UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS

TESIS DOCTORAL

Selenio y mercurio en pescados y productos derivados. Efecto protector del selenio frente al mercurio

Selenium and mercury in fish and fish-based products. Protective effect of selenium against mercury

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

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Programa de Doctorado en Química Avanzada

SELENIO Y MERCURIO EN PESCADOS Y PRODUCTOS DERIVADOS. EFECTO PROTECTOR DEL SELENIO FRENTE AL MERCURIO.

SELENIUM AND MERCURY IN FISH AND FISH-BASED PRODUCTS. PROTECTIVE EFFECT OF SELENIUM AGAINST MERCURY.

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A mis fracasos, por empujarme
a ser mi mejor versión

Nada en este mundo debe
ser temido… solo entendido.
Ahora es el momento
de comprender más,
para que podamos
temer menos.

Marie Curie

ÍNDICE

I. RESUMEN

I. RESUMEN

Actualmente, el valor nutricional y la calidad de los alimentos son campos de gran repercusión social y elevado interés científico, en los que tanto las industrias como las agencias nacionales e internacionales de regulación juegan un papel fundamental a la hora de garantizar la seguridad del consumidor.

Uno de los elementos de mayor interés en cuanto a su determinación en alimentos es el selenio, debido a que es un micronutriente traza esencial para el ser humano y por su efecto protector frente al mercurio, siendo este uno de los metales que provoca una mayor preocupación en relación con la seguridad alimentaria. El selenio se incorpora a los organismos a través de la dieta, siendo el pescado una de las principales fuentes para el ser humano, donde además puede ejercer un efecto protector frente a la toxicidad del mercurio, presente en los pescados de forma natural. La toxicidad tanto del selenio como del mercurio depende de su forma química. El caso del selenio las formas orgánicas como los selenoaminoácidos (selenometionina, selenocisteína y selenometilselenocisteína, entre otros) son menos tóxicas que las formas inorgánicas (selenito y selenato). En el caso del mercurio, el $MeHg^+$ es la forma química más tóxica y la que se bioacumula y biomagnifica debido a su liposolubilidad, además de ser la forma de mercurio predominante en pescados.

Además, es relevante destacar el incremento en el consumo de alimentos derivados del pescado como sustitutos del pescado fresco o congelado. Estos van desde productos derivados, como las huevas, hasta los productos procesados elaborados a partir de músculo de pescado y con base en surimi. Dado que estos alimentos procesados se consumen cada vez con más frecuencia, también pueden llegar a representar una fuente significativa de selenio y mercurio en nuestra dieta, ya que ambos elementos están presentes tanto en estos productos como en las materias primas de las que se originan. Cabe destacar que, actualmente, apenas existen datos en la literatura científica sobre los niveles de selenio y mercurio y sus respectivas especies químicas en este tipo de productos.

Por todo ello, la presente Tesis Doctoral tiene como objetivo, por un lado, la determinación de mercurio y selenio y sus especies, así como su distribución en

biomoléculas (proteínas) en pescados y sus productos derivados y procesados mediante técnicas basadas en espectrometría de masas, lo que permitirá la correcta evaluación de la calidad y seguridad de estos productos en base a los elementos objeto de estudio; y por otro, la evaluación del efecto protector de distintas especies de selenio frente a la neurotoxicidad inducida por el mercurio empleando la línea celular de neuroblastoma humano (SH-SY5Y).

Para ello, la primera parte de la Tesis Doctoral se centra en el estudio de la distribución de selenio y mercurio y sus especies en músculo (parte comestible) de pescados de consumo habitual (atún, pez espada, salmón de piscifactoría y salmón rosado salvaje), productos derivados (huevas de lumpo, de trucha y de salmón rosado salvaje) y productos procesados elaborados a partir del pescado (palitos de cangrejo, gulas y tallarines elaborados a partir de bacalao o salmón), adquiridos en supermercados locales.

En base a los contenidos totales de selenio y mercurio, determinados mediante ICP-MS y DMA, respectivamente, se calcularon las relaciones molares Se:Hg y el índice *Selenium Health Benefit Value* o HBV_{Se}, indispensable para evaluar los riesgos-beneficios asociados a la exposición al mercurio por el consumo de este tipo de alimentos. En todos los casos, las relaciones molares Se:Hg fueron mayores que uno y los valores del parámetro HBV_{Se} positivos, por lo que se puede afirmar que, de manera general, el consumo tanto de los pescados como de los productos derivados y procesados no presenta, *a priori*, efectos negativos en la salud del consumidor asociados con la toxicidad del mercurio. Seguidamente, se llevaron a cabo estudios de especiación de selenio y de mercurio en las muestras de interés. En lo que respecta al selenio, para los pescados considerados, los análisis por HPLC-ICP-MS, y confirmados por HPLC-ESI-MS/MS, evidenciaron la presencia de SeMeSeCys en todos los hidrolizados enzimáticos de las proteínas sarcoplasmáticas, miofibrilares, álcali-solubles y solubles en Tris-HCl, y en el caso de las proteínas solubles en Tris-HCl del atún, también se identificó SeMet. En lo que respecta a los productos derivados y procesados, ambas especies de selenio (SeMeSeCys y SeMet) fueron identificadas en todas las muestras. Por otro lado, en relación con el mercurio, las especies $MeHg^+$ y Hg^{2+} fueron identificadas en las muestras de pescado y en los productos procesados, siendo el $MeHg^+$ la especie mayoritaria en todos los casos. Sin embargo, existen diferencias significativas entre las proporciones de $MeHg^+$ y Hg^{2+} de las muestras analizadas, siendo el atún y el pez espada los que presentan mayores porcentajes de $MeHg^+$.

Debido a que los procesos de elaboración de los productos procesados del pescado pueden afectar a la concentración y distribución de los elementos, se realizó un seguimiento de los contenidos totales de selenio y mercurio y especies de selenio en cada una de las etapas del proceso de producción (materias primas, productos intermedios y productos finales), considerando en concreto tres líneas de producción correspondientes a la elaboración de palitos de cangrejo, gulas y tallarines de salmón. Los resultados obtenidos mostraron que se mantiene la integridad de las especies SeMet y SeMeSeCys desde las materias primas hasta los productos finales comercializados. Además, se llevaron a cabo estudios de bioaccesibilidad de selenio, mercurio y especies de selenio presentes mediante la aplicación de un modelo *in vitro* de digestión gastrointestinal, obteniéndose porcentajes de 20-39 % para el selenio y 9-37 % para el mercurio, no observándose diferencias significativas entre las materias primas y los productos finales. En cuanto a las especies de selenio, aunque se ha comprobado que se mantiene la integridad de las mismas a lo largo de las cadenas de producción, las concentraciones de SeMet y SeMeSeCys en la fracción bioaccesible fueron ligeramente superiores en el caso de las materias primas que en los productos finales. Por otro lado, para completar estos estudios, las fracciones bioaccesibles procedentes de las digestiones gastrointestinales fueron expuestas a la línea celular de adenocarcinoma colorrectal humano (Caco-2). Mediante el ensayo MTT se pudo concluir que dichos extractos gastrointestinales no afectaron significativamente a la viabilidad celular.

Con el propósito de profundizar en la identificación de las especies de selenio presentes en pescados y productos derivados, se llevó a cabo la identificación simultánea de la selenoneína y su análogo sulfurado, ergotioneína, en los pescados, productos derivados y procesados objeto de estudio. La selenoneína tiene un elevado poder antioxidante y juega un papel fundamental en la detoxificación del $MeHg^+$, promoviendo su desmetilación previa formación de los compuestos insolubles estables de Hg-Se.

Para ello, se desarrolló un método de análisis por HPLC-ESI-MS/MS, previa optimización de las condiciones de extracción. Los resultados obtenidos evidenciaron la presencia de selenoneína en el músculo del atún y del pez espada y la presencia de ergotioneína en las muestras de pescado y las distintas variedades de huevas de pescado consideradas. Por lo tanto, parece que ambos compuestos antioxidantes pueden coexistir, al menos, en los peces depredadores. Sin embargo, no se detectaron ni selenoneína ni ergotioneína en productos procesados derivados del pescado, lo que revela que el proceso de elaboración podría afectar a la presencia de estos compuestos.

Para concluir, la segunda parte de la Tesis Doctoral se centró en el estudio del efecto protector del selenio frente a la neurotoxicidad inducida por el mercurio. Para ello, primeramente, se evaluó el efecto protector de distintas especies orgánicas de selenio (SeMet y SeMeSeCys) y de nanopartículas de selenio (SeNPs) frente a la citotoxicidad inducida por el $MeHg^+$ en la línea celular de neuroblastoma humano, SH-SY5Y, mediante ensayos MTT. En el caso de las SeNPs, se emplearon dos tipos, unas comerciales estabilizadas con citrato y otras sintetizadas en el laboratorio, recubiertas con quitosano. Las especies SeMet y SeMeSeCys exhibieron un efecto protector frente a la muerte celular inducida por el $MeHg^+$, especialmente a elevadas concentraciones del tóxico (LC_{50}), produciendo un aumento en la viabilidad celular cuando están presentes en el medio de cultivo. Además, las SeNPs recubiertas con quitosano mostraron una mayor protección comparadas con las citrato-SeNPs cuando se expusieron junto con el $MeHg^+$ en los cultivos celulares.

A continuación, mediante la aplicación de la técnica SC-ICP-MS se pudo determinar que, cuando el selenio (SeMet o SeMeSeCys) es añadido al medio de cultivo en coexposición con el $MeHg^+$, un mayor número de células acumulan menos cantidad de mercurio, comparado con las células expuestas solo a $MeHg^+$. De igual modo, también se evidenció que, en concreto, la coexposición de SeMet con $MeHg^+$ produjo una disminución de los niveles máximos del tóxico que pueden ser acumulados por las células.

Cabe destacar que las conclusiones derivadas de este estudio no hubieran sido posibles de obtener utilizando únicamente el ICP-MS en modo convencional, ya que este no tiene en cuenta la enorme variabilidad celular, lo que pone de manifiesto el gran potencial que tiene la técnica SC-ICP-MS en este tipo de estudios contribuyendo a una mejor comprensión del potencial papel neuroprotector del selenio frente a la toxicidad inducida por el $MeHg^+$.

II. SUMMARY

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Nowadays, food safety and quality are fields of great social impact and high scientific interest, in which both industries and international and national regulatory agencies play a key role in guaranteeing consumer safety.

One of the most interesting elements to determine in food is selenium as it is an essential trace micronutrient for humans and it also plays a protective role against mercury toxicity. On the other hand, mercury is one of the metals that causes greater concern in relation to food safety. Selenium is incorporated into organisms through the diet, being fish one of the main sources for humans, where it can also have a protective effect against mercury toxicity, which is also naturally present in fish. The toxicity of selenium and mercury is highly dependent on their chemical forms. In case of selenium, organic selenium species such as selenoamino acids (selenomethionine, selenocysteine and selenomethylselenocysteine, among others) are more toxic than inorganic ones (selenite and selenate). In relation to mercury, $MeHg^+$ is the most toxic chemical form as it is efficiently bioaccumulated and biomagnified through trophic chain due to its liposolubility, apart from being the predominant form of mercury in fish.

In addition, it is important to highlight the increasing trend in the consumption of fish-derived and processed food products as substitutes for fresh or frozen fish. Most of these products are made of surimi and they can also represent a significant source of selenium and mercury in our diet, since they are present in both the raw materials from which they are elaborated and in the finished products marketed. Nowadays, the data in the scientific literature concerning selenium and mercury levels and species content in this type of products is still scarce.

Therefore, the objective of this Doctoral Thesis is, on the one hand, the determination of selenium, mercury and their chemical species and the distribution of selenium and mercury bound to biomolecules (proteins) in fish and fish-based products by means of mass spectrometry-based techniques in order to correctly assess the safety and quality of these products in relation to selenium and mercury; and on the other hand, the evaluation of the protective effect of different selenium species against mercury-induced neurotoxicity using the human neuroblastoma cell line (SH-SY5Y).

For this purpose, the first part of the Doctoral Thesis focuses on the study of the distribution of selenium and mercury and their species in the muscle (edible part) of highly consumed fish (tuna, swordfish, farmed salmon and wild salmon), derived products (lumpfish roe, trout roe and wild salmon roe) and processed products made from fish (crab sticks, surimi-derived elvers and noodles made from cod or salmon), purchased in local supermarkets.

Based on the total selenium and mercury contents, determined by ICP-MS and DMA, respectively, Se:Hg molar ratio and Selenium Health Benefit Value or HBV_{Se} index were calculated, which is essential for correctly evaluating the risks-benefits associated with exposure to mercury from the consumption of this type of food. In all cases, the Se:Hg molar ratios were greater than one and the values of the HBV_{Se} parameter were positive, therefore it can be concluded that the consumption of both fish and fish-derived and processed products would not pose a risk for the consumer associated with mercury toxicity. Regarding selenium speciation studies in muscle of fish, results from HPLC-ICP-MS, and confirmed by HPLC-ESI-MS/MS, evidenced the presence of SeMeSeCys in all enzymatic hydrolysates of sacroplasmic, myofibrillar, alkali-soluble and Tris-HCl-soluble proteins, and in the case of Tris-HCl soluble proteins of tuna, SeMet was also identified. Concerning fish-derived and processed products, both selenium species (SeMeSeCys and SeMet) were identified in all samples. On the other hand, with respect to mercury, $MeHg^+$ and Hg^{2+} species were identified in fish samples and processed fish products, being $MeHg^+$ the major species found in all cases. However, there are significant differences between the proportions of $MeHg^+$ and Hg^{2+} in the samples analyzed, with tuna and swordfish having the highest percentages of $MeHg^+$.

Since the elaboration of processed fish products could affect the levels and distribution of selenium and mercury species, total selenium and mercury contents and chemical species were monitored at each of the steps involved in the production process (raw materials, intermediate products and final products). Three production lines corresponding to the preparation of crab sticks, surimi-derived elvers and salmon noodles were considered. The results obtained showed that the integrity of the SeMet and SeMeSeCys species is maintained from the raw materials to the final marketed products.

In addition, bioaccessibility studies of selenium, mercury and selenium species present were carried out by applying a gastrointestinal *in vitro* model, obtaining percentages of bioaccesibility of 20-39 % for selenium and 9-37 % for mercury, without significant differences between the raw materials and the finished products. Concerning selenium species, although it was found that their integrity is maintained throughout the production steps, the concentrations of SeMet and SeMeSeCys in the bioaccessible fraction were slightly higher in the case of raw materials than in the final products. Moreover, the bioaccessible fractions from the gastrointestinal digestions were exposed to the human colorectal adenocarcinoma cell line (Caco-2). By means of the MTT assay it was possible to conclude that these gastrointestinal extracts did not significantly affect cell viability.

In order to get a deeper insight in the presence of selenium species in fish and fish-based products, the simultaneous determination of selenoneine and its sulfur analog, ergothioneine, was carried out in the fish, fish-derived and processed products. Selenoneine has a high antioxidant activity and plays a fundamental role in the detoxification of $MeHg^+$, promoting its demethylation prior to the formation of stable insoluble Hg-Se compounds.

For this purpose, a method of analysis based on HPLC-ESI-MS/MS was developed, after optimizing the extraction conditions. The results obtained showed the presence of selenoneine in the muscle of tuna and swordfish and the presence of ergothioneine in all the muscle fish samples and the different fish roe varieties considered. Therefore, it appears that both antioxidant compounds may coexist, at least, in predatory fish. However, neither selenoneine nor ergothioneine was detected in processed fish products, revealing that the processing could affect the presence of these compounds.

To conclude, the second part of the Doctoral Thesis focuses on the study of the protective effect of selenium against mercury-induced toxicity in cells. For this purpose, first, the protective effect of different organic selenium species (SeMet and SeMeSeCys) and selenium nanoparticles (SeNPs) (commercial citrate-stabilized and synthesized chitosan-coated) against $MeHg^+$-induced cytotoxicity in the human neuroblastoma cell line, SH-SY5Y, was evaluated by MTT assays. SeMet and SeMeSeCys exhibited a protective effect against $MeHg^+$-induced cell death, especially at high concentrations of the toxicant (LC_{50}), producing an increase in cell viability when they are present in the culture medium. Furthermore, chitosan-coated SeNPs showed enhanced protection compared to citrate-SeNPs when exposed together with $MeHg^+$ in cell cultures.

The application of SC-ICP-MS technique allows us a deeper study of the protective effect of selenium against mercury. Results from the co-exposure of selenium (SeMet or SeMeSeCys) with $MeHg^+$, evidenced the existence of a greater number of cells accumulating less mercury, compared to cells exposed only to $MeHg^+$. Similarly, it was also observed that co-exposure of SeMet with $MeHg^+$ resulted in a decrease in the maximum levels of the toxicant that can be accumulated by the cells.

It should be noted that the conclusions derived from this study would not have been possible to obtain using only conventional ICP-MS, since this technique does not take into account the cellular variability, which highlights the great potential of the SC-ICP-MS technique in this type of studies, contributing to a better understanding of the potential neuroprotective role of the selenium species considered against $MeHg^+$-induced toxicity.

III. INTRODUCCIÓN

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1. Selenio y mercurio: esencialidad y toxicidad. Interacción selenio-mercurio

1.1. Relevancia biológica del selenio

Pocas veces el nombre de un elemento ha sido tan bien escogido, ya que, así como el cuerpo celeste que lleva su nombre (la palabra "selenio" deriva de *Selene*, que proviene de la palabra griega Σελήνη, cuyo significado es luna), el selenio nos muestra dos caras: una oscura (elemento tóxico) y la otra brillante (elemento traza esencial).

El selenio (Se) es un micronutriente esencial para los seres vivos ya que interviene en numerosos procesos biológicos formando parte de las selenoproteínas. Fue descubierto en 1817 por el químico sueco Jöns Jacob Berzelius como un elemento tóxico, detectado como un residuo rojizo generado en la pared de las cámaras de plomo utilizadas en la producción de ácido sulfúrico a partir de piritas de cobre (Hurd & Kipling, 1964; Tinggi, 2003). No fue hasta 1957 cuando se describen por primera vez los efectos beneficiosos que presentaba en los seres vivos, concretamente a la hora de prevenir la necrosis hepática causada por deficiencia de vitamina E en ratas (Schawrz & Foltz, 1957). A partir de este momento, el selenio y sus propiedades beneficiosas suscitaron un gran interés entre la comunidad científica. Posteriormente, en 1973, la enzima glutatión peroxidasa (GPx) se identificó como la primera selenoproteína. Esta proteína esta constituida por un tetrámero con cuatro átomos de selenio por molécula, que desempeña un papel fundamental en la protección de las células contra el daño oxidativo (Rotruck *et al.*, 1973).

La esencialidad o toxicidad del selenio, como la de cualquier elemento, depende en gran medida de la forma química en la que se encuentre, además de su concentración. En general, las especies inorgánicas de selenio (selenito, Se (IV), y selenato, Se (VI)) son más tóxicas que las especies orgánicas (selenometionina, SeMet, selenocisteína, SeCys, selenometilselenocisteína, SeMeSeCys, γ-glutamilselenometilselenocisteína, γ-Glu-Se-MeSeCys, y selenoneína, entre otras) y las especies volátiles (dimetilselenio, DMSe, y dimetildiselenio, DMDSe) (**Figura 1**), siendo el Se (VI) la especie de mayor toxicidad (Tian *et al.*, 2017; Vinceti *et al.,* 2018).

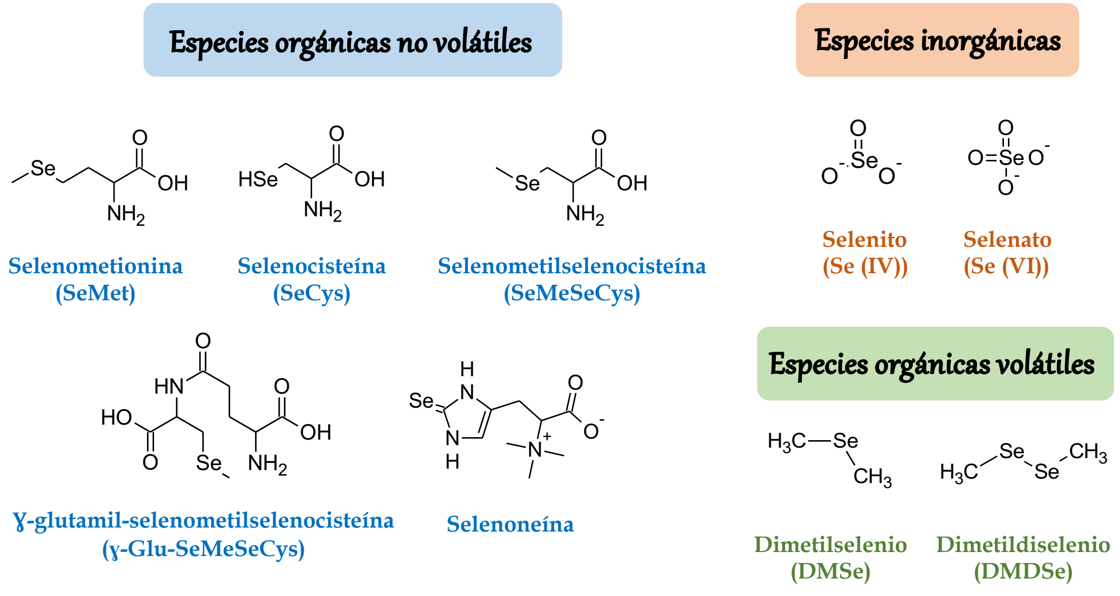


Figura 1. Estructura química de las principales especies de selenio.

