was found between the intake and the plasma levels in both species. Unlike the rest of pollutants included in this study, the PAHs are readily metabolized in vertebrates (Walker et al., 2006), and although high differences in metabolizing capabilities among species have been reported between dogs and cats (Saengtienchai et al., 2014), in this case the differences found seem to be mainly attributable to the higher intake in dogs (Fig. 1).

According to the literature only one previous study has reported the plasma levels of PAHs in dogs (Ruiz-Suárez et al., 2015), and none in cats. The levels reported in dogs by Ruiz-Suárez et al. (2015) were similar to those found in this series.

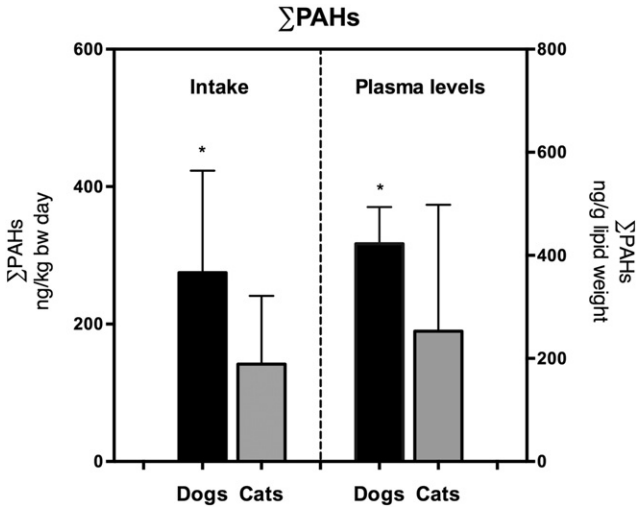

Fig. 1. Comparison of the intake of ΣPAHs through food in dogs and cats (left), and of the plasma levels of ΣPAHs in both species (right). The bar height represents the median and the lines extending vertically from the bar indicate the interquartile ranges.

3.2.2. OCPs

Although, as described above, levels of OCPs in feed and their dietary intake were significantly higher in dogs than in cats; the plasma levels found in cats showed an inverse pattern, with median values almost doubling those in dogs (Table 3 & Fig. 2). The greatest difference found was for ΣDDTs, whose mean plasma levels in cats were about 23 times higher than in the dogs (Fig. 2, inset), even though the similar

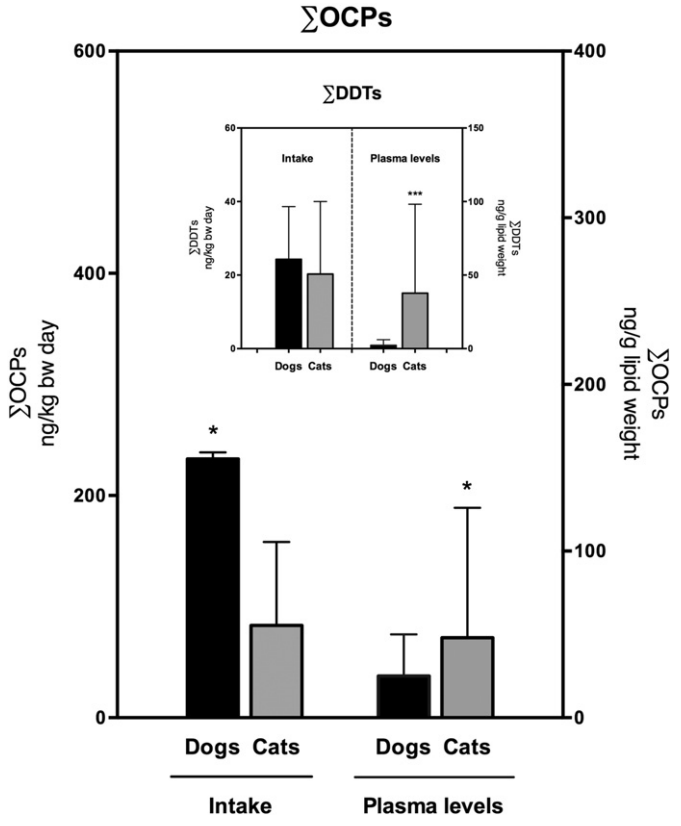

Fig. 2. Comparison of the intake of ΣOCPs through food in dogs and cats (left), and of the plasma levels of ΣOCPs in both species (right). The bar height represents the median and the lines extending vertically from the bar indicate the interquartile ranges. Inset: representation of the detailed comparison of the subgroup of DDTs.

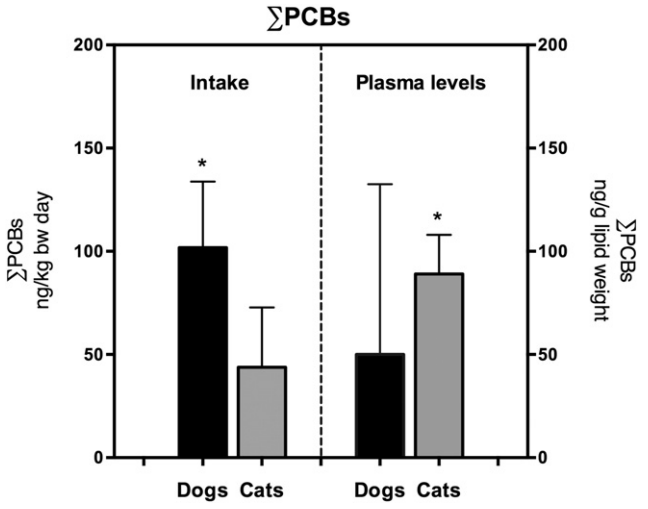

Fig. 3. Comparison of the intake of ΣPCBs through food in dogs and cats (left), and of the plasma levels of ΣPCBs in both species (right). The bar height represents the median and the lines extending vertically from the bar indicate the interquartile ranges.

dietary exposure to these contaminants in both species. This was no an unexpected result, as other authors have also found low or even almost undetectable levels of these pesticides in dogs (Ali et al., 2013; Kunisue et al., 2005; Ruiz-Suárez et al., 2015), other canines (Shore et al., 2001), and some other species of vertebrates, such as foxes or polar bears (Hoshi et al., 1998; Polischuk et al., 2002). Some of these authors have pointed to the possibility that dogs (and maybe other species) would have an uncommon capacity of metabolizing these pesticides. However, it was also possible that these animals were exposed to lower levels of contaminants through diet. In this work we have demonstrated that these animals do not accumulate OCPs in the same manner than cats (or other vertebrates), although the dietary exposures were comparable to each other. Furthermore, according to our results this does not seem to be only a particularity of DDT. So, we also report here that dogs, although they are 5 times more exposed to HCHs through diet than cats, they exhibit much lower plasma levels (Table 3), which would also suggest a higher metabolic capability for these other pesticides in dogs.

3.2.3. PCBs

Similarly to what was observed with the group of OCPs, also plasma levels of PCBs were significantly higher in cats than in dogs ($p < 0.05$) (Table 3), although dietary intake was calculated over twice in dogs than in cats (Table 2). These differences were observed both, for ΣPCBs and ΣM-PCBs, but not for ΣDL-PCBs, nor $\Sigma TEQ_{DL\text{-}PCBs}$. In Fig. 3 we graphically show this inverse relationship. Interestingly, Ali et al. (2013) reported higher levels of PCB metabolites (OH-PCBs) in dogs than in cats, and Kunisue et al. (2005) suggested that cats hardly metabolize and eliminate PCBs. Elaborating on this, other authors have shown that dogs are capable of metabolizing some PCB congeners by means of the cytochrome P-450 (CYP2B) (Ariyoshi et al., 1992; Duignan et al., 1987), so that they can eliminate them more quickly and more efficiently than other mammalian species (Sipes et al., 1982a,b). Reversely, in the study of Ali et al. (2013) the authors reported lower levels for 4OH-PCB 146 (a major metabolite of PCB 153) in cats compared with dogs, suggesting that cats hardly metabolize PCB 153, and Kunisue and Tanabe (2009) indicated that cats may preferentially metabolize lower chlorinated OH-PCBs and retain these metabolites in their blood. Unfortunately, due to technical limitations we were not able to determine the levels of OH-PCBs in these groups of animals. However, other authors have used the concentration ratio of PCB 153/PCB 180 as an indicator of specific metabolism of PCB 153 (Storelli et al., 2009), and found a big difference between dogs and cats (0.3 vs. 1.8), which suggests the ability of dogs to metabolize this highly persistent congener. When we applied this indirect indicator of metabolism our results were consistent with those of Storelli et al. (2009), with significantly lower ratios in dogs than in cats (median = 0.52 vs. 2.33, $p < 0.001$).

Taken together, our results indicate that there are great disparities between the capacities to metabolize organochlorine contaminants between dogs and cats. Thus, we agree with previous authors in that cats may serve as better sentinels of human exposure to environmental organochlorine compounds and for the control of geographical variations of these pollutants than canine species (Dirtu et al., 2013; Kunisue et al., 2005; Storelli et al., 2009). However, we reinforce here the findings of Ruiz-Suárez et al. (2015), who indicated that dogs are not good sentinels of the exposure to POPs, since we add evidence that this species pose higher metabolic capabilities to deal with these compounds than the majority of mammals.

4. Conclusions

The findings of this study reveal that dogs exhibit lower plasma levels of OCs than cats, even though it was estimated that this species is exposed to higher dietary levels when fed on commercial feed. All the analytes detected in the feed were detected in the plasma samples of both, dogs and cats, but the levels of many organochlorine compounds were from 2 to 23 less abundant in dog plasma samples than in cat plasma samples. Taken into account that it has been reported that more than 90% of exposure to POPs is through food in vertebrates, our results suggest that dogs, unlike the majority of mammals, seem to be able to efficiently metabolize and eliminate some POPs. Therefore domestic cats, instead of dogs, may represent a better model to assess human exposure to these chemicals.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank Mrs. María de los Reyes Suárez Hanna for her technical assistance. We also thank the veterinarians Dr. Elena Carretón, Dr. Alberto Montoya, and Dr. Alejandro Suárez for providing dog and cat blood samples.

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Science of the Total Environment 472 (2014) 146–153

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Assessment of the exposure to organochlorine pesticides, PCBs and PAHs in six species of predatory birds of the Canary Islands, Spain

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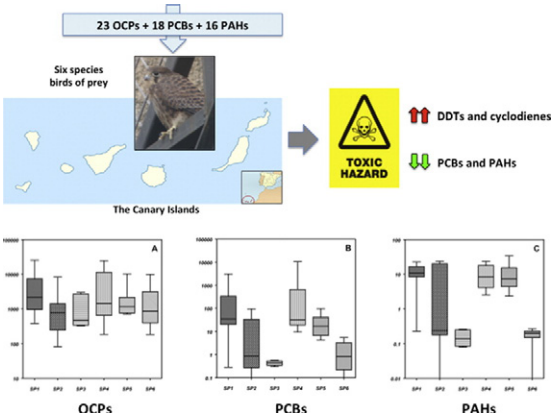
HIGHLIGHTS

- Monitoring of 57 POPs in six species of birds of prey
- High levels of DDTs and dieldrin consistent with previous reports of this area
- Non hazardous-low levels of PCBs found in these animals
- First report on polycyclic aromatic hydrocarbons in these species
- Levels of PAHs below the values considered as toxic in predictive models

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 October 2013
Received in revised form 2 November 2013
Accepted 3 November 2013
Available online 27 November 2013

Keywords:
Birds of prey
Polycyclic aromatic hydrocarbons
Polychlorinated biphenyls
Organochlorine pesticides
Liver
Canary Islands

ABSTRACT

In the present study, we investigated the concentrations and distributions of 57 anthropogenic pollutants, including 23 organochlorine pesticides (OCs), 18 polychlorinated biphenyls (PCBs) and 16 polycyclic aromatic hydrocarbons (PAHs) in liver samples from 102 birds of prey of six species that were found dead or that had died during their stay in the Wildlife Recovery Centers of the Canary Islands (Spain) between 2009 and 2012. The dual goal of this work was to determine the occurrence of these contaminants in these six species of birds of prey, and also whether they can be used as bioindicators for monitoring environmental pollution in the region. We found that *Accipiter nisus*, *Falco pelegrinoides* and *Falco tinnunculus* were the most contaminated species. The profiles of contamination among the species were extremely similar in the case of organochlorine contaminants, with DDT and its metabolites as the most abundant compounds. The contamination by DDT and its metabolites, as well as contamination by dieldrin, could be considered high in these animals, much higher than reports from other regions of the planet, which is in agreement with previous reports from our group regarding humans, food and other animals from this area. In contrast, the contamination by PCBs could be considered extremely low and was probably below the threshold of toxicity for these contaminants. The content of carcinogenic/mutagenic PAHs in these animals was clearly dependent on the feeding pattern of the species; however, the levels were also well below the values that were considered toxic in predictive models. This study represents the first report of contamination by PAHs in all these species and is also the first report of PCB levels in Barbary Falcons.

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http://dx.doi.org/10.1016/j.scitotenv.2013.11.021

1. Introduction

Persistent organic pollutants (POPs) are toxic chemicals that are resistant to degradation in the environment and biota. Due to their fat solubility and resistance to chemical and biological degradation, ingestion of certain classes of POPs by animals leads to bioaccumulation throughout their lives, generally in the fatty tissues, and to biomagnification in the food chain (Gioia et al., 2013; Safe, 1994). Among the POPs, organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) are highly prevalent in vertebrates. Because of their efficient metabolization, strictly speaking, polycyclic aromatic hydrocarbons (PAHs) cannot be considered as POPs, but due to their high prevalence in the environment and their lipophilicity, PAHs are usually considered as POPs. The majority of POPs, such as PCBs and OCPs, are currently banned from use and are no longer produced or used around the world; therefore, their levels have been constantly declining through the years (Addison et al., 2013; Ryan et al., 2013; Schuster et al., 2011). Nevertheless, relevant amounts of these pollutants still persist in the environment, and certain species, especially those top predators, are especially contaminated (Bourgeon et al., 2013; Olafsdottir et al., 2001), and it has been described that these pollutants lead to adverse health effects on living beings (Hamlin and Guillette, 2010). Birds of prey are especially prone to POP accumulation due to their high position on the food chain and high rates of food consumption (Fernie and Letcher, 2010; Tillitt et al., 1992). Many adverse effects have been described in relation to birds' exposure to POPs, such as eggshell thinning, decreased reproductive success (Ratcliffe, 1967), reduced normal sexual behavior, smaller brain size, or neurotoxicity (Iwaniuk et al., 2006), among others.

The Canary Islands are located 1600 km away from southwest Spain, in the Atlantic Ocean, and 63 miles away from the nearest point on the North African coast (southwest of Morocco) (Fig. 1). Geographically, the Islands are part of the African continent; however, from historical, economic, political and sociocultural points of view, the Canaries are European. Notably, large quantities of organochlorine pesticides have been used in the past in this archipelago because of the important role of agriculture in the economy of the region (Diaz-Diaz and Loague, 2001). For this reason, the levels of POP contamination in the human population of the archipelago have been deeply studied. Thus, whereas this population shows lower levels of PCB contamination than many populations from developed countries (Henriquez-Hernandez et al., 2011), chronic exposure to DDT and its metabolites, as well as to some cyclodienes, seems to currently persist (Luzardo et al., 2006; Zumbado et al., 2005). These findings correlate with the presence of these pollutants in food items that are consumed in this region, particularly those food items that are locally produced (Almeida-Gonzalez et al., 2012; Luzardo et al., 2012, 2013b). Moreover, potential adverse health effects of POPs have been described in marine wildlife in nearby areas (Camacho et al., 2012, 2013).

Due to the relative isolation of the archipelago and its climate, the flora and fauna in the islands are completely different from those of the European and African continents. Here, many endemic species and subspecies are found in areas of high ecological value. There are 7 species of diurnal birds of prey and 2 nocturnal nesting birds of prey in the Canary Islands. Four of these species are endemic to the Canary Islands, and two species are endemic to the Macaronesian region (which includes the Azores, Madeira, Canaries and Cape Verde) (Lorenzo et al., 2012). These raptors have suffered a population decline in recent decades and have several threats to their survival, including power lines, malicious or accidental poisonings, slow reproductive rates, high tourist pressure on the territory (the archipelago has four national parks that receive 5.5 million visitors a year (MAGRAMA, 2013)), as well as the extensive use of pesticides in the past and also currently, among others. In particular, pesticides, specifically organochlorines, can cause reproductive failure in birds and are responsible for the population decline of several birds of prey worldwide. Despite the potential risks to birds of prey, little exposure data for pesticides or pollutants exist for wild populations, particularly those populations utilizing terrestrial food webs (Yordy et al., 2013). To address this gap in the data, we have evaluated the concentrations of 57 POPs, including organochlorine pesticides (OCs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs), in liver samples of 102 birds of prey, which belong to six different species from the Canary Islands, and we have compared these levels with the thresholds of toxicity that have been established for these pollutants in order to elucidate if raptor species of the archipelago are exposed to toxic amounts of anthropogenic pollutants, which could represent a threat for their conservation. In addition, the accumulation patterns of these pollutants in the liver of these animals are utilized as additional "biological indicators" for assessing the current level of environmental contamination by POPs in this archipelago.

2. Materials and methods

2.1. Sample collection and ethics statement

Liver samples were obtained from necropsies of 102 birds of prey from 6 species that were admitted to the Wildlife Recovery Centers (WRCs) of Tafira (Gran Canaria, Spain) and La Tahonilla (Tenerife, Spain) between October 2009 and December 2012. Birds that were sampled included 12 Common Buzzards (*Buteo buteo*), 16 European Sparrowhawks (*Accipiter*

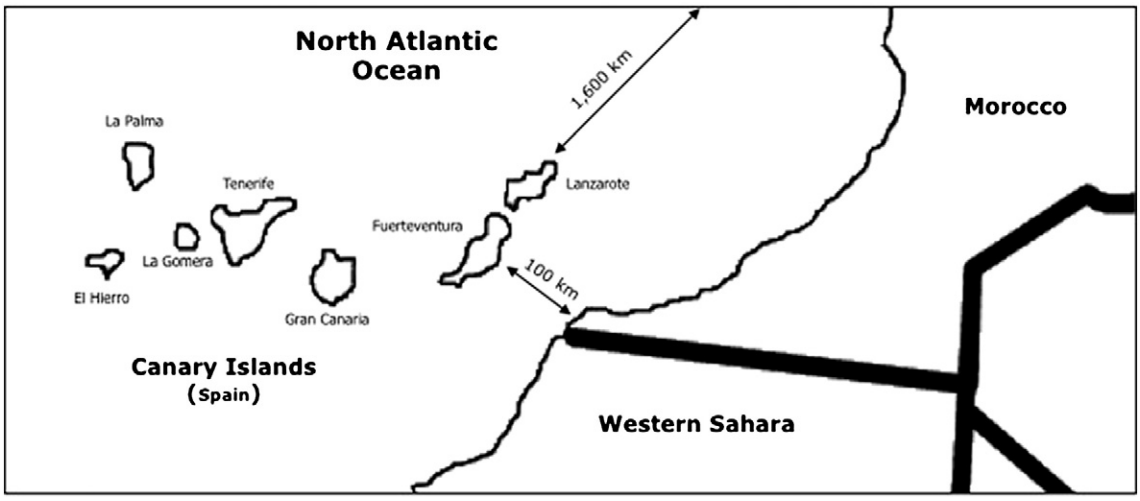

Fig. 1. Canary Islands archipelago in northwestern African coast (arrows: minimal distance from Spanish and Moroccan coasts).

nisus), 20 Barbary Falcons (*Falco pelegrinoides*), 20 Common Kestrels (*Falco tinnunculus*), 14 Long-eared Owls (*Asio otus*), and 20 Barn Owls (*Tyto alba*). The birds died naturally or were euthanized within one week of admission, and necropsies were performed within 72 h postmortem. No animal was killed for the purposes of this study. The main cause of death was determined by examining the birds macroscopically in the WRCs, and, when necessary, radiological, histological, or toxicological analyses were performed. The causes of death for all the animals that were included in this study included different types of trauma. Livers were excised and stored at −20 °C until sample preparation.

2.2. Chemicals and reagents

Dichlorometane, hexane, ethyl acetate and cyclohexane were of the highest purity available (>99.9%) and were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure (UP) water was produced from a Milli-Q Gradient A10 (Millipore, Molsheim, France). Diatomaceous earth was purchased from Sigma-Aldrich (St. Louis, USA). Bio-Beads SX-3 were purchased from BioRad Laboratories (Hercules, USA). Standards of OCPs, PCB congeners, surrogates (PCB 12, PCB 202, *p,p′*-DDE-d_8, acenaphtylene-d_8) and internal standards (ISs, tetrachloro-m-xylene, trans-heptachlor epoxide, and benzo[a]pyrene-d_{12}) were purchased from Dr Ehrenstorfer (Augsburg, Germany). Standards of PAHs were purchased from Absolute Standards, Inc. (Connecticut, USA). All standards were pure compounds (purity from 97% to 99.5%). Stock solutions of each compound at 1 mg/mL were prepared in cyclohexane and stored at −20 °C. Diluted solutions from 0.05 ng/mL to 100 ng/mL were used for calibration curves.

2.3. Analytes of interest

In total, 57 analytes, which belonged to three relevant groups of POPs, were selected for this study. The 23 OCPs and metabolites included the diphenyl-aliphatics (methoxychlor, *o,p′*-DDT, *p,p′*-DDT, *o,p′*-DDE, *p,p′*-DDE, *o,p′*-DDD, *p,p′*-DDD, and dicofol); the persistent and bioaccumulative contaminant hexachlorobenzene (HCB); the four isomers of hexachlorocyclohexane (α-, β-, δ-, and γ-HCH); the cyclodienes heptachlor, dieldrin, aldrin and endrin, chlordane (cis- and trans-isomers) and mirex; and endosulfan (α- and β-isomers) and endosulfan sulfate. With respect to the PCBs, we included 18 congeners: the dioxin-like congeners (DL-PCBs) (IUPAC numbers 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189), and the six marker congeners of non-dioxin-like PCBs (M-PCBs) (IUPAC numbers 28, 52, 101, 138, 153 and 180). Finally, we also included in the suite of analytes that is on the list of the 16 EPA priority PAHs that are often targeted for measurement in environmental samples (naphthalene, acenaphtylene, acenaphtene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo [a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene and benzo [ghi]perylene).

2.4. Sample preparation

Because the contaminants included in this study are completely lipid-soluble and, therefore, are found in the lipid fraction of tissues, we first extracted the fat from the liver tissue. In total, 5 g of liver was finely chopped with scissors and homogenized in 5 mL ultrapure water with a disperser (Ultra-turrax, IKA, China). This homogenate was spiked with the 10 ppm-surrogate mix in acetone to a final concentration of 100 ppb and was mixed with 30 g of diatomaceous earth to absorb moisture. The method of extraction and clean up followed the method that was previously recommended by the European Standard for the determination of pesticides and PCBs in fatty foods (EN European Norm, 1528-2, 1996, EN European Norm, 1528-3, 1996) and whose validity has been previously proven in our laboratory for different fatty samples (Almeida-Gonzalez et al., 2012; Luzardo et al., 2012, 2013a). This method combines an automated Soxhlet extraction method (FOSS Soxtec Avanti 2055) with a purification step using gel permeation chromatography (GPC) and gives acceptable recoveries that ranged between 74.5% and 104.7%. Additional clean-up steps were not required, and the 1 mL-extracts in cyclohexane that were obtained at the end of the GPC were used for the chromatographic analysis.

2.5. Procedure of chemical analysis

Gas chromatography analyses of 57 contaminants, 4 surrogates and 3 ISs were performed in a single run on a Thermo Trace GC Ultra, which was equipped with a TriPlus Autosampler and coupled to a Triple Quadrupole Mass Spectrometer Quantum XLS (Thermo Fisher Scientific Inc., Waltham, MA, USA), using appropriate internal standards (ISs) as previously described and validated in our laboratory (Camacho et al., 2012, 2013; Luzardo et al., 2013c). A fused silica capillary column BPX5 (crosslinked 5% phenyl methylpolysiloxane, SGE Inc., USA) with a length of 30 m, a 0.25 mm i.d. and a film thickness of 0.25 μm was used as the stationary phase. Helium (99.999%) at a constant flow rate of 1.0 mL/min was used as the carrier gas. The temperatures were programmed as follows: the initial oven temperature of 60 °C was maintained for 1 min, ramped at 12 °C/min to 210 °C, then raised at 8 °C/min to 320 °C with a 6 min hold time. The total run time was 61 min. The injector and transfer line were set to 270 °C and 310 °C, respectively. The standards and samples were injected (1 μL) in the splitless mode. Thermo Fisher Xcalibur software (Ver. 2.0.1) was used for the instrument control, data acquisition, and data analysis. After the retention times were determined in full scan mode (range m/z 45–650), a timed selected reaction monitoring (SRM) method was developed to analyze the 57 target compounds plus four surrogates and three internal standards in one single run. A calibration curve was constructed from 0.05 to 100 ng/mL with all the compounds, with the exception of the surrogates and internal standards, contained in each calibration standard mixture. Argon (99.99%) was used as the collision gas, and the collision cell pressure was set to 0.2 Pa. The triple quadrupole mass spectrometer was operated under the following conditions: ionization with electron impact at 70 eV in MRM with an emission current of 50 μA. The ionization source temperature was set to 220 °C. A filament multiplier delay of 5 min was established to prevent instrument damage. The electron multiplier voltage was set to 1500 V. The scan width was 0.15, and the scan time was 0.05 s. Peak widths of m/z 0.7 Da were set for both the first (Q1) and third quadrupole (Q3). The analytical performance of this method (confirmation criteria, precision, linearity, limits of quantification (LOQs), and repeatability) has been previously studied and published (Luzardo et al., 2013c). We added 20 μL of the IS mixture, which was prepared at 1 ppm in cyclohexane, immediately before the GC–MS/MS analysis. Because no matrix effects have been observed with this method, all quantifications were performed against a 10-point calibration curve using cyclohexane (0.05 to 40 μg L^{-1}).

2.6. Quality control

The recoveries of the 57 analytes and surrogates were acceptable with this method because the recoveries were invariably above 74%. All the individual measurements were corrected by the recovery efficiency for each analyte. All the measurements were performed in triplicate, and the values used for calculations were the mean of the three measurements. In each batch of samples, two controls were included with every 12 samples. For the chromatographic procedure we used a reagent blank, which consisted of a vial containing only cyclohexane that was spiked with the surrogates and ISs. Besides an internal laboratory quality control of the whole procedure was included (QC), which consisted of melted butter that was spiked at 20 ng/g for each of the analytes that was processed with the same method as the samples. The batch analyses were considered valid when the values of the

analytes in the QC and the reagent blank were within a 10% deviation of the theoretical value.

2.7. Statistical analyses

The database management and statistical analyses were performed using the PASW Statistics v18.0 software (SPSS Inc., Chicago, IL, USA). Because the POP levels (individually or grouped) did not follow a normal distribution, the results were expressed with the median and the 5th and 95th percentiles of the distribution. Differences in POP concentrations among two groups or more were tested with the non-parametric Mann–Whitney U-test and the Kruskal–Wallis test. The categorical variables were presented as percentages and were compared between variables with the chi-square test. P values of less than 0.05 (two-tail) were considered statistically significant.

3. Results and discussion

3.1. Accumulation pattern of OCPs

Organochlorine pesticide residues were detected in all the samples of birds that were analyzed in this study (Table 1). A mean of 5 residues per sample was found (range 3 to 14). The presence of α-HCH, δ-HCH, heptachlor, aldrin, endrin, α-endosulfan, β-endosulfan, and metoxichlor was not detected in any sample of liver. In contrast, 3 compounds were detected in 100% of samples: *p,p′*-DDE, dieldrin, and β-HCH. In recent studies from different regions of the world, other authors have reported a similar pattern of contamination, where *p,p′*-DDE and HCH isomers were the chief contributors to the total OC content (Dhananjayan, 2013b; Gomez-Ramirez et al., 2012; Yordy et al., 2013). We observed a high dispersion in the level of residues among species (Fig. 2A). *A. nisus*, *F. pelegrinoides* and *F. tinnunculus* were the most contaminated species, with total OCP contents of 2160, 1419 and 1154 ng/g wet weight, respectively (Table 1). Notably, dicofol was present with high frequency in our set of samples and at concentrations ranging from 1.0 to 9.3 ng/g wet weight. This residue is not usually included in the set of analytes that is measured in environmental samples; therefore, we cannot compare our results with many previous studies. However, this pesticide has been found in wild bird eggs with similar frequency and concentrations (Malik et al., 2011). The high frequency presence of this residue might be explained by its thorough use in the cultivation of tropical fruits in the Canary Islands in the past, and also because this pesticide, which is an important source of DDT, was produced in Spain until late 2008.

In general, we found lower levels of contamination by OCPs in these birds from the Canaries than OCP levels in birds from other regions of the world. However, the high levels of DDTs and dieldrin that were found are two remarkable differences that should be highlighted. We found much higher levels of these pesticides (Table 1) than in prey birds from the USA (range from 1.8 to 3240.8 ng/g wet weight) (Yordy et al., 2013), mainland Spain (median 6248.19 ng/g lipid weight) (Gomez-Ramirez et al., 2012), Italy (range from 110.2 to 215.7 ng/g wet weight) (Licata et al., 2012), or even from India (range from 3.0 to 520.0 ng/g wet weight) (Dhananjayan, 2013b), where the use of DDT remains allowed for malaria control. However, these results are in agreement with our studies in human blood samples that were taken 15 years ago (Luzardo et al., 2006; Zumbado et al., 2005), and more recently, in amniotic fluid samples (Luzardo et al., 2009), or in some food items that were produced in this archipelago (Almeida-Gonzalez et al., 2012; Luzardo et al., 2012). The current level of contamination by DDTs and dieldrin is surprisingly high because in Spain, as well as all other developed countries, the usage of OCPs ceased in the late 1970s or the beginning of the 1980s. These results could be explained, at least in part, by two facts: the extreme environmental persistence of these compounds and the proximity to the African continent. The first factor is most likely to be the major cause because many organochlorine pesticides have been used in this area in the past. There are no reliable data regarding the consumption of these products; however, until the political awareness of pesticide consumption in recent years, the pesticide consumption in the islands was as high as 12 times (>200 kg/Ha, 42% insecticides) than that of the rest of the country (Diaz-Diaz and Loague, 2001; MAGRAMA, 2006). Several circumstances could explain this high use of pesticides, such as the weather conditions that favor the occurrence of pests and the fact that many farmers abuse pesticides by implementing scheduled preventative treatments, overdosing, and continue to ignore basic instructions regarding their use. Therefore, it is more than likely that this situation was similar or even worse in the past, when organochlorines were available as the only option. Alternatively, the high level of pollution that we have found can also be explained in part by the proximity of these islands to the African continent. Several prohibited OCs remain under use in developing countries, and high levels of contamination are found in these countries because of this or due to the existence of considerable stocks of uncontrolled obsolete pesticides, which makes it possible for the people and wildlife living there and in the neighboring areas to be under inadvertent exposure (Gioia et al., 2013; Najera et al., 2011; Nweke and Sanders, 2009). The fact that DDT is currently used as an insecticide in Morocco, which is only 100 km away from the Canary Islands archipelago, supports this hypothesis.

3.2. Accumulation pattern of PCBs

We detected residues of PCBs in all the samples that were analyzed within a range of 2 to 15 different congeners (Table 2). Only PCB 169

Table 1
Concentrations of organochlorine pesticides (median and percentiles 5th and 95th of the distribution) in liver tissues of six species of birds collected from the Canary Islands, Spain (2009–2012). Results are expressed in ng/g wet mass and in ng/g lipid weight [between brackets].

Bird species	∑DDT	Dieldrin	∑HCH	HCB	Dicofol	∑OCP
Accipiter nisus	1955.8 [41,785.6] (141.6–24772.3)	88.5 [1890.4] (47.1–1423.9)	190.2 [4058.4] (28.8–426.8)	2.7 [57.3] (n.d.–634.1)	1.0 [21.4] (n.d.–3.53)	2160.4 [46,146.2] (383.5–25,249.8)
Asio otus	475.8 [10,727.4] (60.1–7838.9)	189.7 [4276.9] (112.3–465.4)	141.8 [3199.0] (42.6–258.0)	0.4 [9.1] (n.d.–5.63)	2.7 [60.4] (n.d.–24.83)	770.6 [17,369.3] (249.2–8223.3)
Buteo buteo	403.9 [8120.4] (121.6–2642.4)	106.5 [2141.2] (72.1–123.3)	135.4 [2729.7] (43.4–597.4)	0.1 [2.4] (0.08–0.22)	9.3 [187.5] (3.9–11.7)	467.1 [9416.7] (328.1–2949.4)
Falco pelegrinoides	974.07 [18,044.6] (70.6–23,064.2)	78.4 [1452.4] (18.1–471.4)	207.6 [3886.3] (59.8–1049.8)	0.3 [5.2] (0.03–209.3)	6.9 [129.6] (0.4–24.4)	1419.2 [26,567.4] (184.7–24,418.9)
Falco tinnunculus	699.6 [14,247.4] (35.6–9213.8)	217.3 [4425.3] (66.8–570.3)	210.1 [4278.7] (15.4–1227.8)	1.2 [24.0] (0.2–3.7)	4.2 [85.4] (n.d.–12.5)	1154.7 [23,475.1] (692.9–10,036.6)
Tyto alba	597.9 [12,705.4] (30.7–9575.9)	180.3 [3831.4] (21.4–402.5)	72.4 [1538.5] (14.0–747.7)	0.01 [0.2] (n.d.–0.6)	2.9 [62.7] (n.d.–13.2)	848.2 [18,024.3] (183.7–9731.1)

n.d., non-detected.
HCB, hexachlorobenzene.
∑DDT, sum of *p,p′*-DDE, *p,p′*-DDD + o,*p′*-DDT, *p,p′*-DDT, o,*p′*-DDE, o,*p′*-DDD.
∑OCs: sum of all analyzed OCs.

Table 2
Concentrations of polychlorinated biphenyls (median and percentiles 5th and 95th of the distribution) in liver tissues of six species of birds collected from the Canary Islands, Spain (2009–2012). Results are expressed in ng/g wet mass and in ng/g lipid weight [between brackets].

Bird species	∑M-PCBs	∑DL-PCBs	$\sum TEQ_{DL\text{-}PCB}$ [a]	∑PCBs
Accipiter nisus	33.1 [707.0] (0.3–2906.5)	4.4 [94.0] (0.1–449.5)	0.2 [4.3] (0.1–13.48)	35.6 [760.4] (0.3–3090.9)
Asio otus	0.8 [18.1] (0.1–86.4)	0.2 [4.5] (0.1–10.8)	0.05 [1.1] (0.0–0.3)	0.9 [20.3] (0.1–92.81)
Buteo buteo	0.7 [14.7] (0.2–0.5)	0.2 [4.0] (0.1–0.3)	0.1 [2.0] (0.1–0.4)	0.8 [16.1] (0.3–0.7)
Falco pelegrinoides	28.8 [533.4] (8.5–4032.5)	6.1 [57.4] (2.2–1507.3)	0.2 [3.7] (0.1–46.1)	32.3 [598.2] (9.5–10,645.8)
Falco tinnunculus	16.4 [333.9] (3.9–89.7)	1.7 [34.6] (0.4–7.9)	0.1 [2.0] (0.0–0.3)	16.8 [342.1] (4.2–95.8)
Tyto alba	0.4 [8.5] (0.1–4.8)	0.1 [2.1] (0.1–0.9)	0.1 [2.1] (0.0–0.4)	0.6 [12.8] (0.1–5.5)

∑M-PCBs, sum of marker PCBs (congeners 28, 52, 101, 118, 138, 153, and 180).
∑DL-PCBs, sum of dioxin-like PCBs (congeners 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189).
∑PCBs, sum of all measured PCBs (congeners, 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180, and 189).
[a] Expressed in pg/g wet weight.

was not detected in any sample. However, in our set of samples, PCB numbers 28, 118, 138, 153 and 180 were the most prevalent congeners, with PCB numbers 28, 118 and 153 detected in 100% of samples (3.8, 2.7 and 3.4 ng/g, respectively) and with PCB numbers 138 and 180 detected in 97.1% of the samples (1.4 and 2.1 ng/g, respectively). More than 95% of the content of PCBs came from M-PCBs. This result is consistent with studies that have found PCBs 180 and 153 as the most predominant congeners (Gomez-Ramirez et al., 2012) and with other studies that have also found high levels of contamination by PCB 28 (Wan et al., 2011).

Regarding PCB contamination in this region, previous studies by our group in humans and in food items have revealed that the level was relatively low (Almeida-Gonzalez et al., 2012; Henriquez-Hernandez et al., 2011; Luzardo et al., 2012), which was expected as the Canary Islands are a relatively non-industrialized area. We expected to find also very low levels of PCBs in these samples of birds living in the same region, but as shown in Table 2, the levels found were not so low as it would be expected. However, the levels of contamination by POPs can be enormously different among populations depending on the geographical region in which they live, and the local environment greatly influences the levels of contaminants that are found (El-Shahawi et al., 2010). However, it seems obvious that there are other unknown factors that influence the PCB levels in these birds. Nevertheless, the levels in our series are much lower than those levels that were found in raptors from other regions: USA (range from 1.34 to 10,500.0 ng/g wet weight) (Yordy et al., 2013), Belgium (range from 285.0 to 8690.0 ng/g wet weight) (Jaspers et al., 2008), Italy (range from 131.0 to 3050.0 ng/g wet weight) (Licata et al., 2012), Greenland (median 11,000 ng/g lipid weight) (Jaspers et al., 2013), Southern Spain (median 544.7 ng/g lipid weight) (Gomez-Ramirez et al., 2012) and it may be possible that these animals were not at toxicological risk for PCBs.

As in the case of OCPs, we observed significant differences in the levels that were found among species (Fig. 2), and again we found that *A. nisus*, *F. pelegrinoides*, and *F. tinnunculus* were, in that order, the species that displayed the highest levels of ∑PCBs (Fig. 2). Variations in the diet as well as the species-specific accumulation and metabolism of PCBs may be the most important causes that explain these differences, as it has been described for other species including human beings (Beyer and Biziuk, 2009; D'Ilio et al., 2011; Gasull et al., 2011). Differences among species have also been found by other authors (Jaspers et al., 2008); however, the pattern of raptors from the Canaries is completely different from other regions. Thus, in the study by Jaspers et al. (2006), *T. Alba* and *A. nisus* were the most contaminated of the analyzed species, with 150,000 and 140,000 ng/g of ∑PCBs, respectively.

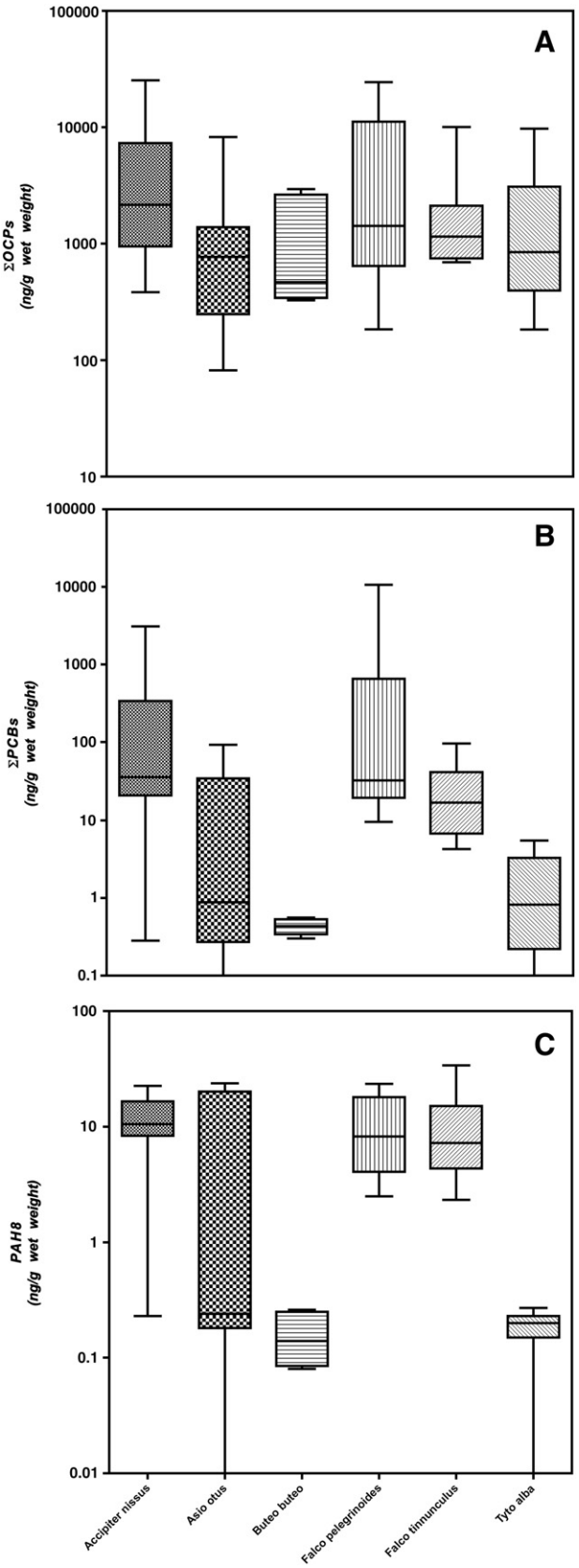

Fig. 2. Box plot indicating the levels of ∑OCs (panel A), ∑PCBs (panel B), and ∑PAH8 (panel C) in the six species that were included in the study. The line inside the box represents the median, the bottom and top of the box are the first and third quartiles of the distribution, and the lines extending vertically from the boxes indicate the variability outside the upper and lower quartiles.

In our study, the levels found in both species were much lower, and *T. alba* was the species that displayed the lowest levels (0.8 ng/g wet weight, Table 2). To our knowledge, this report is the first that tests the levels of contamination by PCBs in Barbary Falcons (*F. pelegrinoides*).

3.3. Accumulation pattern of PAHs

Of the 16 PAH compounds that were analyzed, 15 were detected in one or more of the six species that were analyzed. Only dibenzo[a,h]anthracene was not detected in any of the samples, and thus, the remaining 15 compounds were considered for ∑PAHs. Additionally, 3 compounds were detected in 100% of the samples: phenanthrene, fluoranthene, and pyrene, consistent with other authors who showed that phenanthrene is the most frequent PAH in samples from predatory birds in Britain (Pereira et al., 2009). Regarding concentrations, naphthalene was the compound that reached the highest values by far (median 1152 ng/g, range 2.2 to 63,240.8 ng/g wet weight).

As in the case of organochlorine compounds, we found great differences in the total content of PAHs among both different species and individual samples from each species (Fig. 2). Thus, the median ∑PAH concentration among the six species ranged from 48.9 to 53995.3 ng/g wet weight; however, the individual values ranged from 9.2 to 80053.2 ng/g wet weight (Table 3). Notably, the species that showed less contamination by ∑PAHs were those species whose diet was primarily composed of birds (*F. pelegrinoides* < *F. tinnunculus* < *A. nisus*), whereas the other three species, which feed mainly on small mammals and reptiles were those species that showed the highest levels of the sum of these contaminants (Table 3). The extremely high values of ∑PAHs that we have found in some species and individuals are primarily due to the high content of low molecular weight PAHs, particularly naphthalene, which contributed more than 99% of ∑PAHs in the most contaminated species (Fig. 3B). Therefore, it seems a plausible hypothesis that there could exist a source of naphthalene that is probably related to the similar feeding patterns of these three species. However, we do not have data on feedstuff to support this hypothesis, and further research is needed to elucidate this issue. When we separate the PAHs according to their molecular weights, and we consider only the sum of the more weighted ones (mainly to exclude the less toxic, but highly abundant, naphthalene), the differences among species become less evident (range from 10.5 to 35.5 ng/g wet weight), and the pattern of contamination among species varies. This pattern is paradoxical because the less contaminated species are precisely those species that feed on mammals and reptiles. This pattern is maintained when we consider only those PAHs for which oral carcinogenicity data were available (∑PAH8: benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene), as well as when we used the classical toxic equivalency factor (TEF) approach to the risk characterization of the PAH mixtures, and expressed these mixtures as benzo[a]pyrene toxic equivalents ($TEQ_{B[a]p}$, Table 3). As other authors have described in other environmental samples, the contribution of $TEQ_{B[a]p}$ to the total dioxin-like activity was much higher (>1000 times) than that of $TEQ_{DL\text{-}PCBs}$ (Eljarrat et al., 2001). In any case, according to the concentrations of carcinogenic PAHs that were detected in the liver tissues of the birds that were included in the present study, the risk of acute toxic effects to these birds can be considered moderate when compared with the model for predicting the toxicity of polycyclic aromatic hydrocarbon mixtures (Reeves et al., 2001).

These values of our study (without including naphthalene) can be compared with those values that were found by other authors in the liver of birds from other regions of the world. Thus, the levels in our study are lower than those levels that were found in birds from India, Australia, USA and Britain (overall range from 79 to 382 ng/g wet weight) (Custer et al., 2000; Dhananjayan, 2013a; Kayal and Connel, 1995; Shore et al., 1999). It seems logical that our levels were much lower than those levels that were found in oiled seabirds from the North Atlantic (mean 245 ng/g wet weight) (Troisi et al., 2006) but higher than those levels found in seabirds from the Canaries and the Mediterranean Sea (mean 13.1 ± 11.7 ng/g wet weight) (Roscales et al., 2011). Notably, the studies that report the levels of these contaminants in the liver of predatory birds, or even in terrestrial birds, are scarce in the literature. Thus, these comparisons should be taken with caution because they have been made with the data that were obtained from species that live in a different environment and that have a completely different feeding pattern. To our knowledge, this report is the first of PAHs levels in tissues of all of the six species that were included in the present study, and it is expected that the data that were generated in this study will serve as reference values for future studies.

Taken together, we observed that the total load of contaminants greatly varies depending on the species and that the total load of contaminants can also be extremely different among individuals of the same species (Fig. 2). However, the profile of contaminants (the percentage of each pollutant or group of pollutants) is particularly similar in the case of organochlorines. Thus, the total load of ∑DDTs invariably accounted for more than 60% of total organochlorines (Fig. 3A). Dieldrin and the sum of HCH isomers were also major contributors to the total load of organochlorine contaminants. However, as suggested above, the profile of contamination by PAHs seems to be related to the feeding pattern of the species. Thus, this profile is particularly similar among the bird-eating raptor species but is different from the profile of those species that feed on small mammals and reptiles (Fig. 3B).

Table 3
Concentrations of polycyclic aromatic hydrocarbons (median and percentiles 5th and 95th of the distribution) in liver tissues of six species of birds collected from the Canary Islands, Spain (2009–2012). Results are expressed in ng/g wet mass and in ng/g lipid weight [between brackets].

Bird species	∑PAHs $_{lw}$	∑PAHs $_{hw}$	∑PAH8	TEQ $_{BaP}$	∑PAHs
Accipiter nisus	380.9 [8136.0] (49.5–2216.6)	35.5 [758.28] (0.9–82.7)	10.5 [224.3] (0.2–22.6)	5.4 [115.3] (0.1–12.4)	421.5 [9003.2] (90.3–2217.4)
Asio otus	3966.3 [89,400.4] (1267.4–15,412.2)	0.6 [13.54] (0.0–83.1)	0.2 [4.5] (0.0–23.7)	0.1 [2.3] (0.0–17.5)	3966.7 [89,409.4] (1345.7–15,413.0)
Buteo buteo	53,994.7 [1,085,293.5] (6816.5–80,052.8)	0.4 [8.0] (0.3–1.0)	0.2 [4.0] (0.1–0.3)	0.1 [2.0] (0.0–0.2)	53995.3 [1,085,305.5] (6816.8–80,053.2)
Falco pelegrinoides	27.3 [505.6] (4.0–270.0)	17.4 [322.3] (5.2–58.0)	8.2 [164.8] (2.5–23.5)	2.2 [40.2] (0.6–5.8)	48.9 [949.8] (9.2–290.5)
Falco tinnunculus	543.9 [11,073.8] (130.5–4277.0)	27.0 [549.7] (7.0–114.4)	7.2 [146.6] (2.3–33.9)	4.2 [85.5] (1.2–15.6)	611.0 [12,439.9] (138.2–4356.6)
Tyto alba	6480.6 [137,712.7] (2335.2–31,383.1)	0.5 [10.7] (0.0–0.6)	0.2 [4.2] (0.0–0.3)	0.1 [2.1] (0.0–0.2)	6481.1[137,723.4] (2335.7–31,383.5)

∑ di-, tri- cyclic PAHs (low molecular weight PAHs): sum of naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene.
∑ tetra-, penta-, hexa cyclic PAHs (high molecular weight PAHs): sum of fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, Benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, benzo[g,h,i]perylene, and benzo[a,h]anthracene.
∑ PAHs, sum of all compounds.

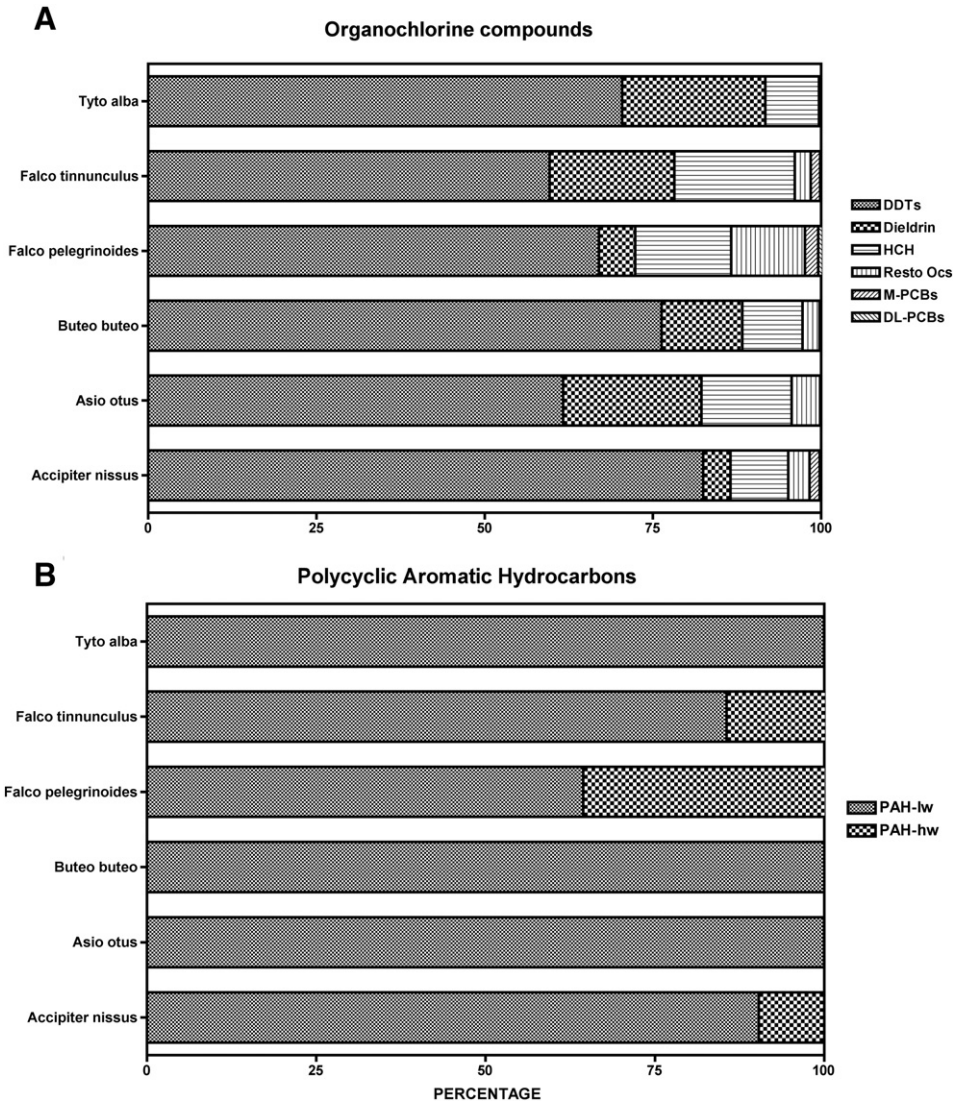

Fig. 3. A. Percentage composition of organochlorine contaminants: ∑DDT, dieldrin, ∑HCH, ∑other OCs, ∑M-PCBs and ∑DL-PCBs (Panel A). B. polycyclic aromatic hydrocarbons: ∑PAH of low molecular weight and ∑PAH of high molecular weight in the six species of birds of prey that were collected from the Canary Islands, Spain.

4. Conclusions

The levels of 57 POPs were determined in 102 birds belonging to six different species from the Canary Islands (Spain). We observed significant differences in the content of contaminants among species, although the variability of individuals within each species was high. The profiles of contamination among species were extremely similar in the case of organochlorine contaminants, including DDT, dieldrin, and HCH isomers as the most abundant compounds. The contamination by DDT and its metabolites, as well as contamination by dieldrin, can be considered high in these animals, with much higher levels than reports from other regions of the world, which is in agreement with previous reports from our group regarding humans, food and other animals from this area. The high levels of organochlorine pesticides, especially DDTs and dieldrin, add more evidence to the previously reported high level of contamination by these compounds in the Canary Islands. In contrast, the contamination by PCBs can be considered low, with M-PCBs contributing the most to the total load of these contaminants. These PCB levels are not considered a toxic risk for these animals. These results are also in consonance with previous reports of environmental and human samples from the same area. The same consideration can be made for the PAH content in these species because the levels are well below the values that are considered toxic in predictive models. The PAH content of these animals seems to be somewhat dependent on the feeding pattern of the species, with the content of low-molecular-weight compounds much higher in those animals that feed on small mammals and reptiles than in the raptors that feed on other birds. However, further research is needed to elucidate this topic. This study represents the first report of contamination by PAHs in all these species. Taken together, the results of this study indicate that the level of contamination of these birds is moderate to low for PCBs and PAHs and high for OCPs, being in consonance with previous reports from samples in the same area. This indicates that carcasses of birds of prey of the archipelago may be used as an additional indicator to assess the level of contamination of this area.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank the veterinarians of the Wildlife Recovery Centers of Gran Canaria (Pascual Calabuig and Dolores Estévez)

and Tenerife (Santiago Mayans) for their collaboration in the necropsies and in the sampling of the animals. We would also like to thank Mrs. María de los Reyes Suárez Hanna for her technical assistance.

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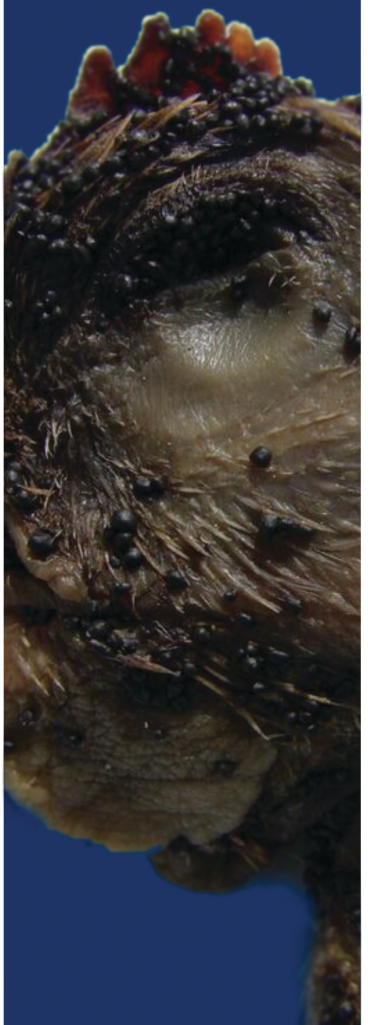
BLOQUE C

EL ENVENENAMIENTO INTENCIONADO



Resumen

En este bloque se presentan los trabajos derivados de estudios en donde la característica común es la intencionalidad, entendiéndose esta como la puesta en el medioambiente de productos de manera consciente y que como fin último se produzca el envenenamiento de un animal, ya sea primario o secundario, intencionado o no.

En el caso de los rodenticidas anticoagulantes (método comúnmente utilizado para el control de roedores), estos reciben un tratamiento por separado al ser culpables en muchas ocasiones de provocar envenenamientos secundarios en aves de presa a través del consumo de roedores. Este es el primer estudio que se lleva a cabo en Canarias en busca de estos rodenticidas ,ya sean de primera o segunda generación, en aves rapaces. El total de aves analizadas asciende a 104, repartidas entre las siguientes especies *Buteo buteo*, *Accipiter nisus*, *Falco pelegrinoides*, *Falco tinnunculus*, *Asio otus* y *Tyto alba.* En el 61% de los hígados analizados se detectó al menos un tipo de rodenticida. El rodenticida que mayor frecuencia de detección obtuvo fue la bromadiolona. No detectándose en ningún caso anticoagulantes de primera generación. Las frecuencias de detección variaron según las especies, al igual que hubo diferencias si las agrupamos en diurnas o nocturnas, estás últimas con concentraciones superiores. También existieron diferencias entre aquellas que basan su alimentación principalmente en pequeños roedores, obteniendo concentraciones mayores frente a aquellos que se alimentan de otras aves. De todas las especies la que mayor contaminación mostró fue el cernícalo (*Falco tinnunculus*).

En muchas ocasiones, durante la toma de muestra no existían evidencias o lesiones macroscópicas resultado de una exposición a este tipo de sustancias, así todo, en torno al 35% de los animales superaron los umbrales de toxicidad. Estos resultados sugieren que el uso de estas sustancias entran en la cadena alimentaria y llegan hasta las poblaciones de rapaces, pudiendo comprometer la supervivencia de estas.

Claro está que no podíamos cerrar este bloque sin referirnos a la casuística recibida durante los años de realización de este Tesis Doctoral. Partiendo de la base de que el envenenamiento de fauna, silvestre o no, es algo por desgracia bastante común en nuestro País, en Canarias hasta hace unos años, la información disponible al respecto era escasa. En un periodo de 32 meses comprendidos entre 2010 y 2013, se confirmó que un total de 225 animales fueron envenenados con pesticidas. Confirmándose intencionalidad en 117. Los pesticidas que con mayor frecuencia formaron parte de estos episodios fueron: carbofurano, aldicarb, bromadiolona y brodifacoum. Aún estando prohibidos el aldicarb y el carbofurano en la Unión Europea, ambos se vieron involucrados en el 75% de los casos, y cuando nos referimos a cebos, el porcentaje ascendía hasta casi el 100%.

Los resultados derivados de estos estudios además de confirmar que aún estando prohibidos muchos de estos productos, todavía se siguen utilizando (ya sea debido a remanentes que todavía estén en circulación, o la existencia de un mercado ilegal), ratifican la idea o mejor dicho la realidad del veneno en Canarias.

Science of the Total Environment 485–486 (2014) 371–376

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Assessment of anticoagulant rodenticide exposure in six raptor species from the Canary Islands (Spain)

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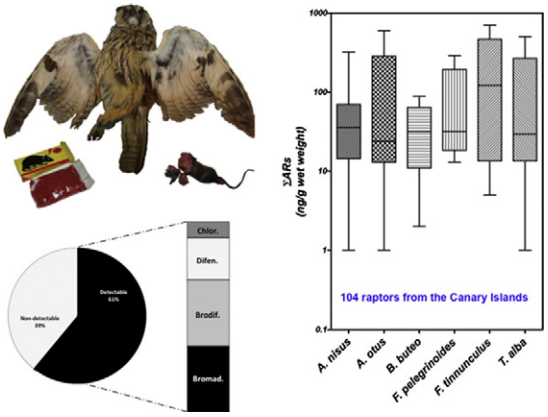
HIGHLIGHTS

- Monitoring of seven anticoagulant rodenticides in six species of birds of prey
- 35% of raptors exceeded the toxicity threshold.
- Higher levels in nocturnal and mammal-eater birds of prey
- High levels in birds of prey that feed on other birds

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 February 2014
Received in revised form 19 March 2014
Accepted 20 March 2014
Available online 16 April 2014

Editor: D. Barcelo

Keywords:
Exposure assessment
Anticoagulant rodenticides
Raptors
Wildlife
Brodifacoum
Bromadiolone

ABSTRACT

Anticoagulant rodenticides are highly toxic compounds that are widely used for pest control of rodents, but that also may threaten the wildlife's health. This work aimed to assess the exposure to first- and second-generation anticoagulant rodenticides (ARs) in six birds of prey species from the Canary Islands (Spain). The concentrations of seven widely used ARs were determined by LC–MS/MS in 104 liver samples of six species of birds of prey (*Buteo buteo*, *Accipiter nisus*, *Falco pelegrinoides*, *Falco tinnunculus*, *Asio otus*, and *Tyto alba*). We determined that 61% of the livers had detectable residues of at least one AR. The most frequently detected AR was bromadiolone, which was detected in 60.3% of the positive cases. The detection frequencies of these compounds varied widely, depending on the species. More than 75% of the *A. nisus*, *T. alba*, and *A. otus* individuals had detectable rodenticide residues in the liver. However, *F. tinnunculus* exhibited the highest concentrations of AR, with median values above 100 ng/g w.w. We did not detect first-generation ARs in any of the samples. When grouped, nocturnal species exhibited higher AR concentrations than diurnal species ($P < 0.001$). The residue levels were higher among small mammal-eaters than bird-eaters ($P < 0.01$). While most animals exhibited no macroscopic signs of coagulation disorders, approximately 35% exceeded the threshold levels of toxicity, which suggests that these compounds could weaken these animals in their natural environment. In conclusion, the control of rodent populations by ARs suggests that these compounds will enter the food chain and thus threaten the vulnerable

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http://dx.doi.org/10.1016/j.scitotenv.2014.03.094

populations of raptors on the Canary Islands. Our findings require authorities to ban or strictly control the use of these rodenticides in the natural environment for the conservation of raptors and other predatory species.

1. Introduction

In the agricultural sector, rodent populations remain one of the primary causes of economic losses in crops not only prior to harvesting but also during storage (Colazo, 1997). Public health authorities also target rodent populations because these animals can transmit zoonotic diseases, such as leptospirosis and plague (Bharti et al., 2003; Collins et al., 1996; Schelotto et al., 2012).

The most preferred and widely used method for rodent population control is the use of anticoagulant rodenticides (ARs), which are chemical products that interfere with normal blood clotting and cause death by inducing diffuse hemorrhages. The first rodenticide anticoagulants started being used in the 1940s, and these chemicals are currently referred to as the first-generation anticoagulants. Because of widespread use and the continuous exposure to these products, resistance to first-generation ARs developed in rodents. This motivated the development of new chemical formulas, and second generation rodenticides (SGARs) appeared in the market in the 1970s. These new chemicals include bromadiolone, brodifacoum, difenacoum, flocoumafen, chlorophacinone, and diphacinone (Murphy, 2007; Pelfrène, 2010). The SGARs are much more powerful and persistent than the first-generation ARs and are considered toxic after a single dose (Pelfrène, 2010). The primary mechanism of toxicity for these substances is the inhibition of vitamin K epoxide reductase. This enzymatic inhibition blocks vitamin K regeneration, and as a result, the vitamin K-dependent coagulation factors II, VII, IX and X are incorrectly synthesized and do not bear the post-translational carboxylation required for activation. This impairs normal blood coagulation and predisposes the animal to death due to bleeding (Murphy, 2007; Pelfrène, 2010).

In the European Union (EU), these products are freely sold and distributed. Even more, governmental organizations encourage their use and finance the purchase of these products to farmers and ranchers. This situation leads to an extensive use of these products by unqualified personnel that may apply the rodenticides directly to open spaces. This has been reported as a usual practice and facilitates free access to these chemicals for many animals (SEO/Birdlife, 2012). It should also be noted that after rodents have consumed a lethal dose of ARs, they do not get sick or die instantly but do so over the course of several days (generally 2 to 4 days), experiencing a gradual change in their habits that can include erratic behavior or spending more time in open spaces; thus, they become easy prey for raptors (Cox and Smith, 1992; Stansley et al., 2013). During the period when rodents feed on the baits, they can consume approximately 8–10 times the LD50 of the products most commonly used in rodent control campaigns (Stansley et al., 2013). All of these factors lead to AR exposure in many non-target species, and this has been documented for various raptor species worldwide (Albert et al., 2010; Dowding et al., 2010; Elmeros et al., 2011; Guitart et al., 2010; Hughes et al., 2013; Lambert et al., 2007; Stansley et al., 2013; Stone et al., 2003). In some cases, raptors feed on the rodents against which these substances are used, but some species also feed on granivorous birds that sometimes have accidentally ingested cereal baits (Sanchez-Barbudo et al., 2012). As a result, several studies confirm the presence of AR residues in the tissues of raptors (Albert et al., 2010; Hughes et al., 2013; Rattner et al., 2011, 2012; Sanchez-Barbudo et al., 2012; Thomas et al., 2011; Walker et al., 2008), and it appears that in many cases, this exposure leads the birds to a secondary poisoning that can cause them to weaken or die (Hughes et al., 2013; Sanchez-Barbudo et al., 2012; Stone et al., 1999; Thomas et al., 2011).

Due to the relative isolation and climate of the Canary Islands, the flora and fauna of the islands are completely unique from those of the European and African continents. On this archipelago, many endemic species and subspecies are found in areas of high ecological value. There are 7 species of diurnal birds of prey and 2 nocturnal nesting birds of prey on the Canary Islands. Four of these are subspecies that are endemic to the Canary Islands, and two other species are endemic to the Macaronesian region (which includes the Azores, Madeira, Canaries and Cape Verde regions) (Lorenzo et al., 2012). Several anthropogenic circumstances have provoked a population decline of some of these species in recent decades which, along with their characteristic slow reproductive rates, are threatening their survival: power lines, malicious or accidental poisonings, and high tourist pressure on the territory (the archipelago has four national parks that receive 5.5 million visitors a year (MAGRAMA, 2013)), as well as the extensive past and present uses of pesticides in agriculture, among others. In particular, the rodenticides have been widely used in these islands in recent years because the local public administration has provided these products to the farmers for free (BOP, 2011). Although it has been demonstrated that the exposure of raptors to these chemicals is related to potential risks to their health and that this exposure could be threatening the raptor populations of these islands, there are no data documenting the rodenticide exposure for the populations of birds of prey from this region. To address this gap, we have designed this study with the aim of assessing first- and second-generation AR exposures in six species of raptors on the Canary Islands to determine if raptor species of the archipelago are exposed to toxic quantities of these substances, which could potentially represent a threat to their conservation.

2. Material and methods

2.1. Sample collection and ethics statement

Liver samples were obtained from necropsies of 104 birds of prey from 6 species that were admitted to the Wildlife Recovery Centers (WRCs) of Tafira (Gran Canaria, Spain) and La Tahonilla (Tenerife, Spain) between October 2009 and December 2012. The series included 9 common buzzards (*Buteo buteo*), 14 European sparrowhawks (*Accipiter nisus*), 16 Barbary falcons (*Falco pelegrinoides*), 21 common kestrels (*Falco tinnunculus*), 23 long-eared owls (*Asio otus*), and 21 barn owls (*Tyto alba*). The birds died naturally or were euthanized within one week of admission. Dead animals were kept frozen until the moment of the necropsy. No animal was killed for the purposes of this study. The main cause of death was determined by examining the birds macroscopically at the WRCs, and, when necessary, radiological or toxicological analyses were performed. The causes of death for all of the animals that were included in this study consisted of different types of trauma. The whole livers, the primary organ for the accumulation of rodenticides (Dowding et al., 2010), were excised and stored at −20 °C until sample preparation. Part of the liver samples used in this study was retained from a previous study of anthropogenic persistent pollutant exposure in raptors (Luzardo et al., 2014a).

2.2. Chemicals and reagents

Dichloromethane, hexane, ethyl acetate and cyclohexane were of the highest purity available (>99.9%) and were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure (UP) water was produced from a Milli-Q Gradient A10 (Millipore, Molsheim, France). Diatomaceous earth was purchased from Sigma-Aldrich (St. Louis, USA). Bio-Beads SX-3 was purchased

from BioRad Laboratories (Hercules, USA). Standards for ARs (warfarin, coumatetralyl, bromadiolone, brodifacoum, difenacoum, difethialone, and chlorophacinone) and an internal standard (IS, (±)-Warfarin-d_5) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All standards were pure compounds (purity from 98% to 99.5%). Stock solutions of each compound at 1 mg/mL were prepared in cyclohexane and stored at −20 °C. Diluted solutions from 0.1 ng/mL to 500 ng/mL were used for calibration curves.

2.3. Sample preparation and chemical analysis

The procedures for AR extraction and chemical analysis have been previously validated in our laboratory (Luzardo et al., 2014b). Briefly, 2 g of liver was homogenized in 5 mL of ultrapure water. Ten microliters of the IS (50 μg/mL in acetone) was added to each vial to reach a final concentration of 500 ng/mL. Ten grams of diatomaceous earth was added to absorb the moisture in the sample, 10 mL of dichloromethane/ethyl acetate/acetone (50/30/20) was added, and the sample was left in agitation for 5 min. The sample was centrifuged (4000 *g*), and the supernatant was concentrated, redissolved in acetonitrile, filtrated, and subjected to a purification step by freezing centrifugation. The resulting supernatant was used for the quantification of ARs.

The separation and identification of extracted analytes were performed by LC–MS/MS using a Thermo LC Accela Ultra instrument (Thermo Fisher Scientific Inc., USA) equipped with an analytic Accucore C18 column (2.6 μm, 150×3 mm; Thermo Fisher Scientific Inc., USA) as the stationary phase. The mobile phases were (A) ultrapure water as the aqueous phase and (B) methanol (HPLC–MS grade) as the organic phase. The flow was set at 800 μL/min. The injection volume was 25 μL, and the total run time was 5 min. The gradient was programmed as follows: 0–1 min: 50% A; 1–1.5 min: 50% A → 5% A; 1.5–3.5 min: 5% A; 3.5–3.7 min: 5% A → 50% A; 3.7–10 min: 50% A (for equilibration). The ARs were detected using a TSQ Quantum Max QqQ mass spectrometer equipped with the H-ESI II heated electrospray ionization source (Thermo Fisher Scientific Inc., USA). The mass spectrometer and the ionization source were programmed according to the following parameters: skimmer offset (4 V), sheath gas pressure (10 arbitrary units, a.u.), ion sweep gas pressure (8 a.u.), capillary temperature (250 °C), spray voltage (3500 V), and vaporization temperature (200 °C). The spectrometer was programmed in negative ionization mode. We initially determined the retention times of each compound in the full scan mode (range: m/z 45–500), and then we constructed a time-selected reaction monitoring (SRM) method by directly infusing pure standard methanolic solutions into the source. The gas in the collision cell was argon (99.99%) at a pressure of 0.25 Pa. The mass spectrometry settings, validation parameters, and toxicity values (Mineau et al., 2001) for the analytes included in the method are shown in Table 1.

2.4. Statistical analyses

Database management and statistical analysis were performed with the PASW Statistics v 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Using non-parametric statistics as data lacked normality and homogeneity of variances, mean rodenticide concentration was compared between raptor species using Kruskal–Wallis and Mann–Whitney U tests for general and pair-wise comparisons, respectively. The categorical variables were presented as percentages of detection, and differences in percentages were evaluated using the Chi-square test. P values <0.05 (two-tailed) were considered to be statistically significant.

3. Results and discussion

Of the 104 liver samples analyzed, 61% had detectable residues of at least one AR (Fig. 1A), and 36.5% contained more than one compound (six livers had detectable residues of three ARs and one liver had residues of four different compounds). None of the two first-generation ARs included in the study (warfarin and coumatetralyl), nor the diphacinone and difethialone, were detected in any of the samples (Fig. 1A). Bromadiolone, brodifacoum, difenacoum, and chlorophacinone were commonly detected in all six species (Table 2). The most frequently detected compound was bromadiolone (60.3% of positive cases). Conversely, the least common substance was chlorophacinone (14.7% of the positive cases). However, as shown in Table 2, the detection frequencies of these compounds varied widely depending on the species. Considering the sum of all residues, more than 75% of the individuals belonging to *A. nisus*, *T. alba*, and *A. otus* showed rodenticide residues in the liver, whereas the detection frequencies were lower in *B. buteo* and *F. pelegrinoides* (approximately 30%, Table 2). The frequencies of AR occurrence in the Canary Islands raptors were similar to those previously reported for *T. alba*, *B. buteo*, *A. nisus*, and *F. tinnunculus* in Britain (Dowding et al., 2010; Hughes et al., 2013) and for *T. alba*, *B. buteo*, and *F. tinnunculus* in France (Lambert et al., 2007). The occurrence in the Canary Islands raptors was also similar to the detection frequencies of rodenticide residues in other species of raptors from the European and American continents (Albert et al., 2010; Stansley et al., 2013; Thomas et al., 2011; Walker et al., 2008). However, it should be noted that the pattern of AR detection in raptors from the Canary Islands was more similar to that found in European countries other than Spain (mainland), where the most frequently detected compound was chlorophacinone (70%), while brodifacoum and difenacoum were only marginally detected (Sanchez-Barbudo et al., 2012). In our

Table 1
Toxicities of the pesticides detected by LC–MS/MS, method settings and results from recovery experiments.

				Mass spectrometry settings							Validation parameters			
No.	Compound	Toxicity (LD_{50}, mg/kg)[a] in birds	Log kow[b]	RT (min)	CV (V)	First transition m/z ➔ m/z	CE (V)	Second transition m/z ➔ m/z	CE (V)	IPs	LOD (μg/mL)	LOQ (μg/mL)	Average recovery % (RSD)	IS
LC–MS/MS method 1														
1	Coumatetralyl	38.3	3.46	1.57	65	291.1 ➔ 140.9	28	291.1 ➔ 247.0	22	4	0.01	0.03	89.2 (13.6)	1
2	Warfarin	942.0	2.70	1.71	56	307.1 ➔ 116.9	39	307.1 ➔ 250.0	24	4	0.005	0.02	92.7 (8.3)	1
3	Chlorophacinone	430.0	5.50	1.76	123	373.1 ➔ 116.0	50	373.1 ➔ 200.9	25	4	0.01	0.03	87.9 (12.4)	1
4	Difenacoum	50.0	7.62	1.83	90	443.2 ➔ 134.9	36	443.2 ➔ 293.0	33	4	0.005	0.01	91.3 (11.7)	1
5	Brodifacoum	4.5	8.50	1.88	108	521.1 ➔ 135.0	44	521.1 ➔ 186.9	39	4	0.005	0.01	97.4 (5.8)	1
6	Bromadiolone	138	7.02	2.02	96	525.1 ➔ 180.9	37	525.1 ➔ 249.9	37	4	0.005	0.01	94.3 (8.9)	1
7	Difethialone	0.9	5.17	2.08	100	537.1 ➔ 150.9	45	537.1 ➔ 370.9	36	4	0.01	0.03	86.9 (13.4)	1
Internal standard														
IS1	(±)-Warfarin-d_5			1.71	56	312.1 ➔ 116.9	39	312.1 ➔ 250.0	24	4	–		–	–

RT: retention time (min); CV: cone voltage; CE: collision energy; IPs: identification points; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation.
[a] Average data from different species. These data have been taken from Mineau et al. (2001), the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and the Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).
[b] Octanol–water partition coefficients.

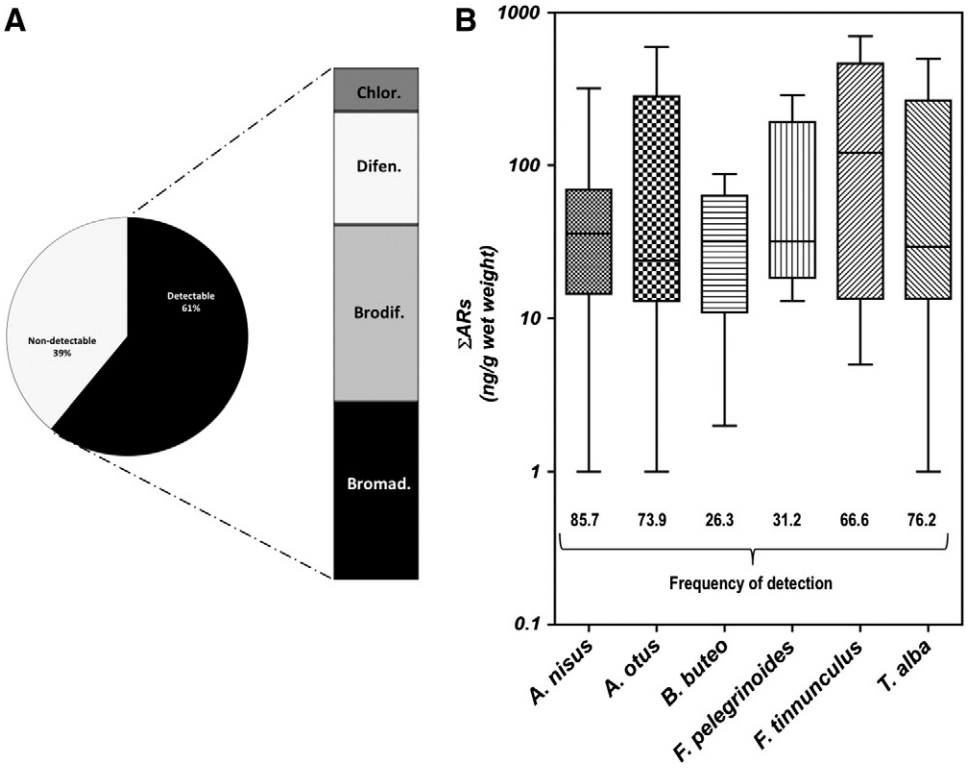

Fig. 1. Panel A. The percentages of animals with detectable and undetectable ARs and the distribution of the detected compounds. Panel B. The frequencies of detection and a box plot indicating the levels of ∑ ARs in the six species that were included in the study. The line inside the box represents the median, the bottom and top of the box are the first and third quartiles of the distribution, respectively, and the lines extending vertically from the boxes indicate the variability outside the upper and lower quartiles.

study, when more than one residue was detected, the most frequent combination was bromadiolone + brodifacoum (34.6%), followed by bromadiolone +diphenacoum (19.2%) (data not shown). Taken together, these results suggest that the marketing and use of these products in the Canary Islands are different from the rest of Spain, and our findings are consistent with the active ingredients of the products that are subsidized by the government (BOP, 2011).

Regarding the liver concentrations of ARs, *F. tinnunculus* showed the highest levels, with median values above 100 ng/g w.w. (Fig. 1B). Taken individually, bromadiolone was the substance that achieved the highest concentration in the entire series (geometric mean = 146.3 ng/g w.w., range = 0.2–516.1 ng/g w.w.) (Table 2). Brodifacoum was detected at high concentrations, especially among *F. tinnunculus* individuals (geometric mean = 57.4 ng/g w.w.). These results agree with other published studies showing that difenacoum, bromadiolone, and brodifacoum were the most prevalent substances and occurred in the highest concentrations (Christensen et al., 2012).

Because it has been previously reported that AR exposure levels can be determined by nocturnal or diurnal behavior (Sanchez-Barbudo et al., 2012), we explored the AR contamination profile in the Canary raptors according to their diurnal or nocturnal habits. We observed that the nocturnal species (*A. otus* and *T. alba*, n = 44 samples) showed higher concentrations of the sum of rodenticides (ΣARs) than the diurnal species ($P < 0.001$, Fig. 2A). These results agree with other studies that have shown the highest levels of AR exposure occurring in nocturnal raptors, especially with respect to second generation ARs (Sanchez-Barbudo et al., 2012). We also found that both the frequency of AR detection and the number of residues per animal were higher among nocturnal species (Fig. 2B and C, respectively). It should be highlighted that the percentage of AR detected in both nocturnal and

Table 2
Concentration (ng/g wet weight), 95% confidence interval, frequency of detection, and range of anticoagulant rodenticides in the livers of six raptor species from the Canary Islands, Spain.

Species	∑ARs[a]			Bromadiolone		Brodifacoum		Difenacoum		Chlorophacinone	
	Mean ± SD (range)	Freq.	No. res.[b]	Mean ± SD (95% confide.)	Freq.	Mean ± SD (95% confide.)	Freq.	Mean ± SD (95% confide.)	Freq.	Mean ± SD (95% confide.)	Freq.
Accipiter nisus (n = 14)	57.7 ± 88.0 (n.d.–321.9)	85.7%	1.6 ± 0.7	31.9 ± 22.6 (−16.9–80.9)	35.7%	13.2 ± 8.1 (−4.2–112.0)	35.7%	3.6 ± 2.0 (n.d.–28.2)	35.7%	0.34 ± 0.57 (−0.6 ± 2.1)	21.4%
Asio otus (n = 23)	132.2 ± 177.6 (n.d.–598.2)	73.9%	1.6 ± 0.6	77.2 ± 29.6 (15.9–138.5)	39.1%	15.8 ± 5.4 (4.6–26.9)	43.5%	2.8 ± 1.3 (0.2–5.4)	26.1%	0.5 ± 0.4 (−0.5–1.5)	4.3%
Buteo buteo (n = 9)	36.8 ± 32.4 (n.d.–88.9)	26.3%	1.6 ± 0.6	2.6 ± 1.9 (−1.5–6.5)	10.5%	4.9 ± 4.1 (−3.7–13.5)	10.5%	2.9 ± 2.6 (−2.4–8.3)	15.8%	0.3 ± 0.2 (−0.3–0.8)	5.3%
Falco pelegrinoides (n = 16)	91.5 ± 115.9 (n.d. ± 298.8)	31.2%	1.2 ± 0.4	26.2 ± 18.6 (−13.5–66.0)	18.8%	0.8 – 0.8 (−0.9–2.6)	6.3%	1.4 ± 1.4 (−1.6–4.5)	6.3%	0.1 ± 0.1 (−0.1–0.2)	6.3%
Falco tinnunculus (n = 21)	219.0 ± 237.5 (n.d.–702.7)	66.6%	1.7 ± 0.6	79.8 ± 34.4 (8.0–151.4)	42.9%	57.4 ± 34.6 (−14.7–129.5)	42.9%	8.2 ± 6.9 (−6.2–22.6)	23.8%	0.6 ± 1.8 (−0.6–1.8)	4.8%
Tyto alba (n = 21)	134.4 ± 163.1 (n.d.–500.1)	76.2%	2.0 ± 0.8	75.8 ± 23.9 (25.9–125.6)	61.9%	12.5 ± 5.9 (0.1–24.9)	38.1%	12.6 ± 9.5 (−7.3–32.4)	33.3%	1.2 ± 1.0 (−0.9–3.3)	14.3%

[a] Sum of all anticoagulant rodenticides.
[b] Mean number of anticoagulant residues per sample.

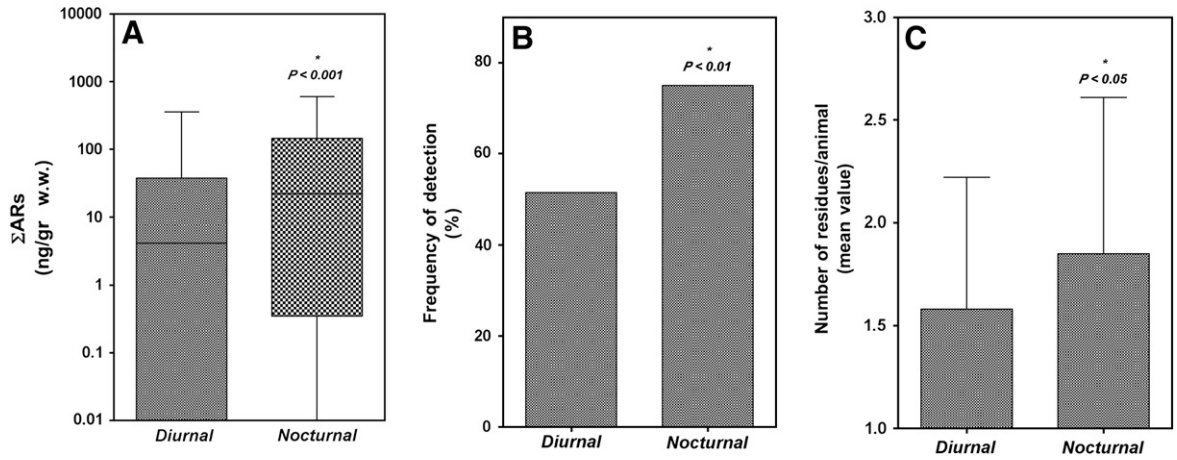

Fig. 2. A comparison between raptors grouped according to their diurnal and nocturnal behavior. Panel A. Liver concentration of ∑ARs. The line inside the box represents the median, the bottom and top of the box are the first and third quartiles of the distribution, respectively, and the lines extending vertically from the boxes indicate the variability outside the upper quartiles. Since the first quartile was 0 in all the cases, there are no lines extending outside the lower quartiles. Panel B. Frequencies of detection of ∑ARs. Panel C. Number of residues per animal (mean ± SD).

diurnal raptors from the Canary Islands was higher than that described in mainland Spain (Sanchez-Barbudo et al., 2012). The levels were double in the case of the diurnal raptors.

The profile of AR contamination was also analyzed with respect to the eating habits of the species under study. Notably, when we considered all of the birds that primarily feed on small mammals and reptiles as a group (*A. otus*, *T. alba*, *and B. buteo*), we found a higher median AR contamination level than that of the species whose diet was primarily composed of birds (*F. tinnunculus* > *F. pelegrinoides* > *A. nisus*) ($P < 0.01$, Fig. 3A). No significant differences were observed regarding the frequency of detection or the number of residues per animal detected in these two groups (Fig. 3B and C). Our results indicate that the contamination profile is dependent on the feeding habits of the raptors surveyed in this study. The fact that small mammal-eating birds accumulate AR residues is not a surprising result because ARs are designed to kill small mammals (such as mice and rats), and these small mammals can accumulate considerably high levels of the chemicals in their livers and digestive tract. However, it is remarkable that raptors that feed on other birds have relatively high levels of these contaminants. Surprisingly, it has been previously reported that some of these species, such as *A. nisus*, have similar exposure rates compared to species that prey on rodents (Hughes et al., 2013). Because birds are not the target species of ARs, this observation is an indicator of the penetration achieved by these contaminants in the food chain. Many granivorous birds may actually ingest the anticoagulant baits by cereal impregnation, in which the chemical is adhered to the grain with oil and dyed with a color, usually blue or red, to act as a deterrent to birds and for identification purposes. However, several studies have demonstrated that in spite of the deterrent measures, many granivorous birds feed on these baits and may suffer either a primary poisoning or a subtle but chronic exposure as a result (Borst and Counotte, 2002; Lemus et al., 2011; Sanchez-Barbudo et al., 2012). Thus, raptors that hunt these birds would also be exposed to significant amounts of these compounds. However, the insectivorous birds theoretically would represent a minor source of ARs for the raptors. Nonetheless, it has been shown that some of the invertebrates that are important parts of the diet for insectivorous birds may act as anticoagulant reservoirs because these invertebrates feed on the baits without experiencing adverse effects (Johnston et al., 2005a,b; Spurr and Drew, 1999). Thus, raptors that feed on insectivorous birds may also be exposed to considerable amounts of ARs.