En cuanto a las especies orgánicas de selenio, la SeMet y la SeCys poseen una estructura similar a la metionina (Met) y a la cisteína (Cys), respectivamente, salvo por la sustitución del átomo de azufre por un átomo de selenio. Como consecuencia, cuando el selenio se incorpora de forma específica a las proteínas, como SeCys (aminoácido número 21), estas son consideradas selenoproteínas. Sin embargo, cuando se incorpora de forma inespecífica como SeMet, en lugar de la Met, son consideradas proteínas que contienen selenio (Letavayová *et al.*, 2006).

Se han descrito un total de 25 genes de selenoproteínas en humanos, distribuidos en diferentes órganos y tejidos, con distinta función y efectos beneficiosos (Peeler & Weerapana, 2019; Zhang *et al.*, 2023), conteniendo selenio en forma del selenoaminoácido SeCys, codificado por el triplete UGA (Townsend *et al.*, 2003), aunque la totalidad de sus funciones biológicas no ha sido aún esclarecida. A continuación, se describen las funciones esenciales de las selenoproteínas más importantes, las cuales aparecen resumidas en la **Tabla 1**.

- *Selenoproteínas de la familia de la glutatión peroxidasa (GPx) y la tiorredoxina reductasa (TRx)*: participan en los sistemas de defensa antioxidante celular y en el mantenimiento de los estados redox intracelulares para mantener la viabilidad celular (Allan *et al.*, 1999; Spallholz *et al.*, 1990).

- *Selenoproteínas de la familia de la yodotironina deiodinasa (Dio)*: catalizan la eliminación de los grupos de yodo presentes en las hormonas tiroideas, activándolas o desactivándolas (Schomburg & Köhrle, 2008).
- *Selenoproteína R (SelR o MsrB)*: se ha relacionado con el control redox de la Met en diferentes proteínas, incluida la actina para la regulación de su ensamblaje/desensamblaje (Hung *et al.*, 2013).
- *Selenofosfato sintetasa 2 (SPS2)*: es una de las enzimas que intervienen en la biosíntesis de selenoproteínas a través de la catálisis de la producción de selenofosfato (Beckett & Arthur, 2005).
- *Selenoproteínas de 15 (Sel15) y 18 kDa (Sel18)*: sus funciones se relacionan con la protección anticancerígena de las células secretoras y el sistema endocrino, respectivamente. La Sel18 destaca además por ser preservada en estado déficit de selenio (Behne *et al.*, 1998; Gladyshev & Hatfield, 1999).
- *Selenoproteína M (SelM)*: está altamente expresada en el cerebro, protege frente al estrés oxidativo y regula los niveles intracelulares de calcio. Además, se le asignan funciones neuroprotectivas (Pitts *et al.*, 2013).
- *Selenoproteína P (SelP)*: es una glicoproteína monomérica que contiene 10 residuos de SeCys, una característica importante que la distingue de otras selenoproteínas. Además, es una proteína transportadora de selenio, representa aproximadamente el 40 % de la concentración total de selenio en el plasma humano, siendo la principal. SelP también tiene propiedades redox y se asocia con la protección de la función endotelial (Olson *et al.*, 2008; Leiter *et al.*, 2022).
- *Selenoproteína T (SelT)*: regula los niveles de calcio en el retículo endoplasmático y ejerce una función redox clave que permite a las células hacer frente al estrés oxidativo y garantizar la homeostasis (Anouar *et al.*, 2018).
- *Selenoproteínas W (SelW) y N (SelN)*: participan en el desarrollo y mantenimiento muscular. La función biológica de la SelW se relaciona con la calcificación de los músculos esqueléticos, situación que puede ser revertida al suplementar selenio (Holben & Smith, 1999).

Tabla 1. Características de las principales selenoproteínas identificadas en mamíferos. Función y efecto protector frente a ciertas enfermedades.

Selenoproteína		Distribución en los tejidos	Función	Efecto protector
Familia de la glutatión peroxidasa	GPx1	Todos los órganos	Actividad antioxidante; reducción celular del H_2O_2; reserva de selenio	Cáncer; diferenciación condrogénica; diabetes tipo 2; depresión; enfermedad de Keshan; cataratas
	GPx2	Tracto gastrointestinal	Actividad antioxidante; protección de la mucosa del tracto gastrointestinal y células endoteliales del daño oxidativo	Cáncer; inflamación intestinal
	GPx3	Plasma, riñón, leche materna	Reducción los hidroperóxidos lipídicos y el H_2O_2	Cáncer; fibrosis miocárdica; remodelación ventricular
	GPx4	Todos los órganos, tiroides y cerebro	Actividad antioxidante; disminución el hidroperóxido de fosfatidilcolina; supresión de la ferroptosis celular	Cáncer; osteoartritis; cardiomiopatía; lesión por isquemia-reperfusión; función cerebral
	GPx6	Epitelio nasal y glándula de Bowman	No se conoce	Enfermedad de Huntington
Familia de la tiorredoxina reductasa	TRx1	Mitocondria	Actividad antioxidante; regeneración de la tiorredoxina; supresión de la ferroptosis celular	Hipertensión arterial pulmonar idiopática; carcinoma hepatocelular; artrosis; epilepsia generalizada genética; enfermedad de Keshan
	TRx2	Citosol	Regeneración de la tiorredoxina; regulación de la proliferación celular y apoptosis	Cáncer; glaucoma primario de ángulo abierto; enfermedades cardiovasculares
	TRx3	Testículos	Actividad antioxidante; supresión de la piroptosis	Reproducción masculina; colitis; carcinogénesis
Familia de la yodotironina deiodinasa	Dio1	Hígado, riñón y tiroides	Activación T3	Metabolismo de la hormona tiroidea; inhibición de la hepatosteatosis; cáncer renal
	Dio2	Sistema nervioso central, placenta, glándula pituitaria y tejido adiposo	Activación T3	Artrosis; obesidad; retraso mental
	Dio3	Sistema nervioso central, piel y placenta	Desactivación T3	Artrosis; desarrollo mental; sepsis y choque séptico

Tabla 1 Continuación. Características de las principales selenoproteínas identificadas en mamíferos. Función y efecto protector frente a ciertas enfermedades.

Selenoproteína		Distribución en los tejidos	Función	Efecto protector
Selenoproteína R	SelR, MsrB	Varios tejidos y cerebro	Actividad antioxidante; efecto anti-inflamatorio; regulación respuesta inmune	Carcinoma hepatocelular; respuesta inflamatoria
Selenofosfato sintetasa 2	SPS2	Varios tejidos	Catalización de la producción de selenofosfato	Cáncer
Selenoproteína 15 kDa	Sel15	Varios tejidos y cerebro	Antioxidante en células secretoras	Cáncer; estrés oxidativo
Selenoproteína 18 kDa	Sel18	Varios tejidos y cerebro	Reserva de selenio	Deficiencia de selenio
Selenoproteína M	SelM	Varios tejidos y cerebro	Defensa contra el estrés oxidativo; regulación de calcio	Glioblastoma; enfermedad del hígado graso no alcohólico; déficits sinápticos; disfunción cognitiva
Selenoproteína N	SelN	Músculo esqueletal, hígado, corazón, estómago y cerebro	Proliferación celular; regeneración	Miopatías
Selenoproteína P	SelP	Hígado	Actividad antioxidante; transporte de selenio	Cáncer; convulsiones y ataxia; enfermedades cardiovasculares
Selenoproteína T	SelT	Varios tejidos	Regulación de calcio	Glioblastoma; enfermedades cardiovasculares
Selenoproteína W	SelW	Músculo esqueletal, corazón, testículos, bazo, cerebro	Funciones redox	Osteoporosis; anemia

En la **Tabla 1** se detallan algunas enfermedades donde el selenio puede ejercer un efecto protector. De hecho, entre las implicaciones biológicas del selenio más relevantes destacan la prevención frente al daño oxidativo por sus propiedades antioxidantes (Peretz *et al.*, 1992; Tinggi, 2003), el mantenimiento del normal funcionamiento del metabolismo del cerebro (Hawkes & Hornbostel, 1996; Zhang *et al.*, 2023) y su efecto protector frente a enfermedades, como el cáncer (Rayman, 2000; Rayman, 2012), enfermedades de carácter inmunológico, como el VIH (Burbano *et al.*, 2002), enfermedad de la glándula tiroides (Vanderpas *et al.*, 1990; Soriano-García, 2012), enfermedades cardiovasculares (Nève, 1996) o enfermedades causadas por metales pesados, como la enfermedad del Alzheimer (Hawkes & Hornbostel, 1996), entre otras. A continuación, se abordan con más detalle:

Prevención frente al daño oxidativo:

La homeostasis redox, fundamental para mantener la actividad de los procesos biológicos, se ve afectada por el estrés oxidativo. Esto es debido al desequilibrio que puede tener lugar en la producción de especies reactivas de oxígeno (ROS) que superan la capacidad de eliminación de los sistemas de defensa antioxidantes y que, en última instancia, provocan daños estructurales y funcionales en el ADN, los lípidos y las proteínas. En consecuencia, las mitocondrias pueden sufrir grandes daños estructurales ya que son una fuente importante de ROS. Los hidroperóxidos, especialmente el peróxido de hidrógeno (H_2O_2), son las principales ROS en la regulación redox y son responsables de la señalización celular, las reacciones enzimáticas, el metabolismo energético y el ciclo celular (Zhang *et al.*, 2023).

El selenio contribuye significativamente a la eliminación de ROS en múltiples tejidos como son el corazón (Shengyu *et al.*, 2022), el hígado (Wang *et al.*, 2022), el riñón (Zhang *et al.*, 2022), la tiroides (Marcocci *et al.*, 2012) o el cerebro (Maraldi *et al.*, 2011), por su efecto antioxidante, el cual se asocia con la resistencia a la inflamación, apoptosis y autofagia. Las selenoproteínas, especialmente las familias de la GPx y de la TRx, son cruciales en la defensa antioxidante, desempeñando un papel fundamental en la prevención del daño oxidativo y en el mantenimiento de la homeostasis redox (Spallholz *et al.*, 1990; Allan *et al.*, 1999).

Efecto sobre el mantenimiento de la funcion cerebral:

El metabolismo del cerebro depende en gran medida de los niveles de selenio y la deficiencia de este puede causar lesiones cerebrales irreversibles. Aunque el contenido de selenio en el cerebro humano sea inferior al de otros órganos como el riñón, hígado, bazo, páncreas y corazón (Chen & Berry, 2003), el cerebro es el órgano de preferencia, en detrimento de otros, para mantener los niveles óptimos de selenio cuando hay déficit del mismo, recibiendo un suplemento prioritario. Este hecho pone en evidencia su importancia en la función cerebral (Hawkes & Hornbostel, 1996). Las selenoproteínas más expresadas, es decir, transformadas en una secuencia de ADN concreta y que, por tanto, desempeñan funciones esenciales en el cerebro, son la SelP, la GPx4 y la SelW (Zhang *et al.*, 2023).

La deficiencia de SelP está vinculada a déficits neurológicos y deterioro en las funciones motoras. Esta selenoproteína se expresa en más del 90 % de la región cerebral a niveles elevados. Numerosos estudios han demostrado que SelP promueve la actividad neuronal y que la neurogénesis en el tálamo, tronco encefálico e hipocampo se ve afectada negativamente cuando está subexpresada (Scharpf *et al.*, 2007; Caito *et al.*, 2011; Leiter *et al.*, 2022).

La selenoproteína GPx4, por su parte, es esencial para la actividad neuronal y se encuentra en el núcleo, las mitocondrias y el citoplasma de la célula, siendo una molécula clave en la inhibición de la ferroptosis celular (Hambright *et al.*, 2017; Chen *et al.*, 2020). Sus propiedades redox contribuyen al mantenimiento de la función mitocondrial y la inhibición de la apoptosis. La deficiencia de GPx4 dificulta el mantenimiento de la actividad neuronal y puede provocar atrofia cerebelosa (Kahn-Kirby *et al.*, 2019; Chen *et al.*, 2020). Estudios de inactivación específica de GPx4 en neuronas de ratones adultos condujeron a una neurodegeneración masiva (Wirth *et al.*, 2010; Yoo *et al.*, 2012). De igual modo, se ha encontrado una correlación entre los niveles de GPx4 y las enfermedades de Parkinson y de Alzheimer (Yoo *et al.*, 2010; Bellinger *et al.*, 2011; Hambright *et al.*, 2017).

Finalmente, aunque no se conoce con exactitud la función biológica de la SelW, se sabe que muestra actividad redox representativa y que es la que se encuentra más sobreexpresada en el cerebro, de entre todas las selenoproteínas (Chung *et al.*, 2009).

Protección frente al cáncer:

Estudios epidemiológicos han relacionado una adecuada ingesta de selenio con un menor riesgo de padecer cáncer, siendo esta enfermedad significativamente más frecuente en países donde hay carencia de selenio en la población (Sundström *et al.*, 1984; Rayman, 2000). Sin embargo, algunos ensayos clínicos han evidenciado que el efecto beneficioso del selenio frente al cáncer se da cuando el sujeto presenta inicialmente niveles bajos de este y se le suplementa, pero no cuando el individuo ya presenta de partida unos niveles adecuados de selenio (Clark *et al.*, 1996; Flowers *et al.*, 2022; Zhang *et al.*, 2023). Aunque los estudios son altamente dependientes tanto de la forma química y la biodisponibilidad del selenio como del tipo de cáncer y su estadio, sí hay evidencias del efecto protector del selenio frente a ciertos cánceres, como el cáncer de mama, pulmón, esófago, hígado, próstata, colon, tiroides, vejiga, gástrico y de piel (Clark *et al.*, 1996; Rayman, 2000; Rayman, 2012; Flowers *et al.*, 2022).

Las especies de selenio responsables de este efecto protector son fundamentalmente las especies monometiladas, como el metilselenol y el seleniuro de hidrógeno (Ip *et al.*, 2000; McKenzie *et al.*, 2006). Los compuestos más frecuentemente suministrados en tratamientos contra el cáncer son el selenito, la SeMet y la SeMeSeCys, puesto que se metabolizan fácilmente en el organismo para dar lugar a las especies monometiladas (Tapiero *et al.*, 2003). La especie SeMeSeCys es la que presenta una mayor capacidad anticarcinogénica, como se ha evidenciado en estudios realizados en ratas frente al cáncer de mama (Ip & Dong, 2001).

En la actualidad, la relación entre el selenio y el cáncer sigue siendo un tema de investigación de alto interés e importancia debido a la controversia que en algunos casos generan los resultados obtenidos, así como a la necesidad de tratamientos que prevengan, disminuyan o erradiquen esta enfermedad.

Protección frente a otras enfermedades:

Dado que las selenoproteínas desempeñan un papel fundamental para el buen funcionamiento del sistema inmune, y que se ve altamente afectado por el estrés oxidativo, el selenio también está ligado con la incidencia y progresión de enfermedades víricas como el VIH, el virus de Koxsackie, el poliovirus, las hepatitis B y C o el virus de la gripe (Sappey *et al.*, 1994; Taylor *et al.*, 1997; Yu *et al.*, 1997; Beck *et al.*, 1998; Yu *et al.*, 1999).

En lo que respecta a otras enfermedades autoinmunes, como es el caso de la tiroiditis o enfermedad de Hashimoto, también se han observado efectos beneficiosos bajo la suplementacion de selenio (Toulis *et al.*, 2010). En este sentido, se ha observado una disminución tanto del hipotiroidismo como del hipertiroidismo (Marcocci *et al.*, 2011).

La deficiencia de selenio también se ha asociado con enfermedades cardiovasculares debido a la capacidad de la GPx para evitar la oxidación lipídica y reducir la agregación plaquetaria (Nève, 1996). La primera evidencia de la relación del selenio con enfermedades cardiovasculares se tuvo a partir de la inclusión de selenio en la dieta de ciertas ovejas Muth para protegerlas frente a la enfermedad del músculo blanco, que afecta al músculo cardiaco, entre otros. La aparición de cardiopatías en Keshan (China) y su similitud con dicha enfermedad del músculo blanco, llevó a relacionar la carencia de selenio con la aparición de dicha cardiomiopatía, conocida desde entonces como enfermedad de Keshan, ya que, en dicha zona, los suelos poseían concentraciones bajas de selenio. Se han constatado mejoras en pacientes suplementados con selenio que antes presentaban concentraciones bajas de este en sangre y suero (Loscalzo, 2014; Liu *et al.*, 2021).

Aunque algunos estudios no han encontrado evidencias significativas de la relación entre el selenio y las enfermedades cardiovasculares (Virtamo *et al.*, 1985; Jenkins *et al.*, 2018), la teoría comúnmente aceptada es que el selenio ejerce efecto protector frente a las enfermedades cardiovasculares al afectar a las respuestas inflamatorias, al metabolismo lipídico, al estrés oxidativo, a la autofagia y a la apoptosis en el sistema cardiovascular (Zhang *et al.,* 2023).

Por otro lado, el selenio ha demostrado tener un papel importante sobre la resistencia a la oxidación y en la quelación, inhibiendo la toxicidad de metales pesados como el mercurio, cadmio, arsénico, cromo, talio, plomo y plata (Yuan *et al.*, 2016; Zwolak, 2020; Aaseth *et al.*, 2021). La acumulación de estos metales en organismos puede inducir efectos adversos, incluyendo toxicidad hepatorrenal, neurotoxicidad, toxicidad reproductiva e inmunotoxicidad. El estrés oxidativo se presenta como el principal mecanismo tóxico de los metales pesados que, además, favorece el desarrollo de la enfermedad del Alzheimer (Rahmanto & Davies, 2012).

Se ha observado que tanto las enzimas GPx como TRx, esenciales para la defensa antioxidante, son el principal objetivo de los metales pesados. En este sentido, por ejemplo, se ha comprobado que la concentración de selenio en el celebro de pacientes afectados por la enfermedad del Alzheimer es hasta un 40 % inferior con respecto a los que no padecen dicha enfermedad (Hawkes & Hornbostel, 1996).

En la **Figura 2** se muestra la curva dosis/respuesta para el selenio en adultos (Dumont, 2006), la cual ilustra la regla de Bertrand (Bertrand, 1912), con los valores de deficiencia, óptimos y de toxicidad en función de la dosis suministrada. La parte central del gráfico es una meseta que representa el mantenimiento del nivel de selenio óptimo a través de la regulación homeostática. La regla de Bertrand es válida para todos los oligoelementos esenciales, aunque cada uno tiene su propia curva dosis/respuesta específica.

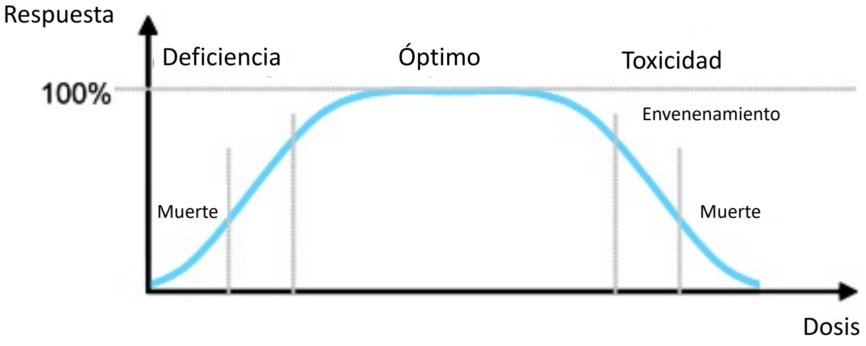


Figura 2. Curva de dosis/respuesta para el selenio en adultos (adaptada de Dumont, 2006).

Una deficiencia de selenio puede producir una reducción en la síntesis y expresión de las selenoproteínas y, por tanto, afectar a las correspondientes funciones biológicas en la que están implicadas (Rayman, 2012). En este sentido, el valor mínimo de referencia para alcanzar la máxima actividad de las GPx en plasma es de 95 µg Se L^{-1}. De igual modo, el valor de referencia mínimo considerado para evitar riesgos de sufrir cáncer o enfermedades cardiovasculares por déficit de selenio es 45 µg Se L^{-1} (Duffield et al., 1999; Rayman, 2000).

En cuanto a los síntomas de la intoxicación por selenio, estos incluyen uñas quebradizas con aparición de decoloración blanca amarillenta o roja, pérdida de cabello, lesiones cutáneas, mal aliento, afecciones gástricas, hipotensión o aflicción respiratoria, entre otros (Wilber, 1980; Combs, 2001).

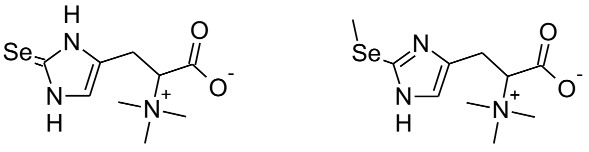
El parámetro UL (*Tolerable Upper Intake Level*) indica la máxima cantidad de ingesta diaria posible sin que se observen efectos tóxicos para la salud. Para el caso del selenio en Europa, en adultos se ha establecido en 300 μg día^{-1} (*Scientific Comittee on Food*, 2000). Existen otras normativas más específicas que tienen en cuenta el peso del individuo, como por ejemplo el NOAEL *(Non Observed Adverse Effects Level*), establecido por la Organización Mundial de la Salud (WHO (*World Health Organization*), 2003) en 4 μg de selenio por kg de peso corporal por día.