Initially, all of the animals used in this study were diagnosed with trauma (mainly motor vehicle collisions, window collisions, electrocution, flight collision, and falling out of the nest). After the necropsy, we only had direct evidence of AR poisoning as the cause of the death in one case (diffuse hemorrhages evidencing a lack of blood clotting factors during the necropsy in one individual of *A. otus*). This finding was consistent with other studies that determined the AR levels in raptors:

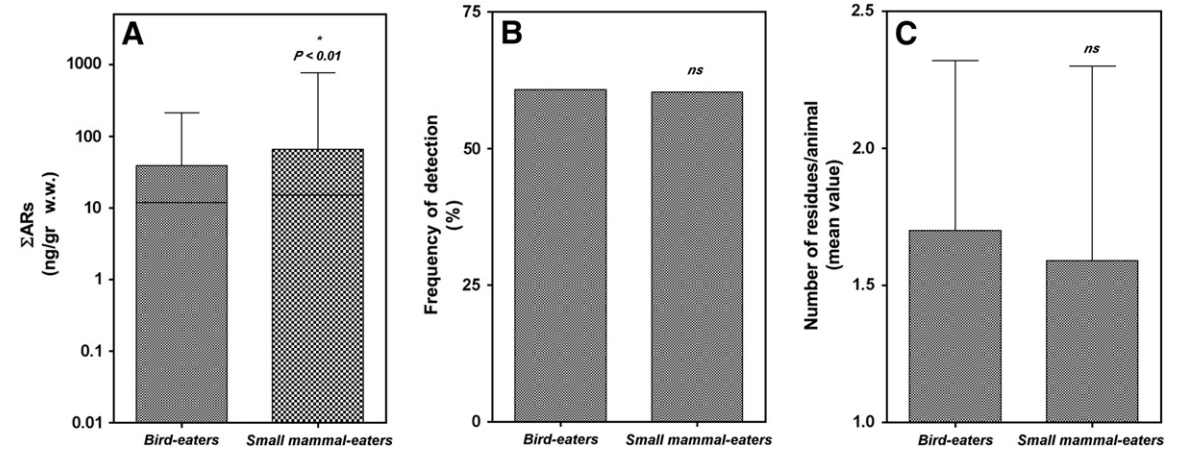

Fig. 3. A comparison between raptors grouped according to their feeding habits. Panel A. Liver concentration of ∑ARs. The line inside the box represents the median, the bottom and top of the box are the first and third quartiles of the distribution, respectively, and the lines extending vertically from the boxes indicate the variability outside the upper quartiles. Since the first quartile was 0 in all the cases, there are no lines extending outside the lower quartiles. Panel B. Frequencies of detection of ∑ARs. Panel C. Number of residues per animal (mean ± SD).

no direct relation between necropsy findings and ARs was found in most cases (Sanchez-Barbudo et al., 2012; Stansley et al., 2013). However, the lack of direct evidence for blood clotting disorders, which are the most obvious effects of these compounds, should not automatically be interpreted as a lack of toxicity in these animals. For example, chronic exposure to low levels of oral anticoagulants has been associated with reduced bone density and a higher frequency of bone fractures and osteoporosis due to a deficiency in the synthesis of osteocalcin, which is not carboxylated in the presence of these compounds (Pearson, 2007). In fact, low liver toxicity thresholds (0.1–0.2 mg/kg) have been established for these compounds in some species of raptors (Stansley et al., 2013; Thomas et al., 2011), which are the only available data that can be used for comparisons to date. It is remarkable that these thresholds were exceeded in a large percentage of the animals used in our study (34.8%). Moreover, it should be noted that a wide variation in AR sensitivity exists between different species (Erickson, 2004; Thomas et al., 2011). Although the diagnostic interpretation of liver AR residues in the absence of other clinical findings is problematic, the possibility exists that a chronic exposure to these compounds may be causing a change in the health status of these animals, which would predispose them to weakness, sickness and accidents (Albert et al., 2010).

In conclusion, our study shows that the use of ARs to control rodent populations in the environment implies that these products will enter the food chain, thus representing a relevant threat for wildlife, especially raptors. It is necessary for authorities to increase the management and regulation of these substances. Without addressing the problem of inadvertent rodenticide exposure in the Canary Islands wildlife, any wildlife recovery or reintroduction program may not be undertaken successfully.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank the Canary Islands Government (Dirección General de Protección de la Naturaleza, Gobierno de Canarias (Nº 104/2013) for their financial support. The authors are also thankful to the veterinarians of the Wildlife Recovery Centers of Gran Canaria (Pascual Calabuig and Dolores Estévez) and Tenerife (Santiago Mayans) for their collaboration in the necropsies and in the sampling of the animals, and also to Mrs. María de los Reyes Suárez Hanna for her technical assistance.

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Science of the Total Environment 505 (2015) 1093–1099

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain)

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HIGHLIGHTS

- Report of pesticide poisoning of animals in the Canary Islands (Spain)
- 225 animals poisoned, intentionality confirmed in 52% of cases
- Frequent involvement of protected wild species
- The highest incidence of poisoning reported in any region from European Union
- European Union's banned pesticides identified in 75% of animals

ARTICLE INFO

Article history:
Received 27 September 2014
Received in revised form 26 October 2014
Accepted 26 October 2014
Available online 11 November 2014

Editor: D. Barcelo

Keywords:
Banned pesticides
Intentional poisoning
Carbofuran
Aldicarb
Poisoning

ABSTRACT

Although nowadays the intentional poisoning of domestic and wild animals is a crime in EU, in the past the poison was used in rural areas of a number of European countries to kill animals that were considered harmful for human activities. In Spain evidences indicate that intentional poisonings continue to occur throughout the entire country nowadays. This situation seems to be of particular concern in the Canary Islands (Spain), where this study was performed. Our results confirmed that 225 animals were poisoned by pesticides over the study period (32 months; 2010–2013). The intentionality of the poisoning was confirmed in 117 cases. It has to be highlighted that the other 108 animals also died by pesticide poisoning, although the intentionality was only suspected. This incidence is currently the highest reported in any region from European Union. The pesticides carbofuran, bromadiolone, brodifacoum and aldicarb were the most frequently detected involved. Among the affected species, it has to be highlighted that endangered species are frequently affected in poisoning incidents. Notably, chemicals banned in the EU (carbofuran and aldicarb) were identified in approximately 75% of cases, and in almost 100% of baits, which suggests that these pesticides are still available to the population. Several circumstances may explain these results. Firstly, little control over the sale and possession of pesticide products, and the potential existence of an illegal market of pesticides banned in the European Union in the neighbouring African continent. In addition, the limited awareness of the population about the dangerousness of these compounds, for the environment, animals, or even people, make the situation very worrying in these islands. Stronger regulations, control of legal and illegal pesticide use, development of educational programs and legal action in poisoning incidents are needed to decrease the impact of pesticide misuse on wildlife and domestic animals.

1. Introduction

It is well documented that agricultural pesticides cause a large number of accidental poisonings in target and non-target species. In the literature it has been described that wild animals are particularly

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http://dx.doi.org/10.1016/j.scitotenv.2014.10.093
0048-9697/

affected by accidental pesticide exposure (Berny, 2007; Berny and Gaillet, 2008; Guitart et al., 1999; Kwon et al., 2004; Martínez-Haro et al., 2008; Ogada, 2014; Slaninova et al., 2009; Wagner et al., 2013); additionally, domestic animals (Berny et al., 2010; Guitart et al., 2010a; Hornfeldt and Murphy, 1997), and humans are frequently poisoned by pesticides (Eddleston et al., 2002; Litchfield, 2005). However the situation is even worst when pesticides are used intentionally to kill animals, mainly through the use of poisoned baits. Of the chemicals that baits can be laced with, pesticides are commonly used (Brown et al., 2005; Ogada, 2014). It has been estimated that pesticides are used illegally in up to 68% of all suspected poisoning animal cases (Berny, 2007). Epidemiological studies revealed that pesticides account for approximately 52.5% of bird poisonings and that pesticides are a major cause of wild mammal deaths (Guitart et al., 2010b). Because of their high toxicity, several restrictions have been applied to many compounds that are currently banned or severely restricted in the EU (EC, 2003, 2006).

The use of poisoned baits is an illegal, massive, non-selective method affecting many species, in addition to those against whom they are directed. Its use is a serious threat to public health and biodiversity in Europe. In the EU, this illegal practice represents one of the biggest conservation problems for some endangered species, often becoming the main cause of non-natural death. Currently intentional poisoning is an act of animal cruelty and a criminal offense in the European legislation.

In Spain and other Mediterranean countries, where conflicting rural uses may motivate the illegal use of poisoned baits, the problem is particularly serious. Although the current Spanish legislation condemns and prosecutes the use of poisoned baits, this practice has been legally used (and even promoted) to eliminate animals in Spain over the past century (BOE, 1953). Thus, the poison was used in rural areas to kill animals that were considered harmful for different activities, including hunting, farming, agriculture, pigeon breeding or apiculture (De la Bodega, 2012). In addition, poisoned baits were used in urban areas against stray cats and dogs. Due to such circumstances, the habit of using poisoned baits is deeply rooted in the population and is not perceived as a serious risk, even currently, in rural areas.

Currently, no governmental body in Spain can provide reliable statistics on the actual incidences of animal poisoning. However, various reports have examined the effects of poisons on endangered wildlife populations (González et al., 2007; Hernandez and Margalida, 2008; Hernández and Margalida, 2009; Margalida et al., 2008). Moreover, veterinary toxicology services from several universities have published statistics indicating that animal poisoning remains a common practice in Spain (Berny et al., 2010; Guitart et al., 1999; Motas-Guzman et al., 2003). Many poisoning cases were caused by strychnine (rodenticide) until the year 2000, when its use was prohibited (Hernandez and Margalida, 2008), whereas from 2000 to 2007, most primary and secondary poisoning cases were caused by anticholinesterase agents, mainly aldicarb and carbofuran, and by anticoagulant rodenticides (Fajardo et al., 2012; Guitart et al., 1999; Hernandez and Margalida, 2008; Martínez-Haro et al., 2008; Motas-Guzman et al., 2003). In the EU, the marketing of products containing aldicarb was withdrawn in 2003 (EC, 2003), and products containing carbofuran in 2007 (EC, 2007). Therefore, a reduction in poisoning episodes involving these substances should be expected. However, studies on other compounds indicate that legal and commercial restrictions have not influenced the intentional illegal use of these substances as poisons (Kervegant et al., 2013; Martínez-Haro et al., 2008).

In our laboratory, samples poisoning of wild and domestic animals are received since 2003. Our preliminary evidence indicated that pesticides were often involved in poisoning episodes, and in many cases there was also evidence of the intention thereof, with the use of banned substances. With these antecedents in mind, we planned this study in which samples of all cases of poisoning of animals detected in the Canary Islands since the beginning of 2010 were taken for chemical analysis. A careful and systematic collection of all data of each episode was performed. Our main objective was to test the hypothesis that in this archipelago exists a continued use of pesticides banned in the EU for the intentional poisoning of wildlife. This paper presents and discusses the results of those cases where intentional poisoning was suspected initially, and also those cases of poisoning of unknown origin. Cases of accidental poisoning with a clear accidental origin were expressly excluded. The collection of cases lasted for a period of 32 months. Our results have confirmed a high incidence of intentional illegal poisoning in this region as well as the continued use of pesticides that were banned in the EU for several years.

2. Materials and methods

2.1. Sampling and ethics statement

We analysed 544 samples (tissues and baits) from 188 poisoning cases that involved the death of 225 animals. The number of animals involved per incident ranged from 1 to 14. Samples analysed included liver, blood, stomach contents, vomiting, brain, kidney, flesh and bones, and plastic containers. Investigated animals belonged to 14 different species (187 dogs and cats, 22 reptiles, 75 raptors/scavengers, 11 wild mammals, and 11 wild non-raptor birds). Sampling was performed between January 2010 and September 2013 in the Wildlife Recovery Centres (WRCs) and the Veterinary Hospital of the Veterinary Faculty of the ULPGC, where clinical veterinary facilities of the Canary archipelago submitted cases for analyses. During the necropsy samples were collected and kept frozen (−20 °C) until they were submitted to the Service of Clinical and Analytical Toxicology (SERTOX) of the University of Las Palmas de Gran Canaria (ULPGC, Canary Islands, Spain), where remained frozen at the same temperature until analysis. Most animals were found dead in the countryside or urban areas or died while at veterinary facilities. No animals were killed for the purposes of this study, and no experiments were performed on or with samples from living animals.

2.2. Data selection

Circumstantial, medical and pathological records of all suspected animal poisoning cases submitted to the SERTOX were reviewed. Only cases in which one or more pesticides were identified in samples from animals (mainly blood, liver, brain or gastric contents), and these pesticides were quantified at a concentration considered toxic for that species (http://toxnet.nlm.nih.gov/), were considered positive cases for this study. We also included in this study all those baits, which were suspicious to have been laced with toxic substances. Data analysis included the time and location of the poisoning, seasonality, type of species, number of animals affected, and pesticide(s) detected. A major challenge in this type of cases is to distinguish whether they are intentional or accidental poisonings. For this task we have had the help of the veterinary experts from the WRCs of the Canary Islands. The veterinary assessment of the potential intentionality was based on: a) visualization of abnormal substances, baits or materials and identification of possible exposure route or method of administration; b) estimate of time of exposure to the toxic, based on the progression of physical symptoms or physiological changes; c) direct evidence of intentionality, such as remains of bait or unusual food items in the mouth, stomach contents or vomit; d) exclusion of potential natural or accidental poisoning sources. Besides the above, the veterinarians gave a written report about pain or possible animal suffering associated with a particular poison, which may be necessary to file animal cruelty charges against the perpetrator. In many cases environmental protection agents of the Government of the Canary Islands or officers of the Service of Nature Protection of the Police (Guardia Civil) conducted the forensic investigation of the crime scene to provide additional evidences of poisoning. On the basis of all the above, the cases were initially classified as: a) allegedly intentional poisoning; b) poisoning of unknown origin; c) accidental poisoning. These latter cases were excluded from this study.

2.3. Chemical analyses

Procedure for the extraction, purification and quantification of pesticides in samples from animal poisoning incidents have been fully validated in our laboratory and previously published (Luzardo et al., 2014). This procedure was developed for the quantification of 117 pesticides, which were selected because of their high toxicities in animals or their frequent use in animal poisonings (Table 1) (Berny, 2007; Berny et al., 2010; De la Bodega, 2012; Mineau et al., 2001). Briefly, the samples (2 g) were subjected to a solid-extraction with 10 ml of a mixture of dichloromethane/ethyl acetate/acetone (50/30/20) followed by one (gel permeation chromatography, GPC) or two purification steps for highly degraded matrices (GPC plus freezing centrifugation). The purified extract was divided into two aliquots that were re-dissolved in cyclohexane and acetonitrile prior to chromatographic analyses. We used a Thermo Trace GC Ultra with split/splitless injector for the chromatographic analyses coupled to a triple quadrupole TSQ XLS mass spectrometer (Thermo Fisher Scientific Inc., USA) for the analysis of 91 non-polar pesticides, using a column of 30 m × 0.25 mm, 0.25 μm film thickness (BPX5, SGE Inc., USA) as the stationary phase and helium (99.999%) as the carrier gas. A timed selected reaction monitoring (t-SRM) method was constructed to analyze all the target compounds plus ISs in a single run (61 min). The most polar pesticides were analysed by two complementary methods using a Thermo LC Accela Ultra instrument coupled to a TSQ Quantum Max triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., USA). An analytic Accucore C18 column (2.6 μm, 150 × 3 mm; Thermo Fisher Scientific Inc., USA) was used for the separation of anticoagulant rodenticides, and an analytic Synergi Hydro-RP column (4.0 μm, 150 × 4.6 mm; Phenomenex, Torrance, USA) was used for the analysis of the rest of polar pesticides. A t-SRM method was contructed by directly infusing pure standard methanolic solutions into the source to analyze 26 target compounds plus ISs in two separate runs. The calibration curve ranged from 0.5 to 500 ng/mL and included all of the compounds in each calibration standard level. The quantification was performed by the internal standard method. An example can be found in Supplementary Fig. 1. In each batch of samples, three controls were included: a reagent blank consisting of a vial containing only cyclohexane and two internal laboratory quality controls (QC) consisting of melted meat fat and chicken liver, both spiked at 20 μg/kg of each of the analytes, which were processed using the same method as the samples. The batch analyses were considered valid when the values of the analytes in the QC were within a 10% of deviation of the theoretical value. All the details of the methodology and validation procedure can be found in Luzardo et al. (2014).

2.4. Statistical analysis

Database management and statistical analysis were performed with the PASW Statistics v 17.0 statistical software (SPSS Inc., Chicago, IL,

Table 1
Toxicities of the pesticides included in the screening.

	Toxicity [a]			Toxicity [a]			Toxicity [a]	
Compound	LD_{50} birds	LD_{50} mammals	Compound	LD_{50} birds	LD_{50} mammals	Compound	LD_{50} birds	LD_{50} mammals
Acephate	125.0	321.0	Dichlorphos	8.8	61.0	Metolcarb	100.0	109.0
Aldicarb	3.8	1.9	Dicrotophos	1.2	11.0	Metomil	20.5	24.9
Aldrin	7.2	65.0	Dieldrin	13.3	65	Mevinphos	1.4	4.0
Allethrin	2030.0	370.0	Difenacoum	50.0	50.0	Monocrotophos	0.8	15.0
Amitraz	–	100.0	Difethialone	0.9	4.0	Nuarimol	200.0	2450.0
Azinphos ethyl	34.4	12.0	Dimefox	1.7	3.5	Omethoate	125.0	50.0
Azinphos methyl	8.5	10.0	Dimethoate	45.6	220.0	Oxamyl	4.2	30.0
Bendiocarb	21.0	35.0	Dioxathion	200.0	10.0	Parathion ethyl	1.3	0.9
Benfuracarb	92.0	102.0	Disulfoton	2.4	5.0	Parathion methyl	5.0	57.0
Bifenthrin	1975.0	54.5	Ediphenphos	350.0	100.0	Phenthoate	58.6	138.0
Brodifacoum	4.5	2.5	Endosulfan sulphate	52.4	18.0	Phorate	1.0	20.0
Bromadiolone	138	16.5	Endosulfan, alpha	35.0	26.0	Phosalone	–	112.0
Bromophos ethyl	20.5	125.0	Endosulfan, beta	35.0	26.0	Phosmet	18.0	40.0
Bromoxynil	50.0	78.0	Endrin	1.7	3.0	Phosphamidon	1.8	6.0
Cadusafos	16.0	71.4	EPN	2.4	20.0	Phoxim	5.6	250
Carbaryl	56.0	150.0	Ethion	45.0	13.0	Pirimicarb	45.5	100.0
Carbofuran	22.4	10.2	Etoprophos	4.2	34.0	Pirimiphos ethyl	3.0	25.0
Carbophenothion	5.8	14.0	Famphur	1.8	59.0	Pirimiphos methyl	30.0	1150.0
Carbosulfan	120.0	115.0	Fenamiphos	2.4	10.0	Profenofos	1.9	116.0
Carboxin	42.2	430.0	Fenitrothion	11.0	142.0	Propachlor	91.0	392.0
Chlordane, cis	220.0	50.0	Fensulfothion	0.3	2.2	Propaphos	2.5	61
Chlordane, trans	220.0	50.0	Fenthion	1.4	46.2	Propetamphos	49.0	130.0
Chlorfenvinphos	13.0	20.0	Flucythrinate	2708.0	76.0	Propoxur	19.9	51.2
Chlormephos	65.0	12.5	Fonofos	10.0	3.0	Pyrazophos	118.0	184.0
Chlorophacinone	430.0	7.5	Formothion	630.0	175.0	Quinalphos	20.0	75.0
Chlorpyrifos	5.2	60.0	Heptachlor	125.0	50.0	Resmethrin	75.0	250.0
Chlorpyriphos methyl	13.0	2000.0	Heptenophos	17.0	117.0	Sulfotep	25.0	22.0
Chlorthiophos	45.0	20.0	Imidacloprid	152.0	98.0	Sulprofos	65.0	70.0
Cifluthrin	250.0	300.0	Isazophos	244.0	27.0	Tebufenpyrad	2000.0	210.0
Coumatetralyl	38.3	42.5	Isobenzan	1.0	5.0	Tefluthrin	267.0	22.0
Cyanazine	400.0	141.0	Isofenphos	3.0	91.5	TEPP	1.3	2.3
Cyanophos	3.0	215.0	Isoxathion	21.6	112.0	Terbufos	15.0	3.5
Cyproconazole	150.0	352.0	Leptophos	268.8	65.0	Tetrachlorvinphos	100.0	4200.0
Dazomet	424.0	415.0	Lindane	127.0	25.0	Thiometon	100.0	37.0
DDT	1135.0	200.0	Malathion	400.0	53.0	Thionazin	2.4	5.0
Deltamethrin	1000.0	22.0	Mephospholan	2.8	11.0	Triazophos	4.2	57.0
Diallate	167.0	395.0	Metamidofos	14.3	18.5	Trichloronat	1.6	10.0
Diazinon	2.0	76.0	Methidathion	80.0	25.0	Warfarin	942.0	6.5
Dichlone	–	160.0	Methiocarb	2.4	16.0			

[a] Average data from different species. These data have been taken from Mineau et al. (2001) and the National Library of Medicine internet resources ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp) and Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

USA). Kolmogorov–Smirnov test was employed for testing the normality of the distribution of the variables. Differences between variables were performed using Student's *t*-test. Categorical variables (percentages) were compared by the chi-squared test. P values <0.05 (two-tailed) were considered to be statistically significant.

3. Results

We finally included in this study 455 samples from 225 deceased animals and 24 baits. 117 animals were initially classified as having been poisoned on purpose according the veterinary services. The rest (108 animals) could not be initially classified nor as intentionally nor as accidentally poisoned. All the baits were initially classified as intentionally delivered in the environment. The number of deceased animals in cases where a pesticide was detected at toxic concentrations (Mineau et al., 2001) (Table 1) was significantly higher than from negative cases, this is, cases where no pesticide was detected (mean 2.73 ± 0.31 vs. 1.82 ± 0.22, $P < 0001$).

The species of domestic animals affected included cats (number of pesticide-poisoned/analysed animals, n = 84/103), and dogs (n = 42/84). The wildlife species involved in suspicious poisoning incidents included Eastern Canaries lizards (*Gallotia stehlini*, n = 22/22), common buzzards (*Buteo buteo* = 16/20), European turtle doves (*Streptopelia turtur*, n = 9/11), common ravens (*Corvus corax* = 13/16), European hedgehogs (*Erinaceus europaeus* = 11/11), common kestrels (*Falco tinnunculus*, n = 15/17), barn owls (*Tyto alba*, n = 8/9), long-eared owls (*Asio otus*, n = 5/6), Egyptian vultures (*Neophron percnocterus*, n = 0/3), and Barbary falcons (*Falco pelegrinoides*, n = 4/4). In addition, pesticides were detected in 81.82% of 24 baits (meat, fish, bones and flesh, bread, milk, or plastics and containers). The pesticides that have been detected in each species or the baits are presented in Table 2.

Pesticides were present in 61.34% of the analysed cases (76.47% of the animals submitted for testing). Fourteen different pesticides were detected with a mean of 1.23 ± 0.58 pesticides per incident. The chemicals most frequently detected were carbofuran (43.12% of the incidents, n = 119 dead animals), bromadiolone (13.62%, n = 18), aldicarb (12.23%, n = 51), and brodifacoum (10.56%, n = 17). The other pesticides were oxamyl (8.32%, n = 11), methomyl (8.43%, n = 19), difenacoum (6.85%, n = 10), carboxin (4.07%, n = 9), chlorpyrifos (3.11%, n = 7), diazinon (2.04%, n = 4), fenthion (2.04%, n = 3), propoxur (1.81%, n = 2), fenazaquin (0.90%, n = 2), and fipronil (0.90%, n = 1). No significant differences in the toxic concentrations of any of the pesticides among species were found. However we found a highly significant relationship between the number of dead animals and the pesticide involved. The most lethal pesticides in our study were aldicarb and carbofuran, which involved the death of 3.92 ± 0.78 and 2.52 ± 0.46 animals per incident, respectively ($P < 0.01$). We also found that Eastern Canaries lizard and domestic cat were the species where higher number of individuals died per incident (6.11 ± 1.82 and 3.76 ± 1.25 animals per incident respectively).

Interestingly, four of the 14 pesticides detected were banned products in the EU, including aldicarb, carbofuran, diazinon, and fenthion. Carbofuran and aldicarb were responsible for the death of approximately 75% of the animals from positively identified cases (Fig. 1). These pesticides were detected in the baits and in domestic and wild animals (Table 2). We considered very relevant the fact that 93.75% of positive baits had been laced with either carbofuran or aldicarb (Fig. 1, and Table 2). It was also considered very relevant that in 97.43% of the cases that were initially classified as intentionally poisoned pesticides were positively identified and quantified at levels that revealed the substance was the cause of the death. Moreover in 103 of these 117 animals (88.03%) the compound detected was a banned substance (carbofuran, aldicarb or diazinon).

We have to note that all of the species were not affected by pesticides identically. Cats, dogs, and wildlife species such as Eastern Canaries lizards, European hedgehogs and common buzzards were significantly affected by banned pesticides ($P < 0.005$). On the contrary, the nocturnal birds of prey (barn owls and long-eared owls) were mainly affected by products that are legal in the EU (anticoagulant rodenticides, $P < 0.01$).

4. Discussion

We report in this work the relevance of pesticide poisoning as a cause of death in domestic animals and wildlife in the Canary Islands (Spain) according to data obtained from SERTOX. Our results indicate that the incidence of intentional poisonings in this archipelago is very high, probably higher than that of other European regions.

It has to be highlighted that products that were banned several years ago were involved in a significant percentage of the cases. Notably, the involvement of chemicals banned in the EU (such as carbofuran and aldicarb) in the death of animals does not seem to be decreasing as expected.

Table 2
Identification of pesticides in animals and baits from poisoning episodes occurred in the Canary Islands during the period 2010–2013.

	Principal toxicant(s)				
	Carbofuran	Aldicarb	Other AcHE Inhibitors	Anticoag.	Others
Wild animals					
Eastern Canaries Lizard (*Gallotia galloti*)	6	16			
Barn Owl (*Tyto alba*)				8	
Common Kestrel (*Falco tinnunculus*)	3	4	3	4	1
Long-eared Owl (*Asio otus*)	1			4	
Common Buzzard (*Buteo buteo*)	9	3	2	1	
European Hedgehog (*Erinaceus europaeus*)	11				
Common Raven (*Corvus corax*)	8		5		
European Turtle-dove (*Streptopelia turtur*)				4	6
Barbary Falcon (*Falco pelegrinoides*)	1		2	1	2
Eurasian Coot (*Fulica atra*)	1				
Long-eared Bat (*Plecotus teneriffae*)					2
Domestic animals					
Cats	59	7	16	1	
Dogs	12	19	7	4	
Goat					**1**
Baits and suspicious materials					
Meat	3	1			
Feed	3		1		
Bones and flesh	1	3			
Other	4				

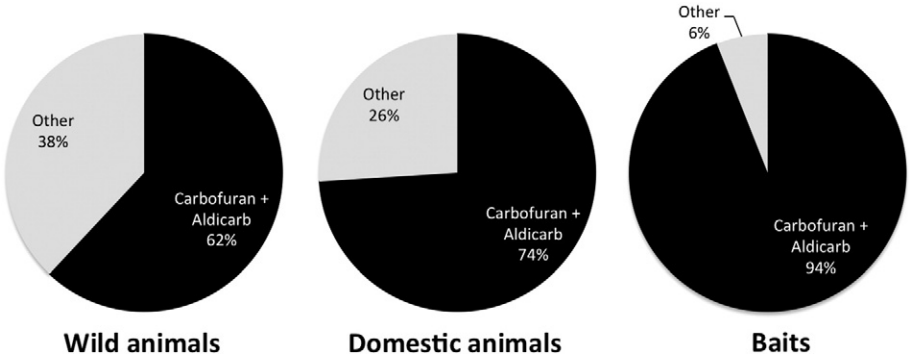

Fig. 1. Frequency of the implication of carbofuran and aldicarb in the animal poisoning incidents.

Because this practice is greatly harmful to biodiversity as well as an important public health problem, it is necessary that authorities enact effective measures on the marketing of toxic chemicals, control of banned chemical stocks, the implementation of educational programs, and effective criminal prosecution of poisoners to prevent, or at least minimise, the incidence of this harmful practice.

The high incidence of pesticide poisoning cases in this territory, and the high mortality of the exposed animals are remarkable. The data presented here are much higher than other results published in Europe (Guitart et al., 1999; Motas-Guzman et al., 2003), which reported far fewer cases per year. However, there are no official animal poisoning records, and recent information from many regions is lacking. From the literature it can be assumed that the figures reported in those studies only represent an approximation of the actual incidence of wildlife mortality because it has been estimated that less than 10% of poisoning cases are detected and sent to a forensic laboratory (De la Bodega, 2012; Guitart et al., 1999). This is especially relevant for wildlife because sick animals often become less visible and many die in nests, burrows or inaccessible places. Nevertheless, our findings indicate that the actual incidence of mortality from poisoning in the Canary Islands is very high and undoubtedly higher than that of other European regions.

It has drawn our attention that the percentage of cases in which there was evidence of intentional poisoning was very high in the Canary Islands, as we confirmed the presence of a pesticide at toxic levels in the great majority of the cases that were initially classified as intentional poisonings by the veterinary services. Notably, substances that were banned several years ago were detected in approximately 75% of the positive cases, and this percentage raised to almost 90% in the cases classified as intentional poisonings, and to almost 100% in the baits. In Spain and other Western countries, these highly toxic products have been often involved in poisonings, but the references are dated prior to the permanent ban of these pesticides (Guitart et al., 1999; Hernandez and Margalida, 2008; Hernández and Margalida, 2009; Motas-Guzman et al., 2003). After the ban of other pesticides, such as strychnine (Hernandez and Margalida, 2008) or paraquat (Kervegant et al., 2013), poisonings continued for several years because stocks of these chemicals that had been acquired before the prohibition were illegally used until the supply was exhausted. Nevertheless, the incidence of poisoning with these banned chemicals has exhibited a progressive decreasing yearly trend each year until ultimately ceasing to occur (Hernandez and Margalida, 2008; Kervegant et al., 2013). According to our data, the opposite situation is occurring in the Canary Islands because an increased incidence in animal poisonings with carbofuran, aldicarb, and other banned compounds (such as diazinon and fenthion) has been detected. The reasons for these findings are not clear; however, in a recent study, some companies agreed to the sale of banned substances when the authors attempted to purchase 5 g on the internet, which demonstrates the existence of an illegal market of banned pesticides (De la Bodega, 2012) that may also be present in the Canaries.

Carbofuran and aldicarb are granular insecticides that were banned throughout the European Union (EC, 2003, 2007) because of their high toxicity, low handling safety and ecotoxicological effects. Table 3 presents the "potential of lethality" for these compounds, which indicates the number of different animal species that would theoretically die with 5 g of product, based on the LD_{50} value and average body weight for each species (De la Bodega, 2012). Both pesticides have been widely used in the agricultural sector of the Canary Islands, including in the cultivation of banana (carbofuran) and vegetable crops (aldicarb); therefore, significant amounts of obsolete product may be present in private farms that have not been destroyed and could be motivating their intentional use against domestic and wild fauna. In addition to the existing market in internet, an illegal market for these chemicals may exist in the neighbouring African continent where a number of highly toxic pesticides are still permitted (De la Bodega, 2012).

Various reasons have been proposed to explain why pesticides, and poisons in general, have been used to kill animals indiscriminately. When analysing the causes, some sociocultural factors should be considered. The first issue is determining how tolerant a society is to of animal abuse, which is a factor that varies greatly between countries and regions, and also how important is for this society the conservation of the environment and natural resources. In Spain, certain forms of animal abuse are extensive and even accounted for in the legal system, such as the use of animals during the approximately 20,000 popular festivals held each year throughout the country that often results in the death or torture of different types of animals, mainly bulls, cows and calves. Therefore, in many Spanish regions, there is a social habituation to certain forms of animal abuse (Chew and Armstrong, 2001). Furthermore the presence of abandoned animals in large numbers without effective control measures performed by the administration creates numerous problems for ranchers, hunters, farmers, and in

Table 3
"Potential of lethality" of carbofuran and aldicarb.

	5 g of product	
	Species	Number of animals that could die
Carbofuran	Common Buzzard (*Buteo buteo*)	926
	Common Kestrel (*Falco tinnunculus*)	4167
	Cat (*Felis silvestris*)	257
	Human being	13
Aldicarb	Common Buzzard (*Buteo buteo*)	11,211
	Common Kestrel (*Falco tinnunculus*)	50,012
	Cat (*Felis silvestris*)	3246
	Human being	154

residential and tourist areas of the country. Poison sometimes becomes the quickest option to end the problem. Besides, it has been recently reported that the Canary Islands and the Balearic Islands are regions in Spain where the rate of abandoned animals is higher (>60 dogs and cats / 10,000 inhabitants) (Segú et al., 2010) and among the highest rates in Europe compared with countries where data have been reported (Tasker, 2008). Additionally, other factors may be influencing the intentional use of pesticides against animals, including a high accessibility to toxic pesticides, or the lack of controlling the possession of prohibited chemicals. This ease of access to chemicals is coupled with a certain sense of impunity for poisoners because, although the crime of using poison to control predators is included in the European legislation (including Spain), material and human resources devoted to criminal prosecution are usually lacking. Recently the "Strategy for eradication of illegal use of poison in the non-urban areas of the Canary Islands" has been approved by the regional government with the aim of prosecuting this illegal activity (BOC, 2014) but to date there have not been convictions for this crime in the Canaries.

Several of the above cited circumstances simultaneously concur in many European regions and are especially relevant in the Canary Islands, where this study was performed. Therefore, authorities should take different measures to correct the circumstances that motivate the intentional poisoning of animals to curb, or at least minimise, this serious problem that severely threatens biodiversity, animal welfare and public health.

5. Conclusions

We present the results of a prospective analysis of all cases of poisoning of wild and domestic animals that have occurred over a period of 32 months in the archipelago of the Canary Islands. Our preliminary experience seemed to indicate that there was a high involvement of pesticides in these cases and, more worryingly, the involvement of prohibited pesticides. In order to test this hypothesis we designed this study including only those cases in which there was direct evidence of intentional poisoning, or cases of unknown origin, being expressly excluded those cases with a clear accidental aetiology. Our results indicated that 117 animals were intentionally poisoned, which represents 52.5% of the animals that were included in the study, and that frequently wildlife was affected animals (mainly birds of prey and scavengers). The set of results presented in this study is the highest reported to date in any territory of the European Union. Our study confirms the involvement of banned pesticides is high in this archipelago (mainly carbofuran and aldicarb), as this product was identified in 75% of animals and 97% of the baits. This set of results is extremely worrying, as it implies that there is still access to these products of extremely high toxicity, which are being used in an uncontrolled manner. This is particularly acute in a territory such as the Canary Islands, where there are 11 protected natural areas with the highest level of protection under the European Union (4 National Parks and 7 Parks Rural), so that the high use of poison poses a great risk to biodiversity conservation. It is imperative that the authorities allocate material and human resources to the prosecution of this crime, and the parallel development of education and awareness campaigns in order to minimize environmental impact of this illegal and harmful practice.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.10.093.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank the Canary Islands Government (Dirección General de Protección de la Naturaleza, Gobierno de Canarias) for their financial support. Authors are also thanked to veterinarians of the Wildlife Recovery Centers of Gran Canaria (Pascual Calabuig and Dolores Estévez) and Tenerife (Santiago Mayans) for their collaboration in the necropsies and in the sampling of the animals, and also to Mrs. María de los Reyes Suárez Hanna for her technical assistance.

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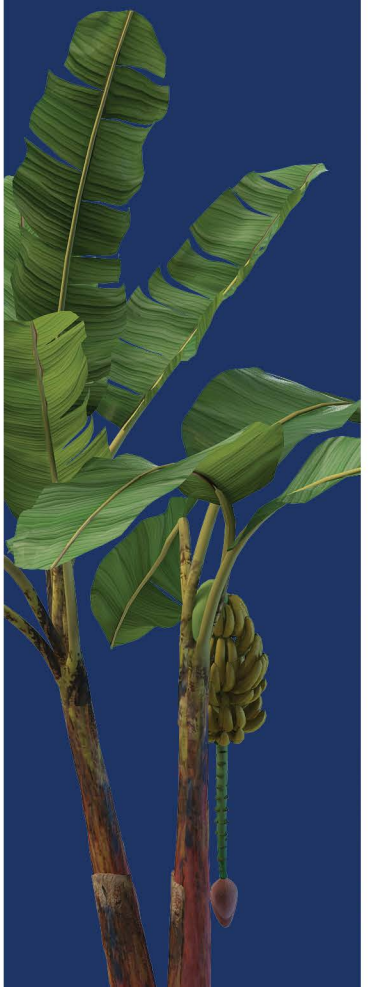
CONCLUSIONES



CONCLUSIONES

1. El trabajo de nuestro grupo de investigación ha permitido, en lo estrictamente metodológico, el desarrollo de un método multirresiduo que facilita la extracción, identificación y cuantificación de un gran número de tóxicos de interés medioambiental a partir de una única muestra biológica, con las ventajas que ello conlleva, tanto en el consumo de tiempo como de recursos económicos. En concreto, especial relevancia tiene la optimización del método QuEChERS como un método muy útil para la extracción de tóxicos ambientales presentes en muestras biológicas complejas.

2. Con respecto a la presencia de tóxicos ambientales ubicuos en fauna doméstica, nuestro trabajo ha puesto de manifiesto que, pese a compartir hábitat y ambiente con la especie humana, el perro doméstico no es un buen indicador de exposición (centinela) de la población humana con la que convive ya que el perfil de contaminantes orgánicos persistentes mostrado por ambas especies es muy diferente. Por el contrario, los gatos parecen ser mejores marcadores de la exposición humana que los perros ya que su perfil de contaminantes persistentes es muy diferente al de los perros y más parecido al de la especie humana. Quizás diferencias en las fuentes de contaminantes (exposición a través de dieta) o diferencias en las capacidades de metabolización o eliminación de estos compuestos puedan justificar estas diferencias interespecies.

3. De modo similar, en lo que a la presencia de tóxicos ambientales ubicuos en fauna silvestre se refiere, nuestro trabajo ha puesto de manifiesto que, como era de esperar, prácticamente el 100% de los animales estudiados presentaban niveles detectables de CTPs. Se ha de destacar la homogeneidad interespecie y los altos niveles de contaminación por POCs, mientras que los niveles de PCBs e HAPs fueron bajos en comparación con estudios en otras regiones. Nuestro trabajo pone de manifiesto que el análisis de muestras procedentes de aves silvestres se puede utilizar como un indicador útil para evaluar el nivel de contaminación de una región geográfica.

4. En lo que concierne a tóxicos liberados de forma intencionada al medio natural hemos de concluir que, nuestros trabajos revelan que las aves rapaces están comúnmente contaminadas por rodenticidas anticoagulantes lo que indica que este tipo de compuestos tienen capacidad para incorporarse en la cadena alimentaria, representando por lo tanto una seria amenaza para la fauna, en especial para las aves rapaces. Y asimismo refleja que son plaguicidas, en concreto los ya prohibidos carbofurano y aldicarb, los tóxicos más frecuentemente implicados en episodios de envenenamiento en nuestras islas.

5. Un incremento en el seguimiento y persecución del uso ilegal del veneno, el desarrollo de programas educativos que favorezcan la sensibilización de esta problemática y la aplicación de acciones legales en casos de envenenamiento, son necesarios para disminuir el impacto de los pesticidas en los animales silvestres y domésticos.

CONCLUSIONS

1. The work of our research group has allowed us to developing a multiresidue method that facilitates the extraction, identification and quantification of a large number of interesting environmental toxicants from a single biological sample. That method involves some advantages in terms of saving time and economic resources. In particular, special importance has the optimizing of the method known as QuEChERS as a useful tool for the extraction of environmental toxicants present in complex biological samples.

2. Regarding the presence of ubiquitous environmental toxicants in domestic animals, our work has shown that, despite sharing habitat and environment with humans, the domestic dog is not a good indicator of exposure (sentinel) of the human population because the profile of POPs shown by both species is very different. By contrast, cats appear to be better markers than dogs because the persistent contaminants profile is very different from dogs and more similar to humans profile. Perhaps differences in sources of pollutants (exposure through diet) or differences in metabolism or elimination capabilities of these compounds can justify these interespecies differences.

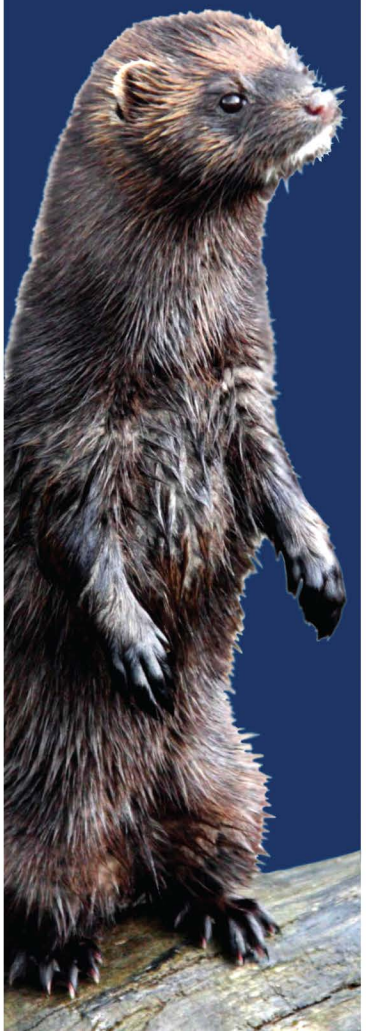
3. Similarly our work has shown, as we expected, that almost 100% of the wildlife animals studied had detectable levels of POPs. It is necessary to emphasize the interspecies homogeneity and high level of POCs contamination, while levels of PCBs and PAHs were low compared to studies in other regions. Our work shows that the analysis of samples from

wild birds can be used as a useful tool to assess the level of contamination of a geographical region.

4. Considering the deliberate release of poison into the environment we must conclude that our studies shows that raptors are commonly contaminated by anticoagulant rodenticides indicating that these compounds have the capacity to be incorporated into the food chain, thus representing a serious threat to wildlife, especially for birds of prey. Our studies also reflect that pesticides, in particular those already prohibited like carbofuran and aldicarb, are the subtances most frequently involved in episodes of poisoning in our islands.

5. An increase in monitoring and prosecution of illegal use of poison, the development of educational programs to promote awareness of this problem and the implementation of legal action in cases of poisoning are needed to reduce the impact of pesticides on wildlife and domestic animals.

ANEXO I



Resumen

Cómo ya adelantamos en la introducción y con motivo de la obtención del título de Doctor con mención Europea, se realizó una estancia en un centro de investigación europeo distinto al país de origen del doctorando tal y como especifica la normativa vigente. Durante la estancia se acordó realizar un estudio dirigido a la detección y cuantificación de los rodenticidas anticoagulantes en visones americanos (Neovison Vison). La importancia de este trabajo radica en que como vimos en la introducción general, los rodenticidas anticoagulantes son la primera opción en cuanto a la lucha contra las poblaciones de roedores se refiere, y la intencionalidad con la que estas sustancias llegan al medio y la capacidad de las mismas para entrar en la cadena alimentaria, convierten a este grupo de sustancias en un importante objeto de estudio para muchos grupos de investigación, entre ellos el nuestro. Este trabajo muestra similitudes al realizado con anterioridad por nosotros, puesto que el visón americano al igual que las aves rapaces, se encuentran situados en lo más alto de la cadena trófica.

El visón americano, es una especie que como su propio nombre indica proviene de América. Su presencia en otros lugares y en el caso particular de Escocia es accidental. La especie actúa como especie invasora convirtiéndose en responsable de innumerables problemas

medioambientales. Entre ellos la predación sobre la rata topera, sobre las aves que nidifican en el suelo y sobre peces. Como depredador generalista que es, el visón americano adapta su alimentación dependiendo de las presas disponibles. Una vez que agota una fuente de alimento, simplemente cambia a otra. De ahí la gran importancia que supone para la conservación de muchas especies las campañas de erradicación contra este tipo de depredadores.

Como resultado de la campaña realizada en Escocia que responde al nombre de “Mink Iniciative”, los hígados de estos animales fueron remitidos al “Science and Advice for Scottish Agriculture (SASA)” y custodiados en el mismo centro a la espera de ser analizados. Los resultados obtenidos se muestran en el documento adjunto. En él podemos observar que una vez más dichas sustancias se encuentran ampliamente distribuidas en el medioambiente y como de manera indiscriminada se introducen en la cadena alimentaria hasta llegar a los eslabones más altos.

El presente artículo se encuentra enviado a la revista Science of the Total Environment, y se encuentra en proceso de revisión.

Rate of exposure of a sentinel species, invasive American mink (*Neovison vison*) in Scotland, to anticoagulant rodenticides

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Competing Financial Interests Declaration: There are no actual or potential conflicts of interest to declare for any author.

Keywords: Mink; *Neovison vison*; anticoagulant rodenticides; exposure risks; age; land use; Scotland; sentinels.

ABSTRACT

Anticoagulant rodenticides (ARs) are highly toxic compounds that are almost exclusively used for the control of rodent pests. Despite their defined use, they are nonetheless found in a large number of non-target species indicating widespread penetration of wildlife. Attempts to quantify the scale of problem are complicated by non-random sampling of individuals tested for AR contamination. The American mink (*Neovison vison*) is a wide ranging, non-native, generalist predator that is subject to wide scale control efforts in the UK. Exposure to 9 ARs was determined in 99 mink trapped in NE Scotland, most of which of were of known age. A high percentage (78.8%) of the animals had detectable residues of at least one AR, and more than 50% of the positive animals had two or more ARs. The most frequently detected compound was bromadiolone (75% of all animals tested), followed by difenacoum (53% of all mink), coumatetralyl (22%) and brodifacoum (9%). The probability of mink exposure to ARs above a reported toxicity threshold of 0.2 mg/kg increased by 4.9 % per month of life up to a maximum at 35 months of age, but was 2.36 times higher for mink caught in areas with a high density of farms; this applied equally for both sexes. The number of AR compounds acquired also increased with age and with farm density. No evidence was found for sexual differences in the rate and number of ARs. The wide niche and dietary overlap of mink with several native carnivore species, and the fact that American mink are culled for conservation throughout Europe, suggests that this species may act as a sentinel species, and the application of these data to other native carnivores is discussed.

1. INTRODUCTION

Anticoagulant rodenticides (ARs) are the most common type of chemicals used to control rodent pests, although due to the development of resistance, there has been a shift in use away from the "first generation anticoagulant rodenticides" (FGARs) in favour of the "second generation anticoagulant rodenticides" (SGARs; Pelfrène, 2010). Both chemical groups act by blocking the vitamin K cycle, which is essential for the production of blood-clotting factors; however, SGARs are more effective, more toxic and have longer half-lives than FGARs (Eason et al., 2002; Pelfrène, 2010; Stone et al., 1999). For example, rodents poisoned by SGARs may accumulate as much as 8-10 times the LD50 of these chemicals in their liver tissue in the time between the first ingestion of the rodenticide and their death (Cox and Smith, 1992; Dowding et al., 2010; Sanchez-Barbudo et al., 2012).

ARs are typically formulated within a cereal based bait, and any animal that consumes the bait, including non-target species such as granivorous birds (Sanchez-Barbudo et al., 2012) or insects (Godfrey, 1985; Booth et al., 2001; Spurr & Drew, 1999), may act as a source of contamination to other species that predate or scavenge them. Confirmation of the presence of residues of ARs in non-target species has been found in predatory and scavenging raptors (Hughes et al., 2013; Lambert et al., 2007; Lopez-Perea et al., 2014; Ruiz-Suarez et al., 2014; Stone et al., 2003; Walker et al., 2008), and mammals (Dowding et al., 2010; Elmeros et al., 2011; Sanchez-Barbudo et al., 2012; Tosh et al., 2011), but reliably quantifying the extent of wildlife intoxication and hence the impact of changes in AR use, is challenging.

Exposure to ARs is commonly monitored opportunistically via samples of dead animals. In Scotland, opportunistic encounters of specific dead species, most commonly raptors, are submitted into the Wildlife Incident Investigation Scheme (WIIS). Opportunistic sampling may however have limitations in quantifying exposure in wildlife if individuals submitted are unrepresentative of the whole population of the focal species. Animals submitted dead may be predisposed towards AR exposure. For instance, starving animals may be more inclined to forage close to areas with high levels of human activity where their encounter rate with poisoned rodents, partly intoxicated or dead, is likely to be much higher than in remote areas, and the chance of someone finding and submitting a carcase is also relatively high. Even animals submitted as a result of a road traffic accident (again with a high human encounter rate) might be a biased sample since partially intoxicated individuals could be more vulnerable to collisions with vehicles. Furthermore, potentially confounding factors, such as the age and sex of sampled individuals or the geographical provenance and land use, may give a biased perception of the extent of contamination. Thus, where individuals analysed for ARs contamination are collected haphazardly, it may be unwise to extrapolate the rate of contamination from the obtained sample to the wider population and estimate the true risk ARs pose. An ideal sampling regime would provide estimates of the exposure risk to species exposed to ARs per unit time that could be reliably compared between e.g. ecosystems, land use types and regulatory regimes.

In Northern Scotland, a large-scale participatory project to control invasive non-native American mink (*Neovison vison*) has been underway since 2006, yielding 970 individuals by 2013, over 20,000km^2 (Bryce et al., 2011; Melero et al., 2015). This provided us with an unbiased sample source of a-priori healthy carnivore individuals on

which exposure to ARs could be analysed. Given the generalist diet of mink (see review in Melero *et al.* 2014), the species can be considered a sentinel species of exposure to chemicals for other sympatric carnivores species. In the UK, this includes many native carnivores, some fully protected such as otters (*Lutra lutra*; SSI, 2007; Strachan, 2007), badgers (*Meles meles*; PBA, 1992; Rainey et al., 2009), pine martens (*Martes martes;* SSI, 2007; WCA, 1981; Croose et al., 2014), and Scottish wildcats (*Felis silvestris;* SSI, 2007; Kilshaw et al., 2015), as well as with non-protected carnivores, such as red foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*) and polecats (*Mustela putorius*) (see Harris et al., 2008 for distribution of non-protected carnivores).

In this study we aimed to quantify levels of AR exposure in Scottish wild carnivores using mink as a sentinel species. In addition, we examined potential factors involved in the rate of exposure to ARs, by examining how exposure per unit time varied by land use and the sex of the individuals, and by considering how relationships between mink age and exposure was affected by these covariates.

ARs are commonly used to prevent rodent damage to stored agricultural crops and feed (Hughes et al., 2012 & 2014), where they are permitted for use indoors, and for some compounds, for use outdoors away from buildings. Therefore, in terms of land use, the accessibility of mink to farms was expected to be the main environmental covariate affecting mink exposure to ARs via predation of available, contaminated prey. Thus, AR exposure was compared to the connectivity to farms, a metric reflecting the potential influence of these sources of AR weighted by their sizes (number of fields per farm) and their distance to each mink at their capture location. In addition, given mink are strongly sexually dimorphic, with the smaller females preying upon smaller items

than males (Dunstone and Birks, 1987), we considered whether male and female mink had different levels of exposure. Further, because culled mink were accurately aged, and because of the long half-lives of ARs metabolites, we estimated the per time unit rate of accumulation of SGAR via ingestion of contaminated prey (the slope of exposure and age relationships), which we suggest has the potential to serve as a robust metric suitable for multi-site comparisons of the risk ARs pose to predators in the natural environment.

2. MATERIALS AND METHODS

2.1. Sample collection

Liver samples were obtained from necropsies of a total of 99 mink selected amongst 979 that were captured between 2007 and 2013 in rural areas of northern Scotland as part of an invasive non-native species control project (Bryce et al 2011; graphical abstract). All mink were captured in cage traps typically placed on floating platforms and sacrificed in accordance with the Wildlife & Countryside Act, 1981 and only secondarily used for answering applied ecology research questions (e.g. Melero et al. 2015; Oliver et al., in review). The whole livers, the primary organ for the accumulation of rodenticides (Dowding et al., 2010; Fournier-Chambrillon et al., 2004), were excised and stored at -20°C until sample preparation and analysis. Place of capture, year of capture and sex were recorded on site or during necropsy. Based on the appearance of dental pulp of canine teeth at X-ray, mink were aged as younger or older than 10 months (Helldin, 1997). Those judged to be older than 10 months were further aged using tooth cementum analyses performed by Matson Laboratory LLC (MT, USA).

2.2. Analytes of interest

The ARs examined in this study include those that have been most commonly used in rodent control activities in the UK: warfarin; coumatretralyl; diphacinone; chlorophacinone (all FGARs), and bromadiolone; difenacoum; flocoumafen, brodifacoum and difethialone (SGARs).

2.3. Preparation of matrix-matched calibration curves

Standards for ARs were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All standards were certified reference materials (purity ranging from 98% to 99.5%). Stock solutions of individual pesticides were prepared from certified reference material into methanol (≈400 µg/ml) and aliquots taken to compose standards mixture (5µg/ml) of warfarin, coumatetralyl, diphacinone, chlorophacinone, bromadiolone, difenacoum, flocoumafen and brodifacoum. From this, an intermediate solution at 0.4 µg/ml was prepared by diluting 2 ml of mix stock to a final volume of 25 ml with methanol. This intermediate solution was used to prepare solvent standards at different concentrations: 0.05 µg/ml, 0.02 µg/ml, 0.004 µg/ml, 0.002 µg/ml; all in methanol.

To prepare rodenticide matrix-matched standards, 2.5ml of each solvent standard concentration plus 0.25 ml of a concentrate of chicken liver (4 g/ml in methanol) were introduced into 5 ml volumetric flask with methanol (containing 5mM Di-buthylammonium Acetate (DBAA) to obtain the standards at 0.025 µg/ml, 0.01 µg/ml, 0.002 µg/ml and 0.001 µg/ml with a final matrix concentration of 0.2 g/ml. Another

mixture of rodenticides was prepared as above, to be used as a confirmation mixture. Both, the matrix-matched and the solvent standards were prepared every 7 days to ensure the correct quantification of samples. Linear calibration curves were constructed using QuanLynx software (Waters Corporation, MA, USA), which correlates peak areas and concentration.

An experiment was conducted to check the validity of the chicken liver for preparing matrix-matched calibration curves. All the procedures described above were repeated using residue free mink liver (n = 18) instead of chicken liver, and the quantification was compared to each other. As shown in Figure 1, the results in both matrices were well correlated (Pearson correlation test; $R^2 = 0.984$, $p < 0.0001$), and thus, the employment of chicken matrix-matched standard curves for the quantification of ARs in mink liver was validated.

2.4. Sample preparation and clean-up

Liver tissue was finely chopped and a portion (≤ 4 g) was weighed into a beaker (100 ml) then 40 ± 1 mg of solid ascorbic acid was added and mixed thoroughly using a glass rod. Sufficient amount of anhydrous sodium sulphate was added to absorb moisture. The mixture was left to dry for 20-30 minutes until friable then transferred in to an extraction bottle (250 ml) and 100 ± 10 ml of extraction solvent chloroform/acetone (1:1 v/v, 0.075 % ascorbic acid) was added. The bottle was securely capped and placed on a shaker for at least an hour at 145 strokes per minute. The crude extract was filtered off through a Whatman No1 filter paper (18.5 cm) with washings into a round bottom flask (150 ml) and evaporated just to dryness by rotary evaporation (bath temperature

not exceeding 40ºC). The dry residue was redissolved in approximately 2ml of cyclohexane/ethyl acetate (1:1 v/v) and the resulting extract was transferred quantitatively to a volumetric flask (4 ml) and made up to volume with the same solvent mixture.

Automated gel permeation chromatographic (GPC) clean-up was undertaken using a Gilson 233-XL/402 system and Bio-bead SX-3 column (340 x 25 mm). The Bio-bead column was prepared as previously described (Hunter and Sharp, 1988) except that the solvent mixture employed was cyclohexane/ethyl acetate (1:1 v/v). The GPC flow rate used was 5 ml/min.

Liver tissue extracts were filtered through glass fiber syringe filters (25 mm, 1.2 μm) and 2 ml applied to the GPC column (approx. 2 g of extract). The first 70 ml of eluate was discarded, and the next 100 ml collected. The cleaned-up extract was evaporated just to dryness (bath temperature not exceeding 40 ºC) and re-dissolved, with the aid of ultrasonication in 5 mM methanolic DBAA solution (10 ml) for analysis by Liquid Chromatography Mass Spectrometry (LC-MSMS). When sample weight was < 4 g, the final volume of 5 mM methanolic DBAA was calculated to maintain the ratio of 0.2 g/ml.

2.5. Chemical analysis

Chromatographic analyses were performed using an Acquity UPLC system coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Waters Corporation, MA, USA). The chromatographic separation was performed using a 50 x 2.1 mm, 1.7 μm

analytic column (Waters Acquity UPLC BEH C18) at 35ºC. Mobile phases were (A) water/methanol 95/5 v/v, 5mM ammonium acetate, and (B) methanol, 5 mM ammonium acetate. The flow was set at 480 µl/min. The volume injection was 5 µl. The total run time was 7 minutes and the gradient was programmed as follows: min 0, 70 % A; min 0.52, 70 % A; min 0.66, 40 % A; min 1.05, 40 % A; min 3.31, 15 % A; min 4.90, 15 % A; min 5.00, 0 % A; min 6.00, 0 % A, min 6.05, 70 % A; and min 7.00, 70 % A.

Retention times of each compound were initially determined in the full scan mode (mass range: *m/z* 45-600). The time-selected multiple reaction monitoring (MRM) method was constructed by infusion of methanolic solutions of pure standards directly into the source. Optimum cone voltage and collision energy values were determined for each analyte. The molecular ion species was identified i.e. $[M-H]^-$ and selected as the precursor ion. The precursor ion → product ion transitions listed in Table 1 were used for screening, confirmation and construction of associated calibration curves. Analyses were performed using electrospray ionization in the negative mode. An interchannel delay of 0.005 s, an interscan time of 0.02 s, dwell time 0.02 s and span corresponding to 0.2 Da were used. Argon of 99.9 % purity (BOC Manchester, UK) was used as a collision gas (2.89×10^{-3} mbar cell pressure). A nitrogen generator (Peak Scientific, Renfrew, UK) and compressor system (Atlas Copco, Cumbernauld, UK) were used to supply nitrogen as the dessolvation, cone and nebulizer gas. These were set at universally applied values of approximately 500 l/h (dessolvation gas flow rate) and 50 l/h (cone gas flow rate). The ion source was operated at 120 ºC, the dessolvation temperature held at 500 ºC and the capillary voltage was maintained at 0.3 kV. The LC/MS/MS instrument was controlled and the data processed using MassLynx 4.1 and

QuanLynx Application Manager software (Waters Corporation (Micromass), Manchester, UK).

The limit of determination (LOD) was set at 0.005 mg/kg for all the ARs. When necessary, the samples were diluted in order to fit within the limits of the calibration curve. Acceptable recoveries fell within the range 60%-140% with the mean being between 70%-90% at low and high levels. However as recoveries for chlorophacinone and diphacinone were between 20%-80%, the method is therefore considered qualitative/semi-quantitative for these two compounds. Measured residue values were not corrected for recovery rates.

2.6. Quality control of AR measurements (QA/QC)

All of the measurements were performed in duplicate, and mean values were used for the calculations. In each batch of samples, two 4-point calibration curves were injected: one at the beginning and the other at the end of the batch. A low-level calibrator (0.002 µg/ml) was included every four samples. Each batch also contained a routine liver matrix sample spiked at high-level (0.1 mg/kg) processed at the same time as samples. In addition, a liver matrix sample spiked at low-level (0.02 mg/kg) was included with every fourth batch analysed. Two blanks were also included in each batch of samples i.e. a reagent blank, containing 100% methanol, and a matrix blank (procedural blank). The results were considered to be acceptable when the quantification of the analytes in the QC was within 40% of the deviation of the theoretical value.

2.7. Statistical analyses

Two variables were defined to quantify the rate of contamination in mink: the rate of contamination and the cumulative number of rodenticides in mink liver each likely to reflect the ingestion of contaminated prey. Rate of contamination was defined as the frequency of finding levels of ∑AR rodenticides above a previously reported toxicity threshold (0.20 mg/kg; (Grolleau et al., 1989; Newton et al., 1999). The cumulative number of rodenticides was the sum of the different ARs compounds found per mink ($\sum N_{AR}$).

To test the hypotheses of sexual dimorphism in feeding habits and the bio-accumulation of ARs with age, we tested the effects of sex and age (in months) on the rate of contamination and the cumulative number of rodenticides. To test the effect of the availability of potentially contaminated prey from farms, we used the connectivity index S^F following Hanski and Thomas (1994), defined as:

$$S_i^F = \sum_{j=1}^{n} \exp\left(-\frac{d_{ij}}{d'}\right) A_j$$

where S^F is the connectivity of each mink i to the surrounding matrix (sum) of all farm holdings j , d_{ij} is the distance (km) between each mink i to each farm holding j, and A_j is the size of the farm, defined as the number of fields per farm holding j. Connectivity increases with the number of fields within a farm but decreases exponentially with the distance to the farms weighted by the parameter d' (also known as $\alpha = 1/d'$), which reflects the mobility of mink. Higher values of d' indicate that farms in a larger neighbourhood influence AR contamination of mink, reflecting the scale of mink foraging. We estimated the value of d' based on a profile likelihood approach whereby

models for the frequency of contamination and the cumulative number of rodenticides, analysed independently as response variables (see below), were iteratively fitted to the data using values of S^F estimated using a range of values for *d'* (1–120 km) chosen to best reflect mink movements (Oliver et al, in review). The most likely value of *d'* was obtained by the model with the lowest model deviance value.

All statistical analyses were performed using generalised linear models (GLM). The rate of contamination by ARs was fitted to a binomial distribution (zero for levels <0.2mg/kg, one for levels >0.2mg/kg) with a logit link or cloglog when needed to accommodate a high rate of zeros. The cumulative number of ARs was fitted to a Poisson distribution with a log link. For all models the null hypotheses was that the contrast between estimates compared to the baseline factorial category (i.e. female = 0). For each analysis, a global model was first defined and model selection was conducted by sequentially dropping non-standardised covariates based on AIC. Model averaging and estimates weighting across the most likely models ($\Delta AIC < 1$) were used to incorporate model uncertainty in the parameter estimates using the R package MuMIn (Bartoń, 2014). Analyses were carried out in R 3.2.0 using package lme4 (Bates et al., 2014).

3. RESULTS

Of the 99 animals sampled in this study, 54 were captured in the period 2007 – 2008; 15 in the period 2009 - 2010; and 30 in the period 2011 – 2012. Forty eight percent (n=45) were male and 52% (n=54) were female. Most mink were juveniles (less than 10 months of age, n = 57; median age = 6 months old, average = 10.83, range = 2 to 59).

Mink were tested for exposure to a total of 9 AR (4 FGARs and 5 SGARs), with 79% (n=78) of the animals exhibiting detectable residues of at least one of these compounds in their livers; 56% with two or more compounds; 21% with 3 or more, and 5% with 4 compounds (average 1.56 compounds, range 0-4 for the whole sample). The most common SGARs found were bromadiolone and difenacoum, one or both being present in all mink with residues (n = 77) with the exception of one animal, which contained only brodifacoum. The single-feed, more toxic SGARs (brodifacoum, flocoumafen) were found in 10% of mink (n = 9); difethialone was not detected. Coumatetralyl was the only FGAR detected (n = 22; Figure 2 and Table 2), but was only found in liver samples that also contained at least one SGAR. The average concentration of AR was 0.23 mg/kg (median = 0.11, $p25^{th}$ – $p75^{th}$ = 0.009 and 0.357 mg/kg, respectively), with almost 50% of positive cases (n = 37) exhibiting levels of ∑AR above a reported toxicity threshold of 0.20 mg/kg (Figure 2 and Table 2).

The probability of mink exposure to ARs above a proposed toxicity threshold of 0.2 mg/kg increased by 4.9 % per month of life but the rate of contamination was 2.36 times higher in locations with the proportionally more farms (3 quartile of connectivity) relative to those in areas with fewer farms (first quartile of connectivity), due to the connectivity-age interaction (Table 3). Male and female mink had similar contamination rates irrespective of age (additive effect of ARs and age sex interaction were non-significant; Tables 3 and 4). Model predictions were that virtually all mink are contaminated by at least 0.2mg/kg by the time they reach 35 months old (Figure 4a). The influence of farm connectivity was best explained 2.5 km away (0- 3.7 km 95% CI) from the source of contamination (hence, within each mink's territory; best *d'*= 13; 0.5-

42 CI; Figure 3), a distance at which the rate of contamination was already reduced to a third.

The cumulative number of ARs present in mink also increased with age and connectivity to farm, but with a smaller estimated area size influencing the rate of acquisition of new ARs to a distance of 2.5-3 km (0.5-7 km 95% CI, *d'*). The accumulation of ARs was slower for males; although the difference was not significant. There was no evidence of any asymptote in the number of ARs encountered in a mink lifespan over the range of mink age available (Figure 4b, Table 3), although clearly there are only a limited number of ARs to which mink may be exposed, and the confidence intervals for this statistic are large, suggesting that some older animals may only be exposed to a single AR.

4. DISCUSSION

Unlike most other studies that either do not provide age data, or use broad age categories, the accurate aging of mink in this study allowed us to model AR exposure with age, along with sex, and proximity to farms at known densities. Overall, 79% of mink (n=99) culled in northern Scotland exhibited detectable residues of AR compounds in their livers; with over half exposed to two or more compounds, and a fifth, three or more compounds. Mink were increasingly likely to have acquired AR as they age, with virtually all mink contaminated by 3 years old. Mink living in the more densely farmed area acquired AR contamination above the reported toxicity threshold of 0.2mg/kg at the rate of 9.09 % per month, which was significantly higher than 4.9 % in

the least intensely farmed parts of the study area. By 2 years old, mink were predicted to be contaminated with two distinct AR compounds.

Although livers were tested for nine different AR compounds, only five were found, with bromadiolone being the most frequently found (75% of all animals tested). Difenacoum was the second most frequently detected AR (53%) in the mink, followed by coumatetralyl (22%), brodifacoum (9%) and flocoumafen (2%). The pattern of AR exposure, with comparatively low exposure to the FGARs and especially the single-feed SGARS, reflects the known usage patterns of ARs on arable, grassland and fodder crop farms (that tend to support grazing livestock) over broadly the same time period that mink were collected (Hughes et al., 2012; Hughes et al., 2014).

In this study we used healthy mink that were trapped and culled, rather than a potentially biased sample of opportunistically collected carnivores, including individuals found dead, moribund or road-killed. The expected direction of any bias arising in opportunistic samples would be to overestimate true contamination rates. Despite this, and worryingly, the observed frequency of AR contamination in this study (79%) was higher than those reported in other mustelids in the UK before 2000, and higher than the frequency reported in some other European countries. For example, 36.0% (n=50) of European polecats (*Mustela putorius*) which had been killed on roads in Wales and England, 30.0% (n=10) of weasels (*Mustela nivalis*) and 22.5% (n=45) of stoats (*Mustela erminea*) that had been killed by gamekeepers on shooting estates in England sampled in 1996/97, were reported to have detectable amounts of ARs in their livers (McDonald et al., 1998; Shore et al., 2003). Unfortunately, non-standardised sampling between studies precludes establishing whether this reflects a high degree of

penetration of these chemicals in the trophic chain in Scotland compared with the rest of the UK, or changing patterns of AR use over time. The latter option is supported by a recent study of foxes (shot and opportunistically sampled from road kills) collected from across the UK (n: Northern Ireland=155; England & Wales=29; Scotland=44), which suggested similar levels of contamination in Scotland as the rest of the UK (Tosh et al., 2011). However, only 15% of trapped American mink (n=47) and 10% of Eurasian otters opportunistically collected (n=11; 20 respectively) were exposed to ARs in France (Fournier-Chambrillon et al., 2004; Lemarchand et al., 2010); while 39% of Scottish otters (n= 23) were found exposed to ARs between 2004 and 2015 (EA Sharp, SASA, pers. comm.; unpublished data). Whether the high contamination rate observed in Scottish mink reflect real differences or merely sampling effect caused by e.g. differences in the age of samples, and hence the length of exposure, of the typically small number of individuals sampled is not known. A further ground for caution in comparing prevalence are differences in assay sensitivity (limits of detection, recovery of compound, and whether or not corrections for recovery are applied) mean that at present, comparisons between studies should be treated with extreme caution. Using assay sensitivities broadly comparable with those of the current study, we found that 97% (n=61) of stoats and 93% (n=69) of weasels collected opportunistically in Denmark and 78% (n=58) of fishers (*Martes pennanti*) in California, that had been trapped, radiotagged and carcases collected if later found dead, contained ARs residues (Elmeros et al., 2011; Gabriel et al., 2012). These degrees of exposure are comparable with that found in stoats (100%, n=11 and 85%, n=115) after intensive rodent eradication operations using broadcast baiting methods in New Zealand (Alterio, 1997; Eason et al., 2002 respectively).

Almost 50% of the positive cases (n=37) detected residues above a previously reported toxicity threshold (0.20 mg/kg), which has been associated with mortalities in mustelids and other mammals (Grolleau et al., 1989; Newton et al., 1999), although the relationship between mortality and ∑AR is complex (Eason et al., 2002; Rattner et al., 2014). Furthermore, it has been reported that individual animals, once they have recovered from sub-lethal exposure, may develop compensatory tolerance to ARs (Eason et al., 2002), and that American mink may have an inherent resistance to these chemicals (Kaukeinen, 1982). Thus exposure in this species might be indicative of a more deleterious impact on other carnivores.

A key contribution of this study is the first estimates of the rate of contamination above 0.2mg/kg of carnivores per unit time, calculated from the slopes of the contamination age relationships. The rates of exposures are high, in the order of 4.9 % per month. This exposure rate results in virtually all mink being exposed to ARs by 3 years of age, and assumes cumulative exposure due to the long half-lives of these compounds. For example, the hepatic half-life of bromadiolone, the most frequently detected active ingredient found in this study, has been estimated in rats at between 170 and 318 days (see Erickson and Urban, 2002 for review). While few mink live to 3 years in culled populations, other carnivores routinely do (e.g. otters and martens) such that if extrapolated, our result suggest widespread penetration of potentially toxic levels of AR and therefore, potential population impacts. The monthly rate of acquisition of ARs by mink was significantly related to connectivity to farm holdings, our chosen measure of the intensity of farming activities in the locality of sampled mink. While our estimates of the parameter d' that best predicted the frequency of contamination, and the number of ARs, were relatively imprecise (0-42 and 0.5-7 95% CI), they indicated that the most

influential farms as source of ARs were at ≤ 3.7 and ≤ 3 km respectively, therefore within mink home range and foraging distances (Zuberogoitia et al., 2006; Melero et al., 2008a). The interaction between age and farm connectivity strongly suggest that farming practices represent a major source of contamination of ARs in this species. AR residues in foxes have also been positively associated with farming practices in Germany; specifically with livestock (pig) densities (Geduhn et al., 2015). Although the current analysis did not test the relationship between total ARs, and occurrence or distribution of the different farming sectors, in 2014, 57% of Scotland's pigs were reared in parts of northern Scotland where many of the sampled mink were trapped (see graphical abstract; http://www.gov.scot/Topics/Statistics/Browse/Agriculture-Fisheries/agritopics/Pigs). Our analyses do not rule out the influence of other contributors to the rate of contamination, since even mink caught in areas with low farm density were contaminated. The contribution of rodent control by gamekeepers, where rats are a significant pest of game rearing activities should be assessed in future studies (Mcdonald and Harris, 2000; Sánchez-García et al., 2015). Also, urban sources of ARs will arise from sewer baiting of rats by Local Authorities, and use in domestic and industrial circumstances to control ingress of commensal rodents (Battersby et al., 2002).

Carnivores can be aged relatively easily using canine x-ray and section, and it would be highly desirable for future studies to report age-corrected estimates of AR exposure, where possible using exposure rate per unit time for comparing prevalence of AR non-target contamination between regions and different regulatory regimes.

The precise routes of exposure remain unconfirmed, although dietary analysis of mink has shown that they will take target rodents such as rats (*Rattus norvegicus)*, as well as non-target rodents such as field voles (*Microtus agrestis),* wood mice *(Apodemus sylvaticus),* water voles *(Arvicola terrestris)* and shrews (*Sorex spp.*) (Akande, 1972; Cuthbert, 1979; Melero et al., 2014). Recent studies from Germany have found high AR residues in non-target species which were trapped at various distances from AR bait boxes. The highest maximum residues were found in field mice (*Apodemus*), followed by voles (*Microtus, Myodes*), then shrews (*Sorex, Crocidura*). However, 21% of *Apodemus* species contained AR residues; 7% and 26% respectively in *Microtus* and *Myodes* species*;* and 28% and 66% respectively in *Sorex* and *Crocidura* species (n total = 732). The majority of rodents with AR residues were trapped within 15 metres of the bait boxes (Geduhn et al., 2014; 2016). These data strongly support previous reports of secondary exposure risks via non-targets (Brakes and Smith, 2005).

Of particular concern is the incidence of the most potent, single-feed SGARs, brodifacoum (9% of all mink) and flocoumafen (2%) (Table 2), which at the time of mink trapping, were only approved for indoor use (EC, 2004; EC, 2007). Given these restrictions, routes of exposure suggest regular movement of rodents in and out of buildings which is plausible only for house mice, wood mice and rats, or unapproved use outside of buildings.

Similarly high rates of exposure and high concentrations of ARs found in this study, have been reported in foxes and some raptors from Scotland (Tosh et al., 2011; Hartley et al., 2013; Hughes et al., 2013). These levels of exposure may suggest possible risks to other non-target species, although the current data may be more indicative of risks to

mustelids of high conservation status, especially given the degree of dietary overlap between mink and native mustelids (Gorman, 2008). Where there is both niche and dietary overlap, it is possible that native mustelids may be exposed to toxic levels of ARs. Analyses performed on 23 Eurasian otters from Scotland over a similar time period found that 39% were exposed to ARs, and that just under 9% exhibited ∑AR above 0.20 mg/Kg (E. Sharp, pers. comm.; unpublished data). While the diet of otter and mink overlap (Clode and MacDonald, 1995; Melero et al., 2008b), otters specialize mainly on aquatic prey, while mink can exploit both aquatic and terrestrial species (see Melero *et al.* 2014). Although there are no published data from Scotland on AR residues in European pine marten (*Martes martes*), concerns have been raised regarding AR impacts in protected American fishers (*Martes pennanti*) (Gabriel et al., 2012).

4. CONCLUSIONS

This study has demonstrated a relatively high level of AR exposure in mink. The long half-lives of the SGARs in particular (WHO, 2007; Vandenbroucke et al., 2008), means that across the lifetime of most mink, AR residues increase in both frequency and typology. This relationship is highly affected by the presence of farms in terms of number and the size of the farms found in the area around mink locations.

Mustelids are particularly susceptible to AR contamination probably as a result of their varied prey base, which includes target and non-target rodents (Shore et al., 2003; Gabriel et al., 2012; Melero et al., 2014). These data support the use of mustelids, and in particular the American mink, as a sentinel of environmental AR contamination in rural areas. The rigour of comparisons of the degree of penetration of wild carnivore

populations by AR could be increased if done in a standardised manner by using feral mink that are widely culled for conservation. Indeed the wide distribution of the American mink across the UK and Europe, and their non-native status in these areas (Bonesi and Palazon, 2007), means that capture and removal is of benefit to native wildlife. Our results suggest that correcting prevalence for age, hence the time of exposure to AR, would greatly increase the power of comparisons.

Under Directive 98/8/EC concerning the placing on the market of biocidal products, several anticogulants were described as "high toxic, non-selective and can pose a high risk of primary and secondary poisoning to non-target animals and children". For these reasons, European Member States are required to implement and assess the success of risk mitigation measures (EC, 2009). The measurement of AR residues in American mink has the potential to provide an intra-continental reference database, against which risk mitigation measures may be judged at the national and international level.

5. FIGURE LEGENDS

Figure 1: Correlation of anticoagulant rodenticides in chicken liver and mink liver matrices.

Figure 2: Average concentration of AR compounds (± SE) found in all mink livers sampled.

Figure 3: AIC profile obtained by fitting the model of the frequency of contamination by ARs (black), and the cumulative number of ARs (grey) in relation to the connectivity (S^F) data estimated across values of the parameter *d'* between 0 (i.e. capture location) and 120km.

Figure 4: Model predictions for (a) the frequency of contamination of AR, and (b) the cumulative number of AR in mink in relation to mink age (in months) for males (black) and females (grey), keeping connectivity and age at their median values. Continuous lines relate to the estimated fit of the best model weighted for models within the best values of *d'* (13 and 4 km; dashed lines denote the 95 % CIs.

6. ACKNOWLEDGEMENTS

Field sample collection was funded by NERC grants NE/E006434/1 and NE/J01396X/1 to XL and a Marie Curie FP7-PEOPLE-2011-IEF 300288-grant to YM. We thank the Scottish Mink Initiative, staff, funders and multiple mink volunteers for the continued effort, samples and data. We are especially thanked to Eduardo Salazar Villaverde for his assistance in the preparation of the figures. Also, to Kenneth McNeill for providing data on farm and building densities.

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Table 1. LCd MS/MS method settings of the anticoagulant rodenticides

Rodenticide	MRMd screen (m/z→m/z)	Cone voltage (V)	Collision energy (eV)	MRMd confirmation (m/z→m/z)
Warfarin	307 → 161	60	20	307 → 250
Coumatetralyl	291 → 141	40	30	291 → 247
Diphacinone	339 → 167	50	25	339 → 144
Chlorophacinone	373 → 201	40	25	375 → 203
Bromadiolone	525 → 250	80	40	527 → 250
Difenacoum	443 → 293	70	30	443 → 135
Flocoumafen	541 → 161	70	35	541 → 289
Brodifacoum	521 → 135	70	35	521 → 187
	523 → 135	70	35	523 → 187

Table 2. Liver concentration (mg/kg) of different anticoagulant active ingredients in all mink and mink with detectable residues

		All mink sampled		Mink with detectable AR residues	
Compound	%	Mean ± SD	Median (range)	Mean ± SD	Median (range)
FGAR					
Warfarin	0.0	NA	NA	NA	NA
Chlorophacinone	0.0	NA	NA	NA	NA
Coumatetralyl	22.2	0.015 ± 0.050	0.000 (0.000 – 0.300)	0.067 ± 0.089	0.026 (0.004 – 0.300)
Diphacinone	0.0	NA	NA	NA	NA
Multi-feed SGAR					
Bromadiolone	74.7	0.186 ± 0.251	0.063 (0.000 – 1.296)	0.249 ± 0.263	0.141 (0.006 – 1.296)
Difenacoum	52.5	0.022 ± 0.049	0.004 (0.000 – 0.315)	0.042 ± 0.062	0.016 (0.003 – 0.315)
Single feed SGAR					
Brodifacoum	9.1	0.003 ± 0.018	0.000 (0.000 – 0.171)	0.038 ± 0.054	0.015 (0.004 – 0.171)
Flocoumafen	2.0	0.0002 ± 0.002	0.000 (0.000 – 0.017)	0.012 ± 0.008	0.012 (0.006 – 0.017)
ΣARs	**77.8**	**0.227 ± 0.276**	**0.110 (0.000 – 1.296)**	**0.288 ± 0.281**	**0.194 (0.004 – 1.296)**

Abbreviations: AR: anticoagulant rodenticide; FGAR: first generation anticoagulant rodenticide; SGAR: second generation AR; ΣARs: sum of all ARs; NA: not applicable.

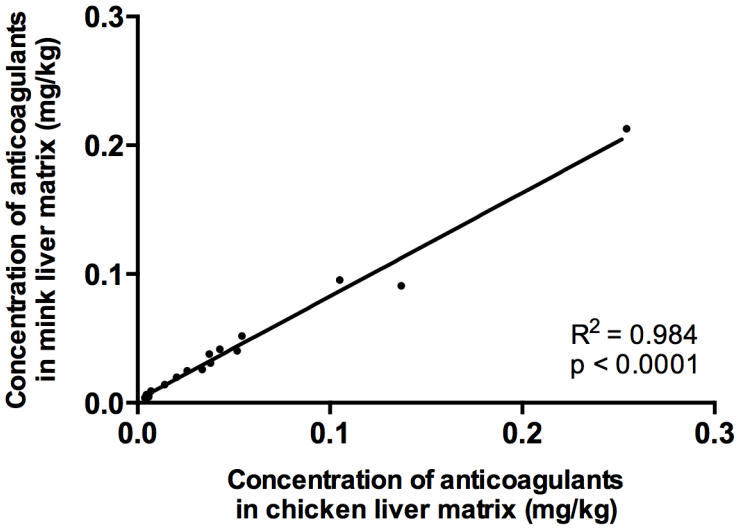
Table 3. Parameter estimates of the effect of covariates with their associated standard errors for variables included in the best model weighted for the models including the best values of *d'* for (a) the frequency of contamination by AR, and (b) the cumulative number of AR. S^F is the connectivity of mink to the surrounding farm holding matrix.

Parameter	Estimate	SE	Z	pd value
(a)Frequency of contamination by AR				
age	0.14	0.06	2.37	0.02
S^F	0.0002	0.0001	1.55	0.12
sex	d 0.0005	0.0003	d 1.29	0.20
Age* S^F	d 2ed 5	7ed 6	d 1.93	0.05
(b)Cumulative number of AR				
age	0.06	0.03	2.35	0.02
S^F	0.0002	5ed 5	2.83	<0.001
sex	d 0.01	0.02	d 0.94	0.35
Age* S^F	d 8ed 6	4ed 6	d 2.38	0.02

Table 4. Model selection based on AIC and ΔAIC for (a) the frequency of contamination by AR and (b) the cumulative number of rodenticides AR in mink. . S^F is the connectivity of mink to the surrounding farm holding matrix. Best models are marked in bold.

Rank	Covariates	df	AIC	ΔAIC
(a) Frequency of contamination by AR				
1	**Age*S^F+sex**	**90**	**117.84**	**0**
2	Age+S^F+sex	90	121.28	3.44
3	Age*S^F*sex	90	122.80	4.96
(b) Cumulative number of AR				
1	**Age*S^F+sex**	**90**	**282.23**	**0**
2	Age+S^F+sex	90	286.68	4.45
3	Age*S^F*sex	90	286.69	4.46

Figure 1


Concentration of anticoagulants in mink liver matrix (mg/kg)
0.3
0.2
0.1
0.0
0.0
0.1
0.2
0.3
Concentration of anticoagulants in chicken liver matrix (mg/kg)
R2 = 0.984
p < 0.0001

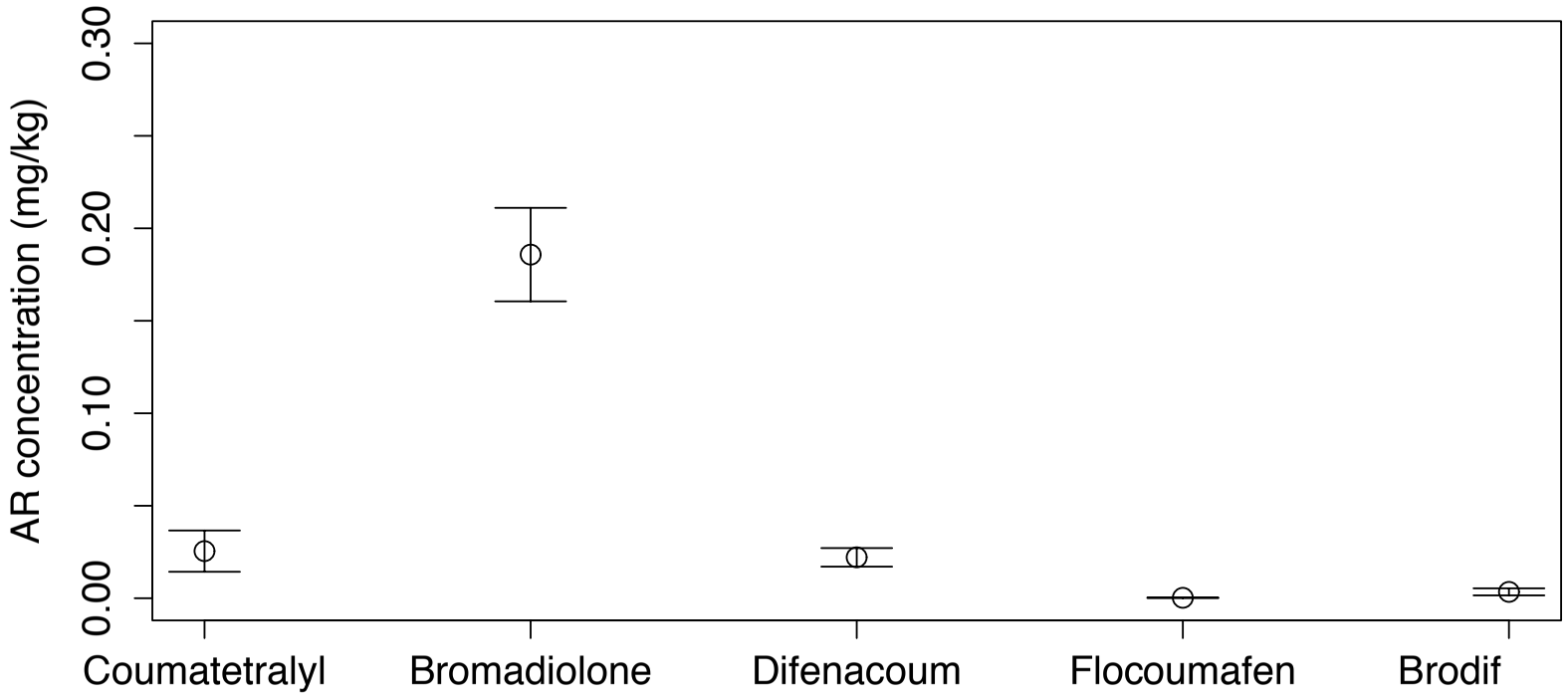


Figure 2. Average concentration of AR compounds (± SE) found in mink livers (mg/kg)

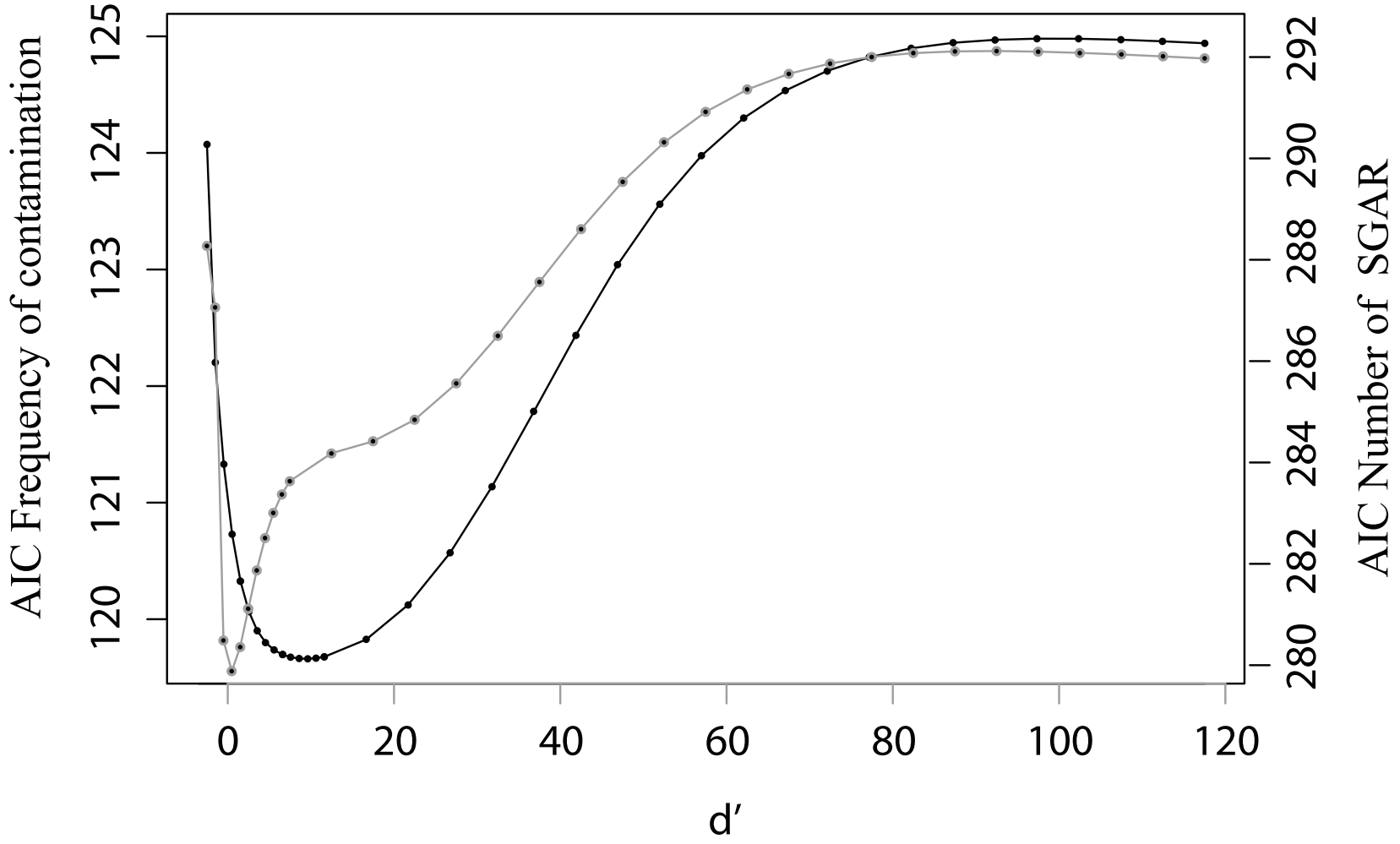


Figure 3. AIC profile obtained by fitting the model of the frequency of contamination by ARs (black), and the cumulative number of ARs (grey) in relation to the connectivity (S^F) data estimated across values of the parameter *d'* between 0 (i.e. capture location) and 120km.