1.2. Selenoneína y ergotioneína

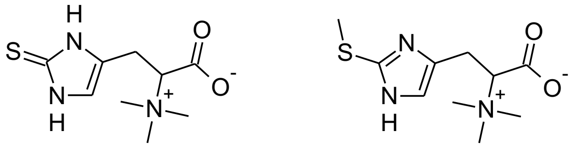
En los últimos años, además de las especies de selenio consideradas tradicionalmente, las investigaciones se han centrado en el estudio de la selenoneína (2-selenil-N$_\alpha$, N$_\alpha$, N$_\alpha$-trimetil-L-histidina). La selenoneína es análoga al compuesto ergotioneína, ((2S)-3-(2-sulfanilideno-1,3-dihidroimidazol-4-il)-2-(trimetilazaniumil) propanoato), donde se ha sustituido el azufre por selenio (**Figura 3**) (Yamashita & Yamashita, 2010), evidenciándose grandes similitudes en sus propiedades, como por ejemplo la gran capacidad antioxidante que presentan (Yamashita *et al.*, 2010).

Sin embargo, debido a la estructura química de la molécula selenoneína y a las mayores propiedades antioxidantes del grupo selenol frente al grupo tiol, la selenoneína debería ser incluso más beneficiosa que la ergotioneína (Lim *et al.*, 2019).

En este sentido, la selenoneína se ha identificado en algunas matrices biológicas de origen animal como: piel de la ballena beluga (Achouba *et al.*, 2019), hígado de las tortugas marinas (Anan *et al.*, 2011) y delfines (Pedrero *et al.*, 2014), sangre de atún (Yamashita *et al.*, 2010), músculo de diferentes peces como atún, pez espada, sardina, caballa o dorada, entre otros (Yamashita *et al.*, 2011), y en sangre (Klein *et al.*, 2011; Achouba *et al.*, 2019; Kroepfl *et al.*, 2019; Little *et al.*, 2019; Achouba *et al.,* 2023) y orina humanas (Klein *et al.*, 2011).



Selenoneína **Se-metilselenoneína**



Ergotioneína **S-metilergotioneína**

Figura 3. Estructura química de la selenoneína, la ergotioneína y sus respetivos compuestos metilados.

La ergotioneína está directamente relacionada con la selenoneína, ya que esta última se transporta a través de las membranas celulares mediante el transportador de ergotioneína, OCTN1, que también está involucrado en el transporte de los complejos formados entre la selenoneína y el metilmercurio ($MeHg^+$) (Gründemann *et al.*, 2005). Ambos compuestos se incorporan así a los tejidos y a las células, actuando como importantes antioxidantes en el ciclo redox. Dado que la selenoneína y la ergotioneína no son sintetizadas por los mamíferos, su principal fuente de obtención para el ser humano es la dieta. La ergotioneína puede encontrarse en setas, judías blancas, trigo o avena (Ey *et al.*, 2007), mientras que los alimentos ricos en selenoneína son principalmente especies marinas como el atún, el pez espada o la sardina (Yamashita *et al.*, 2011).

Las formas reducidas del selenio suelen ser excelentes ligandos para los iones metálicos blandos, como el Fe^{2+} y el Hg^{2+}, y la selenoneína no es una excepción. Esta se une fuertemente a la fracción hemo de la mioglobina y la hemoglobina, protegiendo así a estas proteínas de hierro centrales de la autooxidación, como por ejemplo en condiciones de hipoxia, cuando puede haber cantidades excesivas de ROS dentro de los glóbulos rojos (Yamashita & Yamashita, 2010). De igual modo, esta capacidad para unirse a los iones metálicos también se ejemplifica en la detoxificación del Hg^{2+}, ya que la selenoneína participa en el metabolismo y la eliminación del $MeHg^+$ de la circulación sanguínea. Yamashita *et al.* (Yamashita *et al.*, 2013) demostraron que el transportador

OCTN1 parece ser esencial para que tenga lugar esta protección, aunque los mecanismos todavía no han sido completamente elucidados.

La forma metilada de la selenoneína, la Se-metilselenoneína (**Figura 3**), se ha encontrado en sangre y orina humanas, sugiriendo que podría ser el resultado de la metilación de la selenoneína ingerida del pescado por las enzimas metiltransferasas presentes en el hígado y los riñones (Ohta & Suzuki, 2008; Klein *et al.*, 2011; Achouba *et al.*, 2023). De igual modo, también ha sido identificada la S-metilergotioneína (**Figura 3**) en la sangre humana (Achouba *et al.*, 2023). Estas formas metiladas de selenoneína y ergotioneína sugieren ser el resultado de la vía de biotransformación que conduce a la excreción en la orina (Klein *et al.*, 2011).

1.3. Fuentes de selenio en la dieta

La cantidad total de selenio en el ser humano es de aproximadamente 3-20 mg (Zhang *et al.*, 2023). Como micronutriente esencial, la principal forma de incorporación de selenio es seres humanos es a partir de la dieta, sobre todo a través de la ingesta de pescado, carne, cereales, frutos secos, vegetales y huevos (Xie *et al.*, 2021; Alcântara *et al.*, 2022).

Los cereales son la principal fuente de selenio, aunque su contenido total es relativamente bajo, oscilando entre 0.01-0.55 µg g^{-1}. En los alimentos de origen animal el selenio se encuentra en concentración de 0.08-0.70 µg g^{-1}. Sin embargo, en frutas y en verduras esta concentración no llega a 0.1 µg g^{-1} (Zhang *et al.*, 2023). En contraposición, el alimento con más concentración de selenio son las nueces de Brasil, llegando a presentar concentraciones de hasta 512 µg g^{-1} (Alcântara *et al.*, 2022).

Los niveles de selenio en el organismo, ingeridos a través de la alimentación, son altamente dependientes de la concentración que haya en el suelo donde se cultiven los alimentos que sean fuente de selenio (Combs, 2001; Dumont *et al.*, 2006). Algunos estudios realizados sobre los niveles de selenio en la población española arrojaron una concentración media de selenio en sangre en España de aproximadamente 60-72 µg Se L^{-1} (Rayman, 2000; Combs, 2001). Este valor está por encima de 45 µg Se L^{-1} (valor de referencia mínimo considerado para evitar riesgos de sufrir cáncer o enfermedades cardiovasculares por déficit de selenio), pero se encuentra por debajo de

95 µg Se L^{-1} (mínimo de referencia para alcanzar la máxima actividad de las GPx en plasma) (Duffield et al., 1999; Rayman, 2000).

España se clasifica, al igual que la mayoría de los países de la Unión Europea, por debajo de Estados Unidos y Canadá, con más de un 50 % de su población con niveles deficientes de selenio, junto a países como China, Nueva Zelanda y las Repúblicas de la antigua Yugoslavia (López-Bellido & López, 2013). La población mayor de 60 años es la que presenta más deficiencia, con niveles de unos 45 µg Se L^{-1}, frente a casi 90 µg Se L^{-1} entre los adultos jóvenes (Fernández-Bañares *et al.*, 2002).

1.4. Metabolismo del selenio en humanos

En la **Figura 4** se representa un esquema del metabolismo del selenio en humanos.

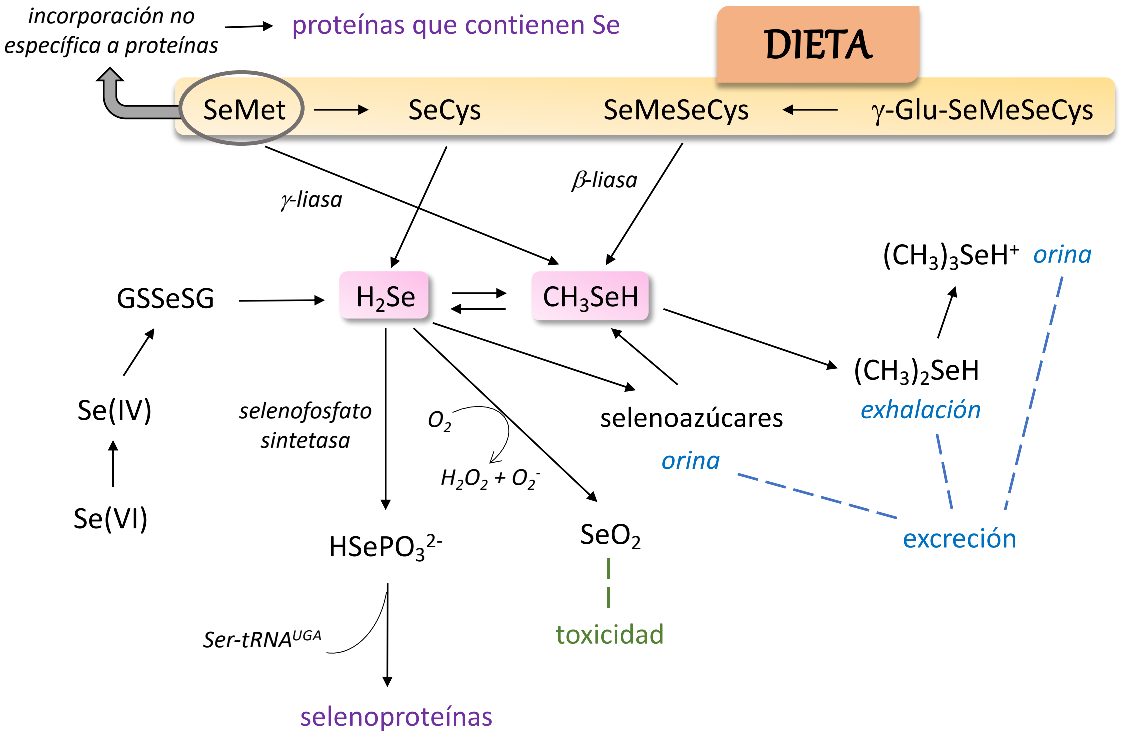


Figura 4. Ruta metabólica para el selenio en humanos (adaptada de Rayman *et al.*, 2008). SeMet: selenometionina; SeCys: selenocisteína; SeMeSeCys: selenometilselenocisteína; γ-Glu-SeMeSeCys: γ-glutamil-selenometilselenocisteína; $(CH_3)_2SeH$: dimetilseleniuro; $(CH_3)_3Se^+$: catión trimetilselenonio; CH_3SeH: metilselenol; H_2Se: ácido selenhídrico; SeO_2: óxido de selenio (IV); $HSePO_3^{2-}$: selenofosfato; GSSeSG: selenodiglutatión; UGA: codón Uracilo-Guanina-Adenina; tRNA: ácido ribonucléico de transferencia; Ser: serina.

Las formas químicas de selenio ingeridas a través de la dieta (SeMet, SeCys, SeMeSeCys y γ-Glu-SeMeSeCys) se transforman en H_2Se para, posteriormente, sintetizar las selenoproteínas o ser excretado. Las formas inorgánicas de selenio también se reducen a H_2Se. El H_2Se puede convertirse en $HSePO_3^{2-}$ por la acción de la selenofosfato sintetasa, para seguidamente dar lugar a la SeCys unida al tARN e insertarse en la cadena polipeptídica de manera específica, mediante el codón UGA (Rayman, 2005).

La SeMet y la SeCys están interconectadas mediante la ruta de la trans-selenización, análogamente a la Met y la Cys por la ruta de la trans-sulfuración. La SeMet, además, puede seguir dos caminos: transformarse a CH_3SeH a través de la acción de la γ-liasa en caso de estar en exceso (camino regular), o incorporarse de forma no específica a las proteínas sustituyendo a la Met, ya que el codón UGA no distingue entre ambas, formando así las proteínas que contienen selenio (camino irregular) (Suzuki, 2005). De esta forma, la SeMet es absorbida más rápidamente por el organismo que cualquier otra forma de selenio, siendo una manera estable de almacenarlo.

La γ-Glu-SeMeSeCys y la SeMeSeCys también están interconectadas, pudiéndose transformar también en CH_3SeH a través de la β-liasa y ser posteriormente convertidos en H_2Se o ser excretados (Navarro-Alarcón & Cabrera-Vique, 2008; Rayman *et al.*, 2008).

Finalmente, la excreción se produce fundamentalmente a través de la orina en forma de selenoazúcares (Gammelgaard *et al.*, 2012) o como vía de detoxificación tras varias metilaciones a través de la orina ($(CH_3)_3SeH^+$) y de la exhalación en la respiración ($(CH_3)_2SeH$) (Francesconi & Pannier, 2004; Navarro-Alarcón & Cabrera-Vique, 2008; Rayman *et al.*, 2008; Ohta *et al.*, 2009).

1.5. Mercurio: toxicidad y fuentes de contaminación

El mercurio ha sido reconocido por la Organización Mundial de la Salud como una de las diez sustancias de mayor preocupación para la salud pública (WHO, 2005, 2008) debido a su efecto tóxico. Por tanto, la importancia biológica del mercurio se limita a su toxicidad, que depende en gran medida tanto de la dosis ingerida como de su forma química (Singh *et al.*, 2023).

El mercurio se emite al medio ambiente tanto por actividades naturales: actividades geotérmicas o liberación de rocas y sedimentos; como antropogénicas: minería, combustión de combustibles fósiles, producción de metales, etc. (Panel & Chain, 2012); y se encuentra en continuo movimiento a través de su ciclo biogeoquímico, que integra la atmósfera, el océano y la tierra (Selin, 2009), representado en la **Figura 5**. En la atmósfera, la forma más abundante de este elemento es el Hg^0 que se oxida a Hg^{2+}, el cual se deposita en las superficies de lagos y océanos. El Hg^{2+} puede seguidamente sufrir diferentes transformaciones: reducirse a Hg^0, metilarse formando $MeHg^+$ y dimetilmercurio ($(CH_3)_2Hg$) o ser absorbido por partículas. La conversión del Hg^{2+} en $MeHg^+$ se produce por la acción de bacterias y depende de factores como la actividad microbiana, la concentración de mercurio biodisponible, el potencial redox, el pH o la temperatura (Burger *et al.*, 2001; Nogara *et al.*, 2019).

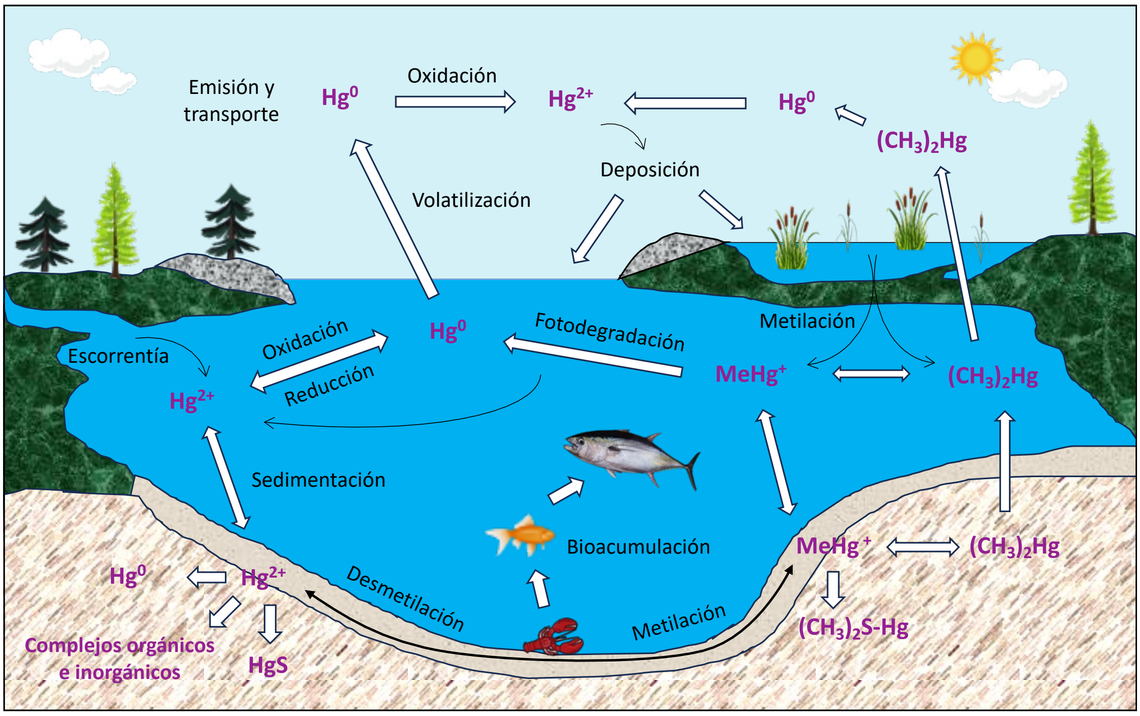


Figura 5. Ciclo biogeoquímico del mercurio.

La toxicidad de las distintas especies de mercurio aumenta de la siguiente manera: $Hg^0 < Hg^{2+} < MeHg^+$. Los efectos tóxicos en el organismo a largo plazo también varían: el Hg^0 puede causar problemas neurológicos y renales, el Hg^{2+} puede causar daño renal, gastrointestinal y afectar al sistema nervioso, y el $MeHg^+$ puede ocasionar daño neurológico irreversible, especialmente en el desarrollo fetal, siendo capaz de atravesar la placenta y provocar daño celular y la consecuente muerte celular (Kim *et al.*, 2014; Singh *et al.*, 2023). Además, las principales patologías que provoca el $MeHg^+$ son deficiencia sensorial y motora, ataxia muscular, pérdida de visión, coma e incluso la muerte (WHO, 2005).

Los sistemas acuáticos están considerados como la parte más importante del ciclo biogeoquímico del mercurio, ya que constituyen un sumidero para este tanto procedente de fuentes naturales como antropogénicas. La incorporación del $MeHg^+$ a los sistemas acuáticos se produce mediante la acción de microorganismos para, posteriormente, bioacumularse en los organismos vivos y biomagnificarse en la cadena trófica. El contenido de $MeHg^+$ aumenta con la edad, el tamaño y el nivel trófico en la cadena alimentaria, teniendo más concentración del tóxico los peces que se sitúan en los niveles superiores de la misma, es decir, peces grandes y depredadores que ingieren especies más pequeñas (Bisi *et al.*, 2012; Ruus *et al.*, 2015; Arcagni *et al.*, 2018).

Las concentraciones de mercurio en la biota marina no solo están relacionadas con las deposiciones atmosféricas provenientes de fuentes naturales y antropogénicas, sino que también se ven influenciadas por las características físicas y químicas intrínsecas de un ecosistema dado, ya que desempeñan un papel importante en la biodisponibilidad del mercurio. En este sentido, existe una asociación entre la presencia de altas concentraciones de mercurio en invertebrados y peces con un pH bajo, un nivel elevado de carbono orgánico disuelto (COD) y altas concentraciones de mercurio en las aguas (Hammerschmidt & Fitzgerald, 2006; Burgess & Meyer, 2008). Sin embargo, la influencia de estos factores en la biomagnificación del mercurio en las cadenas tróficas no está totalmente demostrada. Se desconoce si el proceso de biomagnificación está afectado solamente por los procesos intrínsecos de las cadenas tróficas (tasa de crecimiento, diversidad de especies, longitud de la cadena trófica, etc.) o si, además, las variables fisicoquímicas también modulan dicha biomagnificación (Lavoie *et al.*, 2013).

Salvo exposición ocupacional, la dieta constituye la principal fuente de exposición al mercurio para los seres humanos, especialmente a través del consumo de pescado y marisco (Córdoba-Tovar *et al.*, 2022), donde el mercurio se encuentra mayoritariamente en forma de $MeHg^+$ (Panel & Chain, 2012). Una vez ingerido, el 95% del $MeHg^+$ total es absorbido en el tracto gastrointestinal (Syversen & Kaur, 2012). En el organismo, el efecto tóxico del $MeHg^+$ se ve potenciado por su capacidad de unión a proteínas, biomoléculas y transportadores de aminoácidos (Ajsuvakova *et al.*, 2020). Debido a su elevada afinidad por los grupos sulfhidrilo (-SH), se encuentra unido covalentemente a biomoléculas como la GPx, la Cys, la S-adenosilmetionina (SAM) o la metalotioneína (MT), bajo el mecanismo bioquímico implicado en el transporte de mercurio a través de membranas, su redistribución a través de diferentes tejidos y su excreción (Clarkson & Magos, 2006; Farina *et al.*, 2011a; Spiller, 2018). El 10 % del $MeHg^+$ existente en el organismo se localiza en el cerebro, y el 20 % de este es hidrosoluble y se encuentra como MeHg-GPx (Syversen & Kaur, 2012). El $MeHg^+$ es capaz de atravesar la barrera hematoencefálica unido a las biomoléculas anteriormente mencionadas, sobre todo a la Cys, siendo los astrocitos la primera célula diana del complejo MeHg-Cys en el cerebro (Nogara *et al.*, 2019).

1.6. Interacción selenio-mercurio: mecanismos. Efecto protector del selenio frente a la toxicidad inducida por el mercurio

El mecanismo de toxicidad del mercurio se basa en su capacidad de alterar el control de la homeostasis redox intracelular, provocando, por tanto, estrés oxidativo intracelular. Los principales órganos diana son el cerebro, los riñones y, en menor medida, el hígado (Ganther, 1978; Farina *et al.*, 2011a; Farina *et al.*, 2011b; Sheikh *et al.*, 2011). La interacción del mercurio con el selenio es una característica intrínseca en la toxicidad que produce. Tanto el $MeHg^+$ (en mayor medida) como el Hg^{2+} se unen al sitio de unión de SeCys de la TRx1 en el citosol y de la TRx2 en las mitocondrias, inhibiendo significativamente su función, ya que permanece en estado oxidado (Clarkson & Magos, 2006; Branco *et al.*, 2014; Lu & Holmgren, 2014). La SeCys puede ser reducida por la GPx para restaurar su función, sin embargo, esta también se ve inactivada con concentraciones significativas de mercurio (Raymond *et al.*, 1977; Franco *et al.*, 2009; Branco *et al.*, 2012; Branco *et al.*, 2015). Una solución es la generación de la TRx *de novo*, lo que requiere que una parte de la reserva de selenio en forma de SeCys se inserte en la nueva enzima. Sin embargo, el mercurio también se une al selenio intracelular disponible para formar compuestos insolubles de Hg:Se, MeHg-Se o Hg-Se-Cys, produciendo un estado de deficiencia de selenio intracelular, reduciendo así la disponibilidad de selenio para la producción *de novo* de las TRx y GPx, e inhibiendo aún más su restauración.

Dentro del cerebro (**Figura 6**), el Hg^0 puede oxidarse a Hg^{2+} que puede unirse al seleniuro formando un compuesto inerte (HgSe). El $MeHg^+$, por su parte, puede ser desmetilado, liberando HgSe, por un mecanismo que implica la unión al residuo SeCys de las selenoproteínas. Además, el $MeHg^+$ puede interactuar directamente con el seleniuro, lo que puede facilitar la ruptura de la unión C-Hg, liberando el compuesto inerte HgSe, el cual puede ser retenido en el cerebro sin tener efectos tóxicos para el mismo (Oliveira *et al.*, 2017).

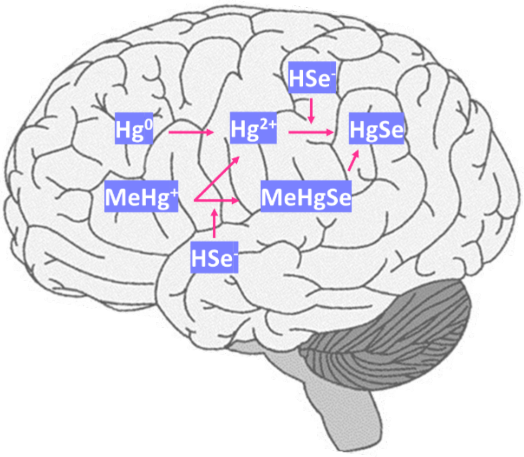


Figura 6. Transformaciones del mercurio en el cerebro.

En este sentido, el selenio puede proporcionar un papel protector en la neurotoxicidad inducida por el mercurio, de diversas maneras y dependiendo de la especie de mercurio. En la literatura científica se describe mediante diversas formas de acción, que se abordan a continuación (Spiller, 2018):

1) Desmetilación de mercurio orgánico a mercurio inorgánico

El proceso de desmetilación constituye uno de los mecanismos de detoxificación de mercurio que se ve favorecido por la presencia de selenio (Ohi et al., 1976; Itano et al., 1984), y que provoca la conversión de las formas tóxicas de mercurio, generalmente, $MeHg^+$, en otras formas menos tóxicas, como puede ser el Hg^{2+}. La mayoría de los estudios sugieren que este proceso no se debe a la presencia de selenio inorgánico, sino que se da cuando el selenio está presente en sus formas orgánicas, como es el caso de la SeMet (Seppänen *et al.*, 1998; Khan & Wang, 2010).

2) Redistribución del mercurio a órganos diana menos sensibles

Otro mecanismo que puede ser clave y que se postula también como posible responsable de la detoxificación del mercurio, es el efecto que tiene el selenio en su redistribución en el propio cerebro. Aunque existen notables discrepancias entre los estudios llevados a cabo sobre el contenido cerebral de mercurio tras la suplementación con selenio, todos concluyen en una disminución de la neurotoxicidad inducida por el mercurio. Varios estudios han mostrado una disminución o no han evidenciado cambios significativos en el contenido cerebral de mercurio tras la suplementación con selenio (Hansen *et al.*, 1981; Andersen & Nielsen, 1994; de Freitas *et al.*, 2009; Glaser *et al.*, 2013; Joshi *et al.*, 2014), mientras que otros han mostrado un aumento del tóxico (Mengel & Karlog, 1980; Glynn *et al.*, 1993; Glynn & Lind, 1995; Newland *et al.*, 2006; Sakamoto *et al.*, 2013; Li *et al.*, 2014). Sin embargo, los estudios que han evidenciado un aumento en el contenido cerebral de mercurio, así como un aumento en el contenido cerebral de selenio, han señalado también una disminución en la neurotoxicidad inducida por el mercurio. Este contraste entre un aumento del mercurio cerebral frente a una reducción de la neurotoxicidad sugiere que probablemente se produce un “secuestro” del mercurio en forma de complejos inertes, como sería el complejo Hg:Se (Newland *et al.*, 2006; Sakamoto *et al.*, 2013; Li *et al.*, 2014). Es decir, aunque se incremente el contenido de mercurio en el cerebro, si hay suficiente selenio que pueda complejarse con dicho mercurio, su efecto tóxico se vería reducido.

3) *Formación de compuestos insolubles, estables e inertes de Hg:Se*

El mercurio y el selenio pueden interaccionar entre ellos y fruto de esa interacción, como se ha comentado anteriormente, se forman compuestos insolubles de Hg:Se, como son el seleniuro bis-(metilmercúrico), el formado a partir del $MeHg^+$ unido a la SeCys, el obtenido tras la unión de la SeIP a clústeres de HgSe, y los biominerales $HgSe_xS_{1-x}$ (Khang & Wang, 2009), aunque los mecanismos de estos procesos todavía están sin elucidar. Estas reacciones pueden ser mediadas por moléculas de bajo peso molecular que contienen selenio, como la selenoneína y como la SeIP (Yamashita *et al.*, 2013; Manceau *et al.*, 2021a). En la SeIP los átomos de mercurio desmetilados se coordinan con cuatro residuos de SeCys formando los complejos $Hg(SeCys)_4$. Parece ser que la coordinación tetraédrica de cuatro átomos de selenio con el mercurio, en moléculas orgánicas, es la más favorecida, lo que también podría ocurrir para la selenoneína, aunque no se conoce con certeza (Palmer *et al.*, 2015).

Manceau *et al.* (Manceau *et al.*, 2021b) identificaron los compuestos MeHg-Cys, $Hg(SeCys)_4$, HgSe (tiemannita) y $Hg(Cys)_2$ en distintos órganos de aves marinas. La tiemannita también ha sido identificada en distintos órganos de varios animales, entre ellos, la ballena beluga (Huggins *et al.*, 2009), distintas especies de delfines (Nakazawa *et al.*, 2011; Gajdosechova *et al.*, 2016), aves de presa (Manceau *et al.*, 2021b; Lancaster *et al.*, 2022; El Hanafi *et al.*, 2023), el turón patinegro (Arai *et al.*, 2004) e incluso en humanos (Korbas *et al.*, 2010; Moser *et al.*, 2020).

4) Reducción de la absorción de mercurio en el tracto gastrointestinal

La capacidad de absorción del mercurio en el tracto gastrointestinal puede verse reducida cuando este se encuentra formando parte de compuestos insolubles de HgSe, que provienen de la interacción entre ambos elementos. Como consecuencia de esto, el mercurio que forma parte de ellos no se encuentra disponible para su absorción en el tracto gastrointestinal. Si bien es cierto que esta interacción sucede cuando el mercurio y el selenio están en sus formas inorgánicas. En este sentido, la presencia de selenio, en forma de selenito, puede llevar a una reducción de la absorción del mercurio inorgánico hasta de un 10 % (Mykkanen *et al.*, 1987; Andersen & Nielsen, 1994; Seppänen *et al.*, 1998).

5) Restauración de la actividad de las selenoproteínas diana y el entorno redox intracelular

Como ha sido comentado anteriormente, la formación de los compuestos insolubles de HgSe tiene como consecuencia un estado de deficiencia de selenio intracelular, viéndose reducida la disponibilidad de selenio para la producción *de novo* de las selenoproteínas TRx y GPx. Sin embargo, numerosas investigaciones han evidenciado la restauración de la actividad de las selenoproteínas TRx y GPx con suplementación de selenio, aunque depende en gran parte de la concentración de mercurio, de las formas químicas suministradas de selenio y existentes de mercurio, y del órgano objeto de estudio (Carvalho *et al.*, 2011; Branco *et al.*, 2012).

2. Presencia de selenio y mercurio en pescados y productos derivados

2.1. Hábitos de consumo de pescado y productos derivados en España

Según los últimos datos aportados por el Ministerio de Agricultura, Pesca y Alimentación del Gobierno de España (https://www.mapa.gob.es/es/default.aspx), el consumo de pescado ha caído cerca de un 30 % en la última década, situándose, en 19.19 kg por persona y año.

Sin embargo, los 8 000 kilómetros de costa que presenta, hacen de España un país con profundas tradiciones en la pesca y las manifestaciones culinarias asociadas a los productos del mar. España es el principal productor de pescado en la Unión Europea, con más de 900 000 toneladas provenientes de la pesca extractiva y 300 000 toneladas de la acuicultura. Además, la industria de procesamiento, incluyendo la conservación y congelación, también es muy potente, produciendo 800 000 toneladas anuales de conservas, semiconservas, salazones, congelados y preparados.

Las especies de pescado mayormente consumidas en España son la merluza y la pescadilla, con un consumo en 2022 de más de 91 kg, según los datos registrados en el Informe del consumo alimentario en España de 2022 (https://www.mapa.gob.es/es/alimentacion/temas/consumo-tendencias/informe-consumo-2022-baja-res_tcm30-655390.pdf). En segunda posición, pero con mucho menor volumen de consumo (54.63 kg), se sitúa el salmón, seguido de las sardinas y los boquerones (49.78 kg). Los siguientes pescados más frecuentemente consumidos por los españoles son: bacalao (49.16 kg), dorada (31.17 kg), lubina (22.83 kg), atún (18.67 kg), rape (12.64 kg) y trucha (8.6 kg).

Por otro lado, en lo que respecta a los productos derivados del pescado, el surimi (concentrado de proteínas elaborado a partir de músculo de pescado) es la base de todos ellos, teniendo un importante lugar en el mercado mundial, con una producción anual de alrededor de 820 000 toneladas. Asia es el continente que lidera dicha producción, produciendo anualmente unas diez toneladas más que Europa. En contexto europeo, España es el segundo mercado del oeste europeo con una producción del 32 % del volumen total, siendo Francia el primero (41 %). Sin embargo, las últimas tendencias muestran una disminución del mercado francés, que se verá superado por el actual aumento del español (https://europa-azul.es/surimi-alimentacion/).

Aunque los líderes en innovación de productos derivados del pescado con base en surimi siguen siendo los países orientales, como Corea, Japón o China, la demanda de este tipo de productos se ha visto incrementada en los últimos años, siendo España el segundo consumidor de Europa de este tipo de productos, con 0.9 kg de productos por habitante y año, aunque lejos aún de los 3 kg de productos por habitante y año consumidos en Francia (https://europa-azul.es/surimi-alimentacion/).

Estos productos derivados del pescado constituyen formas alternativas e innovadoras de introducir el pescado en la dieta. Entre las principales ventajas asociadas con su consumo destacan su versatilidad para la preparación de distintos platos, su digestibilidad, y constituir una forma atractiva para los niños de consumir pescado. Asimismo, son una fuente de proteínas y omega-3. Entre los principales productos comercializados en España nos encontramos con los comúnmente denominados palitos de cangrejo, las gulas o tallarines elaborados con base de pescado. Además, se busca que los productos derivados del pescado puedan representar un comercio sostenible, ya que este tipo de alimentos suele elaborarse con pescados que tienen una menor salida en el mercado, aunque siguen teniendo un alto valor nutritivo. El proceso general de elaboración de estos productos con base en surimi se muestra en la **Figura 7** y se basa en sucesivas etapas de lavado de músculo de pescado para obtener un gelificado de proteínas, que posteriormente es amasado y mezclado con otros ingredientes, hasta obtener el producto final deseado.

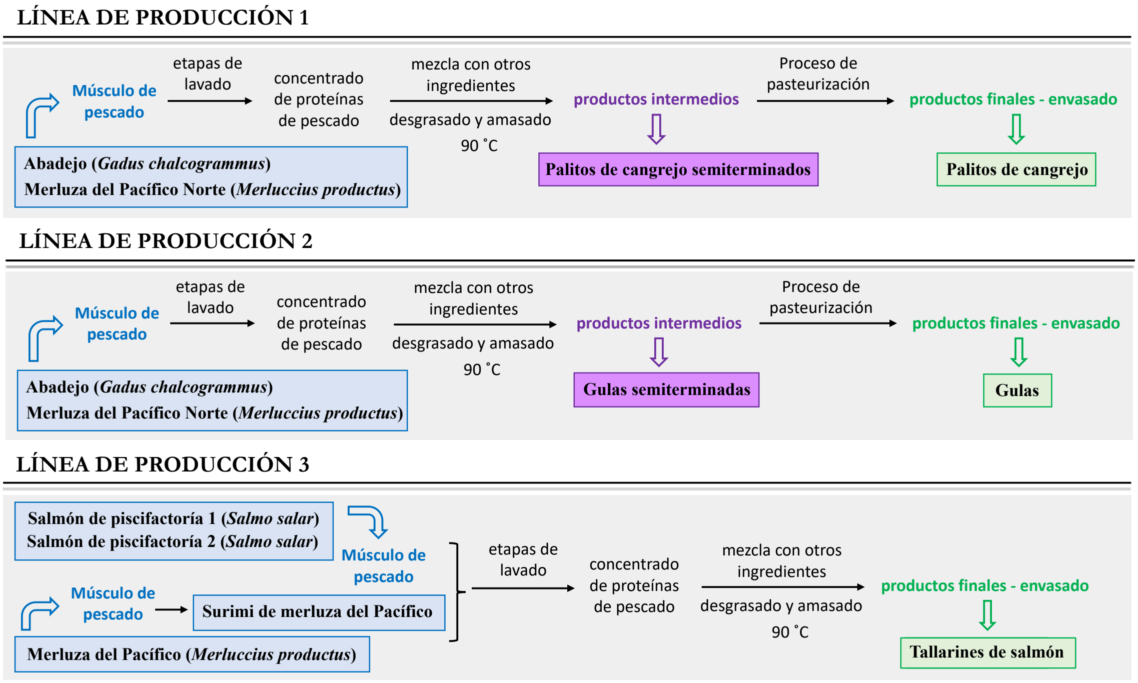


Figura 7. Esquemas del proceso de elaboración de los productos procesados palitos de cangrejo, gulas y tallarines de salmón.

Finalmente, las huevas de pescado también son productos derivados directamente del pescado con un elevado valor nutricional. Destacan por ser una fuente de proteínas de alta calidad, ácidos grasos omega-3, vitaminas y minerales, como selenio, hierro, calcio y zinc.

Los elementos selenio y mercurio estarán presente tanto en los productos derivados del pescado, como en los productos procesados elaborados a partir del pescado, ya que estos analitos se encuentran en los pescados empleados como materia prima para la preparación de estos productos.

2.2. Legislación en los contenidos de selenio y mercurio en pescados

En lo que respecta al mercurio, la EFSA (*European Food Safety Authority*) (Panel & Chain, 2012) fijó una ingesta semanal tolerable de $MeHg^+$ en los alimentos de 1.6 µg por kg de peso corporal. Esto significa que la ingesta semanal de $MeHg^+$ para una persona de 70 kg no debe ser superior a 112 µg, valor bastante elevado teniendo en cuenta los contenidos normales de tóxico que presentan los pescados habitualmente consumidos. En este sentido, también se establece el nivel máximo permitido de mercurio en el músculo de los pescados (parte comestible) en 0.5 mg kg^{-1} (peso húmedo). Los peces depredadores son una excepción, como por ejemplo el esturión (*Acipenser species*), el atún (*Thunnus species, Euthynnus species, Katsuwonus pelamis*) o el pez espada (*Xiphias gladius*), donde el límite máximo se sitúa en 1.0 mg kg^{-1} (peso húmedo).

En relación con el selenio, no se ha establecido ningún nivel máximo permitido en pescados. Sin embargo, la EFSA (EFSA, 2014) fijó una cantidad adecuada de ingesta de selenio (*Adequate Intake*, AI) de 70 µg al día para adultos.

Por otra parte, la FDA (*Food and Drugs Administration*) y la EPA (*Environmental Protection Agency*) han recomendado que las mujeres embarazadas, los lactantes y los niños ingieran entre 226 y 340 g de pescado o marisco a la semana, pero esto se aplica al pescado y marisco con baja concentración de mercurio, como el salmón o las gambas (FDA/EPA, 2004).

Además, la EFSA (EFSA, 2012) realizó una evaluación de los riesgos asociados al $MeHg^+$ y de los beneficios asociados al consumo de alimentos de origen marino, concluyendo que el consumo de entre 510 y 680 g de pescado o marisco a la semana durante el embarazo, iba asociado con aumento significativo del desarrollo neurológico de los niños en comparación con los hijos de mujeres que no consumieron tales cantidades de pescado o marisco durante el embarazo.

2.3. Niveles de selenio y mercurio en pescados consumidos Europa. *Selenium Health Benefit Value* (HBV$_{Se}$) como criterio de seguridad

El músculo del pescado es la parte comestible del mismo, si bien es cierto que la piel, las huevas e incluso las espinas se van consumiendo con mayor frecuencia. La distribución del mercurio no es homogénea en las distintas partes del pescado, encontrándose en mayor concentración en el músculo, seguido de la piel, y siendo las huevas las que presentan menores contenidos del tóxico. Sin embargo, la distribución de selenio sigue un patrón totalmente distinto, ya que se encuentran en mayores concentraciones en las huevas, llegando a representar el doble del contenido encontrado en el músculo o en la piel del pescado. Los diferentes patrones de distribución de los contenidos de selenio y mercurio en las partes comestibles de los pescados son indicativos de las distintas vías de acumulación y metabolismo en el organismo de los mismos (Xu *et al.*, 2018; Zhang *et al.*, 2020).

Por lo anteriormente mencionado, generalmente en los estudios de evaluación de contenidos de selenio y mercurio en pescados suele considerarse el músculo como la parte objeto de análisis. Al evaluar conjuntamente el selenio y el mercurio en esta matriz, se suele proporcionar información de las relaciones molares Se:Hg encontradas, así como del índice *Selenium Health Benefit Value* o HBV_{Se}. Se trata de nuevos criterios de evaluación del riesgo asociado a la exposición al mercurio por el consumo de alimentos como el pescado. Por tanto, resulta más informativo y realista proporcionar los valores de estos parámetros que reportar simplemente las concentraciones indivuales de mercurio y selenio, ya que, como bien es sabido, están relacionados como consecuencia de la interacción existente entre ambos. Si bien es cierto que el selenio muestra un papel protector frente a la toxicidad del mercurio, la exposición al mercurio reduce la actividad de las enzimas dependientes de selenio. Por lo que surge la necesidad de determinar la cantidad de selenio que queda disponible para realizar las correspondientes funciones biológicas en el organismo, una vez que se produce su interacción con el mercurio, donde parte del selenio es secuestrado por el mercurio, y esto se realiza mediante la aplicación del cálculo de la relación molar Se:Hg y el índice HBV_{Se} (Ralston *et al.*, 2016; Ralston *et al.*, 2019).

La relación molar Se:Hg se calcula mediante la **ecuación (1)** (Ralston *et al.*, 2008; Burger & Gochfeld, 2011; Ulusoy *et al.*, 2019):

$$\text{Relacion molar Se: Hg} = \frac{\text{mmol Se kg}^{-1}}{\text{mmol Hg kg}^{-1}} \qquad \textbf{(1)}$$

Por su parte, el cálculo del parámetro HBV_{Se} se lleva a cabo mediante la **ecuación (2)**, basada en las concentraciones molares de ambos elementos en el alimento (Ralston *et al.*, 2016; Ralston *et al.*, 2019):

$$\text{HBV}_{\text{Se}} = \frac{(\text{Se}-\text{Hg})}{\text{Se}}(\text{Se}+\text{Hg}) \qquad \textbf{(2)}$$

Se = contenido de Se en concentración molar (mmol Se kg^{-1})

Hg = contenido de Hg en concentración molar (mmol Hg kg^{-1})

Valores superiores a 1 de la relación molar Se:Hg y valores positivos de HBV_{Se} indican que el consumo del pescado aparentemente no conlleva riesgos de toxicidad en relación a la expoxicion a mercurio. Sin embargo, valores negativos suponen un riesgo potencial para el consumidor. Este parámetro proporciona una información más adecuada y realista que la proporcionada por los contenidos totales de cada elemento por separado. En consecuencia, estos datos son indispensables para una correcta evaluación de la seguridad relacionada con la exposición al mercurio por el consumo de este tipo de alimentos.