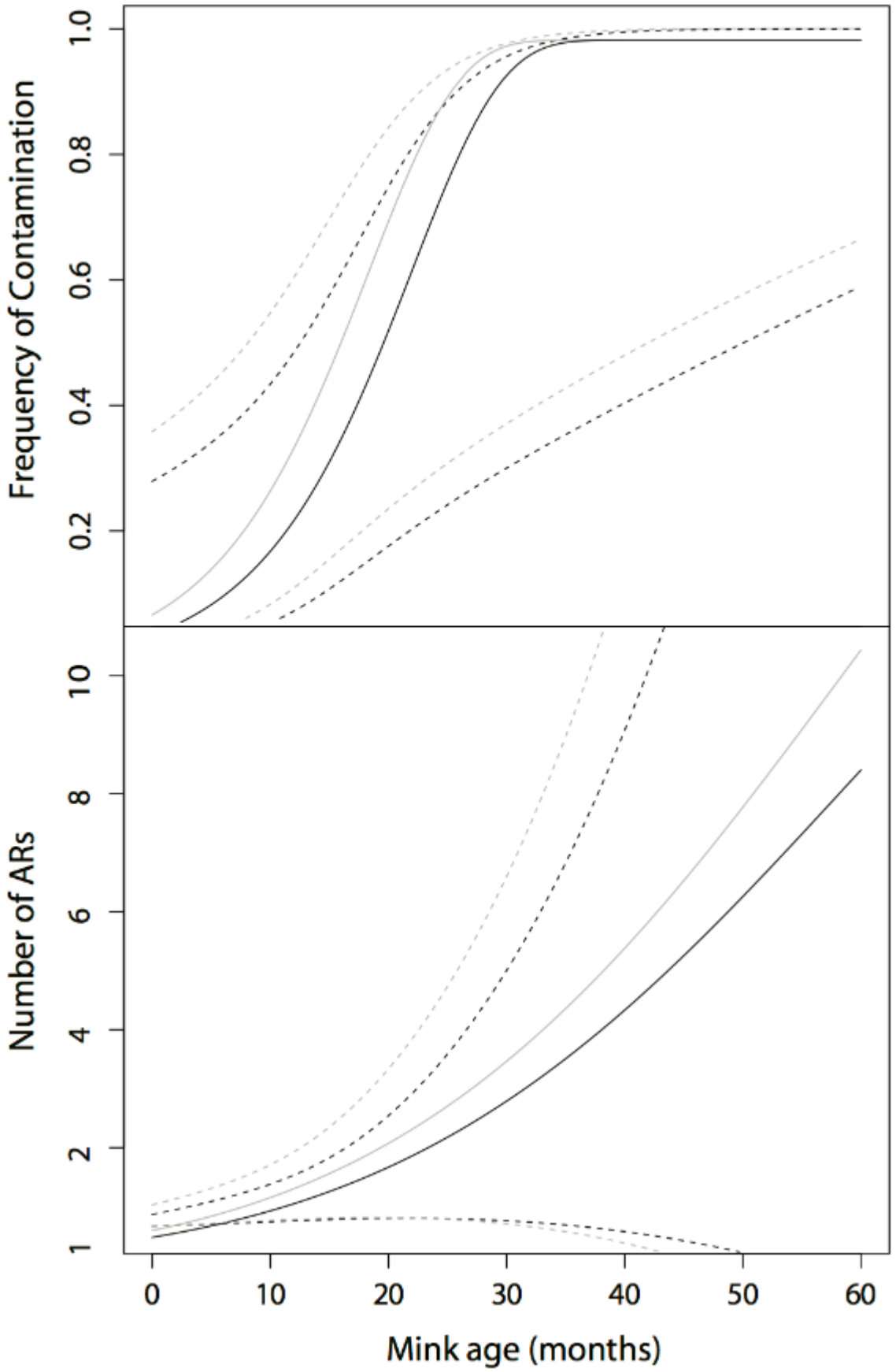
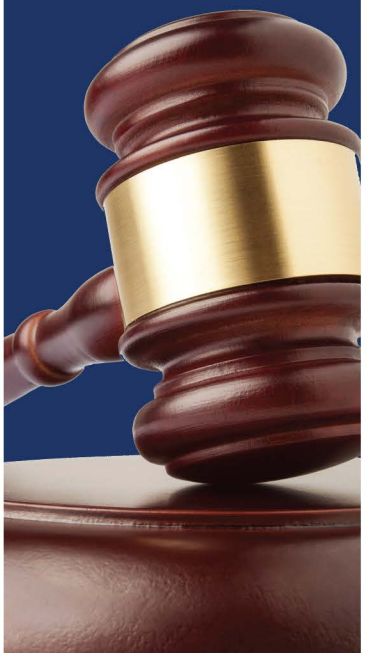


Figure 4. Model predictions for (a) the frequency of contamination of AR, and (b) the cumulative number of AR in mink in relation to mink age (in months) for males (black) and females (grey), keeping connectivity and age at their median values. Continuous lines relate to the estimated fit of the best model weighted for models within the best values of *d'*; dashed lines denote the 95 % CIs.

ANEXO II



Estrategia para la Erradicación del Uso Ilegal de Veneno en el Medio no Urbano de Canarias. (Estrategia Canaria Contra el Veneno).

El problema que ocasiona la presencia de veneno en el medioambiente, sea de manera intencional o no, siempre ha supuesto un grave problema para la conservación o reintroducción de especies.

Es cierto que en España, más específicamente en algunos lugares de la Península Ibérica, la preocupación y el trabajo destinado a la erradicación de dicho problema ha experimentado una tendencia creciente. Dicho pensamiento es debido en su mayoría al esfuerzo que han realizado muchos grupos de investigación tales como el Instituto de Investigación en Recursos Cinegéticos (IREC), el Servicio de Toxicología de la Universidad de Murcia o el Centro de Análisis y Diagnóstico de Fauna Silvestre (CAD) en Málaga entre otros. De igual manera, el desarrollo paulatino de las distintas estrategias regionales de las Comunidades Autónomas cuyo objetivo es aportar medidas adaptadas a las propias particularidades de cada territorio, venían a ratificar una vez más la necesidad imperante de aportar soluciones eficaces a un problema que por desgracia se encuentra arraigado en nuestro país.

En el caso de Canarias, carente de cualquier estrategia y con escasos registros de episodios de envenenamiento, se enfrentaba a una situación difícil de gestionar. Fue entonces cuando debido al conocimiento del trabajo de otros grupos de investigación anteriormente nombrados, junto con los informes emitidos por WWF/Adena, cuando el Servicio de Toxicología Clínica y Analítica de la Universidad de Las Palmas de Gran Canaria (SERTOX) decide poner en

marcha el servicio de diagnóstico y optimizar una serie de métodos encaminados a la resolución de los episodios de envenenamiento y a reducir la situación de incertidumbre en la que nos encontrábamos. Desde SERTOX hemos querido aportar datos que reflejaran la situación real del veneno en Canarias, sin perder de vista que tal y como sabemos, el número de casos de envenenamiento que vamos a registrar sólo suponen una pequeña parte del problema real.

Esta estrategia se presenta como el anexo II, ya que nos enorgullece pensar que gracias al planteamiento y realización de esta Tesis Doctoral Europea, junto al esfuerzo de muchos otros colectivos entre los que destaca La Asociación Veterinaria para la Atención de la Fauna Exótica y Salvaje (AVAFES), SEO/BirdLife, los Colegios Veterinarios de ambas Provincias, Gobierno de Canarias y Cabildos Insulares, se haya aprobado la Estrategia para la Erradicación del Uso Ilegal de Veneno en el Medio no Urbano de Canarias. Dicha estrategia supone la creación de un documento adaptado a nuestras circunstancias y que contempla las herramientas disponibles para poder aportar un plan de lucha contra el veneno que garantice el futuro de nuestra fauna.

III. Otras Resoluciones

Consejería de Educación, Universidades y Sostenibilidad

1489 *ORDEN de 28 de marzo de 2014, por la que se aprueba la estrategia para la erradicación del uso ilegal de veneno en el medio no urbano de Canarias.*

ANTECEDENTES

Primero.- La utilización de cebos envenenados en el medio natural constituye una de las prácticas más lesivas para los ecosistemas en general y para determinadas especies de fauna amenazada en particular. Esta circunstancia justifica que se adopten medidas dirigidas a la erradicación de esta práctica, prohibida por la Ley 42/2007, de Patrimonio Natural y Biodiversidad, que establece en su artículo 62.3 la prohibición de la posesión, utilización y comercialización de todos los procedimientos masivos o no selectivos para la captura o muerte de animales, entre los cuales se encuentra el veneno, según la enumeración recogida en el anexo VII de la misma Ley; por otra parte, la iniciativa se ve reforzada por el hecho de que esta práctica se encuentra tipificada como delito por la Ley Orgánica 10/1995, de 23 de noviembre, del Código Penal, en su redacción dada por la Ley Orgánica 5/2010, de 22 de junio, por la que se modifica la Ley Orgánica 10/1995, de 23 de noviembre, del Código Penal.

Segundo.- La lucha contra el uso del veneno en el medio natural requiere de técnicas y procedimientos especializados, siendo imprescindible para la obtención de resultados positivos la dedicación de medios humanos y materiales específicos, y la coordinación de los distintos agentes y Administraciones con competencias en la materia.

Tercero.- En el marco definido en la «Estrategia nacional contra el uso ilegal de cebos envenenados en el medio natural», aprobada por la Comisión Nacional de Protección de la Naturaleza el 23 de septiembre de 2004, y considerando la información disponible sobre factores de amenaza para las especies incluidas en el Catálogo Español de Especies Amenazadas y en el Catálogo Canario de Especies Protegidas, se ha elaborado la "Estrategia para la erradicación del uso ilegal de veneno en el medio no urbano de Canarias" (Estrategia canaria contra el veneno), con el fin de contribuir a su erradicación.

CONSIDERACIONES JURÍDICAS

Primera.- De acuerdo con lo dispuesto en el artículo 3 del Decreto 86/2011, de 8 de julio, del Presidente, por el que se determinan el número, denominación y competencias de las Consejerías (BOC nº 135, de 11 de julio de 2011), la actual Consejería de Educación, Universidades y Sostenibilidad ha asumido las competencias que en materia de medio ambiente corresponden a la Comunidad Autónoma de Canarias. Asimismo, en virtud de lo establecido en la disposición transitoria primera del Decreto 170/2011, de 12 de julio, por el que se determina la estructura central y periférica, así como las sedes de las Consejerías del Gobierno de Canarias (BOC nº 138, de 14 de julio de 2011), continúa vigente el antes citado Reglamento Orgánico de la Consejería de Medio Ambiente y Ordenación Territorial hasta tanto se apruebe el correspondiente al actual Departamento.

Segunda.- De acuerdo con la previsión contenida en el artículo 3.8 del Decreto 20/2004, de 2 de marzo, por el que se aprueba el Reglamento Orgánico de la entonces Consejería de Medio Ambiente y Ordenación Territorial (BOC nº 52, de 16 de marzo de 2004), entre las funciones de carácter general, corresponden al Consejero, entre otras, todas aquellas funciones en materia de ordenación de los recursos naturales, territorial y urbanística, así como de medio ambiente que estatutariamente corresponden a la Administración de la Comunidad Autónoma de Canarias y que no residan en otros órganos.

En consecuencia, con base en las citadas consideraciones,

R E S U E L V O:

Primero.- Aprobar la «Estrategia para la erradicación del uso ilegal de veneno en el medio no urbano de Canarias» que figura en el Anexo de la presente orden.

Segundo.- Publicar la presente orden en el Boletín Oficial de Canarias.

Santa Cruz de Tenerife, a 28 de marzo de 2014.

EL CONSEJERO DE EDUCACIÓN,
UNIVERSIDADES Y SOSTENIBILIDAD,
José Miguel Pérez García.

A N E X O

ESTRATEGIA PARA LA ERRADICACIÓN DEL USO ILEGAL DE VENENO EN EL MEDIO NO URBANO DE CANARIAS
(Estrategia Canaria contra el Veneno)

1. INTRODUCCIÓN.

El uso de cebos envenenados en el medio no urbano constituye en la actualidad una de las prácticas más lesivas para los ecosistemas en general y para determinadas especies de fauna amenazada en particular. La lucha contra este problema requiere de técnicas y procedimientos especializados, siendo imprescindible para la obtención de resultados positivos marcar una serie de objetivos, llevar a cabo acciones eficaces, así como la dedicación de medios humanos y materiales específicos, y la coordinación de los distintos agentes y Administraciones con competencias en la materia.

Por todo ello, en el marco definido en la «Estrategia nacional contra el uso ilegal de cebos envenenados en el medio natural», aprobada por la Comisión Nacional de Protección de la Naturaleza el 23 de septiembre de 2004, y con la finalidad de contribuir a la erradicación del uso ilegal de venenos en el medio no urbano de Canarias, la Consejería de Educación, Universidades y Sostenibilidad competente en materia de medio ambiente ha elaborado la presente estrategia. Para alcanzar este fin es necesario ampliar y mejorar la información disponible, incidir en la prevención, disuasión y vigilancia del uso ilegal de venenos (especial-

mente cebos envenenados), incrementar la eficacia de las acciones dirigidas a la persecución del delito, controlar la venta de sustancias altamente tóxicas, y las susceptibles de ser usadas para la preparación de los cebos y facilitar y mejorar los mecanismos de coordinación entre todos los colectivos involucrados en la lucha contra el veneno en el medio no urbano, constituyendo todo ello los objetivos de esta estrategia.

Además, se hace necesario desarrollar medidas y protocolos de actuación adecuados para una mejora en la eficacia en la lucha contra el veneno por parte de todos los actores involucrados en su persecución. Para ello se han elaborado cuatro protocolos dirigidos a la vigilancia y el control, a la recogida de las muestras de casos donde presuntamente se halle veneno, al análisis toxicológico de las mismas y a las actuaciones legales que se puedan llevar a cabo en cada caso.

La competencia para las labores derivadas de la presente estrategia corresponderá a la Consejería competente en materia de medio ambiente del Gobierno de Canarias, correspondiéndole la instrucción e impulso de las medidas al Centro Directivo que dicha Consejería designe.

2. DIAGNÓSTICO.

Este diagnóstico ha sido efectuado a partir de los casos detectados de utilización ilegal de veneno entre los años 2006 a 2011, en base a las actuaciones del Servicio para la Protección de la Naturaleza de la Guardia Civil, y de los datos facilitados por el Laboratorio de Toxicología de la Universidad de Las Palmas de Gran Canaria (ULPGC). No obstante, el número de casos que se conocen es casi con seguridad inferior al número de casos que realmente se deben haber producido, ya que los datos que a continuación se contemplan se refieren a casos detectados y analizados, sin que sea posible afirmar que se refieren a todos los episodios de envenenamiento producidos en territorio canario en el periodo señalado.

Por otra parte, la detección de los episodios de envenenamiento está relacionada con las labores de inspección y vigilancia, de tal forma que una mayor eficacia en las acciones encaminadas a la persecución de esta práctica ilegal, da como resultado una mayor aproximación a los casos reales de envenenamiento. Actualmente las prácticas de detección de casos de envenenamiento están bastante desarrolladas en la comunidad canaria y durante los años 2010 y 2011 se han desarrollado algunas medidas dirigidas a reducir esta práctica. Así, se han puesto en marcha protocolos específicos (el más detallado es el relacionado con la recogida de cadáveres y cadena de custodia), se ha impartido información especializada a agentes de medio ambiente y se han efectuado más de un centenar de necropsias y análisis toxicológicos, con el fin de hacer un diagnóstico previo de la dimensión del problema en la comunidad autónoma.

En términos generales los episodios de veneno registrados en Canarias tienen clara relación con conflictos entre fauna y determinadas actividades humanas (caza, agricultura, ganadería y otros sectores).

Las muestras encontradas han sido sometidas a análisis toxicológico en el Laboratorio de Toxicología de la Universidad de Las Palmas de Gran Canaria (ULPGC). A este laboratorio

han llegado durante el año 2011 un total de 50 animales, principalmente aves, 47 ejemplares frente a 3 mamíferos, de los que 26 han dado positivo por envenenamiento. Esto representa un 52% del total de animales recibidos. Esta cifra probablemente no represente la realidad, dado que en muchos casos la muestra recibida en los laboratorios está en avanzado estado de descomposición o incluso momificación, hecho que puede dificultar la detección del veneno por degradación del mismo. En cuanto a los cebos encontrados estos solo han sido 5 casos, suponiendo un porcentaje muy bajo dentro de la casuística global. De estos, 4 fueron confirmados como positivo.

De los análisis realizados se desprende que la sustancia utilizada como veneno en el territorio de Canarias es, en la gran mayoría de los casos, el carbofurano, seguido de la bromadiolona, el brodifacoum y el difenacoum, que se contienen en rodenticidas anticoagulantes de segunda generación. En algún caso se ha detectado la presencia de organofosforados como el tricloronato y el clorpirifós, piretrinas como la cipermetrina metomilo o el fungicida ortofenilfenol.

Aunque las cifras son pequeñas, cabe destacar la afección a especies catalogadas "en peligro de extinción", como el guirre, el cuervo canario o el halcón tagarote, además de otras especies amenazadas como la garza real, el charrán común, el halcón peregrino o el búho chico.

La baja detección de episodios y la no existencia, hasta ahora, de una estrategia y protocolos definidos para un seguimiento y control de esta práctica no permite hacer un análisis exhaustivo sobre la distribución territorial del veneno en Canarias.

3. MARCO LEGAL. FUNDAMENTOS JURÍDICOS.

El uso de veneno es un método masivo y no selectivo de eliminación de depredadores, que está tipificado como delito en el artículo 336 del Código Penal, en su redacción dada por el apartado nonagésimo octavo de la Ley Orgánica 5/2010, de 22 de junio, por la que se modifica la Ley Orgánica 10/1995, de 23 de noviembre, del Código Penal. Esta norma establece que, el que, sin estar legalmente autorizado, emplee para la caza o pesca veneno, medios explosivos u otros instrumentos o artes de similar eficacia destructiva o no selectiva para la fauna, será castigado con la pena de prisión de cuatro meses a dos años o multa de ocho a veinticuatro meses y, en cualquier caso, la de inhabilitación especial para profesión u oficio e inhabilitación especial para el ejercicio del derecho a cazar o pescar por tiempo de uno a tres años. Si el daño causado fuera de notoria importancia, se impondrá la pena de prisión antes mencionada en su mitad superior.

Por otra parte, el uso de veneno o cualquier otro método de destrucción masiva o no selectiva como método de control de predadores o con cualquier otro fin, está expresamente prohibido por la Directiva 2009/147/CEE, de la Unión Europea (artº. 8) relativa a la conservación de las aves silvestres, y la Directiva 92/43/CEE (artº. 15) relativa a la conservación de los hábitats naturales y de la fauna y flora silvestres y por el Convenio de Berna relativo a la Conservación de la Vida Silvestre y el Medio Natural en Europa, convenio suscrito por el Estado español.

La transposición de la normativa comunitaria que prohíbe el uso de veneno para la captura o muerte de animales se encuentra en la Ley 42/2007, de Patrimonio Natural y Biodiversidad, que establece en su artículo 62.3 la prohibición de la tenencia, utilización y comercialización de todos los procedimientos masivos o no selectivos para la captura o muerte de animales, enumerando en su anexo VII al veneno como uno de estos métodos.

Por otro lado, en el año 2004 fue aprobada por la Comisión Nacional de Protección de la Naturaleza, con un amplio consenso, la Estrategia Nacional contra el Uso ilegal de Cebos Envenenados en el Medio Natural en España, que contiene los criterios orientadores para acabar con el problema. Se estructura en tres grandes objetivos: información y mejora del conocimiento; prevención y disuasión y persecución del delito. Para cada uno de ellos establece una serie de criterios orientadores dirigidos a la erradicación del uso ilegal de cebos envenenados.

Dentro de la legislación autonómica, el artículo 43 de la Ley 7/1998, de 6 de julio, de Caza de Canarias, prohíbe, con carácter general, la utilización de todos los procedimientos masivos o no selectivos para la captura o muerte de animales, en particular venenos. En el apartado segundo de este mismo artículo se prohíben los cebos, gases o sustancias venenosas, paralizantes, tranquilizantes, atrayentes o repelentes como medios para cazar. La utilización de estos medios prohibidos y el envenenamiento intencionado de perros de caza se considera por la Ley 7/1998 como una infracción muy grave.

4. FINALIDAD DE LA ESTRATEGIA.

La finalidad de la presente Estrategia es la erradicación del uso ilegal de venenos en el medio no urbano de Canarias, y la adopción de medidas para reducir los efectos de esta práctica.

5. ÁMBITO DE APLICACIÓN Y COMPETENCIAS.

La Estrategia para la erradicación del uso ilegal de veneno será de aplicación en todo el territorio de la Comunidad Autónoma de Canarias.

Respecto a las competencias, corresponde a los órganos que se designen como responsables en materia de medio ambiente por el Gobierno de Canarias, la aprobación, desarrollo y coordinación de la presente Estrategia, excepto en lo referente a las actuaciones propias de la jurisdicción penal y la policía judicial.

La ejecución de la presente Estrategia así como la integración y adaptación de los diferentes protocolos que en ella se recogen corresponderá a los Cabildos Insulares. En este caso, los responsables del Gobierno de Canarias y de los Cabildos Insulares establecerán los mecanismos de coordinación y cooperación necesarios.

Desde el punto de vista municipal, aquellos envenenamientos producidos en los ámbitos urbanos deberían contar con mecanismos de coordinación establecidos desde los cabildos.

6. OBJETIVOS Y LÍNEAS ESTRATÉGICAS.

Se establecen los siguientes objetivos, que llevan asociadas una serie de líneas estratégicas:

Objetivo 1: ampliar y mejorar la información disponible sobre el uso de cebos envenenados y sus consecuencias. Las líneas estratégicas (LE) y acciones encaminadas a la consecución del presente objetivo tienen por objeto la recopilación de la información de los casos de envenenamiento habidos en Canarias, el intercambio de información entre actores implicados o la profundización en las causas que motivan el uso ilegal del veneno.

Línea estratégica 1.1. Crear una base de datos y mapa de riesgos.

Acción 1.1.1. La base de datos contendrá todos los casos de envenenamiento de especies de fauna, especificando con precisión todos los datos disponibles: localización, circunstancias del hallazgo, resultados de las necropsias y análisis toxicológicos, actuaciones legales emprendidas, etc.

Acción 1.1.2. La base de datos contendrá información relativa a los cotos de caza y las explotaciones agrícolas y ganaderas, en especial su titularidad, arrendatarios, los contenidos de los planes técnicos de caza, solicitudes de control de depredadores, solicitudes de pago de daños, ataques por parte de fauna silvestre a bienes agrícolas y ganaderos, y cuanta información relevante se disponga de ellos.

Acción 1.1.3. La base de datos se actualizará y mejorará permanentemente y constituirá en sí misma un mapa de riesgos, que servirá para orientar las labores de búsqueda, vigilancia e investigación.

Línea estratégica 1.2. Coordinar la transmisión de información. Se establecerán mecanismos para garantizar el rápido intercambio de información entre todos los implicados en este plan.

Acción 1.2.1. Identificar los actores implicados en los casos, de forma que haya un acceso directo a todos ellos y un rápido intercambio de la información.

Acción 1.2.2. Colaborar con las clínicas veterinarias para informar sobre los casos de posibles envenenamientos a cada uno de los coordinadores insulares, al coordinador regional y los agentes de la autoridad.

Acción 1.2.3. Transmitir rápidamente la información desde el área responsable en la conservación de la fauna silvestre de cada cabildo insular (Centros de recuperación, técnicos en biodiversidad, en espacios naturales, en caza, etc.) a los coordinadores insulares de la estrategia, al coordinador regional, y a los agentes de la autoridad, de tal manera que permita la adopción inmediata de las medidas necesarias.

Acción 1.2.4. Transmitir rápidamente los resultados de las necropsias y análisis toxicológicos, con el objetivo de tomar las medidas oportunas a tiempo, de forma que no sea necesario esperar al informe definitivo para difundir los resultados.

Acción 1.2.5. Intercambiar de forma fluida la información con la Fiscalía, con el Servicio de Protección de la Naturaleza de la Guardia Civil y, cuando sea necesario, con otras administraciones y con organizaciones no gubernamentales, con el objetivo de coordinar las actuaciones.

Acción 1.2.6. Poner a disposición de asociaciones relacionadas con la conservación de la naturaleza, asociaciones relacionadas con la caza, organizaciones agrarias, y la sociedad en general, la información que permita conocer tanto los casos de veneno en la región como las actuaciones llevadas a cabo para su erradicación.

Línea estratégica 1.3. Mejorar el conocimiento sobre el origen y efectos del veneno.

Acción 1.3.1. Mejorar el conocimiento de las motivaciones o causas que provocan la utilización de cebos envenenados.

Acción 1.3.2. Profundizar en el estudio del impacto que el uso ilegal de venenos provoca sobre especies amenazadas.

Objetivo 2: incidir en la prevención, disuasión y vigilancia del uso ilegal de cebos envenenados. Las líneas estratégicas (LE) y acciones contempladas a continuación tienen como finalidad evitar el uso ilegal de veneno en el medio natural, y se basan en la prevención, disuasión y vigilancia de los casos de envenenamiento, además de la sensibilización de la población, principalmente de los sectores o colectivos en los que el uso de veneno es más habitual.

Línea estratégica 2.1. Potenciar la adopción de medidas preventivas.

Acción 2.1.1. Estudiar y analizar la posible aplicación de medidas de desarrollo rural y de ayudas directas a las fincas y explotaciones agrícolas y ganaderas que promuevan la aplicación de actuaciones para la prevención de daños de la fauna silvestre.

Acción 2.1.2. Asesorar y colaborar con los agricultores y ganaderos para que usen métodos de control y disuasión que minimicen los daños producidos por ratas, conejos, perdices, mirlos, etc., y que no tengan efectos perniciosos sobre la fauna silvestre.

Línea estratégica 2.2. Potenciar la adopción de medidas disuasorias.

Acción 2.2.1. Fomentar el rechazo del veneno tanto por parte de los potenciales usuarios, como por la población local, ya sea por convicción de que su uso es innecesario, por los graves efectos negativos ambientales y sobre las personas que pueden producir, o por las consecuencias sancionadoras de su empleo.

Acción 2.2.2. Incluir en los planes insulares de caza, y en los técnicos de caza, una cláusula de compromiso dirigida a salvaguardar el medio de la presencia o aparición de venenos, especialmente en los acotados (cotos privados de caza, cotos intensivos, campos de entrenamiento, etc.).

Acción 2.2.3. Fomentar con las consejerías (tanto del Gobierno de Canarias como de los Cabildos) competentes en materia de ganadería, agricultura, o caza que en la evaluación de las concesiones de ayudas a la caza, ganadería y agricultura, sirvan como valoración positiva aquellos terrenos o explotaciones que no hayan presentado episodios de envenenamientos.

Línea estratégica 2.3. Potenciar la adopción de medidas de formación y sensibilización.

Acción 2.3.1. Divulgar e informar a los sectores implicados sobre las actuaciones de vigilancia y las sanciones y condenas que se logren en relación con el uso ilegal de cebos envenenados.

Acción 2.3.2. Divulgar e informar a los sectores implicados sobre la importante función ecológica que desarrollan los depredadores en los ecosistemas.

Acción 2.3.3. Fomentar la formación del personal jurídico, técnico y de los agentes de la autoridad, de los departamentos que participan en los procedimientos, así como la del personal de otras administraciones relacionadas con ello.

Acción 2.3.4. Informar y concienciar de forma específica a colectivos relacionados con las actividades cinegética, agrícola y ganadera, a través de los cauces de comunicación de la administración con estos colectivos (envío de las órdenes de caza, circulares, autorizaciones, permisos, reuniones, etc.) y de mecanismos de participación, incluyendo información sobre la legislación vigente, así como sobre las consecuencias del uso de cebos envenenados sobre la salud y el medio ambiente.

Acción 2.3.5. Mejorar la información a las explotaciones agropecuarias de las obligaciones de la condicionalidad de la Política Agraria Común en cuanto al uso del veneno, involucrando a los sindicatos agrarios y asociaciones profesionales en la difusión de la información.

Acción 2.3.6. Realizar una campaña de divulgación en los puntos de venta de los productos, especialmente los que se utilizan para preparar los cebos, para informar sobre los riesgos que conlleva su uso indebido para la salud y para el medio ambiente, además de sus consecuencias legales.

Acción 2.3.7. Fomentar la cooperación y coordinación entre los diferentes sectores implicados, incluyendo a técnicos de las consejerías (tanto del Gobierno de Canarias como de los Cabildos) competentes en materia de turismo, agricultura, ganadería, medio ambiente y política territorial, fiscales, abogados, agentes de la autoridad, ONG ambientales, asociaciones de caza, agrícolas, medios de comunicación, y en general cualquier asociación deportiva o no cuya actividad se desarrolle en el medio natural, a través de la organización de jornadas técnicas, encuentros periódicos o la creación de foros, etc.

Acción 2.3.8. Impulsar campañas de educación ambiental de ámbito insular dirigidas a colectivos y asociaciones relacionados con la actividad cinegética, agrícola y ganadera con vistas a la erradicación del uso inadecuado e ilegal del veneno. En estas campañas, se hará especial hincapié en la Estrategia contra el veneno, el impacto sobre las especies amenazadas que produce el veneno, y los riesgos sanitarios que puede conllevar, así como en las consecuencias penales y administrativas de su utilización.

Acción 2.3.9. Sensibilizar del problema a la población en general y, en particular, a la población escolar, mediante campañas en los distintos medios de comunicación.

Línea estratégica 2.4. Potenciar la adopción de medidas de vigilancia.

Acción 2.4.1. Potenciar la búsqueda, recogida y análisis de cebos envenenados y de ejemplares de fauna silvestre, asilvestrada o doméstica encontrados muertos en el medio no urbano en circunstancias en que exista sospecha de envenenamiento.

Acción 2.4.2. Avanzar en la investigación y probar la efectividad de nuevos métodos de localización de cebos y detección de tóxicos en cebos y cadáveres.

Acción 2.4.3. Establecer planes de vigilancia insulares anuales, con un nivel de esfuerzo adecuado y sobre los que se realizará una evaluación de resultados. La prospección y vigilancia se realizará en base a estos planes de vigilancia y al Protocolo de actuación de los agentes de la autoridad en la vigilancia y acción preventiva contra la utilización de venenos en el medio natural (Protocolo I).

Acción 2.4.4. Promover la formación especializada de los agentes de la autoridad y de los guardas de caza en las tareas de vigilancia, así como la dotación de medios materiales.

Acción 2.4.5. Establecer instrumentos organizativos y de coordinación con el Servicio de Protección de la Naturaleza de la Guardia Civil en la búsqueda y la investigación de venenos y, en especial, en caso de que se sospeche de la existencia de redes de comercialización o distribución ilegal de sustancias utilizadas en la elaboración de cebos envenenados.

Acción 2.4.6. Establecer cauces que proporcionen información y faciliten la colaboración ciudadana con los agentes de la autoridad en labores de prevención y detección de venenos, con especial consideración para las ONG del Programa Antídoto y otras que trabajen en la lucha contra el veneno, fundaciones y otras entidades privadas.

Acción 2.4.7. Garantizar un intercambio fluido de información entre las patrullas y los demás actores implicados en la investigación de los casos, como el personal que realiza los estudios anatomo-patológicos y toxicológicos, jurídicos y Fiscalía.

Objetivo 3: potenciar la eficacia de las acciones dirigidas a la persecución del delito. El empleo no autorizado de venenos en el medio natural está tipificado como delito en el artículo 336 del Código Penal, en su redacción dada por el apartado nonagésimo octavo de la Ley Orgánica 5/2010, de 22 de junio, por la que se modifica la Ley Orgánica 10/1995, de 23 de noviembre, del Código Penal, correspondiendo a las administraciones públicas la persecución de esta práctica ilegal. Por ello, y con el objeto de evitar la impunidad ante esta actuación ilícita y aumentar su vigilancia y control, a continuación se desarrollan líneas estratégicas encaminadas a optimizar la eficacia de las actuaciones de lucha contra el veneno en la administración autonómica y para la coordinación con la vía penal.

Línea estratégica 3.1. Potenciar la creación de una patrulla especializada de agentes medioambientales en materia de biodiversidad, en aquellas islas donde la dotación de personal así lo permita.

Acción 3.1.1. Como medida global para esta línea estratégica se creará, en aquellas islas donde la dotación de personal así lo permita, una patrulla insular -si es posible móvil, no adscritos a ninguna comarca- especializada en materia de biodiversidad. Esta patrulla estará formada por agentes de medio ambiente y realizarán un trabajo global, entre otros en la lucha contra el veneno y se encargarán específicamente en estos casos de:

- Coordinar y realizar las tareas de vigilancia y detección de uso ilegal de veneno.

- Coordinar, supervisar y, en su caso, realizar el traslado de los cadáveres y cebos. En cualquier caso, garantizar la adecuada recogida y conservación de pruebas y la conservación de la cadena de custodia.

- Coordinar y realizar las tareas de investigación de los casos de uso de veneno.

- Participar en la resolución de conflictos relacionados con la fauna silvestre y los venenos.

- Realizar inspecciones para el control de la venta de los productos empleados para preparar los cebos envenenados.

- Realizar tareas de información y sensibilización a los sectores y a la población en general.

Las tareas de estas patrullas se realizarán de forma coordinada con el resto de agentes de la autoridad. Se dotará a los componentes de formación especializada y del material adecuado para desarrollar sus funciones.

En aquellas islas donde la dotación de personal no permitiera la creación de las mencionadas patrullas, se responsabilizará a uno o dos agentes medioambientales de las mencionadas tareas.

Línea estratégica 3.2. Garantizar una adecuada recogida y custodia de pruebas y cadáveres.

Acción 3.2.1. La recogida y custodia de pruebas y cadáveres será realizada siempre por agentes de la autoridad que tengan las funciones de policía judicial (agentes medioambientales o del Servicio de Protección de la Naturaleza de la Guardia Civil), según el Protocolo de actuación para agentes de la autoridad en la recogida de fauna o cebos presuntamente envenenados y de investigación preliminar (Protocolo II).

Acción 3.2.2. Mejorar y promocionar la formación de todos los agentes de la autoridad y personal que pueda intervenir en los casos de envenenamiento en la recogida y mantenimiento de pruebas.

Acción 3.2.3. Dotar del material necesario para la recogida y mantenimiento de la cadena de custodia a todos los agentes implicados.

Línea estratégica 3.3. Garantizar la validez legal de los peritajes y analítica toxicológica.

Acción 3.3.1. Los peritajes y analíticas toxicológicas se realizarán según el Protocolo técnico de actuación en casos de envenenamiento de los centros de recuperación y los laboratorios toxicológicos (Protocolo IV), con el fin de garantizar que todos ellos tengan validez legal.

Acción 3.3.2. Asegurar una correcta dotación de personal y material para la conservación de las muestras y para la realización de peritajes y analíticas toxicológicas.

Acción 3.3.3. Estrechar la colaboración entre los peritos y los servicios jurídicos y/o el Ministerio Fiscal en la investigación de los casos.

Objetivo 4: desarrollar medidas y protocolos de actuación adecuados para una mejora de la eficacia en la lucha contra el veneno por parte de todos los actores involucrados. Las diferentes consejerías que tengan las competencias de medio ambiente en cada uno de los Cabildos de la Comunidad Autónoma de Canarias se comprometen a adoptar dentro de sus posibilidades las actuaciones enmarcadas en la presente Estrategia, aplicando en cualquier caso los siguientes protocolos:

4.1. Protocolo de actuación de los agentes de la autoridad en la vigilancia y acción preventiva contra la utilización de venenos en el medio natural (Protocolo I).

4.2. Protocolo de actuación para agentes de la autoridad en la recogida de fauna o cebos presuntamente envenenados y de investigación preliminar (Protocolo II).

4.3. Protocolo jurídico genérico de actuaciones administrativas y de coordinación con la vía penal derivadas del uso de cebos envenenados en el medio natural (Protocolo III).

4.4. Protocolo técnico de actuación en casos de envenenamiento de los centros de recuperación y los laboratorios toxicológicos (Protocolo IV).

Asimismo, los centros directivos competentes en la elaboración y aprobación de planes de recuperación y conservación de especies amenazadas, planes de gestión de espacios (EE.NN., Red Natura 2000, etc.), planes de caza, etc., deben contemplar en la revisión y/o elaboración de dichos planes los objetivos recogidos en esta Estrategia, e incluir en su caso las acciones que se consideren pertinentes, haciendo especial incidencia en aquellos planes de especies o espacios en los que ha sido constatado el veneno como un factor de amenaza.

Objetivo 5: potenciar el control de la venta de sustancias tóxicas, especialmente las susceptibles de ser usadas para la preparación de los cebos envenenados. Los productos que se utilizan para preparar los cebos son mayoritariamente marcas comerciales de uso agrícola y alta toxicidad. El fácil acceso a estos productos conlleva que sean utilizados de forma ilegal, por lo que es necesaria la adopción de medidas para controlar su venta.

Línea estratégica 5.1. Incrementar el control de sustancias utilizadas como veneno y medidas para dificultar el acceso a las mismas de acuerdo con los departamentos competentes en esta materia, tanto de sanidad como de agricultura.

Acción 5.1.1. Mejorar el control del almacenamiento y comercialización de biocidas y otras sustancias que puedan ser utilizadas para preparar los cebos envenenados, mejorando si es necesario, la normativa autonómica.

Acción 5.1.2. Promover y recomendar, como objetivo final para el control de la comercialización de los productos, la implantación de un sistema de prescripción facultativa obligatoria, con indicación expresa de las cantidades a adquirir para su posterior aplicación.

Acción 5.1.3. Realizar un seguimiento del tipo de productos que se utilizan para preparar los cebos, con el objetivo de detectar nuevas sustancias y poder tomar medidas adecuadas para impedir su uso ilegal.

Acción 5.1.4. Promover investigaciones sobre los efectos que puede producir el uso legal de los productos químicos sobre la fauna silvestre.

Línea estratégica 5.2. Potenciar y recomendar la creación de un Catálogo de Sustancias Tóxicas empleadas como cebos envenenados por la consejería o consejerías competentes en esta materia.

La creación del Catálogo de Sustancias Tóxicas empleadas en los cebos envenenados llevaría consigo la inclusión de dichas sustancias en una de las siguientes categorías:

- Prescindibles: cuando existan en el mercado otras sustancias que cumplan los mismos fines y tengan una menor toxicidad o existan alternativas a su uso.

- Imprescindibles: cuando no existan en el mercado otras sustancias que cumplan los mismos fines y sea necesario su uso para la protección de cultivos o para salud animal.

La inclusión en la categoría de Prescindible conllevará la prohibición de su tenencia, transporte y uso en la Región.

La inclusión en la categoría de Imprescindible conllevará:

a) La obligación de un etiquetado especial que explique la posible comisión de un delito en el caso de mal uso.

b) La necesidad de llevar un registro nominal de venta.

c) La necesidad de inscripción en un registro de los puntos de venta autorizados.

d) Una inspección y revisión por parte de los agentes de la autoridad y de los técnicos de los servicios de plagas de los libros de movimientos de los productos en los puntos de comercialización y uso.

Objetivo 6: facilitar y mejorar los mecanismos de coordinación entre todos los colectivos involucrados en la lucha contra el veneno en el medio natural. Las acciones que se describen a continuación tienen por objeto aunar esfuerzos en la lucha contra el veneno,

procurando la coordinación de las actuaciones llevadas a cabo y de los distintos actores implicados (administraciones regional y de justicia, Guardia Civil, ONG, veterinarios, etc.).

Línea estratégica 6.1. Adoptar medidas en la vía administrativa y de coordinación con la vía penal.

Acción 6.1.1. Las actuaciones en vía administrativa y de coordinación con la vía penal se realizarán según el Protocolo jurídico genérico de actuaciones administrativas y de coordinación con la vía penal derivadas del uso de cebos envenenados en el medio natural (Protocolo III).

Acción 6.1.2. Toda acta de denuncia por el hecho de la utilización de venenos que se reciba en los servicios insulares de medio ambiente dará lugar al inicio de actuaciones encaminadas a la determinación de la responsabilidad sobre los hechos con carácter previo a la incoación del expediente sancionador en la vía administrativa. Este será iniciado tras la acreditación de la existencia de veneno mediante los oportunos informes anatomo-patológicos y/o toxicológicos, con la posterior suspensión del procedimiento en el caso de que los actos puedan ser constitutivos de delito. Incoar el correspondiente expediente a terceros, en caso de que se determine que existen responsabilidades achacables a ellos.

Acción 6.1.3. Realizar la personación de la administración como acusación en los procesos judiciales relacionados con delitos por uso de cebos envenenados, reclamando en su caso el valor de reposición del daño causado en el ecosistema y con el objeto de reiniciar el procedimiento administrativo en el momento en el que finalice la vía penal.

Acción 6.1.4. Fomentar que entidades privadas relacionadas con la lucha contra el uso ilegal del veneno se personen como acusación en procesos penales abiertos por esta causa y procesos sancionadores en vía administrativa.

Acción 6.1.5. Tomar medidas para asegurar, en todo caso, la reparación del daño biológico causado por el uso de veneno, en cumplimiento del artículo 75 de La Ley 42/2007, de 13 de diciembre, del Patrimonio Natural y de la Biodiversidad (LPNB; BOE nº 299, de 14.12.07). Para ello, se adoptarán en cualquier momento medidas encaminadas a la recuperación del equilibrio ecológico que ha sido perturbado por el uso de veneno, independientemente de si hubiera un proceso abierto por el caso en la vía penal.

Acción 6.1.6. Como medida específica de reparación, proceder, cuando existan indicios de acciones de colocación de venenos, a la suspensión temporal de la actividad cinegética o a la anulación de la condición de acotado, que asegure la recuperación del medio y las especies afectadas.

Línea estratégica 6.2. Promover o mejorar la cooperación y colaboración con otras administraciones y unidades con intervención en la lucha contra el veneno.

Acción 6.2.1. Realizar reuniones periódicas de coordinación en las que participen los correspondientes centros directivos del Gobierno de Canarias/Cabildo, los coordinadores insulares y regionales de la Estrategia, los coordinadores de los Agentes de la autoridad y técnicos en materia de caza, sanidad, agricultura y ganadería.

Acción 6.2.2. Impulsar la formalización de convenios de colaboración o sistemas de cooperación entre el órgano competente y otras administraciones para la consecución de las actuaciones consideradas en la presente Estrategia, en particular el Servicio de Protección de la Naturaleza de la Guardia Civil y la Fiscalía de Medio Ambiente.

Acción 6.2.3. Establecer un sistema de colaboración con otros sectores de la sociedad civil involucrados en este problema, como el cinegético, el agrario o las asociaciones de defensa de la naturaleza, a través de la Estrategia Canaria contra el Veneno.

Acción 6.2.4. Establecer instrumentos de cooperación y coordinación con el resto de Consejerías que tengan competencias relacionadas con los problemas derivados del uso del veneno.

Acción 6.2.5. Con el fin de asegurar y facilitar el cumplimiento de las directrices de esta Estrategia, habilitar cauces de coordinación con otras Administraciones con responsabilidad en la gestión de la caza, la actividad agropecuaria y la biodiversidad.

Línea estratégica 6.3. Crear la figura del Coordinador regional de la Estrategia.

El responsable del Centro Directivo donde resida la competencia en materia de biodiversidad, designará un Coordinador regional tras la aprobación de la presente Estrategia, cuyas principales funciones serán:

• Impulsar, coordinar, dirigir y supervisar las actuaciones establecidas en la presente Estrategia, con el apoyo de los coordinadores insulares.

• Proponer las revisiones o correcciones a la Estrategia y los protocolos de actuación que resulten oportunas en relación a su propio desarrollo y al cumplimiento de los objetivos previstos.