En la **Tabla 2** se muestra una revisión bibliográfica de las relaciones molares Se:Hg y Hg:Se, y del parámetro HBV_{Se} para el músculo de distintos pescados. La mayoría de ellos presentan valores de Se:Hg molar ratio mayores que 1 y, por tanto, HBV_{Se} positivos, por lo que constituyen opciones de consumo seguras en cuanto a la toxicidad del mercurio se refiere.

Tabla 2. Comparación de relación molar Se:Hg y Hg:Se, y HBV_{Se} de distintas especies de pescados. Las concentraciones de Se y Hg de partida para el cálculo de los parámetros son en peso húmedo. N es el número de muestras consideradas en el estudio.

Nombre común	**Nombre científico**	**Origen**	**N**	**Se:Hg**	**Hg:Se**	**HBV_{Se}**	**Referencia**
Tiburón azul	*Prionace glauca*	País Vasco, España	11	0.74	1.351	-1	Olmedo *et al.*, 2013
Bacaladilla o lirio	*Micromesistius poutassou*	Galicia, España	11	2.49	0.402	1	Olmedo *et al.*, 2013
		Valencia, España	3	-	0.065	101	Calatayud *et al.*, 2012
		Noruega	75	41.6	-	6.11	Azad *et al.*, 2019
Lenguado	*Solea vulgaris*	Andalucía (costa atlántica), España	11	16.18	0.062	25	Olmedo *et al.*, 2013
Merluza	*Merluccius merluccius*	Valencia, España	11	5.43	0.184	7	Olmedo *et al.*, 2013
		Valencia, España	3	-	0.072	42	Calatayud *et al.*, 2012
		Noruega	92	5.4	-	4.12	Azad *et al.*, 2019
Lubina	*Dicentrarchus labrax*	Murcia, España	11	1.83	0.546	1	Olmedo *et al.*, 2013
Dorada	*Sparus aurata*	Murcia, España	11	0.27	3.641	-1	Olmedo *et al.*, 2013
Caballa	*Scomber scombrus*	Andalucía (costa mediterránea), España	11	25.87	0.039	73	Olmedo *et al.*, 2013
Gallo	*Lepidorhombus boscii*	Andalucía (costa atlántica), España	11	3.91	0.256	7	Olmedo *et al.*, 2013
Salmón	*Salmo salar*	Noruega	11	>100	0.000	>1000	Olmedo *et al.*, 2013
		Valencia, España	3	-	0.094	114	Calatayud *et al.*, 2012

Tabla 2 Continuación. Comparación de relación molar Se:Hg y Hg:Se, y HBV_{Se} de distintas especies de pescados Las concentraciones de Se y Hg de partida para el cálculo de los parámetros son en peso húmedo. N es el número de muestras consideradas en el estudio.

Nombre común	Nombre científico	Origen	N	Se:Hg	Hg:Se	HBV_{Se}	Referencia
Pez espada	*Xiphias gladius*	Galicia, España	11	2.32	0.430	13	Olmedo *et al.*, 2013
		España	5	2.0	-	-	Cabañero *et al.*, 2007
		Valencia, España	3	-	0.663	4	Calatayud *et al.*, 2012
Atún	*Thunnus thynnus*	Islas Canarias, España	11	3.06	0.326	21	Olmedo *et al.*, 2013
		España	5	4.9	-	-	Cabañero *et al.*, 2007
		Galicia, España	10	4.50	0.133	14.53	Melgar *et al.*, 2019
		Turquía	50	5.49	0.219	12.11	Ulusoy *et al.*, 2019
Platija	*Reinhardtius hippoglossoides*	Noruega	546	10.3	-	5.23	Azad *et al.*, 2019
Abadejo	*Theragra chalcogramma*	Noruega	58	8.1	-	4.65	Azad *et al.*, 2019
Bacalao	*Gadus morhua*	Noruega	2105	16.4	-	3.44	Azad *et al.*, 2019
Arenque	*Atlantic herring*	Noruega	1810	39.3	-	6.60	Azad *et al.*, 2019
Rodaballo	*Psetta maxima*	Turquía	21	393	0.002	23.62	Ulusoy *et al.*, 2019
Salmonete	*Mullus barbatus*	Turquía	10	168	0.006	21.88	Ulusoy *et al.*, 2019
Raya	*Raja clavata*	Turquía	30	56.93	0.019	10.90	Ulusoy *et al.*, 2019
Rape	*Lophius piscatorius*	Valencia, España	3	-	0.263	9	Calatayud *et al.*, 2012

Podemos constatar diferencias en los valores reportados de los parámetros de interés (relaciones molares Se:Hg, Hg:Se y HBV_{Se}) en función de la localización geográfica y otros factores (Azad *et al.*, 2019). Las zonas costeras se ven más afectadas por las actividades antropogénicas que el mar abierto, debido a la centralización de las industrias y zonas urbanas, por lo que se espera que estas zonas estén más contaminadas por mercurio que las zonas de alta mar. Además, tienen un intercambio de agua limitado, a diferencia de las zonas de alta mar donde la circulación del agua es mayor debido a las corrientes oceánicas (Jonsson *et al.*, 2014). De igual modo, la salinidad es un factor determinante en la metilación del mercurio en los sedimentos. Las zonas costeras suelen presentar una menor salinidad que las zonas de alta mar debido al aporte de agua dulce de las cuencas terrestres, lo que implica que la metilación del mercurio se produzca en alta mar a un ritmo mayor y que, por tanto, aumente su biodisponibilidad; esto conllevaría una mayor acumulación de $MeHg^+$ en los peces que habitan estas zonas. Otro factor que contribuye a una mayor eficiencia en la metilación del mercurio en las zonas de costa es el oxígeno disuelto, que en estas localizaciones suele ser menor en comparación con el del océano abierto (Azad *et al.*, 2019). De igual modo, otra variable notable que parece afectar a los contenidos de selenio y mercurio encontrados en las distintas especies de peces, es el tamaño y edad de estos y, aunque es escasa la información a este respecto, parece ser que de forma general los contenidos de ambos elementos se ven incrementados con un aumento del tamaño, lo cual es altamente dependiente de la edad (Azad *et al.*, 2019; Zhang *et al.*, 2020).

Finalmente, cabe destacar que, hasta la fecha, en la literatura científica no hay datos disponibles sobre valores de relaciones molares Se:Hg, Hg:Se y del parámetro HBV_{Se} de productos derivados y procesados del pescado.

2.4. Bioaccesibilidad de selenio y mercurio mediante modelos de digestión gastrointestinal *in vitro*. Efecto de la forma química

Las formas químicas predominantes del selenio en pescados son las formas orgánicas, destacando la SeMet, siendo esta además la forma mayoritaria de selenio en los primeros eslabones de la cadena trófica acuática, como por ejemplo en las algas (Hardy *et al.*, 2010). Además, ciertos estudios evidencian que las formas orgánicas de selenio presentan mejor absorción en el tracto gastrointestinal que las especies inorgánicas (Huang *et al.*, 2012; Antony Jesu Prabhu *et al.*, 2020). Una vez absorbido, el selenio es transportado por el plasma y metabolizado en el hígado, dando lugar a las selenoproteínas. Seguidamente, el selenio es transportado a tejidos extrahepáticos y, finalmente, excretado por el riñón (Brigelius-Flohé, 2015; Antony Jesu Prabhu *et al.*, 2020). El hígado y el riñón suelen presentar mayores concentraciones de selenio que el resto de las partes del organismo de los peces, ya que son los órganos metabólicos y excretores de selenio, respectivamente, pero no son órganos de almacenamiento. El órgano principal de almacenamiento de selenio en los peces es el músculo esquelético (Huang *et al.*, 2012).

Además, un factor clave que afecta a la distribución del selenio en los diferentes tejidos y órganos de los peces es la forma química en la que se les suplementa. El selenio orgánico se almacena más rápidamente en el músculo esquelético a partir de vías inespecíficas como la SeMet, mientras que el selenio inorgánico debe ser utilizado para la síntesis de selenoproteínas en el hígado para seguidamente ser transportado a los diferentes órganos y tejidos (Daniels, 1996; Rider *et al.*, 2010; Berntssen *et al.*, 2018; Wischhusen *et al.*, 2019; Antony Jesu Prabhu *et al.*, 2020). En este sentido, se ha observado que la SeMet, con respecto al selenio inorgánico, presenta una mayor acumulación en el hígado y en el músculo esquelético del salmón atlántico (*Salmo salar*) (Lorentzen *et al.*, 1994), del jurel (*Seriola lalandi*) (Le & Fotedar, 2014), del mero (*Epinephelus malabaricus*) (Lin, 2014), y en todo el organismo de la lubina rayada atlántica híbrida (*Morone chrysops x M. saxatilis*) (Jaramillo *et al.*, 2009).

Por su parte, la SeMeSeCys es un aminoácido no proteico considerado la forma de selenio más eficiente en su efecto antioxidante y anticarcinogénico, ofreciendo protección frente a algunos tipos de cáncer (Ip & Dong, 2001).

Por tanto, la forma química en la que se encuentre el selenio va a afectar a la eficiencia en su incorporación en nuestro organismo una vez ingerido el alimento, en este caso el pescado. Esta incorporación estará limitada por la bioaccesibilidad y/o biodisponibilidad del selenio, términos que deben ser bien diferenciados (**Figura 8**).

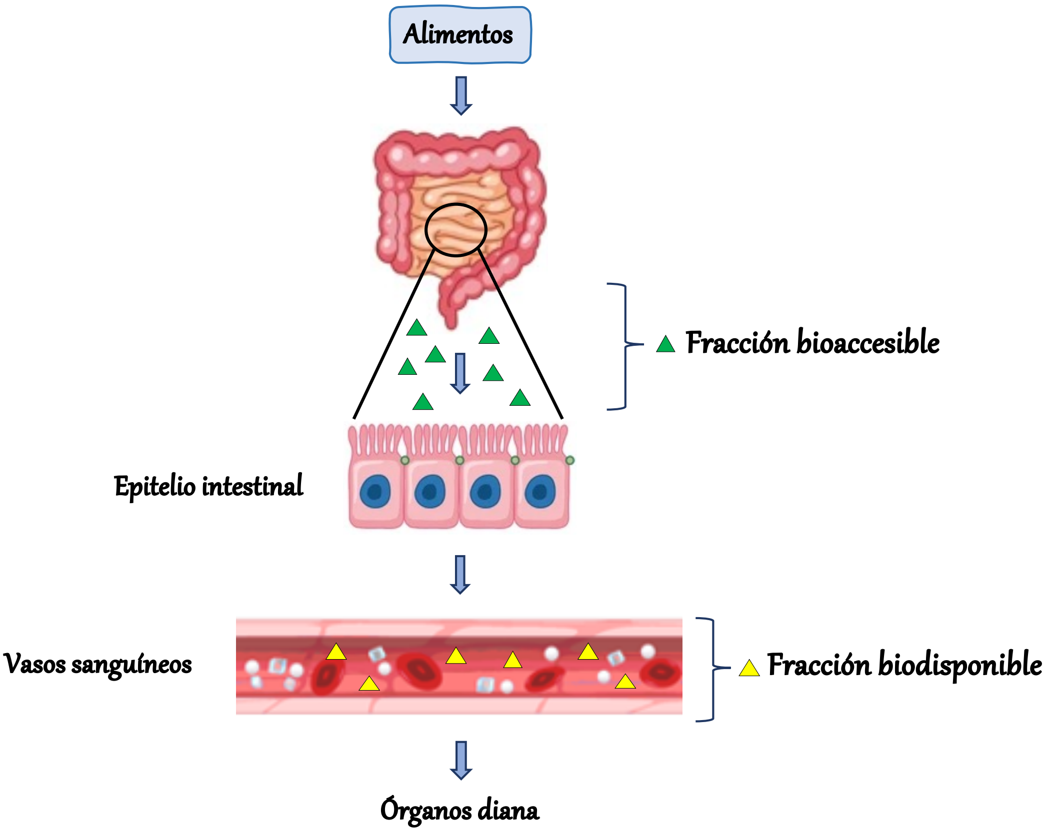


Figura 8. Fracciones bioaccesible y biodisponible de los componentes ingeridos.

La bioaccesibilidad se define como la fracción del compuesto que se libera desde su matriz al medio gastrointestinal y que es potencialmente asimilable, es decir, que puede estar disponible para la absorción intestinal, con posibilidad de entrar en el torrente sanguíneo (Grøn *et al.*, 2003; Oomen *et al.*, 2004).

Por su parte, la biodisponibilidad puede definirse como la fracción de nutriente ingerido que, tras ser absorbido e incorporado al torrente sanguíneo, es utilizada para realizar las correspondientes funciones biológicas en el organismo o para su almacenamiento (Tapiero *et al.*, 2003; Rayman *et al.*, 2008). Los factores más importantes que afectan a la biodisponibilidad de metales a niveles traza en un alimento objeto de estudio son la especie química en la que se encuentra el elemento, la composición de la matriz alimentaria, componentes de la dieta, así como con los ácidos de la bilis y, por último, la absorción de la especie a través de las membranas celulares del epitelio intestinal (Oomen *et al.*, 2004; Semple *et al.*, 2004; Moreda-Piñeiro *et al.*, 2013). Esto último nos daría una idea de la biodisponibilidad del elemento estudiado.

Para evaluar la bioaccesibilidad y biodisponibilidad se suele emplear, por lo general, la simulación de una digestión gastrointestinal *in vitro*, a través de la cual se obtiene la máxima concentración de analito disponible para su absorción en el tracto gastrointestinal (Cabañero *et al.*, 2004), o fracción bioaccesible. Estos estudios constituyen una opción sencilla, y la alternativa más viable a los estudios *in vivo.* Presentan como ventaja su sencillez a la hora de realizarlos en el laboratorio, su rapidez y su bajo coste. Con respecto a los estudios en los que tienen en cuenta la biodisponibilidad, se aplica un proceso de diálisis con membranas en la última etapa del proceso gastrointestinal para evaluar qué cantidad del analito de interés es capaz de atravesarlas. Además, se suele evaluar la absorción en líneas celulares modelo, como puede ser la línea celular Caco-2, proveniente del carcinoma de colon humano. Esta línea celular muestra características comparables con las células del epitelio intestinal humano, y por ello se elige como modelo para evaluar la absorción de compuestos de interés a nivel intestinal (Moreda-Piñeiro *et al.*, 2017).

Como se ha comentado anteriormente, este proceso de digestión gastrointestinal *in vitro* mimetiza, a escala de laboratorio, las condiciones fisiológicas que ocurren en las diferentes etapas del proceso. En la **Figura 9** se representan dichas etapas, las cuales se describen brevemente a continuación:

1. Etapa salival:

Cuando se produce la ingestión de un alimento, este se encuentra en primer término con la saliva, y esta será la responsable de iniciar el proceso digestivo, el cual se produce a un pH de 6.5. En las glándulas salivales, sobre todo en las glándulas parótidas, se produce amilasa, que es una enzima hidrolasa que actúa sobre el glucógeno y el almidón para formar azúcares simples. En esta etapa, donde está implicado el proceso de masticación, los trozos de alimentos se harán cada vez más pequeños, aumentando su área superficial, en un proceso que dura de segundos a minutos.

2. Etapa gástrica:

Una vez finalizado el proceso salival, el bolo alimenticio pasa al estómago, donde el ácido clorhídrico (HCl) es el medio que permite la disolución de muchos de los componentes de los alimentos, a un pH cercano a 1.8. En esta etapa, la pepsina, que es una proteasa que se produce en el estómago, actúa sobre las proteínas degradándolas, proporcionando como resultado péptidos y aminoácidos, siendo efectiva en condiciones muy ácidas. Esta etapa puede durar desde minutos hasta horas.

3. Etapa intestinal:

El proceso de digestión continúa en el intestino delgado, donde los alimentos están sometidos a los jugos intestinales formados por la secreción pancreática, las sales biliares y bicarbonato de sodio ($NaHCO_3$). El papel de estos jugos es romper los polisacáridos, las proteínas y las grasas, transformándolas para favorecer el proceso de absorción. La secreción pancreática constituye una mezcla de diversas enzimas digestivas o proteasas de amplio espectro compuestas por amilasa, tripsina, lipasa, ribonucleasa y proteasa. La lipasa pancreática actúa en la interfase agua/grasa de las gotas de lípidos emulsificadas, formadas por la agitación mecánica en el intestino, en presencia de las sales biliares. Estas sales están constituidas principalmente por la bilis, que se produce en el hígado y se almacena en la vesícula biliar durante la digestión, y funciona como emulsionante de los ácidos grasos, para ser posteriormente digeridos por las enzimas pancreáticas. Esta etapa puede durar desde minutos hasta horas en cada parte del intestino: el duodeno (30-45 min, pH 5-5.5), el yeyuno (1.5-2 h, pH 5.5-7) y el íleon (5-7 h, pH 7-7.5).

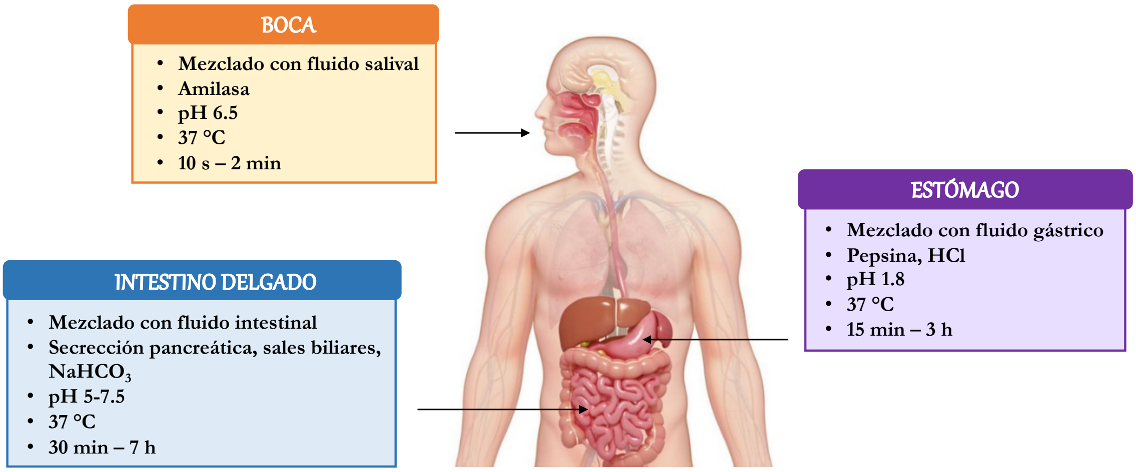


Figura 9. Etapas de la digestión gastrointestinal humana.

Por tanto, para obtener la fracción bioaccessible/biodisponible, se precisa simular todas estas etapas descritas. Varios autores han llevado a cabo métodos de digestión gastrointestinal *in vitro* para la evaluación de la bioaccesibilidad/biodisponibilidad de los contenidos de selenio total, así como de sus especies. Los alimentos en los que, una vez ingeridos, el selenio presenta una mayor biodisponibilidad en el organismo, superando el 85 %, son los cereales, la levadura de cerveza y la mayoría de los productos vegetales. Por otro lado, entre aquellos que se caracterizan por tener las biodisponibilidades de selenio más bajas, se encuentran las carnes y productos cárnicos derivados, no superando generalmente el 15 %. En lo que respecta a pescados y mariscos, la biodisponibilidad del selenio depende mucho del tipo de pescado y de marisco, encontrándose valores tanto bajos, entre 4-30 %, como medio-altos, llegando a alcanzar casi un 100 % (Cabañero *et al.*, 2004; Cabañero *et al.*, 2007; Calatayud *et al.*, 2012; Moreda-Piñeiro *et al.*, 2013). Crews *et al.* evidenciaron que el selenio total bioaccesible en el bacalao cocido rondaba el 60 % del total, además de identificar las especies Se (IV) y SeMet en los extractos de digestión gastrointestinal (Crews *et al.*, 1996). De igual modo, Cabañero *et al.* evaluaron la bioaccesibilidad del selenio en distintas especies de pescados frecuentemente consumidos (atún, pez espada y sardina) mediante un método de digestión gastrointestinal *in vitro*, encontrando diferentes porcentajes de bioaccesibilidad en función del pescado analizado. Observaron mayores porcentajes en los extractos del pez espada y de la sardina (76 y 83 %, respectivamente) en comparación con los obtenidos para el atún (50 %). SeMet resultó la especie mayoritaria de selenio encontrada en los extractos gastrointestinales de los tres pescados analizados, obteniendo bajos porcentajes de bioaccesibilidad, correspondientes a un 16, 14 y 19 % para el pez espada, la sardina y el atún, respectivamente (Cabañero *et al.* 2004, 2007). Finalmente, Calatayud *et al.* también evaluaron los contenidos totales del selenio bioaccesible, resultantes de un proceso de digestión gastrointestinal *in vitro*, obteniendo resultados superiores al 90 % para la merluza, el salmón o el pez espada (Calatayud *et al.*, 2012).