• Elaborar anualmente una memoria de evaluación, mapa de riesgo y base de datos pormenorizado de los resultados del ejercicio finalizado y cuantos informes le sean requeridos al respecto de la utilización ilegal de cebos envenenados.

• Elaborar un programa priorizado de las actuaciones a desarrollar en el ejercicio siguiente.

• Estos documentos se enviarán a los máximos responsables del plan y al órgano de participación correspondiente, que podrán proponer nuevas actuaciones.

• La representación del Departamento competente en el Grupo de Trabajo Nacional de Ecotoxicología constituido en el Comité de Flora y Fauna Silvestres de la Comisión Nacional de Protección de la Naturaleza del Ministerio de Agricultura, Alimentación y Medio Ambiente.

Línea estratégica 6.4. Crear la figura del Coordinador insular de la Estrategia.

Para facilitar la coordinación de actuaciones, la Consejería competente en materia de Medio Ambiente de cada Cabildo Insular designará un Coordinador insular de la Estrategia, cuyas principales funciones serán:

• Planificar la vigilancia con el coordinador de los agentes medioambientales.

• Recopilación, procesado y custodia de la información generada en la aplicación de las actuaciones y transmisión al Coordinador Regional cuando fuera necesario.

• Proponer las revisiones o correcciones al Plan y los protocolos de actuación que resulten oportunas en relación a su propio desarrollo y el cumplimiento de los objetivos previstos.

• Elaboración de los informes técnicos que le requiera la autoridad judicial o instructor.

• Coordinar las actuaciones con los mandos del Servicio de Protección de la Naturaleza de la Guardia Civil u otras autoridades o instituciones de ámbito insular o inferior en la remisión de las correspondientes actas para el inicio de actuaciones previas, en las actuaciones e informes para el inicio de procedimientos sancionadores y suspensión de la actividad cinegética o anulación del acotado.

• Adaptar la aplicación de los protocolos a la realidad de cada Cabildo para asegurar el correcto envío y remisión de las muestras, mantenimiento de la cadena de custodia, etc.

• Elaborar una memoria anual, que servirá para completar la memoria global que debe redactar el coordinador regional.

7. EVALUACIÓN DE LA ESTRATEGIA.

Evaluar una Estrategia como la presente, en la que están implicados varios Departamentos de la Administración autonómica, organizaciones, asociaciones y agentes de la autoridad, es una tarea difícil que requiere la aplicación de procedimientos sistemáticos y rigurosos de recogida de información y análisis.

La evaluación del cumplimiento de los objetivos previstos, de la idoneidad y eficacia de las actuaciones puestas en marcha, así como de los resultados obtenidos, lleva implícito establecer unos indicadores tanto cuantitativos, para conocer el desarrollo de las acciones adoptadas, como cualitativos para conocer si los resultados obtenidos son los previstos. Para la obtención de estos indicadores es necesario realizar la memoria de evaluación:

MEMORIA DE EVALUACIÓN:

Al finalizar cada año, se elaborará por el Coordinador Regional de la Estrategia, una memoria de evaluación sobre las acciones realizadas durante el año para la erradicación del uso ilegal de cebos envenenados en el medio no urbano, teniendo en cuenta los objetivos previstos en la Estrategia.

La memoria deberá contener información sobre:

a) Los recursos existentes en la región para luchar contra el uso ilegal de cebos envenenados y para paliar sus efectos en el medio natural, incluidos los medios técnicos puestos a disposición (laboratorios, equipos de seguimiento, etc.), las patrullas especiales de agentes.

En concreto, se analizarán:

- Las actuaciones de sensibilización y prevención sobre el uso de veneno llevadas a cabo, distinguiendo tipo de actuación y población a la cual fue destinada.

- Las actuaciones llevadas a cabo para la detección y vigilancia de la utilización de cebos envenenados, incluyendo el número de controles o actividades de vigilancia programadas y realizadas; las programadas no realizadas y las no programadas realizadas o el número de muestras tomadas.

- Las actuaciones dirigidas a la persecución del delito en vía penal y administrativa, incluyendo el número de procedimientos administrativos sancionadores o penales abiertos por casos de utilización de cebos envenenados y su estado o resolución final.

b) El número de especies afectadas por uso de veneno, así como la especificación de las sustancias empleadas.

c) Información sobre las actuaciones de coordinación llevadas a cabo con y entre los distintos actores implicados (sectores agrícola, ganadero o cinegético, ayuntamientos y demás departamentos autonómicos, autoridad judicial, agentes de la autoridad y otras instituciones).

d) Documentos, procedimientos, programas, planes o protocolos relacionados con la presente Estrategia.

e) Otras medidas administrativas adoptadas: suspensión cinegética en cotos con casos de veneno, número de ayudas afectadas por criterios de condicionalidad de la PAC; número de actuaciones específicas de recuperación; otras medidas.

8. VIGENCIA Y REVISIONES.

Esta Estrategia dispondrá de una duración indefinida, determinada en todo caso por el cumplimiento de los objetivos establecidos. Cada año podrá realizarse una revisión de ella, a excepción de que determinadas variaciones sustanciales en parámetros que puedan afectar al cumplimiento de los objetivos del Plan obliguen a su modificación con una periodicidad menor.

PROTOCOLO I: DE ACTUACIÓN DE LOS AGENTES DE LA AUTORIDAD EN LA VIGILANCIA Y ACCIÓN PREVENTIVA CONTRA LA UTILIZACIÓN DE VENENOS EN EL MEDIO NATURAL

Índice

2. Fase II: actuación en los casos con claros indicios de uso de venenos.
3.- Fase III: realización de servicios de vigilancia e interceptación de sospechosos.
4.- Fase IV: actuaciones posteriores a la interceptación.
5.- Otras actuaciones.
5.1. Inspección del libro oficial de plaguicidas peligrosos y uso de zoosanitarios.
5.2. Educación ambiental y sensibilización de la población.

Las tareas de vigilancia e investigación preventiva que los agentes de la autoridad realizan para controlar el uso del veneno son fundamentales en la lucha para la erradicación del mismo.

Para que las labores de prevención de los casos de envenenamiento tengan la mayor efectividad posible, se procederá a la investigación y vigilancia de la utilización de venenos en el medio natural del modo en que se describe en el presente protocolo. Para la realización de estas labores se podrán realizar servicios fuera del horario de trabajo habitual cuando sea imprescindible.

1.- FASE I: INVESTIGACIÓN PREVENTIVA.

1.1. Antecedentes históricos.

En caso de aparición de casos de veneno, o para su prevención, se deberá contar con los antecedentes históricos de los posibles casos, para lo que se podría recurrir a las siguientes fuentes para la recopilación de información:

• Administración: información sobre animales silvestres cuya necropsia determina el envenenamiento como causa de la muerte; información sobre la costumbre en determinada zona de la utilización de veneno; revisión de denuncias e informes sobre casos de furtivismo. La detección de lazos, cepos, especies protegidas (como rapaces tiroteadas) pueden constituir vestigios que ponen sobre aviso de un posible uso de veneno en la zona.

• Veterinarios: se facilitará a las clínicas veterinarias documentación en la que se les informe del problema del uso ilegal de veneno, así como de la patología que detectaría la ingestión del veneno en animales domésticos. Se proporcionará a estos centros un número de teléfono para contactar con los agentes de la autoridad y transmitirles la importancia de notificar cualquier caso en el que se sospeche el uso de veneno.

• Pastores: su información es precisa y amplia. En muchas ocasiones son personas que han sufrido en sus animales el uso del veneno.

• Vecinos: fundamentalmente habitantes de casas aisladas enclavadas en el medio natural así como viviendas y urbanizaciones limítrofes con cotos de caza, montes, etc.

• ONG's u otras entidades relacionadas con la protección del medio ambiente.

• Internet: foros y páginas especializadas.

1.2. Localización e investigación del uso de veneno.

La vigilancia para la acción preventiva contra la utilización de venenos, preferentemente deberá ser efectuada por funcionarios públicos, por su condición de agentes de la autoridad y Policía Judicial genérica (caso de los Agentes Forestales/Medioambientales), aunque podrán participar también miembros de patrullas de ONGs u otras entidades privadas.

Para conocer y localizar los casos de envenenamiento se tendrá en cuenta el mapa de zonas de riesgo elaborado con las fechas en que se producen los envenenamientos y las investigaciones de las motivaciones que originan el uso de veneno.

1.3. Inspección y vigilancia en diferentes zonas del medio natural.

En general, la vigilancia debe ser exhaustiva en aquellas fincas donde se estén registrando casos de envenenamiento, aunque cada zona requiere de una serie de atenciones específicas que se exponen a continuación.

• Inspección y vigilancia en cotos de caza y fincas cinegéticas especialmente donde se produzcan sueltas o repoblaciones de fauna cinegética.

En el caso de fincas cinegéticas se recopilarán todos los datos posibles para conocer ampliamente el coto, sus instalaciones, límites, el guarda de la finca, cazadores, gestores, realizándose encuentros con ellos a fin de conocer las diversas problemáticas y poder realizar labores de asesoramiento.

En aquellos casos en que se haya solicitado una autorización de control de depredadores se verificará que se realiza como establece la autorización y que se lleva a cabo sin utilizar métodos masivos y no selectivos. Este control se extenderá a las áreas limítrofes. El agente de la autoridad se personará en la zona, a fin de darse a conocer al gestor o responsable con el objetivo de asesorarlo.

En temporada cinegética se realizarán inspecciones a los cazadores de los cotos que se estén investigando. Asimismo se realizarán durante todo el año visitas de inspección y recorridos a pie por el lugar, inspeccionando zonas y épocas en las que anteriormente ha aparecido veneno, como zonas aisladas, pasos de fauna, zonas con agua, madrigueras o nidos.

Se hará un especial seguimiento en los casos de cotos donde se produzcan sueltas o repoblaciones de fauna cinegética para evitar la posible eliminación con métodos ilegales de depredadores.

• Inspección y vigilancia de fincas donde hay conocimiento de daños a cultivos.

En fincas en donde haya conocimiento de quejas o peticiones de control de especies por daños a cultivos deberán realizarse labores de vigilancia para verificar que no se está utilizando el veneno (control de plagas de topillos, control de conejos que producen daños en los cultivos, etc.).

• Inspección y vigilancia de explotaciones ganaderas, especialmente donde haya antecedentes de daños a la ganadería.

En aquellas zonas (ej. zonas donde existan supuestos ataques de cuervos hacia crías de ganado caprino) donde hay conocimiento o presunción de ataques a la ganadería, hay que extremar la vigilancia para la detección de cebos envenenados, animales necrófagos con síntomas de envenenamiento o cualquier otro indicio que haga sospechar del uso de veneno.

En el caso de fincas o explotaciones agrarias (ej. Fincas con problemas con especies animales que actúan sobre los cultivos) se recopilarán todos los datos posibles para conocer ampliamente la misma, sus instalaciones, límites, gestores, arrendatarios o trabajadores, realizándose encuentros con ellos a fin de conocer las diversas problemáticas y poder realizar labores de asesoramiento. Asimismo se realizarán durante todo el año visitas de inspección y recorridos a pie por el lugar, inspeccionando zonas y épocas en las que anteriormente ha aparecido veneno.

1.4. Recogida de todos los cadáveres de fauna o de sus restos.

Se recogerán todos los cadáveres de fauna o de sus restos que se encuentren en el medio natural, aunque hayan sido víctimas presuntamente de veneno, incluyendo aquellos ejemplares en los que pueda haber sospechas (en especial aquellos que puedan estar colocados bajo tendidos eléctricos, parques eólicos, etc.), para su posterior traslado al centro de recuperación, dependencia administrativa o laboratorio asignado donde se les realizará la necropsia y se informará de la causa real de la muerte del animal. Nunca se descartará a priori el posible envenenamiento de fauna porque sus restos se encuentren en esas instalaciones o infraestructuras.

Si hay sospechas o indicios de muerte por intoxicación se recogerán también los ejemplares de fauna doméstica.

2. FASE II: ACTUACIÓN EN LOS CASOS CON CLAROS INDICIOS DE USO DE VENENOS.

Dentro de esta fase se llevarán a cabo las siguientes actuaciones:

• Se aumentará la presencia en la zona, realizando servicios de recogida de vestigios e información fuera del horario habitual de los agentes de la autoridad preferiblemente sin uniformar para no alertar al envenenador.

• Se intensificará el seguimiento de especies centinela como perros o gatos, aves necrófagas, córvidos, etc. para detectar posibles decrementos de su población.

• Se hará un mayor control de las solicitudes y seguimiento de las autorizaciones para realizar repoblaciones cinegéticas.

• Se realizará un seguimiento de sospechosos, personas que gestionan el área vigilada, guardas, etc.

• Si se ha detectado un cebo/cadáver envenenado se intentará desgranar el "modus operandi" del envenenador, ya que suelen repetir el patrón de comportamiento en cuanto a método y localización año tras año.

• Se buscarán cebos en parajes frecuentados por animales, zonas próximas a agua, refugios, pasos de fauna, madrigueras, etc.

• Se inspeccionarán posibles marcas hechas por el envenenador para localizar los cebos y verificar si han sido depredados. Estas suelen ser piedras amontonadas, ramas secas, hilos o cuerdas atadas en las ramas próximas o cualquier señal que delate el lugar. Se buscarán indicios que nos puedan aportar información sobre la persona que coloca el veneno, como huellas, marcas de neumáticos, cigarrillos, etc.

3.- FASE III: REALIZACIÓN DE SERVICIOS DE VIGILANCIA E INTERCEPTACIÓN DE SOSPECHOSOS.

Dentro de esta fase se llevarán a cabo las siguientes actuaciones:

• Análisis de la información disponible de la persona/s sospechosas: hábitos, vehículos, horarios, etc.

• Planificación del servicio: programación de los horarios de los Agentes actuantes, puntos de observación.

• Reconocimiento de la zona de actuación mediante fotografías y cartografía del lugar o visitando previamente con coche camuflado y de paisano la zona.

• Identificación de los puntos de observación general y directa, intentando cubrir la mayor área posible en donde pueda actuar el envenenador. Se intentará hacer coincidir estos puntos con lugares de cobertura por emisora o móvil para transmitir al resto de los integrantes del operativo los movimientos del sospechoso y las posibles incidencias.

• Vigilancia de la zona hasta descubrir al envenenador "in fraganti" colocando los cebos envenenados. En este momento se procederá a interceptar al infractor por dos agentes de la autoridad, identificándose con la correspondiente acreditación como tales.

• En este acto se filiará al envenenador, pidiéndole colaboración para que se vacíe los bolsillos, la mochila o cualquier otro material que lleve y que pueda contener pruebas que le incriminen en el delito. Asimismo se registrará vehículo, almacenes, chozas, etc. siempre que no tengan la consideración de domicilio. Se decomisarán todos los elementos que puedan suponer una prueba: productos tóxicos, cebos, guantes, etc., que se precintarán y etiquetarán.

• Se intentará que la persona interceptada colabore para evitar la continuación del daño producido, es decir, que indique la colocación de otros cebos o la localización de animales muertos.

• Abandonarán la zona todos los Agentes excepto uno que se mantendrá vigilando por si el envenenador vuelve en un plazo breve de tiempo al lugar de los hechos para retirar algún otro cebo no detectado, fauna muerta u otros métodos de caza prohibidos.

4.- FASE IV: ACTUACIONES POSTERIORES A LA INTERCEPTACIÓN.

• Se celebrará una reunión de trabajo con todo el personal que ha trabajado en el caso, con el objetivo de comentar problemas, deficiencias, o errores para evitarlos en otras ocasiones.

• Si es necesario, se realizará una búsqueda y recogida de otras pruebas o actuaciones.

• Se informará a las Administraciones o entidades afectadas.

• Se iniciarán los trámites administrativos o penales y las actuaciones que se deriven.

5.- OTRAS ACTUACIONES.

5.1. Inspección del libro oficial de plaguicidas peligrosos y uso de zoosanitarios.

Los productos utilizados como veneno son normalmente productos fitosanitarios que se pueden adquirir y utilizar únicamente para usos agrícolas, con unas condiciones y normas muy estrictas para minimizar los daños.

Los establecimientos en donde se vendan productos fitosanitarios deben tener el Libro Oficial de Movimientos de Plaguicidas Peligrosos (LOM), en el que es obligatorio registrar las operaciones de adquisición de este tipo de productos, fecha, nombre del producto e identificación del comprador para poder hacer un seguimiento de estos productos, y en caso de un uso indebido, saber las personas que lo han adquirido.

Por todo ello es muy importante inspeccionar por parte de los agentes de la autoridad el LOM con la finalidad de detectar un posible uso no autorizado de estos productos para producir la muerte de fauna. Asimismo, cuando tengamos sospechas de una persona concreta como supuesto envenenador, nos puede ratificar nuestra hipótesis el revisar este documento y comprobar que esta persona ha adquirido productos fitosanitarios.

En aquellos casos en los que el envenenamiento haya podido ser causado por la utilización ilegal de productos zoosanitarios se llevarán a cabo las labores de inspección y control previstas en la normativa de aplicación (Ley 8/2003, de 24 de abril, de Sanidad Animal y el Real Decreto 488/2010, de 23 de abril, por el que se regulan los productos zoosanitarios).

5.2. Educación ambiental y sensibilización de la población.

Se realizarán reuniones con alcaldes, gestores de cotos, guardas, sociedades de cazadores, asociaciones ganaderas, etc. con el fin de informar a los diversos usuarios del medio natural la ilegalidad y peligrosidad del uso de venenos.

A nivel local se fomentará el contacto directo y personal con Alcaldes y Concejales de Medio Ambiente de la zona de trabajo, con los gestores de los cotos de caza, cazadores, guardas o personal de vigilancia del coto, agricultores, ganaderos, etc. y se asesorará en las gestiones que tengan que realizar ante la Administración, sobre todo si se ven perjudicados

por fauna silvestre en su actividad. Asimismo se les informará sobre los perjuicios del uso del veneno, así como las responsabilidades penales en las que pueden incurrir.

El intentar sensibilizar de este problema mediante labores de educación ambiental va parejo a la prevención de su uso. Para ello, se debería promover la inserción de anuncios en los principales medios de comunicación sobre los perjuicios para la fauna del uso de venenos y sobre la tipificación como delito de esta actividad. Además, se promoverán campañas de educación ambiental dirigidas a la población escolar.

La difusión en los medios de comunicación del trabajo de los agentes de la autoridad en la investigación e interceptación del uso de venenos y, sobre todo, de los casos en donde se imputa a un responsable por estos hechos, se cierran cotos, etc. es primordial por el efecto disuasorio que tiene ante los futuribles infractores y sobre todo en la lucha contra la sensación de impunidad de los envenenadores.

PROTOCOLO II: DE ACTUACIÓN PARA AGENTES DE LA AUTORIDAD EN LA RECOGIDA DE FAUNA O CEBOS PRESUNTAMENTE ENVENENADOS Y DE INVESTIGACIÓN PRELIMINAR DEL DELITO.

Índice

El presente protocolo se activará cuando haya indicios o sospechas de envenenamiento. Ante el hallazgo de un posible caso se realizarán por parte de los agentes de la autoridad competentes las siguientes actuaciones:

1.- ACTUACIONES INICIALES.

En los primeros momentos después de la detección de fauna o cebo envenenados han de llevarse a cabo con la menor dilación posible las siguientes actuaciones:

• Los agentes de la autoridad realizarán una batida por la zona próxima para buscar más cebos o fauna presuntamente envenenada, especialmente en lugares donde pudieran haberse ocultado o enterrado cebos o cadáveres envenenados.

• Se buscarán huellas de calzado en los lugares donde se encontraran los cebos o cadáveres, en el entorno de vivares de conejo, bebederos o comederos de perdices o de otras instalaciones cinegéticas o ganaderas.

• Se buscarán huellas de rodadas de neumáticos en el entorno.

• Se buscarán vestigios y utensilios en las zonas anteriores, como por ejemplo, colillas de cigarros, guantes de látex, envoltorios, restos de ADN o huellas dactilares, y, en general, cualquier indicio que pueda probar la autoría de los hechos.

• Se identificará a la persona o personas que se encargan del mantenimiento de esas instalaciones cinegéticas (normalmente el guarda del coto) o agropecuarias.

• Se registrarán los vehículos de los anteriores a la mayor brevedad. Si aparecen venenos, cebos o fauna envenenada, se acreditará la propiedad y uso de los vehículos.

• Se tratará de conocer si existe algún tipo de instalaciones, bien del guarda, bien del coto, de la asociación de cazadores, del ganadero (naves, cobertizos, etc.) o del agricultor, que se puedan inspeccionar en el primer momento.

Tras el hallazgo de cebos o fauna sobre los que exista la sospecha de haber sido envenenados se realizará una primera inspección técnico ocular del terrero y de los animales encontrados y se iniciarán labores de señalización de las muestras, acotando el terreno de tal forma que solo tengan acceso las personas encargadas de la investigación.

Además se procederá a la realización de un reportaje fotográfico, utilizarán testigos y escalas métricas, sobre:

• Los animales encontrados muertos y los cebos. En caso de que los cebos sean similares únicamente colocaremos una fotografía a modo de ejemplo en las diligencias.

• Indicios (cigarrillos, marcas de neumáticos, etc.). En muchas ocasiones la fotografía es la única forma de preservar la prueba.

• Objetos comisados.

• Almacén o barraca, en casos de registro, donde se observe que no se trata de una vivienda.

• Perspectivas generales del entorno.

• El GPS en el momento de toma de las coordenadas.

Las fotografías se numerarán y clasificarán e incluirán la fecha, autoría y un texto explicativo. Se pueden presentar en papel fotográfico o impreso directamente sobre el acta de levantamiento de muestras, siendo recomendable que las copias sean en color.

2.- PRECINTADO DE LA FAUNA Y/O CEBOS ENCONTRADOS.

La fauna o cebos envenenados deberán ser precintados conforme a las consideraciones siguientes:

En el caso de cadáveres estos deben ser precintados para garantizar la inviolabilidad de la muestra, ya que se trata de una prueba judicial. Para ello basta introducirlo en una doble bolsa y cerrarlo con un precinto. La bolsa debe llevar una etiqueta perfectamente legible en la que se especifiquen de manera sucinta los siguientes datos:

• Identificación del contenido: especie, edad, etc. y si el ejemplar presenta alguna marca o distintivo.

• Localización del sitio de muestreo (dirección, paraje, matrícula del coto o referencia de explotación ganadera, coordenadas GPS).

• Detalles del punto de muestreo.

• Fecha y hora de la recogida.

• Identificación de los agentes de la autoridad que recogen las muestras.

• Número de atestado o de protocolo.

• Numero de muestras.

• Testigos y escalas métricas.

Si se trata de un cadáver esqueletizado y/o momificado, y si hay rastros de sustancias y restos biológicos, deberá recogerse debajo del mismo una muestra de terreno, llegando hasta una profundidad de 15 cm y depositarse en un envase de plástico de boca ancha, con tapón de rosca de fácil abertura y cierre.

Si se trata de cebos envenenados, deben ser introducidos, previa envoltura en papel aluminio, en envases de plástico de boca ancha, con tapón de rosca de fácil apertura y cierre.

Preferiblemente no se utilizarán recipientes de vidrio. En caso de ser utilizados deberán ser igualmente precintados y etiquetados.

3.- ELABORACIÓN DEL ACTA DE LEVANTAMIENTO DE MUESTRAS.

Para asegurar la correcta recogida de pruebas judiciales, y tras el precintado del cadáver y/o cebo envenenado, los agentes de la autoridad elaborarán, según el modelo incluido en el Anexo I del presente Protocolo, un acta de levantamiento de muestras. Junto al acta se incluirá:

• Descripción detallada de los lugares donde se encuentran cebos o animales muertos: paraje, coordenadas, tipo de vegetación o calificación del terreno (agrícola, forestal, urbano),

distancias a elementos significativos que permitan establecer un patrón del envenenador (caminos, instalaciones de acotados o zonas de repoblación, barrancos, pasos de fauna, granjas, basureros ...). Se puede realizar un croquis de la zona para facilitar o ilustrar la localización de todos los elementos.

• Descripción detallada del estado del cebo o animal: estado de conservación, si está depredado o no, si se encuentra en un lugar diferente al que fue colocado o murió y los indicios que lo demuestran.

• Descripción de los síntomas o indicios de las causas de muerte del animal: fauna cadavérica o insectos muertos, momificación, no coagulación de la sangre, posturas forzadas, risa sardónica, vómitos o diarrea cerca del animal. Si presenta o no algún orificio causado por un tiro o lesiones por alguna otra arte de caza, presencia de carreteras o tendidos eléctricos peligrosos para la fauna.

• Características del veneno: tipo de toxicidad, si se comercializa o está retirado del mercado (fecha de retirada, normativa de referencia, motivación, ...). Nombre o denominación del producto comercial presuntamente utilizado. Si durante las investigaciones hay indicios de la marca comercial donde está presente el tóxico utilizado se hará constar el nombre, la empresa o laboratorio que la comercializa en España (Bayer, Rhone Poulenc, ...) y el uso del preparado.

• Descripción de los elementos encontrados que son de interés para el caso y descripción del lugar donde se encontraron.

• Descripción de los indicios observados y significativos para la investigación en los primeros momentos de la investigación (marcas de neumáticos o pisadas, etc.).

• Reportaje fotográfico del cadáver y/o cebos.

• Las condiciones meteorológicas en el momento del levantamiento.

• Cuantos datos se estimen oportunos para la investigación, como contratos de arrendamiento, prospectos o etiquetado del producto utilizado, plan de gestión cinegética, subvenciones recibidas, acta de denuncias anteriores, autorización de control de predadores, etc.

En el acta de levantamiento se indicará el número de expediente que se le ha asignado al caso, que deberá figurar en todos los documentos siguientes que se hagan sobre dicho expediente. Junto al acta se incluirá el Formulario técnico para el inicio del procedimiento sancionador o adopción de actuaciones previas, incluido en el Anexo III.

Una vez levantada acta de la muestra se avisará y entregará copia al Coordinador Insular.

4.- ENTREGA DE MUESTRAS PARA SU ANÁLISIS. ACTA DE CESIÓN DE MUESTRAS.

Una vez todo el material esté correctamente precintado e identificado y se haya rellenado y firmado el acta de levantamiento de muestras, los agentes de la autoridad las entregarán

para su análisis al centro de recepción de referencia. Este centro será, en primer lugar, centro de recuperación de fauna, y en su defecto el laboratorio o dependencia administrativa que haya sido asignada para la recogida de muestras y análisis toxicológico.

En el caso de cadáveres envenenados, si las muestras no pueden analizarse en el momento de su entrega, se recomienda refrigerarlas inmediatamente para que se encuentren en condiciones de conservación adecuadas a la hora de realizar el informe pericial y el análisis toxicológico. Si en este mismo caso se trata de cebos envenenados se procederá a su congelación.

Cuando no sea posible hacer la entrega material directamente en el centro de recepción se podrá llevar a otras dependencias de la administración o utilizar un servicio de mensajería, siempre y cuando se garantice el estricto cumplimiento de la cadena de custodia mediante los precintos y etiquetados adecuados.

Las muestras siempre irán acompañadas, según el modelo incluido en el Anexo II del presente Protocolo, de un acta de cesión de muestras para asegurar una correcta cadena de custodia. Además se hará entrega del acta de levantamiento y de cuanta documentación y descripción de los hechos se haya obtenido.

El acta de cesión de muestras especificará:

• El número de expediente asignado (según acta de levantamiento).

• El departamento o delegación al que son remitidas.

• La especie o muestra que se envía y su estado de conservación.

• El origen de la muestra o cadáver.

• El tipo y número de precinto.

• La clase de envase.

El acta de cesión de muestras debe ir firmada por la persona o agente de la autoridad que envía la muestra, por la persona o sello de la empresa que se encargue de su transporte y la fecha, hora y firma del responsable del laboratorio o dependencia al que se remite o el centro de recuperación al que se entrega, así como el sello de este.

Una copia de esta acta debe ser remitida por duplicado al laboratorio, dependencia o al centro de recuperación de forma que una se devuelva anexa al informe pericial realizado. Otra copia será enviada al coordinador insular.

A partir de la recepción de la citada documentación, podrá iniciarse, si procediera, el oportuno procedimiento sancionador o la adopción de actuaciones previas, según el formulario técnico contenido en el Anexo III del presente Protocolo.

ANEXO I: ACTA DE LEVANTAMIENTO DE MUESTRAS

Nº Expediente:_____/_____

1. DATOS GENERALES

Fecha de actuación		Hora Inicio		Hora Fin	
Parajes					
Nombre terreno/Explotación				**Matrícula**	
Término municipal				**Provincia**	
Actuación unidad canina especializada	Sí		No	**Nº Registro**	

2. AGENTES DE LA AUTORIDAD ACTUANTES

Organismo	AA.MM	Seprona (G.C)	P. Autonómica	Otros
N.I.A/T.I.P				

3. TESTIGOS

Nombre/Apellidos	D.N.I/C.I.F	Cargo	Teléfono

4. IDENTIFICACIÓN Y RECOGIDA DE MUESTRAS

Nº	Identificación muestra	Hora	Coordenadas UTM						Nº Precinto y tipo
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		
				S	X		Y		

5. HECHOS A DESTACAR

Y para que conste, se extiende la presente ACTA en el lugar y fecha indicados, siendo firmada por cuanto en la misma intervienen.

Dirección
Código Postal Teléfono

ANEXO II: ACTA DE CESIÓN DE MUESTRAS

1. DATOS GENERALES	
Nº de expediente	_____/_____
Muestras referentes al acta de toma de muestras nº	
Transporte realizado por	

2. DESCRIPCIÓN DE LAS MUESTRAS	
Tipos de envases	
Nº total de envases precintados	
Estados de conservación	

3. OBSERVACIONES

4. DATOS DE ENVÍO Y ENTRADA EN EL CENTRO DE RECUPERACIÓN/LAB	
Agente/Técnico que envía las muestras	**Entrada en el centro o laboratorio**
Fecha y hora: Fdo:	Fecha y hora: Fdo:

Dirección:
Código Postal Teléfono

ANEXO III: FORMULARIO TÉCNICO PARA EL INICIO DEL PROCEDIMIENTO SANCIONADOR O ADOPCIÓN DE ACTUACIONES PREVIAS

Referencia al acta de toma de muestras nº……..					
Posible causa de la muerte	Envenenamiento	Colisión vallado	Electrocución	Atropello	
	Intoxicación	Colisión alambrada	Desnutrición	Disparos	
	Caída de nido	Colisión aerogenerador	Enfermedad	Otros	
Estado del cadáver	Fresco	Putrefacción	Colicuativo	Postcolicuativo	Esqueletizado
Presencia de indicios de envenenamiento	Garras agarrotadas	Sangre orific. naturales	Fauna cadavérica	Rictus facial	
	Postura convulsiva	Vómitos/diarrea	Lengua mordida	Otros	
Fecha estimada de la colocación de los supuestos cebos:					
Fecha estimada de la muerte de los ejemplares hallados:					
Distancia de los cadáveres a los cebos hallados:					
Fauna cadavérica:					
Tipo de explotación inspeccionada	Cinegética	Ganadera	Otros (indicar)		
Aprovechamiento principal (si procede)	Menor	Mayor			
Tipo de coto(si procede)	Privado	Intensivo	Deportivo		
Aprovechamiento ganadero (si procede)	Extensivo	Intensivo	Mixto		
Explotación ganadera (si procede)	Ovina	Caprina	Vacuna	Porcina	Otras
Muestras halladas junto a urbanizaciones, casas de campo			Sí	No	Desconocido
Muestras localizadas tras notificación del titular explotación/terreno			Sí	No	Desconocido
Muestras localizadas tras aviso de un particular			Sí	No	Desconocido
Muestras localizadas tras llamada anónima			Sí	No	Desconocido
Existencia de guardería o vigilancia en la zona			Sí	No	Desconocido
Presencia de vallas en el exterior que dificultan el acceso			Sí	No	Desconocido
Antecedentes de actividades ilícitas por empleo de medios prohibidos			Sí	No	Desconocido
Localización de muestras en lindes de aprovechamientos/terrenos			Sí	No	Desconocido
Cebos localizados en época de sueltas de perdices o conejos			Sí	No	Desconocido
Muestras junto a comederos, bebederos, majanos, apriscos, madrigueras…			Sí	No	Desconocido
Especies aparentemente aptas para consumo humano			Sí	No	Desconocido
Cebos aparentemente aptos para consumo humano			Sí	No	Desconocido
Otros riesgos para la salud pública (fuentes, áreas de ocio, caminos…)			Sí	No	Desconocido
Cadáveres enterrados o escondidos con ánimo de encubrir su muerte			Sí	No	Desconocido
Rastros de desplazamiento de los cadáveres para inducir a errores			Sí	No	Desconocido
Rodadas de vehículos junto a las muestras			Sí	No	Desconocido
Huellas de personas junto a las muestras			Sí	No	Desconocido
Bolsas/envases y otros recipientes localizados			Sí	No	Desconocido
Caminos asociados a la localización de muestras			Sí	No	Desconocido
Áreas de campeo/dispersión de especies amenazadas			Sí	No	Desconocido
Vertederos cercanos al lugar de las muestras			Sí	No	Desconocido
Inmediaciones de dormideros de aves			Sí	No	Desconocido
Localización de productos sospechosos en almacenes/vehículos			Sí	No	Desconocido
Adquisiciones sospechosas en establecimientos de venta de fitosanitarios			Sí	No	Desconocido
Otras consideraciones a destacar, declaraciones de testigos, guardas, vigilantes y otros:					

PROTOCOLO JURÍDICO III: GENÉRICO DE ACTUACIONES ADMINISTRATIVAS Y DE COORDINACIÓN CON LA VÍA PENAL DERIVADAS DEL USO DE CEBOS ENVENENADOS EN EL MEDIO NATURAL.

Índice

1. CONSIDERACIONES PREVIAS.

El presente protocolo pretende establecer unas pautas generales para disponer de forma inmediata de la más eficaz respuesta administrativa posible a través de medidas de recuperación, cautelares y sancionadoras, así como de coordinación de dichas actuaciones con la vía penal.

Se trata al fin y al cabo de conseguir la aplicación conjunta y única de la Ley en sus diferentes ámbitos (administrativo y penal) sin que la excusa de un procedimiento judicial penal

elimine las imprescindibles actuaciones administrativas, mucho más rápidas y efectivas en cuanto su tiempo de adopción es mucho menor.

2. ACTUACIONES PREVIAS AL INICIO DEL PROCEDIMIENTO ADMINISTRATIVO SANCIONADOR.

2.1. Recepción de los atestados/actas de denuncias por parte de los servicios competentes.

La recepción de los atestados/actas de denuncias por parte de los servicios competentes puede producirse:

a) A instancia de denuncia voluntaria o comunicación de cualquier ciudadano, asociación o profesional, sin la condición de agente de la autoridad.

En este caso, se deberá proceder a la inmediata comprobación de los hechos por personal que goce de la condición de agente de la autoridad, y en su caso, realizar el levantamiento de las oportunas actas de denuncia de acuerdo al Protocolo de actuación para agentes de la autoridad en la recogida de fauna o cebos presuntamente envenenados y de investigación preliminar del delito, y en todo caso, en tal forma que garantice la inviolabilidad de las muestras recogidas, el mantenimiento de la cadena de custodia y la posibilidad de realizar pericias contradictorias, con remisión a centros oficiales autorizados.

Esta labor la pueden realizar por tanto los Agentes Medioambientales y/o el Seprona, teniendo en especial consideración la debida coordinación y eficacia entre cuerpos, a los efectos de asegurar la economía de medios, evitar duplicidades y asegurar una mayor eficacia.

Sin perjuicio de lo anterior, se requerirá la inmediata intervención policial en los casos en que:

a) Se haya sorprendido al autor material del delito, dado que posibilita legalmente proceder a su detención y toma de declaración como imputado, con todas las formalidades derivadas. A este respecto es de reseñar que las primeras declaraciones en sede policial de instrucción suelen ser de gran valor probatorio, dado que el presunto responsable no ha tenido tiempo para fabricar una coartada, ser asesorado con mayor amplitud, o simplemente se ve desbordado por lo abrumador del material probatorio existente contra el mismo.

b) Existan pruebas que inicialmente apunten de forma nítida a un autor material concreto o identificable. Por las mismas prevenciones que las antes manifestadas.

c) Cuando de lo actuado se desprenda racionalmente la posible existencia de cualquier tipo de organización o red dedicada a la distribución, venta o empleo ilegal de las sustancias utilizadas de ordinario en la elaboración de los cebos (entramado delictual u organización criminal). Esto es especialmente necesario cuando el posible ámbito territorial excede de la competencia de los agentes de medio ambiente.

d) Cuando los Agentes Medioambientales precisen de su intervención para mantener su seguridad personal o exista resistencia a la intervención por parte de los sujetos sometidos a investigación.

e) Cuando sea conveniente y razonable obtener órdenes de entrada y registro o cualquier otra fórmula de investigación netamente policial como la obtención de huellas digitales, ADN u otros vestigios para los que los Agentes Medioambientales carezcan de medios o posibilidad de desarrollo actual.

Salvo fórmulas de colaboración y coordinación específicas que puedan ser establecidas con posterioridad a la entrada en vigor del presente protocolo, y en las que especialmente se tendrá en consideración el funcionamiento interno del cuerpo de agentes medioambientales, de forma que no se vea alterada su independencia de actuación, el Coordinador insular será la persona encargada, salvo casos de urgencia que lo impidan o los anteriormente reseñados, de determinar la necesidad o conveniencia de proceder conjuntamente con el Seprona, o de recabar la necesaria intervención del cuerpo policial a través de sus mandos.

b) Por comunicación de actuaciones operadas por el Servicio de Protección de la Naturaleza de la Guardia Civil (Seprona), o cuerpo policial equivalente de rango autonómico.

A este respecto es fundamental recordar al Seprona de forma periódica, a través del Coordinador Regional, lo esencial de que todas las actuaciones e intervenciones que realice sobre hechos en los que aparezcan indicios de relación con el uso ilegal de venenos, sean notificadas a los correspondientes Servicios Insulares de Medio Ambiente y a su Coordinador insular. De esta forma se trata de evitar la aparición de espacios de impunidad amparados por falta de coordinación interadministrativa que determinen falta de seguimiento o reacción administrativa y judicial oportuna.

c) Por atestado/acta de denuncia confeccionado por los agentes medioambientales.

Deberá ser puesto en inmediato conocimiento del Seprona por el Coordinador Insular a los mismos efectos de coordinación administrativa con las fuerzas policiales.

En todo caso, las actuaciones de seguimiento, investigación, acopio de información o apertura o seguimiento de líneas de investigación, que no han alcanzado el estado de atestado instruido para sede judicial o administrativo, deben quedar, al prudente arbitrio de sus autores, sujetas a la necesaria reserva y sigilo, siendo posible compartir dicha información con el resto de autoridades con competencia en la materia de acuerdo a lo que el buen fin de la investigación imponga.

2.2. Envío de las actas de denuncia o atestados al Coordinador Insular y análisis de las muestras.

Una vez recibidas en los Servicios insulares las actas de denuncia de los agentes medioambientales o de los atestados confeccionados por el Seprona, serán inmediatamente entregadas al Coordinador Insular.

El Coordinador Insular deberá transmitir dichas actas y/o atestados a la Sección Jurídica y asegurarse de que se opera la transmisión de las pruebas (cebos, ejemplares de fauna presuntamente envenenada y resto de efectos) en debida forma al Centro Oficial encargado de la realización de necropsias y preparación de muestras, cumpliendo con el protocolo elaborado al efecto. El resto de pruebas o vestigios que no sea preciso remitir a otras unidades, sino tan solo conservar, será debidamente custodiado en condiciones de seguridad, debidamente precintados e identificados hasta la decisión que se adopte en el procedimiento.

Dicho Centro Oficial se ocupará de la realización de las necropsias e informes forenses, análisis y emisión de los correspondientes peritajes. Si los informes que elabore sobre los cadáveres de fauna o animales domésticos son compatibles con envenenamiento, se procederá al análisis de las muestras recogidas por Laboratorio Toxicológico para su exacta determinación, orientando en todo caso el informe de necropsia hacia los tóxicos probables por ser compatibles con las sintomatologías detectadas. Este informe de necropsia será comunicado de inmediato al Coordinador Insular.

El Coordinador Insular informará de la llegada de las actas denuncia/atestado y sus posteriores vicisitudes al Coordinador Regional en la forma que este establezca. Igualmente lo comunicará al Seprona o agentes para la protección de la naturaleza, en concreto y salvo otra fórmula de colaboración establecida, al Jefe del Equipo que en cada isla tenga encomendadas tareas específicas relacionadas con la persecución del empleo de cebos envenenados o al que sea competente territorialmente, dependiendo de quién haya realizado la remisión inicial, para mantener la debida coordinación de actuaciones.

Se considera muy recomendable que el cargo de Coordinador Insular recaiga en una persona con la condición de agente medioambiental y específica formación en la materia, en especial si también asume competencias específicas en materia de venenos.

2.3. Actuaciones previas a la incoación del procedimiento sancionador en vía administrativa.

Una vez que se tenga conocimiento del inicio de actuaciones encaminadas a la redacción de acta de denuncia, y hasta que no consten informes que puedan aseverar la presencia de tóxicos en los cebos o en los ejemplares de fauna, se iniciarán actuaciones previas a la incoación del procedimiento sancionador en vía administrativa, que consistirán, teniendo en cuenta el Protocolo de actuación de los agentes de la autoridad en la vigilancia y acción preventiva contra la utilización de venenos en el medio natural, a título enunciativo en:

Ámbito cinegético:

• Cuando los hechos tengan posible relación con el ámbito cinegético, bien sea por su empleo en acotados o se deduzca así de su mecánica comisiva, el Coordinador Insular organizará a la mayor brevedad, junto con los técnicos que tengan encomendada la sección de Caza y agentes medioambientales, una inspección exhaustiva del acotado y sus proximidades, encaminada en especial a la detección de otros métodos ilegales o no autorizados de captura de animales, pudiendo a tal efecto incorporarse todos los antecedentes documentales que obren respecto de solicitudes de los titulares o explotadores cinegéticos, con sus

correspondientes autorizaciones o denegaciones, y que incluirá también las autorizaciones para sueltas, refuerzos poblacionales o repoblaciones de especies de caza. Dicha inspección se orientará también a la comprobación de cualquier otra posible infracción en el ámbito cinegético o de la protección de la biodiversidad. El Coordinador Insular podrá movilizar los medios que estime oportunos a tal fin, recurriendo a la inspección del terreno mediante perros u otros medios que resulten disponibles.

• Estudio de la adecuación del Plan Técnico de Caza en vigor a la realidad de los cotos, en especial a la realidad de las poblaciones cinegéticas señaladas en el mismo y todo lo referente a predadores y antecedentes sobre control de los mismos que se hayan solicitado por el acotado. Se traerán al procedimiento Plan Técnico de Caza y resolución aprobando el mismo, o se certificará su ausencia o caducidad. Si el aprovechamiento cinegético lo es sobre terrenos públicos se hará constar expresamente dicha circunstancia.

• Análisis de los antecedentes que obren respecto a la aparición de otros episodios de envenenamiento en dichos terrenos, con justificación documental de los mismos y estado actual de tramitación de dichos procedimientos. Se incluirá una sucinta referencia a las especies animales que pudieran ser afectadas por el uso de veneno y de su concreta catalogación, con especial referencia a la presencia en la zona de las categorías en peligro de extinción, vulnerables o sensibles a la alteración del hábitat, así como la calificación de los terrenos en cuanto a figuras de protección ambiental.

• Se traerán al expediente la justificación documental de cualquier tipo de subvención pública que hubiera recibido el acotado o sus titulares por programas de mejora, conservación del medio, fomento de la riqueza cinegética, etc.

Ámbito ganadero:

• Cuando los hechos tengan posible relación con el ámbito ganadero, el Coordinador Insular procederá en coordinación con los técnicos competentes de ganadería y agentes medioambientales, a la inspección de las instalaciones y terrenos afectos, encaminada a la detección de cualquier método no autorizado o ilegal de eliminación de animales, así como a la comprobación de que la explotación se encuentra dentro de la estricta legalidad.