Es importante mencionar que el procesado de algunos alimentos, como puede ser el pulido del grano de los cereales, la retirada de la piel de algunos alimentos o los distintos tratamientos térmicos de cocinado llevados a cabo para otros, pueden suponer un cambio en la biodisponibilidad del selenio (Moreda-Piñeiro *et al.*, 2017). Sin embargo, las técnicas culinarias basadas en tratamientos térmicos para el pescado no parecen tener un efecto negativo en la biodisponibilidad del selenio (Cabañero *et al.*, 2004; Fox *et al.*, 2004).

En relación con el mercurio, los resultados de bioaccesibilidad en pescados encontrados en la literatura científica son muy escasos y variables, obteniendo porcentajes del 17-87 % para el pez espada, 66-81 % para la merluza, 62-68 % para el abadejo y 9-19 % para el atún (Cabañero *et al.*, 2004; Torres-Escribano *et al.*, 2010; Torres-Escribano *et al.*, 2011; Calatayud *et al.*, 2012). La variabilidad encontrada es tal que, incluso para la misma especie de pez, el pez espada, y empleando la misma metodología analítica, Torres-Escribano *et al.* observaron bioaccesibilidades de mercurio que oscilaban entre el 38-83 % (Torres-Escribano *et al.*, 2010).

3. Metodologías analíticas para la determinación de selenio, mercurio y sus especies

En la determinación de selenio y mercurio, y sus especies, en alimentos, el tratamiento de la muestra suele ser uno de los pasos más importantes y críticos ya que son muestras alimentarias cuya matriz es de elevada complejidad. Por tanto, se debe de trabajar de manera rigurosa para evitar la contaminación de la muestra y minimizar las posibles pérdidas del analito, lo cual es crucial al tratarse de análisis de elementos a niveles traza, como es el caso de la determinación de selenio y mercurio en este tipo de alimentos.

Por otro lado, las etapas previas al tratamiento de la muestra, como son la toma de muestra y el almacenamiento de la misma, deben realizarse adecuadamente, llevando a cabo una mínima manipulación de la muestra y almacenándola, generalmente, a baja temperatura. Esto es de especial relevancia en los análisis de especiación donde durante el almacenamiento de la muestra pueden producirse transformaciones de la forma original en la que se encuentre el elemento, así como alterar su concentración. Esto puede producirse como consecuencia de interacciones químicas entre especies, la acción microbiana, la luz, el pH o la temperatura, entre otros (Lindemann *et al.*, 2000).

La metodología analítica para la determinación del contenido total del selenio y mercurio difiere significativamente de la empleada para el análisis de sus especies. En ambos casos, se precisa del uso de técnicas analíticas de elevada sensibilidad, debido a las bajas concentraciones en las que estos analitos están presentes en este tipo de muestras.

Generalmente, antes del análisis de las especies de selenio y mercurio, es necesario conocer los contenidos totales de ambos elementos en los alimentos objeto de estudio. A continuación, se abordarán cada una de las metodologías implicadas en este tipo de análisis, resumidas en la **Figura 10**.

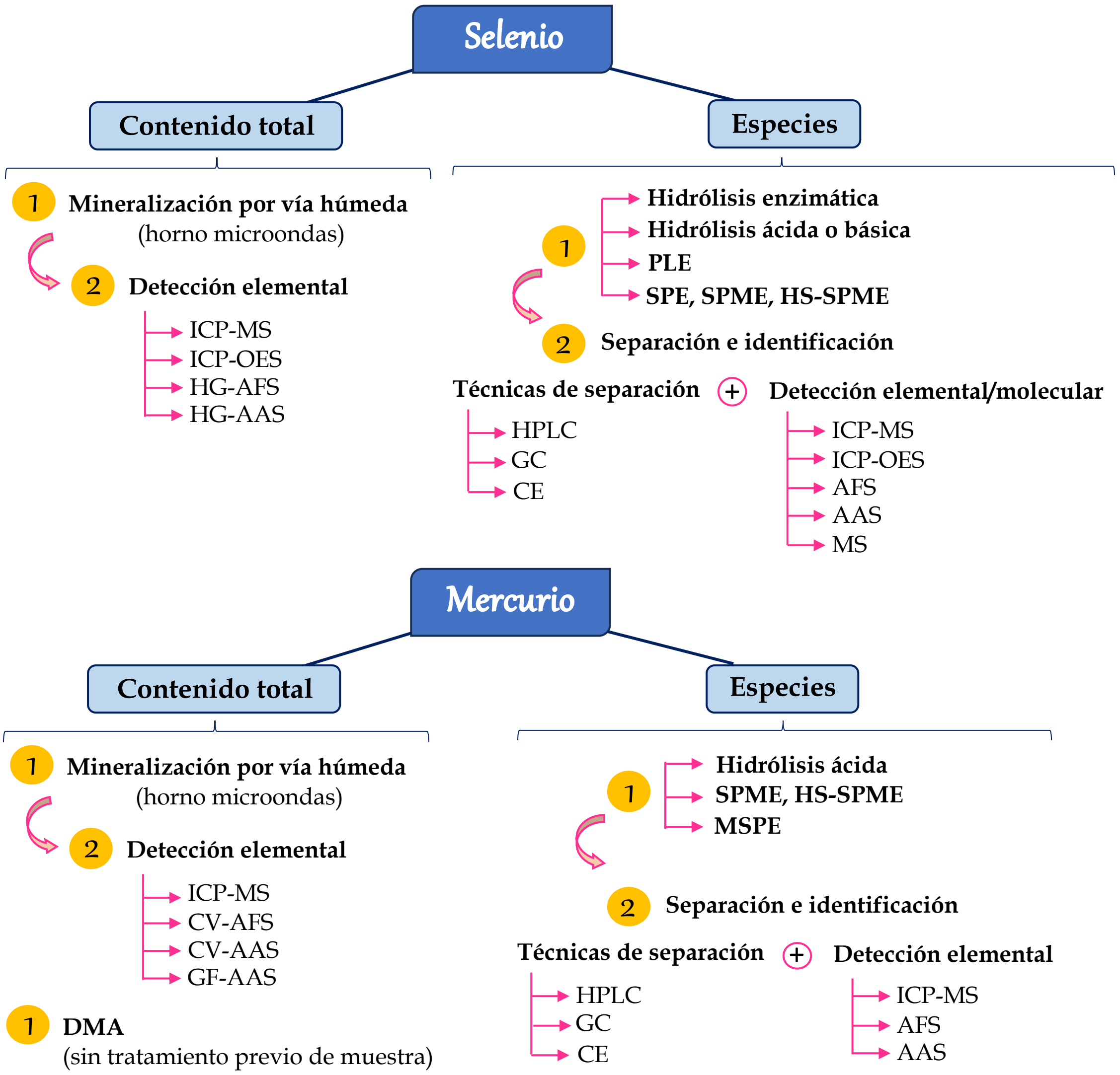


Figura 10. Metodologías analíticas para la determinación de los contenidos totales de selenio y mercurio, así como la separación e identificación de sus especies.

3.1. Determinación del contenido total de selenio y de sus especies

Análisis del contenido total de selenio

El tratamiento de la muestra para la determinación del contenido total de selenio consiste en un proceso de digestión por vía húmeda para liberar el selenio de la matriz a la disolución. Generalmente, se lleva a cabo en un horno microondas y con ello se logra la destrucción de la materia orgánica presente en la muestra. Se suele emplear este sistema cerrado para lograr alcanzar altas temperaturas y presión, lo que disminuye el tiempo de ensayo y cantidad de reactivos, evita la posible volatilización del compuesto de interés y la eficiencia y reproducibilidad se ven incrementadas. La digestión puede llevarse a cabo con distintas mezclas de reactivos, generalmente ácidos oxidantes con otros agentes, con propiedades también oxidantes, como HNO_3-H_2O_2 o HNO_3-H_2SO_4-H_2O_2, o propiedades complejantes, como HNO_3-HCl (Enamorado-Báez *et al.*, 2013).

Posteriormente, la detección y cuantificación del selenio total se puede realizar con distintas técnicas analíticas, entre las que destacan la espectrometría de masas con plasma de acoplamiento inductivo (ICP-MS), la espectroscopia de emisión atómica con plasma de acoplamiento inductivo (ICP-OES), la espectroscopia de fluorescencia atómica con generación de hidruros (HG-AFS) y la espectroscopia de absorción atómica con generación de hidruros (HG-AAS). La técnica de generación de hidruros es una técnica comúnmente utilizada, ya que el selenio es un elemento que puede formar un hidruro estable volátil, el seleniuro de hidrógeno (H_2Se), en condiciones ácidas y reductoras, lo que produce un aumento de la sensibilidad, pero sobre todo de selectividad (Capelo *et al.*, 2006).

En la actualidad, la técnica ICP-MS (**Figura 11**), es la técnica de elección para la determinación de elementos traza como es el caso del selenio debido a su elevada sensibilidad y bajos límites de detección (ng L^{-1}). Además, otras ventajas que ofrece se pueden resumir en una elevada selectividad, precisión, amplio rango lineal, de hasta diez órdenes de magnitud, y la capacidad de poder realizar análisis multielementales, pudiéndose monitorizar hasta 60 elementos en una sola medida (Amais *et al.*, 2020). Sin embargo, en la determinación de selenio por ICP-MS hay que hacer frente a interferencias espectrales, causadas por especies atómicas o moleculares que tienen la misma masa que el analito de interés, y no espectrales, originadas por la matriz de la muestra. En cuanto a las interferencias espectrales, el isótopo más abundante de selenio, ^{80}Se (49 %), tiene la misma m/z que el $^{40}Ar_2^+$, presente en ICP-MS cuadrupolar convencional. Sin embargo, mediante la utilización de celdas de colisión/reacción esta interferencia se reduce, sin afectar significativamente a la sensibilidad. Los gases empleados en las celdas de colisión/reacción para eliminar estas interferencias pueden ser variados (H_2, N_2O, NO_2, CH_4, combinaciones de N_2-NH_3) (Sloth & Larsen, 2000; Mazan *et al.*, 2002; Wallschlager & London, 2004), siendo el hidrógeno el más comúnmente utilizado, capaz de reducir hasta 6 órdenes de magnitud la interferencia producida por el dímero $^{40}Ar_2^+$ (McCurdy & Potter, 2001).

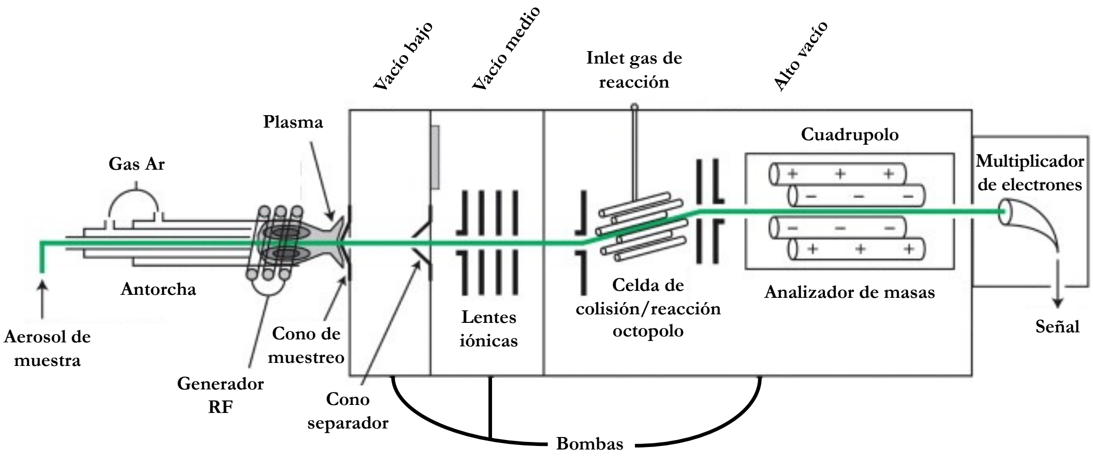


Figura 11. Representación esquemática de un instrumento ICP-MS cuadrupolar (adaptada de Arévalo, 2014).

Identificación y análisis de las especies de selenio

El primer paso en los estudios de especiación consiste en separar las especies del elemento objeto de estudio, en nuestro caso selenio, de la matriz de la muestra. Para ello, se suelen aplicar procesos de extracción basados generalmente en procesos de hidrólisis enzimáticas, ácidas o básicas. El objetivo principal es la extracción cuantitativa de las especies manteniendo su integridad, es decir, sin que sufran transformaciones entre ellas que puedan llevar a una identificación errónea posterior. En el caso de la hidrólisis enzimática, se utilizan enzimas como la proteasa XIV, pepsina, proteasa K o tripsina, con el fin de obtener los aminoácidos de las cadenas polipeptídicas que conforman las proteínas, por tanto, este tipo de hidrólisis será necesaria cuando el objetivo sea extraer los selenoaminoácidos de la muestra, como SeCys o SeMet. Habitualmente requiere de tiempos de extracción elevados (24-48 h), y condiciones de pH neutro para que las enzimas puedan actuar correctamente (Xiong *et al.*, 2023). Sin embargo, estos tiempos pueden reducirse si se hace uso de técnicas como la extracción enzimática asistida por microondas (MAE) (Guzmán *et al.*, 2009; Hinojosa *et al.*, 2009) o si se emplea una sonda de ultrasonidos (UAE). Esta última se basa en el principio de funcionamiento de la cavitación acústica o ultrasónica, generando ondas ultrasónicas de alta potencia y baja frecuencia que consiguen obtener extracciones cuantitativas (Siwek *et al.*, 2006). La hidrólisis enzimática utilizando la sonda de ultrasonidos ha sido empleada en la extracción de SeMet de levadura selenizada (Capelo *et al.*, 2004) y de hígado de pollo (Cabañero *et al.*, 2005a) con tiempos de extracción entre 30 s y 2 min, respectivamente, obteniéndose extracciones cuantitativas. La notable disminución del tiempo de extracción (30 s y 2 min frente a 24-48 h) puede atribuirse a la capacidad de disrupción celular de la sonda de ultrasonidos, que favorece el contacto entre las enzimas y los componentes celulares.

La hidrólisis ácida, que suele emplear ácido clorhídrico (HCl), y la básica, con hidróxido de tetrametilamonio (TMAH), se utilizan en menor medida debido a que son más agresivas al realizarse en condiciones de pH más extremas y conllevan el riesgo de producirse transformación de especies (B’Hymer & Caruso, 2006).

Por otro lado, la extracción con líquidos presurizados (PLE) también constituye otra opción para la extracción de los compuestos orgánicos de selenio con tiempos de extracción menores, aunque es menos utilizada. Se basa en el empleo de uno o varios disolventes a elevada temperatura y/o presión, sin llegar a alcanzar el punto crítico (Moscoso-Pérez *et al.*, 2008). Gómez-Ariza *et al.* (Gómez-Ariza *et al.*, 2004) extrajeron en 10 min con resultados cuantitativos las especies de selenio presentes de muestras de levaduras utilizando una mezcla de H_2O:MeOH 1:1 (v/v).

Finalmente, la extracción en fase sólida (SPE) o la microextracción en fase sólida (SPME), el muestreo de espacio de cabeza o la combinación de ambas técnicas (HS-SPME) son también, por su parte, métodos comúnmente empleados para la extracción de compuestos volátiles de selenio (B’Hymer & Caruso, 2006; Moreno-Martín *et al.*, 2021).

Una vez extraídas las especies de selenio de la muestra, para su determinación se emplean técnicas híbridas, es decir, una técnica de separación acoplada a un detector molecular o atómico de elevada sensibilidad y especificidad. Los acoplamientos más utilizados para la determinación de las especies de selenio son la cromatografía de líquidos de alta eficacia (HPLC), la cromatografía de gases (GC) y la electroforesis capilar (CE) acoplada a ICP-MS, ICP-OES, AFS, AAS o a la espectrometría de masas (MS).

De entre los anteriormente citados, el acoplamiento HPLC-ICP-MS es uno de los más frecuentemente empleados en estudios de especiación donde se consideran las especies de selenio no volátiles. Este acoplamiento es muy sencillo, ya que solamente es necesario acoplar la salida de la columna con el sistema de introducción de muestra en el ICP-MS, siendo clave escoger nebulizadores que sean compatibles (0.2-1.5 mL min^{-1}) con los flujos de las columnas de HPLC. Los mecanismos cromatográficos utilizados suelen ser intercambio iónico (IE), exclusión molecular (SEC) y fase inversa (RP) (B'Hymer & Caruso, 2006), que son comentados a continuación.

La cromatografía de intercambio iónico (IE) se basa en la capacidad de atracción por parte de los grupos cargados de la fase estacionaria hacia los iones de signo contrario de la fase móvil. Puede ser utilizada en dos modos de separación: intercambio aniónico o catiónico, ambos empleados en la especiación de selenio. En este sentido, entre las disoluciones reguladoras más comúnmente utilizadas para la separación de los compuestos de selenio destacan el fosfato (Chassaigne *et al.*, 2002), el citrato (Huerta *et al.*, 2003) y el formiato (Sloth & Larsen, 2000).

Sele *et al.* identificaron mediante esta técnica las especies de selenio orgánicas e inorgánicas presentes en salmones alimentados con dietas suplementadas en selenio (Sele *et al.*, 2018), Moreno-Martín *et al.* estudiaron las transformaciones de las distintas especies de selenio en plantas (Moreno-Martín *et al.*, 2020), Dong *et al.* evaluaron el efecto sobre especies de selenio de distintos cocinados aplicados a patatas enriquecidas en selenio, así como su bioaccesibilidad (Dong *et al.*, 2021) y, finalmente, Hu *et al.* estudiaron el efecto de la suplementación a distintas concentraciones de distintas especies orgánicas e inorgánicas de selenio en setas comestibles (Hu *et al.*, 2019).

La separación por cromatografía de exclusión por tamaño (SEC) se basa en las diferencias en el peso molecular de los compuestos de interés, siendo generalmente aplicada cuando se trata de biomoléculas de pesos moleculares comprendidos entre 10 y 1000 kDa. Sin embargo, no es una técnica de separación apropiada para la separación de compuestos de bajo peso molecular. Constituye una técnica muy interesante a la hora de determinar a qué tamaños de biomoléculas o tamaños proteicos se encuentra unido el selenio en extractos procedentes de muestras biológicas. Sin embargo, en la determinación de selenoproteínas presenta un uso limitado debido a su baja resolución.

Es frecuentemente utilizada como técnica preparativa en separaciones multidimensionales (Chan & Caruso, 2012; Guerra *et al.*, 2012).

La técnica SEC-ICP-MS se ha utilizado, por ejemplo, para estudiar la distribución de compuestos con selenio en suero de vaca (Palacios *et al.*, 2006), hígado de pollo (Cabañero *et al.*, 2005b), plantas (Montés-Bayón *et al.*, 2006; Wojcieszek & Ruzik, 2016), yogur enriquecido en selenio (Alzate *et al.*, 2007), proteínas de pescado (Fernández-Bautista *et al.*, 2022) y distintos órganos de aves marinas (El Hanafi *et al.*, 2022).

Finalmente, la cromatografía de fase inversa (RP) es probablemente la modalidad de HPLC más empleada, en la que la separación tiene lugar en función de la polaridad de los compuestos. En este sentido, los compuestos polares son los que menos se retienen, ya que tienen menos afinidad por la fase estacionaria y más por la fase móvil. El modo *cromatografía de par iónico en fase inversa* se utiliza para la separación de compuestos iónicos en el que se forma un par iónico entre el soluto y el contraión presente en la fase móvil polar. Para la especiación de selenio se pueden emplear varios agentes para formar el par iónico, siendo el ácido trifluoracético (TFA) y el ácido heptafluorobutanoico (HFBA) los más comunes.

Las limitaciones del acoplamiento de la cromatografía de fase inversa con el ICP-MS reside en los disolventes utilizados como fases móviles, ya que el ICP-MS no admite una concentración elevada de disolventes orgánicos, ya que afecta a la sensibilidad y estabilidad del plasma. De igual modo, trabajar en elución por gradiente puede provocar cambios de sensibilidad en el ICP-MS, dificultando la cuantificación. Sin embargo, el uso de nebulizadores de micro- y nano-flujos (0.2-4 μL min^{-1}) permite utilizar contenidos más elevados de disolvente orgánico en la fase móvil (Szpunar, 2005). Otra alternativa para poder trabajar con altas concentraciones de disolventes orgánicos, o incluso al 100 % de disolvente orgánico, es introducir oxígeno en el sistema para evitar la obstrucción de los conos de muestreo y separación por deposición de carbono, sumado al uso de conos de platino en lugar de los convencionales de níquel (Hinojosa *et al.*, 2006).

No solo se ha descrito en literatura la separación e identificación de las distintas especies orgánicas (SeMet, SeCys y SeMeSeCys) e inorgánicas (Se (IV) y Se (VI)) de selenio en distintas matrices mediante este mecanismo cromatográfico (RP) acoplado a ICP-MS (Pedrero *et al.*, 2007; Schram *et al.*, 2008; Vicente-Zurdo *et al.*, 2019), sino que además se ha empleado como una primera técnica de identificación de la selenoneína en sangre y orina humanas (Klein *et al.*, 2011; Kroepfl *et al.*, 2019), en la piel de ballenas beluga y en los glóbulos rojos de los inuit residentes en Nunavik (Achouba *et al.*, 2019), previo análisis a la espectrometría de masas molecular.

Por otro lado, las especies volátiles de selenio más abundantes en las muestras medioambientales y biológicas son los metilseleniuros: dimetilselenio ($(CH_3)_2Se$) y dimetildiselenio ($CH_3SeSeCH_3$), y generalmente son analizados mediante GC-ICP-MS (Pedrero & Madrid, 2009). Meija *et al.* utilizaron la técnica HS-SPME para preconcentrar los compuestos volátiles de selenio, así como sus análogos de azufre para ser posteriormente desorbidos y analizados por GC-ICP-MS, con el fin de contribuir al estudio del metabolismo del selenio en plantas *Brassica juncea* (Meija *et al.*, 2002).

Sin embargo, el acoplamiento GC-ICP-MS es más complejo que en el caso del HPLC-ICP-MS, pues se realiza a través de una interfase o línea de transferencia entre el GC y el ICP-MS. Esta suele estar fabricada de vidrio o cuarzo, ya que proporcionan una superficie inerte, minimizando la adsorción o la condensación de los analitos (B'Hymer & Caruso, 2006).

Además, el análisis de las especies no volátiles de selenio también se puede abordar mediante el acoplamiento GC-ICP-MS aplicando previamente una etapa de derivatización. Yang *et al.* emplearon bromuro de cianógeno (CNBr) como derivatizante para la determinación de SeMet, formando la especie volátil CH_3SeCN para su posterior análisis por GC con detección ICP-MS (Yang *et al.*, 2004). No obstante, el uso de métodos basados en GC para especies de selenio no volátiles requiere de largos tiempos de ensayo y no proporciona buenos resultados en el caso de los oligopéptidos. Por lo tanto, la técnica más comúnmente elegida para el estudio de selenoaminoácidos y selenopéptidos es el acoplamiento HPLC-ICP-MS.

Finalmente, también es necesario mencionar el acoplamiento CE-ICP-MS donde el principal reto reside en la unión del capilar electroforético al nebulizador del ICP-MS, lo que presenta algunas dificultades. En la forma habitual de la CE, un capilar de sílice fundida lleno de tampón tiene ambos extremos sumergidos o en contacto físico con dos depósitos de disolución, lo que proporciona un alto potencial eléctrico a través de estos. El primer problema se presenta al mantener esa conexión eléctrica en ambos extremos del capilar e introducir el flujo en el nebulizador, produciendo un aerosol uniforme para el detector. Otro de los problemas es el caudal bajo al que se trabaja en CE respecto al que emplea el ICP-MS (alrededor de 1 μL min^{-1} frente a mL min^{-1}, respectivamente), lo que requiere un nebulizador de bajo flujo para que la interfaz mantenga una alta eficacia de transporte y suministre una gran cantidad de analito al plasma. Con diferentes diseños de interfases se han logrado solventar los problemas derivados de este tipo de acoplamiento (B'Hymer & Caruso, 2006).

La CE posee buenas características para la separación de especies, como alta resolución, poca cantidad de muestra necesaria (inferior a 1 ng), muy buenos límites de detección (niveles de pg) y la posibilidad del análisis de especies relativamente lábiles (Pedrero & Madrid, 2009).

La técnica CE-ICP-MS se ha empleado para la determinación de especies orgánicas e inorgánicas de selenio (SeMet, SeCys, Se (IV) y Se (VI)) en extractos proteicos de frutos secos (Kannamkumarath *et al.*, 2005), en extractos de levadura (Mounicou *et al.*, 2002), pero también para la separación quiral de los isómeros L- y D- de la SeMet, aunque necesitan previamente ser derivatizados (Day *et al.*, 2002).

Análisis de confirmación mediante espectrometría de masas molecular

Las técnicas anteriormente comentadas y que se basan en sistemas cromatográficos acoplados con el ICP-MS, proporcionan una adecuada sensibilidad y selectividad para la especiación de selenio en muestras biológicas. Sin embargo, en ocasiones la especie de selenio de interés puede coeluir con alguna otra especie de selenio. Es por ello que, en estos casos, es necesario emplear diferentes mecanismos de separación cromatográfica acoplados a ICP-MS para un mismo análisis de forma que se pueda obtener una identificación correcta de las especies. Aun así, para lograr una identificación inequívoca, es necesario el posterior análisis mediante sistemas de detección basados en la espectrometría de masas en tándem (MS/MS). Para ello se emplean técnicas de ionización suave, como la ionización por electrospray (ESI) o la ionización láser asistida por matriz (MALDI), y analizadores tanto de baja resolución (cuadrupolar), como de alta resolución (analizador de tiempo de vuelo, TOF, u Orbitrap). En este último caso, aunque su alto coste sea su principal limitación, los analizadores de alta resolución ofrecen una elevada sensibilidad y exactitud de masa. Estas técnicas son indispensables sobre todo cuando no se dispone de patrones de todos los compuestos de selenio de interés.

3.2. Determinación del contenido total de mercurio y sus especies

Análisis del contenido total de mercurio

Para la determinación del contenido total de mercurio en una muestra también se puede emplear como tratamiento de muestra la digestión por vía húmeda asistida por un horno microondas, como en el caso del selenio (apartado 3.1). Para la detección del mercurio en disolución se pueden emplear distintas técnicas analíticas como son ICP-MS, AFS y AAS, previa generación del vapor frío (CV-AFS y CV-AAS), y AAS con cámara de grafito (GF-AAS). Además, el análisis de mercurio se puede realizar también con el analizador directo de mercurio (DMA) que permite dicho análisis sin un tratamiento previo de la muestra.

Las disoluciones procedentes del proceso de digestión, las cuales, al igual que en el caso del selenio, se han tratado con mezclas de ácidos oxidantes (HNO_3:H_2O_2), contienen todo el mercurio en forma de Hg^{2+}, la única especie capaz de generar el vapor de mercurio atómico (Ruiz, 2016). Aunque las técnicas CV-AFS y CV-AAS son ampliamente utilizadas en la actualidad (Ruiz, 2016; Pérez *et al.*, 2020; Kozaki *et al.*, 2021), ICP-MS y GF-AAS presentan mayor sensibilidad, aunque la instrumentación requerida sea más compleja.

A pesar de las importantes ventajas que presenta la técnica ICP-MS y que han sido comentadas anteriormente (apartado 3.1), uno de los principales inconvenientes evidenciados en la determinación de mercurio por esta técnica es su efecto memoria. Sin embargo, esta aparente limitación se puede solventar fácilmente empleando una disolución diluida de oro (Lemes & Wang, 2009) y, en el caso de querer determinar también oro en la muestra, se pueden utilizar agentes complejantes que contengan azufre como L-cisteína o tiourea (Chen *et al.*, 2000), ya que tienen una gran capacidad de complejar el mercurio, y de esta forma lograr arrastrar ese mercurio residual que se va quedando en el sistema, reduciéndose el efecto memoria anteriormente comentado.

Finalmente, el DMA constituye la única opción en la que se puede llevar a cabo el análisis directo de la muestra sin previo tratamiento, ya sea esta sólida o líquida. El procedimiento de análisis consiste en el secado de la muestra y su posterior descomposición térmica, seguido de la reducción catalítica del mercurio. A continuación, el vapor de mercurio es atrapado en una amalgama de oro, para posteriormente, por desorción térmica, ser analizado por absorción atómica a una longitud de onda de 253.65 nm (Zhang & Zhou, 2020). En la **Figura 12** se esquematiza el equipo DMA, donde se puede observar el proceso detallado anteriormente.

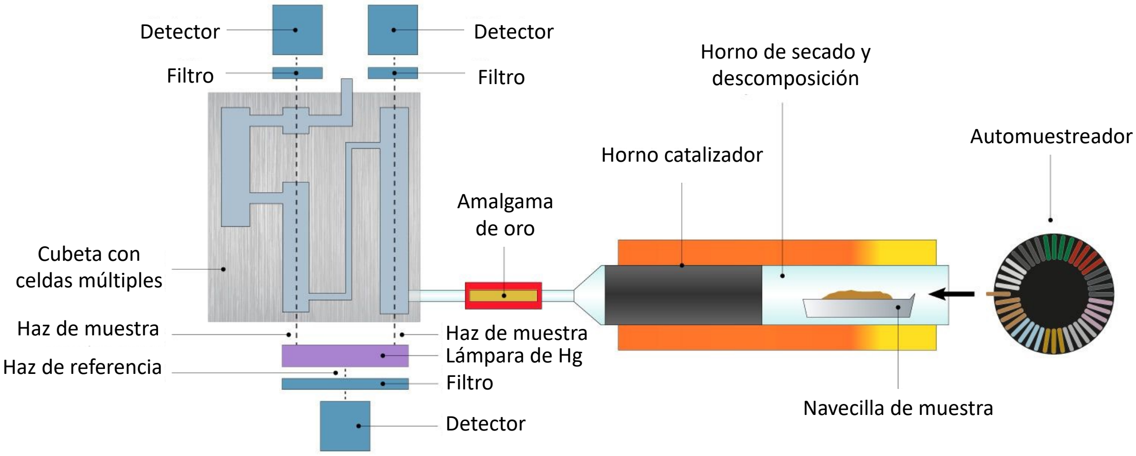


Figura 12. Esquema del DMA-80 evo (adaptado del catálogo de Milestone: milestonesrl.com).

De entre todas las técnicas de determinación del contenido total de mercurio mencionadas, el DMA parece ser la más recomendada debido a las ventajas que presenta, ya que al introducir la muestra en su totalidad en el sistema, se minimizan problemas de pérdida del analito, los tiempos de análisis son cortos (en torno a 5 min por muestra), la determinación es selectiva, relativamente sencilla, no requiere del empleo de gases caros, únicamente de aire comprimido, y los límites de detección alcanzados se sitúan en 0.005 ng de mercurio (Zhang & Zhou, 2020).

Identificación y análisis de las especies de mercurio

Una de las consideraciones más importantes en la extracción de las especies de mercurio presentes en una muestra son las posibles pérdidas de analitos que pueden darse debido a la adsorción de estos en la superficie del material empleado, y a la elevada volatilidad de estos compuestos. La utilización de viales topacios de vidrio para la preparación y el almacenamiento de las muestras minimiza la adsorción del mercurio a las paredes, así como la fotodegradación (Kasper *et al.*, 2015). Además, como en cualquier análisis de especiación, hay que tener especial cuidado con las posibles transformaciones entre especies durante el proceso de extracción de las mismas.

El método de extracción originalmente más utilizado fue el propuesto por Westöö (Westöö, 1967) y consiste en una hidrólisis ácida con HCl para liberar en disolución todas las formas químicas de mercurio de la muestra. Los cloruros neutros de mercurio formados se extraen con benceno, se vuelven a extraer con una disolución acuosa de L-cisteína como complejos de mercurio-cisteína y, finalmente, se extraen de nuevo con benceno mediante acidificación. Las modificaciones más recientes implican la sustitución del benceno por otros disolventes menos tóxicos como el tolueno o el diclorometano y la posterior extracción de los compuestos de mercurio a la fase acuosa mediante L-cisteína o tiosulfato sódico (Bloom, 1989; Thibaud & Cossa, 1989; Hempel *et al.*, 1992).

Además de la hidrólisis ácida, también se puede emplear la hidrólisis básica, que hace uso de bases como hidróxido potásico (KOH) o hidróxido de tetrametilamonio (TMAH). Sin embargo, el empleo de este método de extracción está condicionado a posibles pérdidas de analito, así como a la metilación del mercurio, generando $MeHg^+$ durante el proceso de extracción, dando lugar a resultados erróneos (Liang *et al.*, 2000; Dietz *et al.*, 2001; Qvarnstrom & Frech, 2002).

Tanto la hidrólisis ácida como la hidrólisis básica se pueden utilizar combinadas con el empleo de la MAE o la UAE, para favorecer la extracción y disminuir los tiempos de esta etapa (Ramalhosa *et al.*, 2001).

Otros métodos de extracción frecuentemente utilizados son la SPME y la HS-SPME para la extracción de los compuestos de mercurio Hg^{2+}, $MeHg^+$ y $EtHg^+$, previa derivatización con tetraetilborato de sodio ($NaBC_2H_5)_4$) o tetrafenilborato de sodio ($NaB(C_6H_5)_4$)). La adición de NaCl reduce la solubilidad de los analitos (efecto *salting-out*), favoreciendo su volatilización. Seguidamente, los analitos se extraen utilizando una fibra de polidimetilsiloxano (PDMS) (Li *et al.*, 2020).

Una de las técnicas de extracción más novedosas, y que está incrementando su popularidad y su uso en los últimos años, es la extracción en fase sólida magnética

(MSPE). Consiste en dispersar nanopartículas magnéticas funcionalizadas en matrices acuosas donde se adsorben los analitos mediante agitación o sonicación. Posteriormente, se aíslan de la matriz mediante un campo magnético externo. En el caso del mercurio, para la funcionalización de estas nanopartículas se emplea el grupo funcional mercapto o tiol (-SH). Este método de extracción se ha utilizado para la extracción selectiva y cuantitativa de Hg^{2+} y $MeHg^{+}$ de muestras de agua empleando nanopartículas magnéticas de $Fe_3O_4@SiO_2$ modificadas con γ-mercaptopropil trimetoxisilano (γ-MPTS). Para la desorción del $MeHg^{+}$, se utilizó una disolución de 1.5 M HCl (0.01 % tiourea), y para la desorción del mercurio total (Hg^{2+} + $MeHg^{+}$), una disolución de 1.5 M HCl (3 % tiourea) (Ma *et al.*, 2016). Mediante diferencia de concentraciones se obtienen además los contenidos en Hg^{2+}. Este método también fue aplicado para realizar el análisis de especies de Hg^{2+}, $MeHg^{+}$ y $C_6H_5Hg^{+}$ en el músculo de pecados (Zhu *et al.*, 2017).

Una vez extraídas las especies de mercurio de la muestra, para la separación y determinación de las mismas son comúnmente empleadas las técnicas cromatográficas HPLC y GC, además de la técnica no cromatográfica CE, acopladas a ICP-MS, AFS o AAS. También se pueden emplear CV-AFS y CV-AAS para llevar a cabo estudios de especiación de mercurio, pero en este caso se necesita una etapa previa de reducción de las especies a mercurio elemental. En el caso de reducir el Hg^{2+} a Hg^{0} se necesitan unas condiciones reductoras con $SnCl_2$ (3 % w/v en HCl) (Tirtom *et al.*, 2008; Ruiz-de-Cenzano *et al.*, 2016). Por el contrario, los compuestos orgánicos de mercurio se descomponen para ser reducidos mediante radiación UV y agentes reductores, como L-cisteína o $NaBH_4$/KBH_4 (Torres *et al.*, 2009; Zhang *et al.*, 2011; Chelegão *et al.*, 2016). La cuantificación de las especies orgánicas de mercurio se obtiene mediante diferencia entre el contenido total de mercurio y el contenido de Hg^{2+}.

Como se comentó anteriormente, el acoplamiento HPLC-ICP-MS es muy simple debido a que no se precisa de una interfase tan compleja como, por ejemplo, en el caso del GC-ICP-MS. Además, tampoco es necesaria la etapa previa de derivatización de la muestra. Generalmente, se suele emplear la cromatografía líquida de fase inversa, generalmente columnas C18, utilizando fases móviles como L-cisteína y 2-mercaptoetanol debido a que son ligandos tiólicos para el mercurio. Estas fases se emplean en combinación debido a que la utilización de L-cisteína únicamente no es suficiente para separar las especies de mercurio y el empleo de solo 2-mercaptoetanol alarga mucho los tiempos de retención. También pueden ser utilizadas otras combinaciones de reactivos en la fase móvil como 2-mercaptoetanol, acetato de amonio (en concentración 0.06 mol L^{-1}, que permite mantener un pH favorable de 6.7) y MeOH, este último en pequeña proporción (4-5 % v/v) para aumentar la sensibilidad en la detección del mercurio, así como una disminución del tiempo de retención de sus especies, ya que los complejos de L-cisteína y 2-mercaptoetanol de las especies de mercurio son bastante hidrófobos (de Souza *et al.*, 2010; Rodrigues *et al.*, 2010).

Sin embargo, otros métodos cromatográficos, como la cromatografía en fase normal (NP) requieren altos contenidos de disolventes orgánicos en las fases móviles empleadas (Moreno *et al.*, 2013), que no soporta el ICP-MS en su modo convencional, aunque puede ser adaptado para ello utilizando conos de platino en vez de níquel y utilizando una corriente de oxígeno, como ya se explicó anteriormente en el apartado 3.1.

Por otro lado, si consideramos la GC como mecanismo de separación, los primeros estudios de especiación de mercurio, los detectores más empleados fueron el de captura electrónica (ECD) y de ionización en llama (FID) (Bin *et al.*, 1998; Liu & Lee, 1999). Con el paso de los años, estos detectores se han ido sustituyendo por otros con mayor sensibilidad como el de fluorescencia atómica y los detectores de masas. A día de hoy, las configuraciones más empleadas son GC-AFS y GC-ICP-MS.

La fluorescencia atómica constituye una de las técnicas de determinación de mercurio más sensibles y selectivas existentes. El acoplamiento GC-AFS se puede realizar mediante un método de derivatización *on-line* de las especies (vapor frío) (Díez & Bayona, 2002) o mediante pirólisis (Gómez-Ariza *et al.*, 2004) y para la introducción de la muestra se puede emplear una reacción de alquilación o inyectar directamente la fase orgánica extraída durante la extracción de las especies de mercurio (Horvat *et al.*, 1993; Hammerschmidt & Fitzgerald, 2001; Ebdon *et al.*, 2002; Huang, 2005).

El acoplamiento GC-ICP-MS, por su parte, requiere de una interfase adecuada más compleja, como ya se comentó para el caso de la especiación de selenio mediante esta técnica en el apartado 3.1. Además de la alta sensibilidad y especificidad que presenta, esta técnica acoplada permite además obtener información sobre las posibles transformaciones de las especies de mercurio durante los procesos de pretratamiento de la muestra mediante la medida de relaciones isotópicas (Hintelmann *et al.*, 1997; Tu *et al.*, 2000; Pietilä *et al.*, 2014).

Por último, se puede afirmar que la técnica de separación CE es menos empleada que las anteriores, debido principalmente al elevado límite de detección que presenta, debido al pequeño volumen de inyección. Sin embargo, su acoplamiento a detectores tan sensibles como el ICP-MS, así como empleando etapas de preconcentración en la muestra, pueden resolver ese problema (Da Rocha *et al.*, 2000; Lee & Jiang, 2000).

La separación de los compuestos inorgánicos y orgánicos de mercurio mediante CE es complicada por su relativo poco poder de ionización y que en medios acuosos tiene una carga efectiva cercana a cero. Mientras que las especies inorgánicas de mercurio presentan una solubilidad apreciable en agua, la solubilidad de los compuestos orgánicos de mercurio en agua es limitada, siendo necesario el uso de disolventes orgánicos, como el metanol. Por tanto, se precisa de una etapa previa de derivatización, obteniendo complejos cargados negativamente, resolviendo así el problema de la carga nula (Kubán *et al.*, 2007).

4. Técnicas (bio)analíticas para el estudio *in vitro* del efecto protector de las especies de selenio frente a la neurotoxicidad inducida por el mercurio

En los últimos años se han desarrollado una gran variedad de técnicas para la evaluación de la neurotoxicidad inducida por el mercurio y el estudio del correspondiente efecto protector del selenio, como el ICP-MS (HPLC-ICP-MS, LA-ICP-MS, SP-ICP-MS y SC-ICP-MS), la microscopia electrónica de transmisión (TEM), la espectrometría de masas molecular, la citometría de flujo, los ensayos de capacidad antioxidante (TBARS y DPPH), la técnica western blotting y la electroforesis en gel, entre otros. En este sentido, se ha potenciado el empleo de modelos *in vitro*, como por ejemplo cultivos celulares, en concreto de líneas celulares de fenotipo neuronal, combinadas con ensayos de viabilidad celular (citometría de flujo, MTT, etc.) (Kim *et al.*, 2005; Hossain *et al.*, 2021; Li *et al.*, 2022).

Además, como parte de estos estudios, es imprescindible el empleo de técnicas analíticas extremadamente sensibles capaces de identificar y cuantificar niveles traza de metales y metaloides en cultivos celulares. En este sentido, la técnica ICP-MS y en concreto en modo de célula individual o *single-cell* ICP-MS (SC-ICP-MS) son de especial relevancia en los estudios de neurotoxicidad, ya que permiten determinar la acumulación de agentes neurotóxicos y neuroprotectores a nivel unicelular en modelos celulares, teniendo en cuenta la variabilidad existente dentro de las poblaciones celulares (Rembach *et al.*, 2013; Krebs *et al.*, 2014; Dahlberg *et al.*, 2015; Ramos *et al.*, 2015; Korvela *et al.*, 2016; Ramos *et al.*, 2016; De Jesus & Arrouda, 2020).

4.1. Aplicación de estudios *in vitro* mediante modelos celulares

El mercurio está considerado como un elemento neurotóxico, y los estudios de neurotoxicidad *in vitro* basados en modelos celulares constituyen una primera aproximación en la investigación de posibles efectos neurotóxicos, contribuyendo al esclarecimiento de los posibles mecanismos de acción y toxicidad.