• Se incorporará justificación documental de cualquier otro antecedente o episodio de envenenamiento, así como de las subvenciones que perciba la explotación, distinguiendo en sus cuantías la parte sujeta a eco condicionalidad o mantenimiento de la biodiversidad entendida en toda su amplitud.

• Se contendrá expresa mención a la titularidad de los pastos de los que se sirva la explotación ganadera y su delimitación.

Si como consecuencia del contenido de las actas de denuncia/atestados, y/o de cualquiera de las actuaciones antes descritas, se desprendiera la necesidad de dictar medidas previas a la incoación del expediente administrativo que corresponda, al amparo de lo dispuesto por el artº. 72.2 de la Ley 30/1992, de 26 de noviembre, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, en relación con los artículos

concordantes de la Ley de Caza de Canarias o normas de protección de la biodiversidad o especies, se podrán dictar medidas previas.

En todo caso, el hallazgo de vestigios, cebos, ejemplares de fauna, deberá ser puesto en conocimiento y notificado a los titulares de la explotación y de los terrenos, tan pronto como sea posible.

2.4. Adopción de medidas previas.

Estas medidas estarán basadas en la existencia fundada de indicios racionales de envenenamiento (por los datos iniciales que reflejen los atestados) y la urgencia derivada para la protección inmediata de intereses públicos afectados, en concreto la salud o seguridad de las personas, la protección de determinadas especies animales catalogadas o de la propia biodiversidad, así como para facilitar las labores de investigación y rastreo. A tal efecto, el Coordinador Insular elaborará preceptivamente y a la mayor brevedad un informe sucinto en el que se insten las mismas de forma inmediata y la justificación para su adopción, con independencia de que se auxilie en los informes de otros técnicos o cuerpos competentes que considere necesario.

Estas medidas tienen una vigencia de 15 días, y deberán ser confirmadas, modificadas o levantadas en el acuerdo de iniciación de procedimiento que corresponda, sea sancionador o de recuperación de daños, o de anulación o vedado temporal del acotado.

Algunas de las medidas previas que pueden ser dictadas, a título de ejemplo, serían las siguientes:

a) Suspensión del aprovechamiento cinegético para facilitar las labores de búsqueda, investigación y determinación de responsabilidades.

b) Suspensión del aprovechamiento cinegético para preservar la seguridad alimentaria, prohibiendo la captura de conejo.

c) Prohibición o restricción de acceso a personas por posible presencia de cebos envenenados, lo que será directamente aplicable en áreas de uso público regulado o terrenos de naturaleza pública.

d) Prohibición de pastoreo o tránsito de ganado.

e) Suspensión inmediata de las medidas autorizadas de control de la predación.

Estas medidas guardarán en todo caso la debida proporcionalidad con el número de cebos y ejemplares hallados, su localización dispersa por diferentes ubicaciones dentro de uno o más cotos, la reiteración en su aparición y otras circunstancias que se deriven del expediente, pudiendo establecerse en el ámbito geográfico delimitaciones basadas en los puntos de hallazgo (por ejemplo, perímetros de 2.000 metros desde el punto en que aparecen los cebos para casos aislados, o del centro de una zona bien definida en caso de aparición de numerosos cebos distribuidos). Temporalmente se podrán extender por todo el que resulte imprescindible.

2.5. Informe del Coordinador insular.

Los resultados de los análisis químico-toxicológicos serán recibidos por el Centro de Recuperación u órgano administrativo designado, que a su vez los remitirá junto con los informes realizados, forense, de necropsias y el pericial definitivo, al Coordinador Insular. En los casos en los que se constate la presencia de veneno en los cebos o animales, el Coordinador Insular remitirá a la sección jurídica las actuaciones relativas al caso, junto con su propio informe técnico, para que se prepare el inicio del procedimiento sancionador con medidas cautelares (que pueden consistir en la confirmación y/o ampliación de las medidas previas), y del expediente de medidas recuperadoras, todos ellos en vía administrativa, designando un instructor y procediendo a la notificación en forma a los interesados.

El informe del Coordinador Insular tendrá el siguiente contenido:

• Resumirá los hechos y circunstancias concurrentes en el caso, con inclusión de los antecedentes precisos.

- Localización de los cebos y ejemplares intoxicados o muertos, con especial indicación a su distribución geográfica dentro de la explotación o coto.

- Juicio de permanencia en el campo de los cebos y animales por sus condiciones de conservación.

- Cercanía de los anteriores a instalaciones ganaderas o cinegéticas, así como a instalaciones de las explotaciones (guardería, naves, caminos, vallados).

- Época de aparición (reproducción, veda, reclamo).

- Cantidad de cebos y forma de elaboración.

- Cantidad de tóxico en los mismos.

- Ocultación o manipulación de cualquier tipo de los ejemplares de fauna localizados.

- Presencia de otras artes o medios (autorizados o no) de control de predación.

- Resultado de los registros realizados en instalaciones o vehículos.

- Vallados perimetrales o zonas de acceso restringido.

- Existencia de conflictos previos de cualquier tipo.

• Resaltará y pondrá de manifiesto aquellos aspectos de carácter técnico que tengan importancia para la resolución del expediente.

- Riesgo potencial o real para la salud humana.

- Riesgo potencial o real para especies catalogadas.

- Grado de protección ambiental de los terrenos.

- Actitud y grado de colaboración de los titulares de las explotaciones y sus dependientes o auxiliares.

- Existencia de comunicación de los hallazgos por los anteriores sujetos o no.

- Existencia de llamadas anónimas.

- Antecedentes en otros episodios y estado actual de dichos procedimientos.

• Informará sobre la necesidad de adoptar medidas cautelares y/o de inicio de expediente de imposición de medidas recuperadoras, con justificación técnica de las mismas y su alcance.

• En el caso de haberse impuesto medidas previas, recordar que deberán alzarse, confirmarse o modificarse en el plazo de quince días desde que fueron dictadas.

• En todo caso calificará desde un punto de vista técnico la gravedad e importancia del caso a modo de conclusión.

Una vez realizado todo lo anterior, el Coordinador Insular notificará las actuaciones y remitirá copia o resumen de todo lo actuado al Coordinador Regional.

2.6. Archivo del caso.

Si de los análisis remitidos se deja constancia de la inexistencia de tóxicos, el procedimiento se archivará y las medidas previas deberán alzarse, al menos en cuanto a su fundamentación por empleo de venenos.

2.7. Notificaciones a los interesados.

En todo caso deberá ser objeto de notificación a los interesados en el procedimiento el hallazgo y recogida de todo tipo de muestras y el resto de pasos legales habituales en todo tipo de expediente sancionador.

A criterio del Coordinador Insular se podrán realizar notificaciones complementarias de tipo informativo a Ayuntamientos, veterinarios, asociaciones de cazadores o ganaderos, etc., sobre los hallazgos realizados, informando de la gravedad de los hechos, su posible repercusión sobre el medio natural, la necesidad de extremar las medidas de vigilancia y la obligación de denunciar, en una labor de disuasión y sensibilización ante el problema.

3. ADOPCIÓN DE MEDIDAS PARA REPARACIÓN DEL DAÑO CAUSADO.

Con independencia de la incoación de un expediente sancionador en vía administrativa, o incluso de la instrucción de diligencias judiciales por delito, la Administración puede en

cualquier momento proceder a la apertura de un expediente administrativo de adopción de medidas para reparación del daño causado por la aparición de cebos o ejemplares de fauna protegida envenenados.

3.1. Motivos para la apertura del expediente de medidas de recuperación.

Este expediente se tramitará de forma necesaria e inmediata al conocimiento de los hechos cuando:

a) Por el ámbito geográfico: zonas que por su importancia para la biodiversidad precisan de una inmediata respuesta, como pueden ser a título enunciativo, las siguientes:

a.1) Las definidas como áreas de nidificación, dispersión y alimentación de las especies catalogadas en cada momento como en peligro de extinción.

a.2) Áreas de presencia de especies necrófagas, estrictas o facultativas, cuyas poblaciones estén expuestas a la presencia de cebos envenenados y las que por sus características tróficas resulten especialmente sensibles a la presencia de cebos envenenados.

b) Por las especies afectadas: en aquellos casos en los que se haya producido el envenenamiento de ejemplares de fauna, que por su importancia, aconsejen de inmediato la adopción de estas medidas, como, a título enunciativo, pueden ser:

b.1) Cualquier especie catalogada en peligro de extinción, vulnerable o sensible a la alteración del hábitat.

b.2) Mortalidad significativa a escala local o comarcal de cualquier especie catalogada como de interés especial.

c) Por la entidad o gravedad de la acción: en aquellos casos en que así lo determinen datos de carácter objetivo, que a título enunciativo pueden ser:

c.1) La extensión superficial y/o el número de cebos hallados.

c.2) El número o variedad de ejemplares de fauna silvestre o doméstica afectada.

c.3) La existencia de antecedentes por anteriores episodios en el mismo espacio geográfico.

3.2. Base legal para la adopción de medidas de recuperación.

La ausencia de un desarrollo autonómico específico que regule la reparación de los daños producidos por la aparición de veneno, hace imprescindible, al menos de momento, acudir a la aplicación de normas básicas estatales. El marco actualmente vigente viene constituido por la Ley 42/2007, de 13 de diciembre, de Patrimonio Natural y Biodiversidad. Dicha norma, al igual que lo hiciera en el anterior artículo 34.d) de la Ley 4/1989, dispone en el Título III (Conservación de la Biodiversidad), dentro del Capítulo IV (De la protección de las especies en la relación con la caza y la pesca continental), artículo 62.d), que se podrán

establecer moratorias temporales o prohibiciones especiales cuando por razones de índole biológico o sanitario lo aconsejen.

La adopción de estas medidas precisa de Informe técnico que ponga de relieve el daño al equilibrio natural y la necesidad y proporcionalidad o adecuación de suspensión de la actividad cinegética, su reducción o acomodación a la nueva realidad creada, para posibilitar la recuperación del medio mediante el condicionamiento de la actividad de la caza.

En la práctica pueden establecerse varios informes "tipo" que se adecuen a la entidad y realidad de cada asunto, de forma que el Coordinador Insular pueda recurrir a ellos como una valiosa herramienta que suponga un importante ahorro de medios y tiempo al ser de aplicación directa.

3.3. Medidas de recuperación genéricas que pueden ser decretadas. Ámbito geográfico y temporal.

Las medidas adoptadas con este fundamento legal pueden tener por tanto una gran variedad de contenidos y extensión temporal, por cuanto lo pretendido es la recuperación del daño o impacto, pero se debe mantener la debida coherencia entre distintos pronunciamientos, de forma que el criterio a emplear sea homogéneo y no se dé lugar a la aparición de criterios dispares o arbitrarios. Así, en principio, las medidas genéricas que pueden ser decretadas consistirían en las siguientes, siguiendo un orden decreciente de importancia de acuerdo al suceso que se trate:

1.- Prohibición de toda actividad cinegética sin limitación temporal (a criterio técnico de revisión o comprobación).

2.- Reducción de jornadas de caza o acortamiento de temporada establecida en el Plan Cinegético.

3.- Reducción del número de capturas previstas en el Plan Cinegético o sustitución de dicha limitación por el desarrollo voluntario de medidas de fomento bajo directa supervisión administrativa.

4.- Prohibición de caza de determinadas especies cinegéticas.

5.- Prohibición de control de predadores por determinados periodos o con determinados medios.

6.- Prohibición de determinadas modalidades o del carácter intensivo del coto.

El ámbito geográfico de las medidas: el impacto sobre la biodiversidad no conoce los límites geográfico-administrativos de los aprovechamientos, por lo que la extensión de dichas medidas no debe quedar necesariamente circunscrita al aprovechamiento donde se han detectado las prácticas de envenenamiento, sino que valorando las circunstancias, podrá extenderse a terrenos adyacentes de forma objetiva, a fin de poder facilitar la recuperación de las especies afectadas.

En este sentido, y como criterio general, se parte de la posibilidad de imponer las anteriores medidas a otros terrenos no pertenecientes al aprovechamiento origen de la infracción utilizando radios de 2 km desde los puntos de aparición de los cebos o ejemplares envenenados cuando la entidad de la infracción así lo aconseje por el número de cebos y amplia distribución de los mismos, las especies afectadas o número de ejemplares localizados.

La utilización del término municipal es también adecuada cuando la magnitud de los hechos así lo aconseje.

En el ámbito temporal, y por su propia esencia, se considera que el periodo mínimo por el que deben imponerse, sin perjuicio de la posibilidad de revisión de acuerdo a datos técnicos, es de dos años.

4. INSTRUCCIÓN DEL PROCEDIMIENTO EN VÍA ADMINISTRATIVA.

4.1. Existencia de elementos de prueba contra persona determinada.

La incoación del expediente sancionador contra el presunto autor solo resulta posible si de lo actuado existen elementos de prueba que bien de forma indiciaria o de forma directa apunten a persona/s determinada/s, sean físicas o jurídicas.

En este caso, y hasta la fecha en la generalidad de los casos, el expediente pasa al instructor correspondiente de la Sección Jurídica. Si las pruebas o indicios de todo orden no determinan la posibilidad de mantener la atribución de autoría, se procederá directamente a la remisión de todo lo actuado a la Fiscalía, con indicación de si se ha iniciado expediente de medidas recuperadoras.

También es posible que sea el propio Coordinador Insular o incluso los agentes medioambientales instructores los que procedan a realizar dicha remisión de forma directa, dejando nota de constancia ante los órganos administrativos. Ningún inconveniente legal se puede reprochar a la remisión directa a los órganos judiciales.

Si del contenido del expediente se desprende la muerte o daños para especies catalogadas como en peligro de extinción o vulnerables, dentro de la Ley 4/2010, de 4 de junio, del Catálogo Canario de Especies Protegidas, o del Real Decreto 139/2011, de 4 de febrero, para el desarrollo del Listado de Especies Silvestres en Régimen de Protección Especial y del Catálogo Español de Especies Amenazadas, el expediente se instruirá además como infracción a especies.

Si no se dan las anteriores circunstancias, el expediente se instruirá como infracción de la Ley de Caza de Canarias.

4.2. Inexistencia de indicios suficientes para imputar a persona determinada.

En el caso de que no consten indicios suficientes para mantener la imputación en sede administrativa contra persona determinada, o la incoación se haya producido sobre personas distintas a los titulares de la explotación o de los derechos reales o personales de uso y dis-

frute de los terrenos en los que han aparecido los cebos y/o ejemplares de fauna envenenada, se abrirá de inmediato el expediente sancionador motivado en la falta de cumplimiento de las obligaciones específicas de impedir la aparición de cebos envenenados en el medio natural, que se tramitará de forma independiente a la vía penal hasta resolución. Esto último solo será posible cuando la Comunidad Autónoma de Canarias se haya dotado de los pertinentes recursos legales.

Es de suma importancia recordar que si los presuntos autores de la infracción son los guardas o personal laboral o de servicio dependiente de la explotación, cometiendo los hechos con ocasión del cumplimiento de sus funciones, es posible conceptuar a dichos titulares también como autores de la infracción administrativa por el deber de responder por ellos salvo que acrediten la diligencia debida, por lo que el expediente sancionador administrativo respecto de los titulares puede quedar incoado respecto de los mismos y suspendido a la espera del resultado del proceso penal respecto de sus dependientes. Si el guarda o dependiente fuera condenado en vía penal, una vez recibido el testimonio deberá levantarse la suspensión del procedimiento sancionador sobre la base de los hechos declarados probados (condena al guarda o dependientes), respecto de los que no habrá sanción por aplicación del principio non bis in ídem, y se sancionará al titular como autor de la infracción de colocación de cebos envenenados por responsabilidad asociada a los hechos cometidos por sus dependientes.

En igual sentido, y respecto de las personas jurídicas, dado que en vía administrativa serán sancionadas por la actuación de sus órganos o agentes cuando estos actúen en el desempeño de sus funciones, se deberá proceder de igual forma cuando el presunto autor sea miembro del órgano social o agente de la misma, al efecto de que la sanción administrativa por el uso de cebos pueda ser impuesta con posterioridad a un proceso penal que declare la autoría de alguno de los anteriores.

En ambos casos con respeto y acatamiento de los hechos declarados probados.

Esto es crucial a día de hoy porque en los procedimientos penales en los que se ha sorprendido "in fraganti" al guarda del coto o a trabajadores de la explotación ganadera, se pretende una rápida conformidad por la levedad de la pena a imponer, amparando en la práctica con el silencio a quien ha ordenado la colocación o empleo de dicho medio. La postura del condenado responde en la generalidad de los casos a la estrategia de beneficiarse de un castigo más leve (no hay ingreso en prisión y la multa es ridícula comparada con la administrativa) y que no afecta al "autor intelectual" del delito, que queda amparado por su silencio en razón a la dependencia laboral o económica del mismo. De esta forma, por la obligación de responder en vía administrativa de los titulares respecto de sus dependientes, se puede sancionar en sede administrativa sin las limitaciones del procedimiento penal de una forma contundente.

Evidentemente, en estos casos, habrá de estarse a la espera de la sentencia firme en sede penal y a cuidar especialmente la redacción de la resolución de suspensión en el sentido de que el expediente sancionador incoado a los titulares queda suspendido a la espera de la resolución judicial en sede penal (prejudicialidad penal) por cuanto el hecho básico de la autoría del dependiente debe ser fijado por la jurisdicción penal antes de proseguir con su tramitación ordinaria.

4.3. Remisión de las actuaciones a la Fiscalía Provincial.

En todo caso, el instructor del expediente suspenderá la tramitación del mismo en vía administrativa tras el dictado de las oportunas medidas cautelares, inicio o imposición de las medidas de recuperación, y remitirá vía Delegado insular la totalidad de las actuaciones a la Fiscalía, a fin de que se pronuncie sobre la procedencia de inicio de la vía penal de acuerdo a sus propias normas de procedimiento, en virtud de lo establecido en la Ley de Seguridad Ciudadana y de Régimen Jurídico de las Administraciones Públicas.

Debemos reiterar que la remisión puede realizarse directamente por los agentes de medio ambiente, y así se viene admitiendo en sede judicial de forma pacífica, pero en todo caso garantizado la constancia administrativa de dicha remisión y la tramitación de los expedientes de recuperación, cautelares, sancionadores, etc., que resulten oportunos.

En los escritos de remisión se hará constar de manera expresa la identificación del procedimiento sancionador en vía administrativa, la resolución de incoación, el acuerdo de remisión y una solicitud formal de nota de remisión de la resolución que por cualquier causa ponga fin al procedimiento penal, con envío de testimonio de todo lo actuado, sea por auto de archivo provisional, sobreseimiento libre, sentencia condenatoria o absolutoria, a fin de que puedan proseguir en su caso las actuaciones administrativas previamente iniciadas y evitando la caducidad del expediente o la prescripción de la infracción.

Se informará en todo caso dentro del escrito de remisión a la Fiscalía de las medidas cautelares adoptadas en el procedimiento sancionador incoado, o de la adopción o inicio del procedimiento de adopción de medidas de recuperación, así como de la condición de perjudicados de la Administración Autonómica si han aparecido ejemplares de fauna amenazada o con cualquier régimen de tutela, al efecto de que se puedan realizar a la misma los pertinentes ofrecimientos de acciones de resarcimiento de carácter civil o de personación en los Juzgados competentes para sostener la acusación particular.

Del escrito de remisión a la Fiscalía deberá tener inmediato conocimiento el Instructor y el Coordinador insular, así como la autoridad que haya instruido el atestado. El Coordinador Insular, a su vez, lo pondrá en inmediato conocimiento del Fiscal Delegado de Medio Ambiente para su conocimiento y efectos, así como del Coordinador Regional.

5. ADOPCIÓN DE MEDIDAS CAUTELARES.

Con fundamento en el contenido del informe elaborado por el Coordinador insular, el instructor adoptará la decisión de imponer medidas cautelares (artº. 72 LRJAP) y así lo propondrá al órgano competente.

El objetivo de las mismas será:

• Evitar la continuidad de la infracción.

• Evitar el agravamiento del daño producido.

Algunas de las medidas cautelares posibles y que se pueden adoptar según los casos, son las siguientes, a título enunciativo:

• Suspensión de las medidas de control poblacional, dado que en el caso de utilización de cebos envenenados, especies oportunistas o de amplio espectro trófico pueden quedar mermadas, por lo que las autorizaciones concedidas o el contenido del Plan Técnico de Caza no puede seguir amparando dicha práctica.

• Suspensión de la caza de especies con destino a consumo humano, por la posibilidad de contaminación.

• Suspensión de la caza con perros a fin de evitar accidentes.

• Si de lo actuado y por las circunstancias inicial e indiciariamente acreditadas se desprende con claridad que el uso de cebos envenenados responde a una práctica de gestión cinegética amparada o consentida por sus titulares, suspensión del acotado a fin de evitar la continuidad de dichas prácticas, lo que puede llegar incluso a extenderse a otros cotos gestionados por los mismos, de tenerlos, en todo el ámbito de la Comunidad Autónoma de Canarias.

• Suspensión del pastoreo o tránsito de ganados.

6. PERSONACIÓN DE LA ADMINISTRACIÓN AUTONÓMICA Y ONG EN LOS PROCEDIMIENTOS PENALES.

La personación como parte acusadora (acusación particular) de la Comunidad Autónoma de Canarias precisa de acuerdo expreso de su Consejo de Gobierno, por lo que quedará reservada, a iniciativa y criterio del Coordinador Regional, para ser propuesta en aquellos procedimientos penales en los que por razón del daño ocasionado a la biodiversidad o los valores naturales de la región, o por otras razones de trascendencia pública o importancia medioambiental, se considere inicialmente conveniente que se proceda a la personación en las actuaciones, ejercitando la acusación por los hechos que se pongan de manifiesto, a través de sus servicios jurídicos.

A este respecto, el Coordinador Regional elaborará un informe documentado y razonado en el que se recojan los motivos por los que a su juicio resultaría conveniente proceder a dicha personación, destacando de manera especial la existencia inicial de elementos de prueba suficientes que puedan a su juicio dar viabilidad a dicha acusación, así como las razones de índole objetiva para concluir en la conveniencia de que se mantenga acusación particular por la Comunidad Autónoma. Dicho informe será elevado al Consejero con competencia en materia de medio ambiente a fin de que, previa aceptación, impulse la tramitación que en derecho proceda para tal fin.

Si finalmente hubiera personación a través de sus letrados en el procedimiento propuesto, se pondrá en inmediato conocimiento del Coordinador insular a los efectos oportunos.

Igualmente, a criterio del Coordinador Regional, se podrá cursar carta informativa a las distintas ONG que desarrollen dentro de su ámbito estatutario programas específicos de

lucha contra el uso de cebos envenenados a fin de que, dándoles cuenta de la existencia del procedimiento penal de que se trate, y un breve resumen de los hechos que lo motivan, puedan mostrarse como parte acusadora de acuerdo a las normas reguladoras internas de cada una de ellas.

7. SEGUIMIENTO DE LOS PROCEDIMIENTOS PENALES.

El seguimiento de los procedimientos penales se llevará a cabo por los instructores de los expedientes sancionadores, que se coordinarán a tal efecto de forma directa y personal con los Fiscales de Medio Ambiente. De esta forma se remitirán comunicaciones periódicas en solicitud de información a fin de conocer el estado de tramitación de los mismos.

Si el número de Instructores u otras circunstancias así lo aconsejan, la labor de coordinación recaerá en exclusiva y por cada isla en uno de ellos al efecto designado, que deberá realizar el seguimiento de todos los procedimientos remitidos a sede penal, se hayan incoado los expedientes sancionadores o no.

Los instructores informarán periódicamente al Coordinador Insular del estado de tramitación de los procedimientos en vía penal, y al menos, una vez cada seis meses en listado actualizado, dando este cuenta al Coordinador Regional con la misma periodicidad.

En los casos en los que la Comunidad Autónoma de Canarias se haya personado como acusación, el seguimiento se entenderá por el instructor directamente con el Servicio Jurídico de la Comunidad.

Si constara la personación de alguna ONG en la causa penal, el Instructor podrá solicitar de la misma la colaboración informativa que precise.

8. ACTUACIONES ADMINISTRATIVAS TRAS LA RESOLUCIÓN DEL PROCEDIMIENTO PENAL.

Una vez que haya finalizado por resolución firme el procedimiento penal, se recibirá testimonio completo de las actuaciones emitido por el Secretario del órgano judicial correspondiente, o se recabará del mismo la emisión de dicha copia, como parte interesada.

a.1) Al recibirse testimonio de la sentencia firme dictada, si existiera condena por colocación de cebos y/o muerte de especies de fauna catalogada al guarda, trabajador o dependiente de la explotación, o para alguno de los agentes o integrantes de los órganos sociales de la persona jurídica titular, de ser el caso, se deberá alzar la suspensión del expediente sancionador incoado respecto de los titulares a los efectos de continuar su tramitación por autoría contra los mismos.

a.2) Al mismo tiempo, sobre la base de los hechos declarados probados en la sentencia, y siempre y cuando la misma se refiera al titular cinegético de la explotación o dependientes de los anteriores, se abrirá expediente administrativo para proceder a la anulación del acotado o establecimiento de vedado temporal con fundamento en el aprovechamiento incompatible con el mantenimiento de la biodiversidad.

No se trata de un procedimiento sancionador que castigue unos mismos hechos, sino de la necesidad de dar cumplimiento a lo dispuesto por el artículo 62.3.h) de la Ley 42/2007, de 13 de diciembre, del Patrimonio Natural y de la Biodiversidad, que establece que cuando se compruebe que la gestión cinegética desarrollada en una finca afecte negativamente a la renovación o sostenibilidad de los recursos, las Administraciones Públicas podrán suspender total o parcialmente la vigencia de los derechos de caza. Precepto concordante con los principios que inspiran y presiden la legislación sectorial de caza (Ley y Orden anual de vedas).

Las fases de este procedimiento serán:

1.- A la llegada del testimonio de lo actuado judicialmente se emitirá con carácter previo un informe por el Coordinador Insular en el que, a la vista del procedimiento penal y su conclusión, así como de los antecedentes obrantes en todas las actuaciones administrativas y judiciales hasta el momento desarrolladas, establezca la acreditación del uso de cebos envenenados como método de gestión cinegética, su impacto, presencia de otros métodos prohibidos de captura o muerte de animales o no autorizados, discrepancia entre la realidad cinegética del coto y lo establecido en el Plan Técnico, continuidad, gravedad o intencionalidad de la infracción, etc., estableciendo los fundamentos y criterios técnicos de la propuesta que realice en orden a la suspensión de aprovechamientos y su exacto contenido.

2.- Incoación del procedimiento, por el órgano competente, en la que se propondrán a la vista del anterior informe y documentos a los que se refiera e incorpore, las concretas medidas, que pueden ir desde la suspensión temporal y/o parcial de los derechos cinegéticos hasta, en los casos más graves, la suspensión total de la vigencia de los derechos de caza.

3.- Traslado al titular cinegético para alegaciones y audiencia.

4.- Solicitud de informe al Consejo Insular de Caza, en su calidad de órgano consultivo, no vinculante.

5.- Resolución administrativa y notificación.

a) Si se ha producido archivo/sobreseimiento de las actuaciones por no considerarse delito los hechos sometidos a la jurisdicción penal: se procederá a la reapertura del expediente sancionador y a su tramitación ordinaria imputando autoría o responsabilidad de acuerdo a las pruebas existentes, dado que no ha existido declaración vinculante de hechos por parte de la jurisdicción penal. Se continuará de acuerdo a lo dispuesto en el Punto III de este Protocolo.

b) Si se pronuncia sentencia absolutoria: el Instructor reiniciará el procedimiento en vía administrativa sobre la base de los hechos declarados probados en la sentencia, de forma tal que sean respetados en todo momento.

Si la absolución ha sido debida a meros motivos formales, no de fondo, se reiniciarán las actuaciones administrativas sancionadoras evitando en todo momento la reproducción de dichas irregularidades, y en todo caso sin utilización de las pruebas que hubieran sido declaradas ilícitas.

PROTOCOLO TÉCNICO IV: DE ACTUACIÓN EN CASOS DE ENVENENAMIENTO DE LOS CENTROS DE RECUPERACIÓN Y LOS LABORATORIOS TOXICOLÓGICOS.

Índice

Este protocolo pretende ser una ayuda para la actuación de los veterinarios de centros de recuperación y de los técnicos de los laboratorios forenses y toxicológicos, ante casos de supuestos envenenamientos. Dado que la variabilidad en la disponibilidad de recursos humanos y técnicos, este documento no debe ser contemplado como un protocolo a seguir de forma estricta, pero sí debe ser tomado como un modelo de actuación que puede dar buenos resultados con su aplicación. Esta guía de actuación comienza con la entrada en el Centro Recuperación del animal o los cebos recogidos por los Agentes y termina con la redacción del informe pericial por parte del veterinario del Centro de Recuperación (Figura 1).

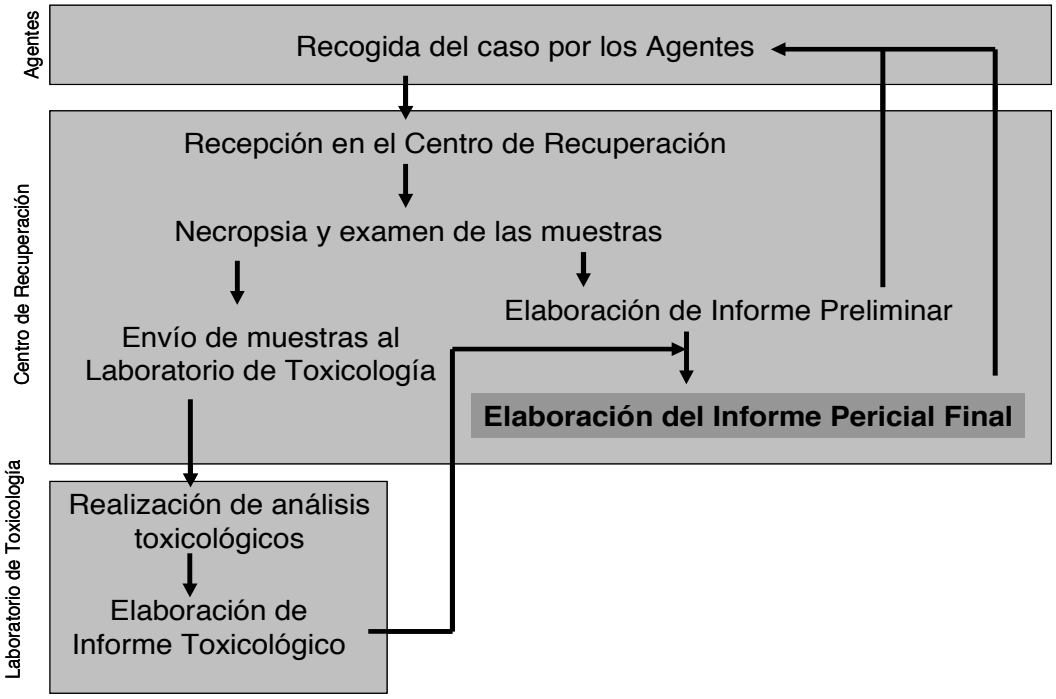


Figura 1. Esquema de la actuación en casos de envenenamiento de los Centros de Recuperación y los Laboratorios Toxicológicos.

1. REALIZACIÓN DE LA NECROPSIA.

El objetivo de cualquier necropsia es establecer de la forma más precisa posible las causas y la circunstancia de la muerte del o de los ejemplares a necropsiar. Por ello, la necropsia debe ser completa, ordenada y sistemática. Debe ser realizada por un veterinario formado y con los conocimientos actualizados en los aspectos fundamentales de la necropsia legal.

1.1. Historial.

Se debe empezar leyendo toda la información recogida en el acta de levantamiento del cadáver, y en caso necesario, contactar con el agente que la ha redactado antes de empezar la necropsia. Es de suma importancia recoger la máxima información posible de los ejemplares:

• Lugar de procedencia, paraje y término municipal, y, si es posible, las coordenadas UTM.

• Circunstancias del hallazgo del cadáver, hora, día, posición ...

• Tratamientos recibidos y manejo del animal desde su recogida, en caso de que se hubiera encontrado aún con vida.

1.2. Examen externo.

El examen externo debe ser exhaustivo y minucioso, e ir encaminado a la determinación de la edad, toma de datos biométricos y detección de lesiones externas.

En primer lugar, todos los cadáveres deberán ser fotografiados con cámara digital de calidad para que las imágenes obtenidas puedan ser útiles en la elaboración del informe pericial. Se recomiendan, al menos, las siguientes vistas:

• Todo el cuerpo, visión ventral.

• Todo el cuerpo, visión dorsal.

• Lesiones o signos que se consideren relevantes.

A continuación llevaremos a cabo el examen externo del animal para estimar la fecha en que se ha podido producir la muerte del animal (descomposición del cadáver y entomología forense) y buscando posibles heridas, traumatismos y fracturas. Se ha de prestar especial atención a signos que puedan evocar situaciones ante-mortem de sintomatología convulsiva, diarreica o hemorrágica, ya que pueden orientar en gran medida el desarrollo del protocolo analítico. Siempre es recomendable radiografiar antes al animal para detectar fracturas e indicios de disparos. Pesaremos al animal, evaluaremos su condición corporal (presencia de grasa y estado de la musculatura pectoral en aves). En el caso de observar restos de vómito o alimento en la boca o alrededores, se recogerá esta muestra por poder presentar el tóxico en concentraciones altas.

El examen externo incluirá:

• Radiografía ventro-dorsal, y de cualquier otra parte del cuerpo que se considere necesaria.

• Examen y palpación de todo el esqueleto, esófago y cavidad celómica.

• Examen completo de pelaje, plumaje, boca, pico, cera y garras.

• Examen de todos los orificios corporales y estado de mucosas.

• Peso y tamaño del animal (medias a tomar en función de la especie).

• Se observará la presencia de ectoparásitos, tipo, identificación, si es posible, localización y nivel de parasitación.

1.3. Examen interno.

Para el examen interno se colocará al animal en decúbito supino, y se procederá la disección de la forma adecuada según la especie animal. De forma general, se iniciará la disección del cadáver por la zona abdominal, y se retirará la piel tanto como sea posible para observar mejor posibles traumatismos y hemorragias. Si se valora la posibilidad de que se trate de un proceso infeccioso, se tomarán muestras de vísceras con material esterilizado y con un mechero Bunsen o de alcohol cerca del cadáver, así mismo se preparará el material necesario para flamear el material si se considerase necesario.

Las vísceras serán examinadas antes y después de ser extraídas del animal. Se recomienda el siguiente orden de extracción de vísceras:

• Corazón.

• Tracto digestivo, desde esófago hasta cloaca en las aves, incluyendo hígado, bazo y páncreas, y cerrando la luz del tracto digestivo mediante el uso de mosquitos para evitar contaminaciones. En mamíferos, según el tamaño del animal, será más indicado extraer el tracto digestivo por partes y las vísceras de forma separada.

• Disección y extracción de lengua, tráquea, bronquios y pulmones.

• Glándulas adrenales, gónadas y riñones.

• Glándulas tiroideas y timo (si fuese necesario).

• Encéfalo.

Dichos órganos se dispondrán en una bandeja limpia y reglada situada en las inmediaciones del mechero. El tracto digestivo se colocará en una bandeja aparte para evitar la contaminación del resto de los órganos.

Seguidamente se procederá al examen exhaustivo de cada órgano/sistema y a la toma de muestras. Además se realizarán fotografías digitales de todas las lesiones que se observen. Se debe prestar especial atención a la presencia de hemorragias, congestión de vísceras, exudados y edemas, y estado de la mucosa gastrointestinal.

Con toda la información recogida, el veterinario redactará un primer informe pericial preliminar en el que, en base a su experiencia, valorará la posibilidad de una intoxicación como causa de la muerte del animal o de la existencia de un posible tóxico en un cebo. Una copia de este informe será enviado al laboratorio toxicológico junto con el impreso de solicitud de análisis (Anexo I) y las muestras a analizar. Este informe preliminar, a la espera de tener las confirmaciones analíticas, puede ser utilizado para ampliar la investigación en el campo por parte de los agentes (por lo que se les debe informar con diligencia) y para tomar las medidas cautelares necesarias.

2. TOMA DE MUESTRAS PARA ANÁLISIS TOXICOLÓGICO.

2.1. Tipo de muestras.

En función de las lesiones observadas y la sospecha de productos implicados pueden ser recomendables diferentes tipos de muestra (Tabla 1). De forma general las muestras de elección que deberían tomarse siempre son:

• **Contenido de esófago y estómago:** la mayoría de los tóxicos son de acción rápida y se llegan a encontrar en alta concentración en el tracto digestivo superior. Una vez absorbidos, tóxicos como organofosforados y carbamatos son rápidamente degradados y pueden no ser

detectables en el hígado. La composición y la naturaleza del contenido digestivo, cuando lo hay, puede aportar información relevante en el proceso. Su manipulación dependerá de la presencia visible o no de material extraño sospechoso. En el caso de observar materiales extraños como polvo, pasta o microgranulados sospechosos, se recomienda introducirlo en un pequeño tubo para que no se mezcle más con el resto de alimento; con ello además facilitaremos la labor del laboratorio a la hora de la detección analítica. Para poder valorar la posibilidad de una ingestión letal es esencial tener información sobre el peso total del contenido gástrico y/o la cantidad de polvo, pasta o microgránulos de producto tóxico presente en dicho contenido. Por el contrario, cuando las sustancias tóxicas se encuentran mezcladas de forma homogénea en el contenido gástrico y, por tanto, el producto no es visible, se manipulará la muestra como un todo. Una vez obtenido el resultado analítico, a partir de la concentración detectada y la cantidad de contenido gástrico, podremos calcular la dosis ingerida. Calculada la dosis de ingesta, teniendo en cuenta el peso del animal, podremos comparar el dato con las dosis letales medias descritas en la bibliografía. No obstante, en muchos casos el tóxico ha podido ser en su mayor parte absorbido o degradado, por lo que la simple presencia en el contenido gástrico junto con las lesiones o sintomatología observadas asociadas a dicho tóxico y otros hallazgos analíticos (ej.: inhibición de la AChE cerebral en el caso de los insecticidas fosforados y carbamatos) son suficientes para llevar a cabo el diagnóstico.

• **Hígado:** por ser un órgano que recibe todo lo absorbido por vía digestiva es recomendable su muestreo. Además, esta recomendación se ve reforzada porque, en ocasiones, puede ser crucial el análisis de hígado para poder demostrar la causa tóxica de la muerte, a pesar de tener un resultado positivo en tracto digestivo. Por otro lado, cuando no hay contenido digestivo o cuando el análisis de este es negativo, el análisis de hígado se hace imprescindible. Algunos tóxicos de acción menos rápida, como rodenticidas anticoagulantes, son acumulados y son principalmente detectados en el hígado. Los metales pesados como el plomo también se acumulan en el hígado y el riñón, estando bien definidas las concentraciones en estos tejidos asociadas con la intoxicación letal en animales.

• **Encéfalo** (no es necesario extraerlo intacto): como la mayor parte de los productos usados en la actualidad como veneno son plaguicidas anticolinesterásicos, es recomendable determinar la actividad de la acetilcolinesterasa cerebral, que estará normalmente muy inhibida en el caso de exposición a carbamatos u organofosforados. Esto permite orientar un análisis químico y al mismo tiempo permite confirmar un efecto del tóxico que se asocia con la muerte del animal (inhibición >50% respecto a valores controles). No obstante, en algunos animales intoxicados por carbamatos se puede encontrar una actividad de colinesterasa cerebral normal, posiblemente debido a una reactivación post-mortem o porque el cuadro ha sido sobreagudo y los efectos en el sistema nervioso periférico han provocado la muerte antes de que el tóxico llegue al sistema nervioso central. Algunos compuestos organoclorados o el mercurio pueden ser acumulados en el encéfalo hasta alcanzar concentraciones letales. Por todo ello, hemos de considerar la prueba de la actividad anticolinesterásica como un complemento útil en el proceso laboratorial y en el diagnóstico, pero no como prueba definitiva de descarte de la intoxicación por anticolinesterásicos.

Otras muestras adecuadas dependiendo de las circunstancias son:

• **Cebos y vómitos:** material sospechoso encontrado cerca del cadáver. Evidentemente la concentración suele ser más elevada que en los animales y la detección del tóxico más probable. Ha de tenerse presente que se trata de una muestra de gran valor en el laboratorio de análisis ya que el producto tóxico suele estar inalterado y, tanto la extracción como la detección analítica se ven muy favorecidas, con un importante ahorro de tiempo y esfuerzo.

• **Sangre:** se restringe la posibilidad de muestreo a animales vivos o recién muertos (corazón), pero es de utilidad para determinar la exposición al plomo y así poner rápidamente un tratamiento con quelantes. Para la determinación de plomo se debe tomar la sangre en tubos con heparina de litio. La sangre también puede ser muestreada en tubos con citrato sódico como anticoagulante para la realización de pruebas de coagulación en el caso de intoxicación por rodenticidas anticoagulantes antagonistas de la vitamina K. También se puede utilizar para realizar un hemograma. La sangre de color marrón puede indicar la intoxicación por nitratos, y en ese caso podemos determinar el porcentaje de metahemoglobina en sangre si esta se analiza en pocas horas o se conserva en nitrógeno líquido.

• **Plasma:** es posible determinar algunos tóxicos, pero sobre todo puede ser útil para determinar la actividad de las colinesterasas plasmáticas por el mismo motivo dado para el muestreo de encéfalo. También se puede utilizar para hacer un perfil bioquímico de rutina.

• **Riñón:** en caso de intoxicación por herbicidas bipiridílicos (paraquat) el tóxico se acumula en el riñón. En estas intoxicaciones es posible observar lesiones pulmonares importantes (congestión, edema). El riñón puede ser también una muestra útil en el caso de que el cadáver haya sido devorado por depredadores y no queden restos de tracto digestivo u hígado. El cadmio se acumula a lo largo de la vida del animal en el riñón, pudiendo alcanzar concentraciones muy elevadas.

• **Grasa:** no es un tejido que sirva para determinar exposiciones agudas, pero sí es útil para monitorizar la exposición a compuestos lipófilos persistentes, como son los compuestos organoclorados. Los plaguicidas más acumulables de esta familia ya no están en uso pero todavía son detectables en los animales. Compuestos halogenados como los bifenilos policlorados, polibromodifenil éteres y otros son altamente persistentes y también es necesario monitorizarlos.

• **Hueso:** el plomo se acumula en el hueso a lo largo de la vida del animal, por lo que es una muestra útil para estudios de monitorización. En determinadas ocasiones, cuando solo se dispone del esqueleto, puede ser utilizado para extraer los restos de médula ósea para realizar el análisis, aunque la probabilidad de demostrar la muerte por intoxicación es muy baja.

• **Pelo, plumas y uñas:** algunos elementos como mercurio y arsénico (e incluso plomo) pueden acumularse en estas estructuras, indicando una exposición crónica o incluso exposiciones agudas letales.

• **Tierra bajo el cadáver:** en casos de cadáveres completamente descompuestos, se pueden tomar los primeros 5 cm de suelo bajo el cadáver, teniendo en cuenta siempre el tamaño del cadáver (cadáveres pequeños, muestras de tierra pequeñas) y la posibilidad de que haya sido depredado y movido del lugar inicial de la descomposición.

2.2. Forma de conservación y envío de las muestras.

Siempre deben tenerse presentes las siguientes premisas a la hora de llevar a cabo los procesos de conservación de muestras para su envío:

• Las muestras deben estar exentas de contaminación química externa (polvo, pelos, tierra, etc.), salvo que, lógicamente, esta sea la muestra a enviar o estos elementos formen parte de la muestra.

• Las muestras deben ser congeladas a -20ºC inmediatamente tras su recolección y enviadas manteniendo estas condiciones hasta su llegada al laboratorio. La única excepción a esta regla se da en la muestra de sangre, la cual se mantendrá a temperatura de refrigeración (aproximadamente +4ºC), con el fin de poder realizar pruebas de coagulación y el hemograma.

• Cada muestra debe ir contenida en un recipiente independiente (tipo envase de muestras de orina o bolsa de plástico con cierre ziploc), debidamente etiquetado con la referencia del caso y la naturaleza de la muestra. Las bolsas tienen la ventaja de ocupar menos espacio y poderse precintar fácilmente con una etiqueta autoadhesiva que incluya la información de la muestra (número de caso y tipo de muestra). El conjunto de muestras de un caso debe ir en una bolsa con precinto numerado tipo brida.

• Todos los contenedores deben estar herméticamente cerrados, tanto si son bolsas o envases de plástico. En el caso de muestras en las que se deban determinar niveles traza de compuestos orgánicos tipo PCBs o plaguicidas podría usarse papel de aluminio para envolver la muestra antes de su introducción en el envase.

• No utilizar conservantes, salvo indicación expresa del laboratorio. En caso de adición de algún conservante deberá incluirse en el informe la información pertinente y enviarse al laboratorio una muestra de este. Este deberá enviarse en un recipiente independiente y debidamente etiquetado de manera que no exista posibilidad de confusión con una muestra.

• En caso de compuestos volátiles, tales como amonio o intoxicación por cianuro, se debe congelar inmediatamente el contenido ruminal, sangre y suero tras la toma, con el fin de evitar su pérdida por volatilización.

• Todos los envases con cada muestra tomada del caso deben introducirse en un embalaje, el cual será precintado de manera que se garantice que cualquier intento de manipulación de las muestras en el contenidas deje rastros inequívocos de tal acción. Este embalaje deberá etiquetarse de la misma forma que los envases contenidos y siempre haciendo referencia al caso y a las actas que lo acompañan.

• El envío al laboratorio de las muestras se hará en cajas de porexpán o similar, convenientemente adecuado con suficientes acumuladores de frío como para garantizar que la muestra no llega totalmente descongelada. Además, habrá de asegurarse el interior con

material de embalaje que impida el golpeo y la apertura de los recipientes durante el transporte. Es recomendable hacer los envíos a principio de semana para evitar que las muestras se queden sin congelar durante el fin de semana.

• También es recomendable incluir muestras fijadas para histopatología con el fin de confirmar el diagnóstico en caso de duda.

2.3. Información adjunta a las muestras.

Junto con las muestras se enviará un sobre con los informes de cada caso en los que se detallen:

• Especie.

• Número de animales del caso.

• Nº de caso del centro de recuperación (única referencia).

• Fecha del hallazgo/muerte.

• Localidad: municipio y provincia.

• Hallazgos de campo: hábitat, tipo de cultivos, ganadería, cotos de caza, torres eléctricas, muerte de otros animales, cronología de aparición de casos, datos del radioseguimiento, tratamientos con fitosanitarios o zoosanitarios ...

• Hallazgos de necropsia. Es recomendable adjuntar el informe de necropsia.

• Fotografías en soporte papel o digital.

Tabla 1. Se señalan las muestras sobre las que se puede determinar el tóxico o alguno de los efectos del tóxico. No obstante, para llevar a cabo un diagnóstico diferencial en caso de no haber una sospecha clara y descartar/identificar los diferentes tóxicos puede ser necesario tomar todas las muestras posibles.

	Prioritarias o generales			**Adicionales o específicas**						
	Contenido gástrico	**Hígado**	**Encéfalo**	Cebo, vómito	Sangre	Plasma	Riñón	Grasa	Hueso	Pelo, plumas, uñas
Organofosforados y carbamatos	**C**	**C**	**C**	C	C	C				
Organoclorados	**C**	**C**	**C**	C	C	C		C		
Estricnina	**C**	**C**								
Rodenticidas anticoagulantes		**C**			R					
Herbicidas dipiridilos	**C**	**C**					C			
Alfacloralosa	**C**	**C**								
Metaldéhido	**C**									
Plomo	**C**	**C**			C		C		C	
Otros plaguicidas	**C**	**C**								
Mercurio		**C**	**C**		C		C			C
Arsénico	**C**	**C**			C		C			C
Cadmio		**C**			C		C			
Otros metales y metaloides		**C**								
Nitratos	**C**				C+					
Cianuro	**C**									

C: congelado a -20ºC, C+: congelado en nitrógeno líquido, R: refrigerado y analizado en pocas horas.

• Tipo de muestras remitidas. Para las diferentes muestras de un mismo caso recomendamos asignar diferentes letras. Por ejemplo, si han muerto dos buitres y se ha tomado muestra de contenido gástrico e hígado le asignaremos el número CRFS001A/07 al contenido gástrico y CRFS001B/07 al hígado del primer buitre, CRFS001C/07 y CRFS001D/07 a las muestras del segundo.

• Sospechas de potenciales compuestos o productos tóxicos que pudieran estar implicados, basándose en cualquier información disponible: casos anteriores, costumbres de la zona, rumores, etc. En todo caso, es conveniente citar el grado de certeza que se tiene de la sospecha.

• Fecha de envío.

• Responsable del envío (escribir nombre entero, D.N.I., firma y sello del centro).

• Persona que efectúa el transporte. En caso de ser una empresa indicación de la empresa y forma de contacto (teléfono, fax o correo electrónico).

• Fecha de recepción.

• Responsable de la recepción (escribir nombre entero, D.N.I., firma y sello del centro).

De este informe se devolverá una copia firmada y sellada al remitente. Se anotarán las incidencias relacionadas con el envío y el muestreo (errores de codificación, mala conservación ...). Se adjunta un formulario (Anexo I) que puede ser utilizado como referencia.

3. ANÁLISIS TOXICOLÓGICOS.

El protocolo en este apartado puede depender del equipamiento y características del laboratorio toxicológico que lleve a cabo los análisis. El que aquí se detalla es una recopilación y unificación de los procedimientos empleados en los tres laboratorios que firman este protocolo.

El laboratorio debe disponer de un libro de registro de entrada de casos donde se anotarán obligatoriamente, al menos, los siguientes datos: fecha de entrada del caso, remitente de la muestra, referencia del caso del remitente, referencia del caso en el laboratorio, muestras remitidas y estado, consideraciones u observaciones sobre el precintado (si es preciso).

3.1. Uso de biomarcadores.

Teniendo en cuenta la historia clínica y los hallazgos de la necropsia, se puede realizar la determinación de la actividad de la enzima acetilcolinesterasa (AChE) cerebral mediante el método de Ellman (Hill y Fleming, 1982 [Hill, E. F. and Fleming, W. J. (1982), Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. Environmental Toxicology and Chemistry, 1: 27-38]). Este biomarcador puede ser de utilidad en muchos de los casos de envenenamiento por anticolinesterásicos, que son la mayoría en la actualidad. Los valores obtenidos se pueden comparar con los obtenidos mediante la reactivación in vitro del enzima mediante dilución de la muestra y con la adición de 2-PAM. La utilidad de esta determinación en cerebro ha sido comentada en el apartado anterior. Este ensayo puede ser llevado a cabo al inicio del estudio de cada caso con el fin de orientar el análisis, o a posteriori para confirmar la muerte por exposición a un anticolinesterásico (inhibición >50%) complementando los demás análisis de laboratorio para el diagnóstico final.

3.2. Detección del tóxico.

En primer lugar, en el caso de los cebos y de los contenidos gástricos, se realiza un examen visual de la muestra para detectar la presencia de formulados granulados u otro signo de presencia de fitosanitarios. En la Figura 2 se muestra de forma esquemática el protocolo seguido en diversos laboratorios toxicológicos en España. Mediante un método general de extracción, purificación y determinación se pueden identificar la mayoría de los tóxicos, pero en el caso de que existan evidencias de tóxicos concretos, o cuando se han descartado diversas posibilidades, puede ser necesario llevar a cabo métodos analíticos más concretos. Estos protocolos de análisis están basados en métodos publicados por los laboratorios de toxicología que han preparado este texto, y de hecho están basados en gran medida en el procedimiento utilizado por la Wildlife Incident Investigation Scheme del Central Science Laboratory de Reino Unido (Brown et al., 2005 [Brown, Peter M.; Turnbull, Gordon; Charman, Sheonaidh; Charlton, Andrew J.A.; Jones, Ainsley. 2005. Analytical Methods Used in

the United Kingdom Wildlife Incident Investigation Scheme for the Detection of Animal Poisoning by Pesticides. Journal of AOAC International, 88: 204-220(17)]). Dependiendo de las disponibilidades técnicas de los laboratorios, existen diferentes alternativas a los métodos aquí descritos que pueden ser también aceptables. De forma genérica, y para garantizar la correcta identificación del tóxico, los compuestos orgánicos deben ser analizados mediante detector de masas acoplado a cromatografía de gases o líquidos. Cuando esto no pueda ser llevado a cabo por no disponer de esta técnica analítica se procurará confirmar el resultado, si es posible, mediante dos técnicas analíticas distintas (Ej.: colorimetría y cromatografía en capa fina para estricnina).

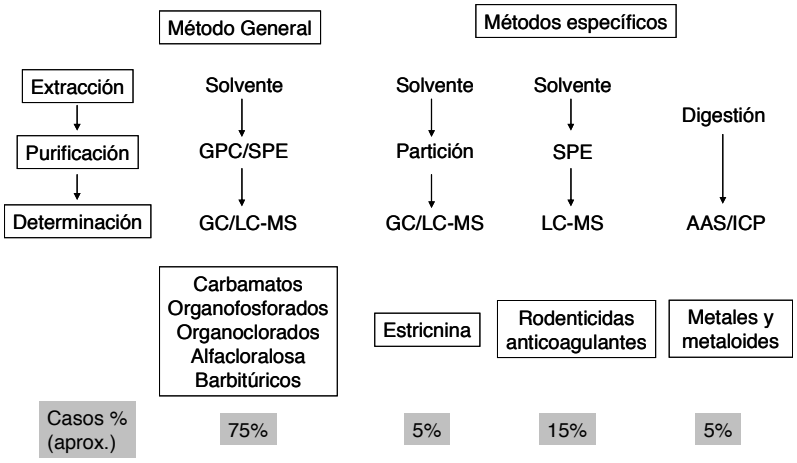


Figura 2. Esquema del procedimiento analítico llevado a cabo en diversos laboratorios toxicológicos.

• Determinación de diferentes tipos de sustancias neurotóxicas (organofosforados, carbamatos, organoclorados, alfacloralosa, barbitúricos): a partir de los cebos o contenidos gástricos se realiza una extracción con diclorometano que, dependiendo del tipo de muestra, se puede analizar sin más purificación tras evaporar mediante rotavapor o flujo de nitrógeno y resuspender en 0,5 ml de acetato de etilo. En el caso del hígado u otro tejido parenquimatoso se homogeniza el tejido con sulfato sódico anhidro y se extrae de la misma forma que el contenido gástrico con diclorometano. En los extractos obtenidos que requieran una purificación previa, esta se puede llevar a cabo mediante cromatografía de permeación en gel a través de una fase de Bio-Beads S-X3 (Bio- Rad) con acetato de etilo y ciclohexano (1:1) como fase móvil. De forma alternativa, y si se tiene algún indicio del tipo de tóxico que se busca, es posible utilizar columnas preparativas de extracción en fase sólida (SPE). En algunos casos, el extracto obtenido mediante la GPC también debe ser purificado adicionalmente mediante técnicas alternativas (Ej.: SPE, Quechers, etc.) antes de llevar a cabo su análisis. Los extractos purificados de cada muestra se pueden analizar mediante cromatografías de líquidos y gases, pero siempre deberían confirmarse los resultados mediante espectrometría de masas, tanto por comparación de espectros de patrones o con las bases de datos comerciales. Algunas técnicas menos específicas y sensibles, como cromatografía en capa fina pueden ser de utilidad para hacer un primer estudio de las muestras, en especial para el análisis rápido de microgránulos y muestras con alta concentración de tóxico.

• **Determinación de estricnina:** aunque puede ser detectable con el mismo procedimiento descrito en el apartado anterior, es recomendable llevar a cabo una extracción más específica con diclorometano y con una posterior purificación mediante extracción líquido-líquido. Los extractos obtenidos se analizarían como en el apartado anterior.

• **Rodenticidas anticoagulantes:** el hígado o el cebo se homogenizan con sulfato sódico anhidro, se extrae con diclorometano u otras mezclas de solventes, se realiza una purificación mediante SPE (puede variar en función del tipo rodenticidas a analizar, indandiona o cumarina) y finalmente se analiza por cromatografía de líquidos acoplada a espectrometría de masas.

• **Metales y metaloides:** se realiza una digestión de la muestra liofilizada de cebo o hígado con ácido nítrico y peróxido de hidrógeno (u otras mezclas de ácidos) en microondas o en tubos de vidrio (o cuarzo) abiertos y se analiza por técnicas específicas (espectrometría de absorción atómica, ICP, voltamperometría).

4. INTERPRETACIÓN DE LOS RESULTADOS ANALÍTICOS Y ELABORACIÓN DEL INFORME TOXICOLÓGICO.

A la hora de elaborar el informe toxicológico deberá incluirse, al menos, la siguiente información, por este orden:

4.1. Datos del caso.

El informe debe ser completo, incluyendo la información referente al caso suministrada por el veterinario del centro de recuperación en su informe de necropsia, en especial, las especies, tipo de muestras y referencias del caso (nº de precintos, nº de actas o nº de caso en el centro de recuperación). Se deberán incluir también referencias a la existencia de documentación adicional (informes de necropsia, informes de otros profesionales, documentos gráficos, etc.) que haya sido enviada al laboratorio junto con las muestras y que se haya utilizado para llevar a cabo la sistemática de análisis e interpretación de resultados.

4.2. Métodos analíticos.

Se detallarán los métodos analíticos utilizados, así como las muestras que han sido analizadas. En algunos casos, no es necesario analizar todas las muestras remitidas, por lo que deberemos justificar, con el debido rigor científico, la selección de muestras a analizar. También se detallará información relativa a la validación de los métodos empleados y el control de calidad aplicado (blancos, patrones utilizados, tasa de recuperación, límites de detección ...).

4.3. Resultados.

En este apartado se aportará la información del tipo de tóxico detectado y la concentración encontrada, en caso de ser necesaria para la elaboración de las conclusiones definitivas. Se indicarán también los tóxicos que han podido ser descartados a partir de las metodologías analíticas utilizadas.

A continuación se incluirá información contrastada con el mayor rigor científico posible (incluir referencias) sobre el o los tóxicos detectados, recomendándose que al menos se suministren los siguientes datos:

• **Dosis letal media** en especies similares a la estudiada para poder **valorar el riesgo** de tratarse de una intoxicación letal en base a la información sobre peso de contenido gástrico, cebo o nº de microgránulos.

• **Aplicaciones comerciales** de esos productos (agroquímicos, zoosanitarios ...) para que los agentes que estudian el caso puedan tener más indicios de los posibles autores del envenenamiento.

• **Mecanismo de acción y sintomatología** asociada, para que el veterinario del centro de recuperación pueda realizar el informe definitivo.

• **Interpretación** final valorando que el animal haya podido resultar letalmente intoxicado o que los cebos puedan ser letales para los animales que los ingieran. También se aportará información sobre la posibilidad de que se pueda tratar de una intoxicación secundaria o que se trate de una intoxicación crónica o aguda. Es fundamental que se haga una correcta interpretación conjunta de todos los datos del caso (clínicos, analíticos, etc.) en apoyo del diagnóstico final. Como ejemplo se presenta un informe toxicológico en el Anexo II.

• **Información complementaria**: cromatogramas y espectros de masas que permitan la valoración de los resultados.

5. INFORME DEFINITIVO DEL VETERINARIO DEL CENTRO DE RECUPERACIÓN.

Una vez el veterinario recibe el informe toxicológico, deberá redactar el **informe pericial definitivo** del caso, en el que en base a los resultados analíticos (toxicológicos u otros como histopatología, microbiología ...) valorará que el animal haya sido intoxicado o que los cebos sean capaces de causar una intoxicación en fauna protegida. Además, en base a la información recogida por los agentes, el veterinario y con los resultados analíticos hay que determinar si se trata de una intoxicación intencionada, accidental, secundaria, etc. También se incluirá en informe la posibilidad de que en la zona en que han aparecido los cebos o los animales muertos puedan resultar intoxicadas especies protegidas en peligro. Se adjunta en el Anexo III un ejemplo de este tipo de informe final.

ANEXO I: SOLICITUD DE ANÁLISIS TOXICOLÓGICO

Dirección del Laboratorio que analizará las muestras
Telf. xxxxxxxxxxx, Fax xxxxxxxxxxxxx, xxxxxxxxxxx@xxxxxxx

Es importante incluir el contacto del laboratorio en el impreso suministrado

Nº de caso del centro de recuperación: Especies: Número de animales del caso: Fecha del hallazgo/muerte: Municipio: Provincia:
Hallazgos de campo:
Hallazgos de necropsia: Sospechas de tóxicos:
Tipo de muestras remitidas y referencias: - - - - Nº de precinto:
Correo-e/Telf. móvil del Agente: Correo-e/Telf. móvil del Veterinario del CR: Fecha de envío: Responsable: Transporte efectuado por: Fecha de recepción: Responsable: Incidencias:

Es importante incluir el contacto del personal implicado en el caso

ANEXO II: INFORME TOXICOLÓGICO

Logos del Laboratorio

Sr. XXXXXX
Organismo XXXX
C/XXXXX
00000 XXXXXXX

INFORME TOXICOLÓGICO. Referencia del Caso en el laboratorio, ref. CR: XXXX

Solicitante: XXXXXX.
Entrada en el laboratorio: DD-MM-AAAA.
Historia clínica: Sospecha de la colocación de cebos envenenados.
Muestras recibidas: Recibimos muestra A (cáscaras de huevo NXXXX) y B (líquido negro NXXXX) con precinto azul nº: XXXXX.
Analíticas solicitadas: Determinación de carbamatos, cianuro, estricnina y compuestos organofosforados.
Métodos analíticos:

Se realiza una extracción con disolventes de las muestras A y B, por separado, tras una homogenización con sulfato sódico anhidro, en una parte del extracto se realiza una purificación selectiva para alcaloides, en la otra una purificación por cromatografía de permeación en gel para plaguicidas, seguido en ambos casos por cromatografía de gases acoplada a espectrometría de masas (Brown *et al.*, 2005[2]).

Resultados:

Tóxico detectado: **Dimetoato** en la muestra A y **dimetoato, diazinón (dimpylate)** y **clorpirifós** en la muestra B.

Concentración: **0,15 µg/g** de dimetoato en la muestra A, **88,66 ng/µl** de dimetoato, **4,04 ng/µl** de diazinón y **1,69 ng/µl** de clorpirifos en la muestra B.

Dosis letal media oral aguda en peso vivo: 60 mg/kg en rata y 42 mg/kg en pato para dimetoato, 66 mg/kg en rata y 3,5 mg/kg en pato para el diazinón y 82mg/kg en rata y 76 mg/kg en pato para clorpirifós (Toxnet).

Formulados:

a) Dimetoato: se comercializa en España como insecticida de aplicación foliar en polvo, líquido o concentrado emulsionable con riquezas entre 3 y 50 % y acompañado de clorpirifós.
b) Diazinón: en la actualidad en España se comercializa como biocida de uso no agrícola en formulados antiparasitarios líquidos para pulverizar (Zooveca) y en collares (Prevender), aunque se comercializó hasta el 2007 (Directiva 2007/25/CE (LCEur 2007,664)) como insecticida agrícola de aplicación foliar en polvo, líquido o granulado con riquezas entre 2,5 al 60 %.
c) Clorpirifós: se comercializa en España como insecticida de aplicación foliar en polvo, líquido o granulados con riquezas entre 1 y 75 % y acompañado de cipermetrín, fosmet y dimetoato.

Interpretación: el dimetoato, diazinón y clorpirifós son plaguicidas organofosforados de baja persistencia e inhibidores de las colinesterasas. Estos enzimas están implicados en la correcta transmisión de los impulsos nerviosos y su inhibición provoca una alteración del sistema nervioso que conduce a la muerte por parada respiratoria. Este mecanismo de acción hace que estos compuestos sean neurotóxicos de acción muy rápida capaces de provocar la muerte habitualmente en 10 ó 30 minutos con dosis superiores a la LD50 y entre 30 minutos y seis horas a dosis más bajas, aunque puede ampliarse hasta 12 ó 24 horas para algunos inhibidores colinesterásicos latentes (Hill, 2004[3]).

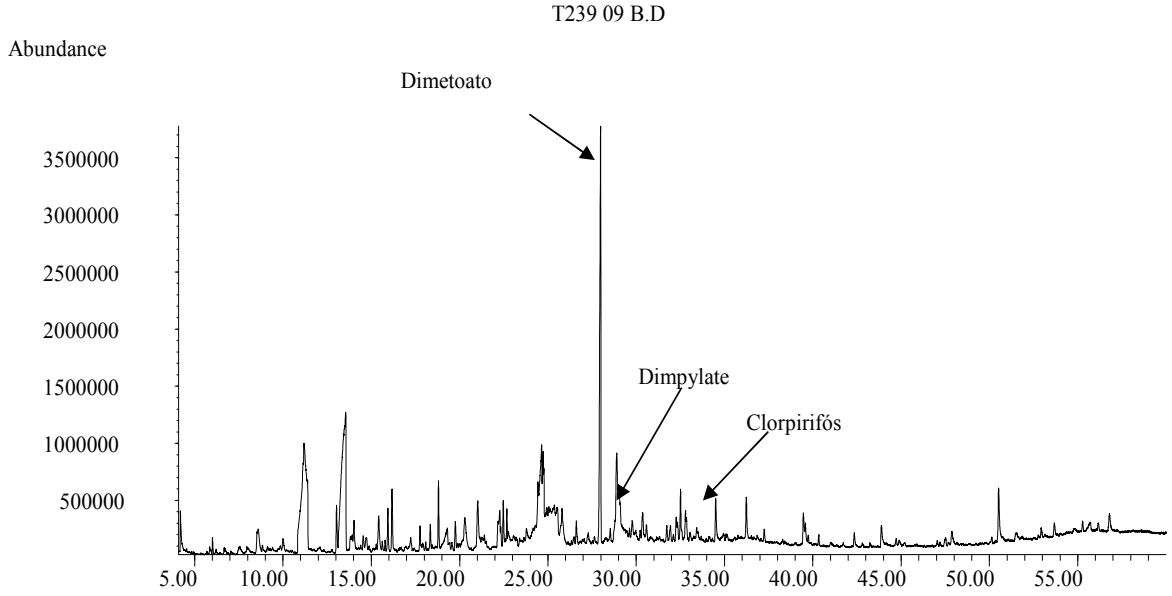
La exposición a organofosforados en aves y mamíferos provoca parálisis de musculatura que condicionan la supervivencia de los individuos y reduce su capacidad de desplazamiento dependiendo de la dosis y el tóxico al que ha sido expuesto (Hill, 2004[3]).

En base a la elevada toxicidad de los compuestos y las concentraciones detectadas en las muestras, podemos confirmar la intencionalidad del uso de los cebos analizados para envenenar animales.

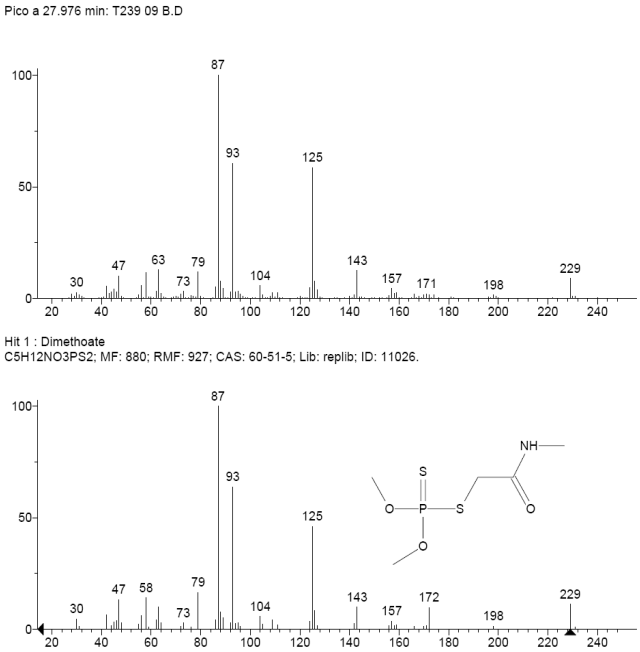
Cromatograma:

3 Hill, E. F. 2004. Wildlife Toxicology of Organophosphorus and Carbamate Pesticides. En *Handbook of Ecotoxicology*. Editores: Hoffman, D. J., Rattner, B. A., Burton, Jr., G. A., Cairns, Jr. J. (ed. Lewis Publishers). pp.: 281-312.

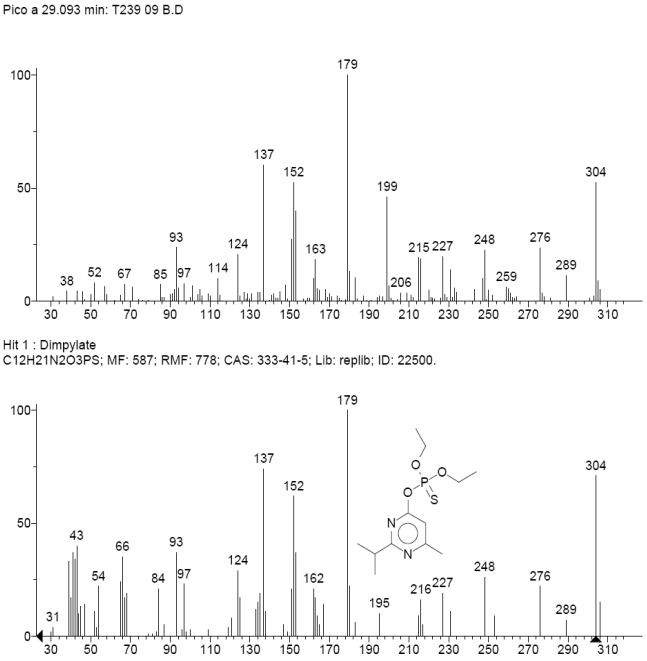
Cromatograma obtenido tras análisis por cromatografía de gases acoplada a espectrometría de masas:



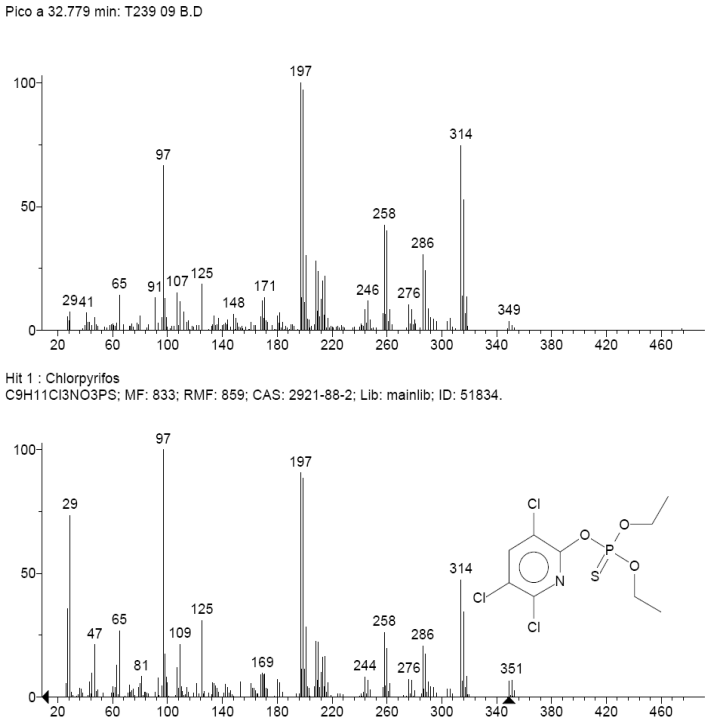
Espectro de las masas presentes en el pico situado a 27,976 minutos e identificación del pico mediante comparación con los espectros de masas recogidos en la espectroteca NIST:



Espectro de las masas presentes en el pico situado a 29,093 minutos e identificación del pico mediante comparación con los espectros de masas recogidos en la espectroteca NIST:



Espectro de las masas presentes en el pico situado a 32.779 minutos e identificación del pico mediante comparación con los espectros de masas recogidos en la espectroteca NIST:



Custodia de las muestras recibidas:

Las muestras serán conservadas a -20 ºC durante tres meses después de emitir este informe, tras lo cual serán destruidas si no se nos comunica el interés por conservarlas durante más tiempo.

Fdo.:
Lic. En

Vº Bº:
Jefe del Laboratorio de Toxicología

En ……, a X de xxx de 20xx

ANEXO III: EJEMPLO DE INFORME PERICIAL FINAL

INFORME DEFINITIVO REFERENTE AL EXÁMEN FORENSE DE DOS AZORES (*Accipiter gentilis*) Y UN SUPUESTO CEBO CON NÚMERO DE REFERENCIA N___/___

Datos de las muestras.

Cadaver A	
Especie:	Azor (*Accipiter gentilis)*
Sexo:	Hembra.
Edad:	Adulto.
Nº y color de precinto:	005013 Verde.
Cadaver B	
Especie:	Azor (*Accipiter gentilis)*
Sexo:	Macho.
Edad:	Juvenil (2 años).
Nº y color de precinto:	005014 Verde.
Muestra A:	Restos oseos, con escaso tejido muscular adherido, de un cadáver incompleto de ave.
Nº y color de precinto:	005015 Verde.
Procedencia:	Paraje……....., término municipal de……..……., isla de……., provincia de……
Remitente:	SEPRONA, AA.MM
Fecha de recepción:	

Resultados de los análisis efectuados

Cadáveres A y B

Examen externo.

Se trata de los cadáveres, de dos ejemplares de Azor (*Accipiter gentilis)* (cadáveres A y B) (Imágenes 1, 2, 3 y 4). Uno de ellos, es un ejemplar adulto, hembra, de 1.064 gr. de peso y el otro es un ejemplar juvenil, macho, de 655 gr. de peso. Ambos presentan un estado de nutrición y musculación óptimo para la especie. Los cadáveres presentan un grado moderado de descomposición (2-5 días de antigüedad aproximadamente). A la palpación no se detecta contenido en el buche.

Imagen 1: Vista ventral del cadáver A, de Azor.

Imagen 2: Vista dorsal del cadáver A, de Azor.

Imagen 3: Vista dorsal de cadáver B, de Azor

.
Imagen 4: Vista ventral del cadáver B, de Azor.

En el caso del cadáver A, el plumaje y demás faneras se encuentran en buen estado. Y en el caso del cadáver B, se observa la rotura de la décima y la séptima plumas primarias del ala derecha, la tercera y la cuarta plumas secundarias del ala izquierda y las dos plumas rectrices centrales.

No se detecta la presencia de heridas o fracturas que hagan sospechar de un traumatismo, en ninguno de los dos casos.

Estudio radiológico.

Se realiza una radiografía de los dos cadáveres de azor. En la radiografía del cadáver B de Azor, se observan tres proyectiles, uno en la extremidad inferior derecha, otro en la izquierda y otro en el ala izquierda (Imagen 5).

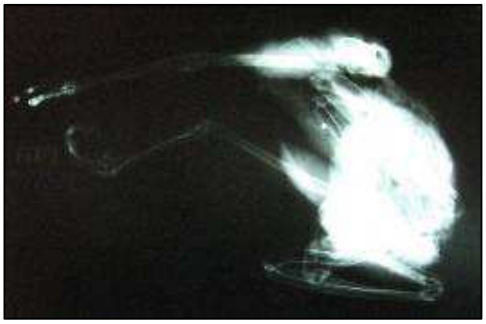


Imagen 5: Radiografía Cadáver B, de Azor.

Examen interno.

Durante el examen interno de las aves, se observa que la grasa abdominal y subcutánea es la normal para la especie.

En ambos casos, se aprecia una marcada congestión hepática y renal. Además, el hígado aparece aumentado de tamaño y con disminuida de consistencia (Imágenes 10 y 11). El digestivo superior, de ambos ejemplares, presenta contenido en su interior. El contenido del digestivo superior, del cadáver A, pesa 18 g, y el del cadáver B, pesa 2.5 g.
En el cadáver A de Azor, el contenido del proventrículo y del ventrículo, se describe como tejido blando de color rosa, compatible con tejido muscular, mezclado con fragmentos óseos y plumas blancas de pequeño tamaño. Entre el contenido del ventrículo, del cadáver A, se identifica la extremidad inferior derecha de un ave, aparentemente de paloma (Imágenes 6 y 7).

En el caso del cadáver B, en el interior del digestivo superior se observa la presencia de 1 ml, aproximadamente, de un líquido marrón claro (Imágenes 8 y 9).

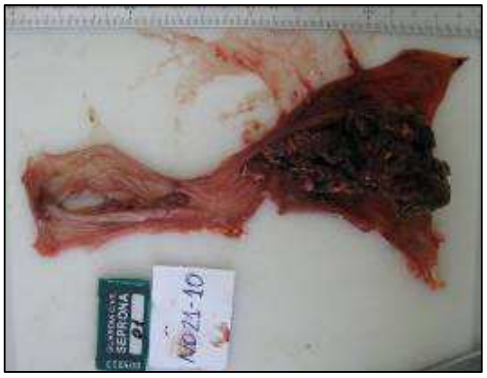


Imagen 6: Contenido del digestivo superior del cadáver A, de Azor.

Imagen 7: Detalle del contenido del digestivo superior del cadáver A, de Azor.

Imagen 8: Contenido del digestivo superior del cadáver B, de Azor.

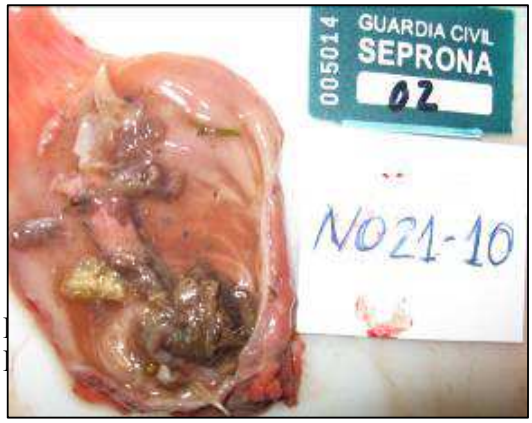


Imagen 9: Detalle del contenido del digestivo superior del cadáver B, de Azor.

En el contenido, de ambos ejemplares, se observa la presencia de granulado irregular, de 1 mm de diámetro aproximadamente, y de color grisáceo.

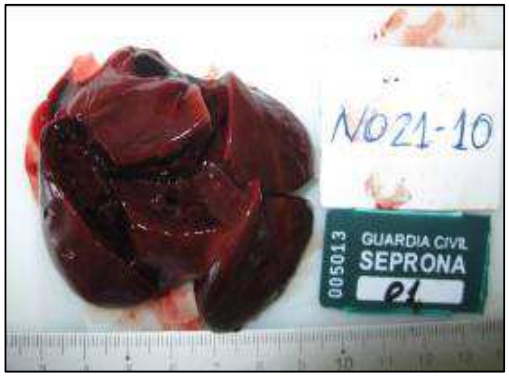


Imagen 10: Hígado congestivo del cadáver A, de Azor.

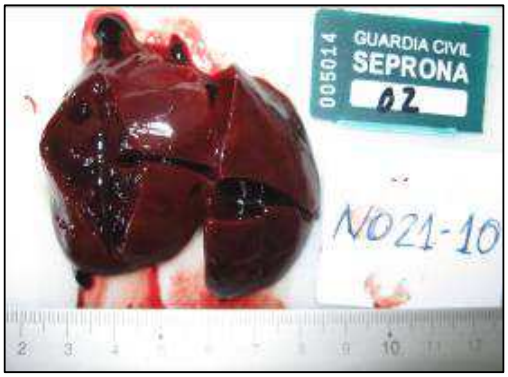


Imagen 11: Hígado congestivo del cadáver B, de Azor.

Durante el examen interno del cadáver B de Azor, se extraen dos proyectiles de plomo, de 2mm de diámetro aproximadamente, de los tres observados en el estudio radiográfico. Estos se localizan en la parte caudal de la de la epífisis proximal del tibiotarso derecho y en la cara caudal del fémur izquierdo, entre la musculatura de la región. Ambos proyectiles están rodeados de tejido fibroso y cicatricial, y no presentan signos de hemorragia o inflamación aguda, alrededor de los mismos.

Muestra A

Examen de la muestra.

La muestra remitida consiste en el cadáver incompleto de un ave, aparentemente de una paloma. El cadáver está constituido por algunos huesos, escaso tejido muscular deshidratado y adherido a dichas estructuras óseas, el corazón y restos de tejido blando de la cavidad celómica.

El cadáver únicamente conserva las tres últimas vértebras cervicales, ambos húmeros, ambos coracoides, las vértebras torácicas, parte del sinsacro, el fémur derecho, seis costillas incompletas del lado izquierdo y la mitad craneal de la quilla y el esternón. En el interior de la cavidad celómica incompleta, se observa el corazón y tejido blando de color rojo oscuro y consistencia friable, compatible con parte del hígado. Adherido a la superficie de los tejidos y musculatura deshidratada, de la superficie visceral, de la cavidad celómica, se observa un granulado sospechoso, irregular, de 1 mm de diámetro aproximadamente, y de color grisáceo azulado.

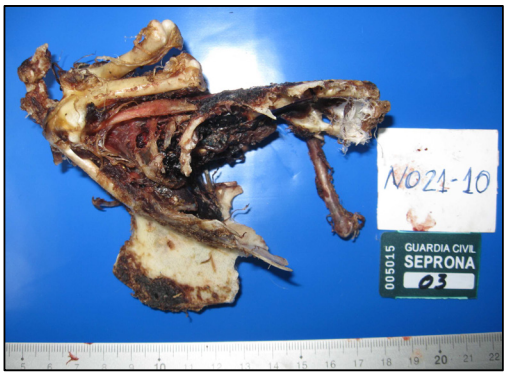


Imagen 12: Muestra A.

Imagen 13: Granulado sospechoso de la muestra A.

Análisis químico-toxicológico

Se remiten las siguientes muestras para su análisis toxicológico:

N021/10 A – Granulado sospechoso, extraído del contenido del digestivo superior, del cadáver B de Azor.
N021/10 B – Líquido extraído del digestivo superior, del cadáver B de Azor.
N021/10 C – Contenido del digestivo superior (2.5 g), del cadáver B de Azor.
N021/10 D – Hígado del cadáver B, de Azor.
N021/10 E – Granulado sospechoso, extraído del contenido del digestivo superior, del cadáver A de Azor.
N021/10 F – Contenido del digestivo superior (18 g), del cadáver A de Azor.
N021/10 G – Hígado del cadáver A de Azor.
N021/10 H – Granulado sospechoso, extraído de la muestra A.
N021/10 I – Corazón, tejido blando compatible con hígado, hueso y tejido muscular deshidratado de la muestra A.

Tóxico detectado: **Carbofurano**, **terbufós** y **fenamifós**.

Concentración:

a) Muestra A (N021/10 A): 37,60 µg/g de carbofurano y 136,5 µg/g de terbufós.
b) Muestra C (N021/10 C): 20,74 µg/g de carbofurano, 61,56 µg/g de terbufós y 9,98 µg/g de fenamifós.

c) Muestra E (N021/10 E): 4,15 mg/g de carbofurano, 379,16 µg/g de terbufós y 21,26 µg/g de fenamifós.
d) Muestra F (N021/10 F): 18,83 µg/g de carbofurano, 23,13 µg/g de terbufós y 14,73 µg/g de fenamifós.
e) Muestra H (N021/10 H): 4,48 mg/g de carbofurano, 750,52 µg/g de terbufós y 58,35 µg/g de fenamifós.
f) Muestra I (N021/10 I): 1,37 mg/g de carbofurano, 802,13 µg/g de terbufós y 529,49 µg/g de fenamifós.

Dosis letal media oral aguda: 5-13 mg/kg en rata y 0,48-0,51 mg/kg en pato para el carbofurano, 1,6 mg/kg en rata y 15mg/kg en codorniz para el terbufós y 8 mg/kg en rata y 1,68 mg/kg en pato para el fenamifós (Toxnet).

Formulados:

a) Carbofurano: en la actualidad en España no se comercializa como fitosanitario o biocida pero se comercializó hasta el año 2007 (LCEur\2007\1034) como concentrado insecticida de aplicación al suelo en microgránulos con una riqueza de principio activo del 5% y como suspensión concentrada de riqueza 20 %.
b) Terbufós: en la actualidad en España no se comercializa como fitosanitario o biocida pero se comercializó como insecticida de aplicación al suelo en microgránulos con una riqueza de principio activo entre 2 y 5%.
c) Fenamifós: en España se comercializa como insecticida de aplicación al suelo en forma de microcápsulas y concentrado emulsionable con riqueza de principio activo de 24% y 40 % respectivamente.

Mecanismo de acción: el carbofurano es un plaguicida carbamato y el terbufós y el fenamifós son compuestos organofosforados. Ambas familias de insecticidas son de baja persistencia e inhibidor de las colinesterasas. Estos enzimas están implicados en la correcta transmisión de los impulsos nerviosos y su inhibición provoca una alteración del sistema nervioso que conduce a la muerte por parada respiratoria. Este mecanismo de acción hace que estos compuestos sean neurotóxicos de acción muy rápida capaces de provocar la muerte de un animal a los pocos minutos tras la exposición. La muerte por exposición a compuestos organofosforados se produce habitualmente en 10 ó 30 minutos con dosis superiores a la LD50 y entre 30 minutos y seis horas a dosis más bajas, aunque puede ampliarse hasta 12 ó 24 horas para algunos inhibidores colinesterásicos latentes y por exposición a carbamatos se produce a los 5-30 min. (Hill, 1995[4]). La exposición a

4 Hill, E. F. 1995. Organophosphorus and carbamate pesticides. In D. J. Hoffman, B. A. Rattner, G. A. Burton Jr., and J. Cairns Jr. (eds.), Handbook of ecotoxicology, pp. 243-273. Lewis Publishers, CRC Press, Inc., Boca Raton, Florida.

carbamatos y organofosforados en aves y mamíferos provoca parálisis de musculatura que condicionan la supervivencia de los individuos y reduce su capacidad de desplazamiento dependiendo de la dosis y el tóxico al que ha sido expuesto (Hill, 2003[5]).

Interpretación: en base a la elevada toxicidad de los compuestos y a las concentraciones detectadas, en los contenidos gástricos, podemos concluir que los azores han resultado intoxicados y la presencia de cebos con los mismos compuestos indica la intencionalidad del envenenamiento.

Conclusiones definitivas.

En ambos cadáveres de Azor, de la óptima condición corporal, del buen estado del plumaje, de los depósitos de grasa abdominal y de la presencia de alimento en el digestivo superior, se puede deducir que la muerte de los animales fue aguda, tras la ingesta de dicho alimento, ya que no se observa pérdida de condición corporal, hecho común en procesos crónicos.

En el cadáver A de azor, la ausencia de proyectiles, de fracturas y de otras lesiones, internas o externas, asociadas, permite descartar el disparo y el traumatismo como causas directas de la muerte.

En el cadáver B de Azor, la presencia de proyectiles en la radiografía, indica que el animal fue disparado. Pero durante el examen interno de dicho cadáver, los proyectiles aparecen rodeados por tejido cicatricial sano, de lo que se puede deducir que son lesiones antiguas y, por lo tanto, que el disparo no es la causa de la muerte.

El cuadro congestivo generalizado de alguno de los órganos internos de ambos cadáveres, es bastante inespecífico, pero es compatible con procesos tales como intoxicaciones con sustancias anticoagulantes (derivados cumarínicos) o con dosis altas de sustancias inhibidoras de la acetilcolinesterasa (carbamatos y compuestos organofosforados).

Los hallazgos de la necropsia, recogidos en el informe preliminar, junto con los resultados del análisis toxicológico y los datos recabados por los agentes judiciales, permiten concluir que la muerte de los dos Azores se produjo por una intoxicación aguda con **carbofurano**, **terbufós** y **fenamifós.**

La presencia de gran cantidad de granulado en la muestra A (cadáver incompleto de ave), indica la intencionalidad de vehicular dichas partículas en el cadáver. Los resultados de los análisis toxicológicos de dicha muestra A (N021/10 H y N021/10 I), confirman que las

5 Hill, E. F. 2003. Jess in Action: Rule-Based Systems in Java". Manning Publications.

partículas vehiculadas en el cadáver, contienen **carbofurano**, **terbufós** y **fenamifós**, que son los mismos compuestos detectados en el contenido del digestivo superior de ambos cadáveres de Azor.

Por todo lo anteriormente mencionado, se puede concluir que se trata de una intoxicación primaria, consecuencia directa del consumo de un cebo envenenado con la finalidad de realizar un control no selectivo e ilegal de depredadores.

Lugar……., a … de …. de ….

Fdo. Ldo……

Colegiado con número……. por el Colegio
Oficial de Veterinarios de……...