Los ensayos *in vitro*, a diferencia de los *in vivo*, ofrecen la posibilidad de controlar el tiempo de exposición y estudiar los mecanismos en condiciones controladas y aisladas, proporcionando información básica sobre las alteraciones celulares y moleculares originadas por la presencia de este tóxico (Barbosa *et al.*, 2015). Además, otras de las grandes ventajas que presentan son la ausencia de problemas éticos (excepto en el caso de emplear células embrionarias humanas), la facilidad en su manipulación y protocolos, los costes reducidos debido a la vida infinita de las líneas celulares, la corta duración de los experimentos, las interacciones directas neurotóxico-célula, las mediciones exactas de las respuestas celulares a los neurotóxicos y la posibilidad de manipulación del entorno celular (Harry *et al.*, 1998; Kumaria & Tolias, 2008; Barbosa *et al.*, 2015). Bien es cierto que poseen algunos inconvenientes, entre los cuales destacan la falta de metabolización y, por tanto, la imposibilidad de identificar metabolitos de interés, así como las condiciones poco realistas de los experimentos, ya que el compuesto neurotóxico se encuentra aislado e inalterado, sin interacción con otros elementos (Kumaria & Tolias, 2008; Barbosa *et al.*, 2015; Lopez-Suarez *et al.*, 2022).

Por su parte, los modelos animales empleados al realizar ensayos *in vivo* carecen de muchas características humanas esenciales, ya que, por ejemplo, el cerebro humano tiene una fisiología extremadamente compleja y distinta a la de otros cerebros animales.

Además, suelen ser ensayos más caros y su uso frecuentemente plantea problemas éticos (Barbosa *et al.*, 2015).

Los modelos *in vitro* más comunes son cultivos neuronales primarios, líneas celulares inmortalizadas derivadas de células neuronales o gliales, co-cultivos, sistemas subcelulares como mitocondrias aisladas y modelos de la barrera hematoencefálica (Balbuena *et al.*, 2010; Barbosa *et al.*, 2015; Chhiber *et al.*, 2020; Cao, 2022). En este sentido, es importante seleccionar la línea celular más adecuada para cada estudio a la hora de utilizar modelos *in vitro*.

Una de las líneas celulares más utilizadas para los estudios de neurotoxicidad es la SH-SY5Y, una línea celular de neuroblastoma humano catecolaminérgico, que puede utilizarse como línea celular humana inmortalizada o diferenciada en un fenotipo específico mediante el uso de agentes de diferenciación como el ácido retinoico (AR), 12-o-tetradecanoil-forbol-13-acetato (TPA) o AMP cíclico dibutirílico (dBcAMP) (Kim *et al.*, 2005; Sarkanen *et al.*, 2007; Faria *et al.*, 2016; Sala *et al.*, 2016; Heusinkveld *et al.*, 2017; Xicoy *et al.*, 2017; Branca *et al.*, 2018; Fernández-Bautista *et al.*, 2023; Vicente-Zurdo *et al.*, 2023).

Kim *et al.* emplearon este modelo celular, SH-SY5Y, para el estudio de los mecanismos del $MeHg^+$ en la alteración de la expresión de las selenoproteínas, en concreto, de la SelW (Kim *et al.*, 2005). Por su parte, Vicente-Zurdo *et al.* utilizaron, aparte de la línea celular SH-SY5Y, la línea celular de neuroblastoma de ratón Neuro-2a, para evaluar la actividad neuroprotectora de las nanopartículas de selenio y de las especies orgánicas de selenio SeMet, SeMeSeCys y $SeCys_2$ (Vicente-Zurdo *et al.*, 2023). Finalmente, Fernández-Bautista *et al.* evaluaron el efecto protector de las nanopartículas de selenio, así como de las especies orgánicas de selenio, SeMet y SeMeSeCys, frente a la neurotoxicidad inducida por el $MeHg^+$, empleando la misma línea celular (SH-SY5Y) (Fernández-Bautista *et al.*, 2023).

Otras líneas celulares neuronales de origen animal empleadas como modelos *in vitro* en este tipo de estudios son MES23.5, producto de la fusión somática de células del mesencéfalo embrionario de rata y una línea celular de neuroblastoma-glioma, y N27, línea celular inmortalizada derivada de neuronas mesencefálicas fetales de rata TH-positivas (Heusinkveld *et al.*, 2017).

Por otro lado, también se han empleado líneas celulares no neuronales para estudios de neurotoxicidad, como por ejemplo HeLa (células de carcinoma de ovario humano inmortalizado), HepG2 y HepaRG (líneas celulares hepáticas), HEK293 (células de riñón embrionario humano inmortalizadas) y PC12 (línea celular derivada de unfeocromocitoma de la médula suprarrenal de las ratas) (Cetin *et al.*, 2022). En concreto, esta última, la línea celular PC12, es ampliamente utilizada para este tipo de estudios ya que, aunque no está aislada de un tumor neuronal, presenta varias características de las neuronas dopaminérgicas maduras (Balbuena *et al.*, 2010; Schmidt *et al.*, 2017). Se han utilizado para investigar los efectos protectores del selenio frente a la neurotoxicidad inducida por mercurio (Hossain *et al.*, 2021), el arsénico (Rahman *et al.*, 2018) y el cadmio (Slanzi *et al.*, 2020).

Alternativamente, los estudios *in vitro* basados en linajes neuronales derivados de células madre humanas o precursores neuronales humanos, constituyen modelos neuronales más complejos y funcionalmente activos. Las células madre pueden experimentar diferenciación y maduración de forma similar a los modelos animales. En este sentido, las células madre embrionarias humanas (ESC, del inglés *Embryonic Stem Cells*) y las células madre pluripotentes inducidas (iPSCs, del inglés *induced Pluripotent Stem Cells*) se utilizan ampliamente como modelos *in vitro* para evaluar también la neurotoxicidad (Slanzi *et al.*, 2020). En concreto, las iPSC son uno de los modelos celulares más empleados para mimetizar la complejidad y funcionalidad de las estructuras neurológicas *in vivo*, ya que son relativamente fáciles de recolectar y tienen en cuenta las variaciones genéticas entre pacientes humanos (Xie *et al.*, 2020), además de poder evitarse los aspectos éticos, a diferencia de lo que ocurre con el uso de las ESC humanas (Prince *et al.*, 2019).

4.2. Evaluación de la viabilidad celular

La viabilidad celular es un parámetro utilizado para estudiar la citotoxicidad *in vitro* de los elementos o compuestos de interés. Entre los ensayos de viabilidad celular más comúnmente empleados destacan el método colorimétrico del bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) y la citometría de flujo.

El ensayo MTT, esquematizado en la **Figura 13**, se basa en la reducción de sales de tetrazolio por células metabólicamente activas, seguida de una medición colorimétrica. En este ensayo, las enzimas mitocondriales de las células vivas reducen el reactivo MTT, una sal de tetrazolio soluble en agua de color amarillo, dando lugar a cristales de color violeta de un compuesto denominado formazán, que son insolubles en agua. A continuación, estos cristales de formazán se solubilizan en dimetilsulfóxido (DMSO) y se mide su absorbancia espectrofotométricamente a 570 nm, estando esta directamente relacionada con el número de células viables presentes en la muestra (Ghasemi *et al.*, 2021).

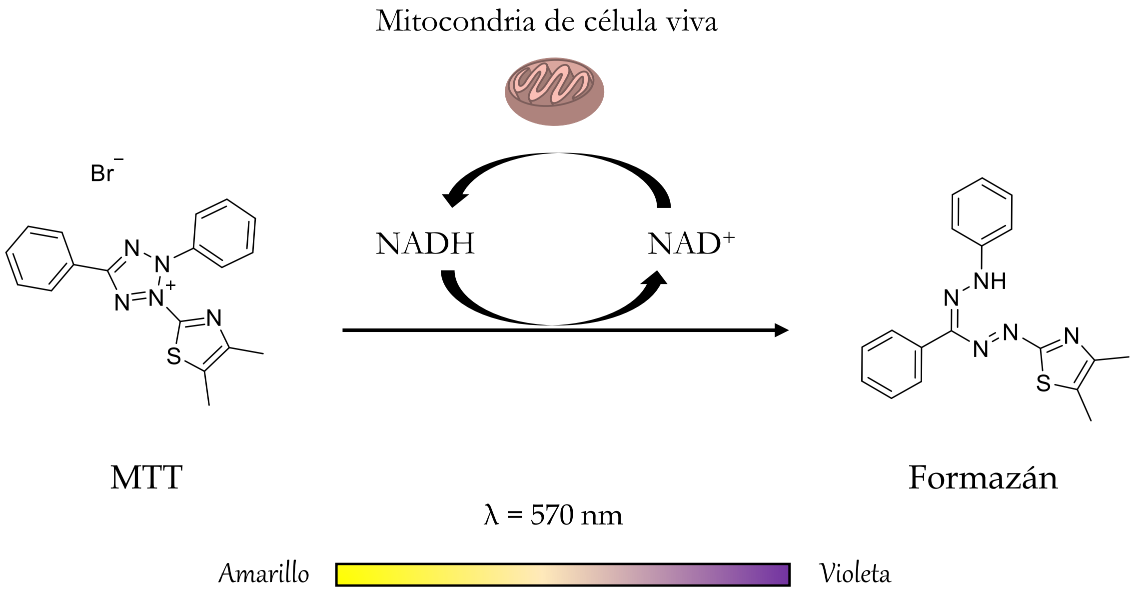


Figura 13. Esquema de reacción del ensayo de viabilidad celular MTT.

Este ensayo se ha utilizado durante décadas como una herramienta rápida y sencilla para estudiar la viabilidad celular, la citotoxicidad de fármacos y la actividad mitocondrial/metabólica de la célula (Stockert *et al.*, 2018). Sin embargo, presenta algunos inconvenientes importantes como son las interferencias químicas, que conducen a resultados inexactos, y limitaciones en la detección de un número bajo de células. Además, el proceso de conversión a cristales de formazán es dependiente de la actividad metabólica y del número de mitocondrias presentes en la célula. Así, una disminución de la concentración intracelular de glucosa, así como células más pequeñas con menor número de mitocondrias, dará lugar a una disminución de la cantidad de cristales de formazán (Van Tonder *et al.*, 2015).

Por su parte, la citometría de flujo es una potente técnica que proporciona información sobre diferentes parámetros de células individuales de cultivos en solución basándose en sus características fluorescentes o de dispersión de la luz. Esta técnica, que es más compleja, aporta resultados más fiables que el ensayo MTT para la evaluación de la viabilidad celular. El ensayo correspondiente emplea fluorocromos, como el yoduro de propidio (PI) que, debido a su hidrofobicidad y su alta masa molecular, únicamente es capaz de penetrar en las células muertas, cuya membrana está rota, y se une a los ácidos nucleicos mediante intercalación en sus bases, generándose compuestos fluorescentes. Utilizando un citómetro de flujo se pueden distinguir las células muertas, que presentarán una alta fluorescencia de PI, de las vivas, con baja fluorescencia de PI. La fluorescencia que presenta el PI en su unión a células no viables se caracteriza por tener una longitud de onda de excitación de 535 nm y de emisión de 615 nm, poseyendo por tanto fluorescencia roja (Mckinnon, 2019; Sathiya Kamatchi *et al.*, 2020).

4.3. Espectrometría de masas con plasma de acoplamiento inductivo (ICP-MS) como herramienta para el estudio de la acumulación de metales/metaloides en células. *Single-cell* ICP-MS

El enfoque convencional para determinar la acumulación de metales/metaloides en cultivos celulares implica una digestión ácida de los mismos, una vez han sido expuestos al elemento traza de interés durante un tiempo determinado, y su posterior análisis mediante ICP-MS. Sin embargo, el principal inconveniente de este procedimiento reside en que la información que nos proporciona es incompleta, ya que solo se obtiene un valor medio de concentración de analito acumulado por la población celular correspondiente, sin tener en cuenta la enorme variabilidad que existe dentro de dicha población celular (Liu *et al.*, 2019; Corte-Rodríguez *et al.*, 2020).

Esta heterogeneidad celular, junto con la necesidad de investigar y elucidar mecanismos complejos en sistemas biológicos, han llevado al desarrollo de técnicas de análisis de elementos traza a nivel de células individuales, como es el caso de la técnica SC-ICP-MS. El análisis mediante SC-ICP-MS abre nuevas perspectivas y posibilidades en los estudios de interacción de las células con metales/metaloides, fármacos e incluso nanomateriales (Meyer *et al.*, 2018; Amais *et al.*, 2020; Gómez-Gómez *et al.*, 2020; Turiel-Fernández *et* *al.*, 2021). Del mismo modo, el estudio de las diferencias célula a célula en la resistencia al estrés y/o la captación de elementos puede ser un aspecto clave a la hora de evaluar la neurotoxicidad o el resultado de una terapia específica (Montes-Bayón *et al.*, 2022). Aparte de la principal ventaja que ofrece, proporcionar información sobre la heterogeneidad celular en cuanto a términos de acumulación de analito dentro de una misma población, también destacan la mínima preparación de muestra necesaria y su elevada sensibilidad, permitiendo la cuantificación de metales/metaloides disueltos y nanopartículas inorgánicas en células individuales a niveles de ultra-traza (Mavrakis *et al.*, 2019; Suárez-Oubiña *et al.*, 2023).

La posibilidad de detectar "objetos" individuales (entendiéndose como objetos individuales células o partículas) mediante ICP-MS fue descrita por primera vez para nanopartículas por Degueldre *et al.* (Degueldre & Favarger, 2003, 2004; Degueldre *et al.*, 2006a, 2006b). Este modo de medida se conoce como *single-particle* ICP-MS (SP-ICP-MS) y permite la identificación de micro/nanopartículas en matrices complejas y la determinación de parámetros cuantitativos como la concentración en masa y en número, a niveles de concentración en masa de hasta ng L^{-1}, así como el tamaño medio y la distribución de tamaño de las dispersiones de nanopartículas.

La técnica SC-ICP-MS sigue los mismos principios que la técnica SP-ICP-MS. Sin embargo, en este caso la información que se puede obtener está relacionada con la cantidad de masa de analito que acumula una célula individual y el número de células que acumulan un determinado analito. Esto es posible ya que las células se transportan en pequeñas gotas generadas por nebulización al plasma, donde se vaporizan, atomizan e ionizan. Cada célula produce una nube de iones de analito que es detecta como un pulso o evento individual por encima de la línea base (**Figura 14**). En este sentido, la frecuencia o número de eventos registrados es proporcional al número de células que han acumulado el analito de interés, y la intensidad de los eventos está relacionada con la masa de analito acumulada en cada célula individual (Meyer *et al.*, 2018). Para que esto se cumpla, es necesario introducir los cultivos celulares lo suficientemente diluidos para evitar la detección de eventos múltiples en un solo pulso, haciendo que cada evento corresponda con una única célula (Corte-Rodríguez *et al.*, 2017). Finalmente, la señal de la línea base corresponde a especies disueltas no acumuladas por las células o a interferencias espectrales relacionadas con el analito.

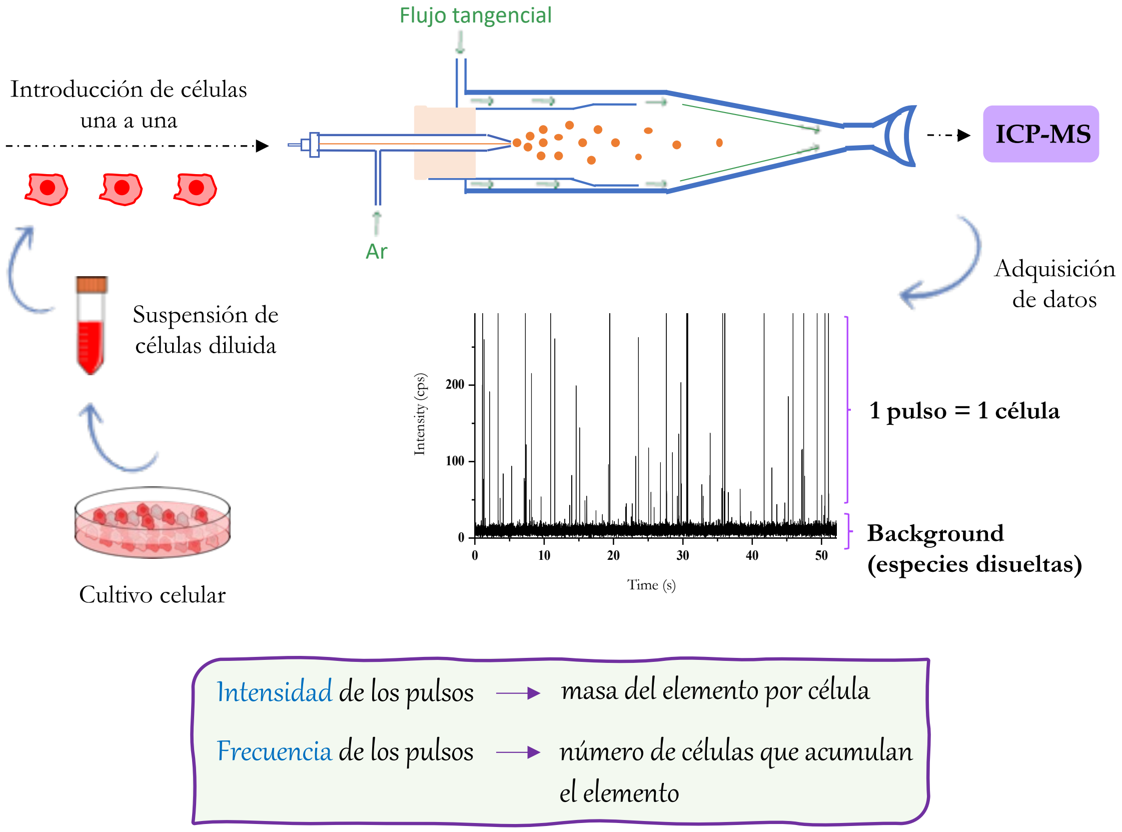


Figura 14. Esquema del procedimiento experimental general para el análisis cuantitativo elemental en SC-ICP-MS.

Dos parámetros clave para poder llevar a cabo análisis mediante SP-ICP-MS o SC-ICP-MS es la frecuencia de adquisición de datos o *dwell time,* que está relacionado con el tiempo empleado en adquirir datos a una masa específica, y el tiempo de asentamiento o *settling time*, es decir, el tiempo empleado para la estabilización del cuadrupolo una vez que ha cambiado de una masa a otra (Abad-Álvaro *et al.*, 2016). Inicialmente, los *dwell times* se limitaban al rango de los milisegundos (entre 3 y 10 ms, con concentraciones en número de partícula inferiores a 10^8 part L^{-1}), permitiendo la monitorización de un solo elemento/isótopo por partícula, registrándose los eventos como pulsos individuales (Laborda *et al.*, 2014). Actualmente, con el desarrollo de instrumentos de cuadrupolo más sofisticados, se están empleando *dwell times* en el rango de los microsegundos (entre 10 y 100 µs) (Hineman & Stephan, 2014; Montaño *et al.*, 2014). Dado que la duración de cada evento de partículas en el detector es de unos 300-500 µs (Olesik & Gray, 2012; Hineman & Stephan, 2014), cuando se utilizan *dwell times* iguales o inferiores a 100 µs, los eventos de partículas pueden registrarse como señales transitorias (eventos de partículas transitorios) en lugar de como pulsos, aunque se pueden monitorizar hasta dos elementos/isótopos por partícula (Montaño *et al.*, 2014). En este sentido, los analizadores de masas TOF permiten una adquisición muy rápida de todo el espectro de masas, proporcionando una capacidad multielemental en los análisis (Montes-Bayón *et al.*, 2022).

Por otro lado, para poder llevar a cabo los análisis mediante SC-ICP-MS es necesario que las células individuales lleguen intactas al plasma. Teniendo esto en cuenta, uno de los principales retos de la técnica SC-ICP-MS es el desarrollo de sistemas de nebulización lo suficientemente poderosos para que permitan mantener la integridad celular y transferir el mayor número de células al plasma, es decir, obtener una alta eficiencia de transporte. El transporte ineficaz de las células en SC-ICP-MS, debido a su mayor tamaño en comparación con las nanopartículas en SP-ICP-MS, es uno de los principales cuellos de botella de la técnica. Con los sistemas convencionales de nebulización, que suelen consistir en una cámara de pulverización ciclónica, se consiguen eficiencias de transporte extremadamente bajas, incluso inferiores al 1 % (Groombridge *et al.*, 2013; Corte-Rodríguez *et al.*, 2020).

Los sistemas más habituales utilizados para la introducción de células individuales al plasma son: (1) sistemas de introducción de muestras basados en nebulización neumática para introducir suspensiones celulares líquidas o (2) ablación por láser para generar un aerosol de las células previamente fijadas en superficies sólidas (Montes-Bayón *et al.*, 2022).

Sin embargo, en los últimos años se han desarrollado sistemas que garantizan una alta eficiencia de transporte de células intactas al ICP-MS, basados en la combinación de micronebulizadores neumáticos con cámaras de pulverización de consumo total, que poseen una entrada doble de gas de reposición colocada específicamente para proporcionar un flujo tangencial a las paredes de la cámara de nebulización, evitando la colisión celular y la deposición en las paredes (**Figura 14**). En este sentido, ya existen sistemas de introducción de muestra comerciales, como el Sistema de Introducción Celular de Alta Eficacia (HECIS, del inglés *High Efficiency Cell Introduction System*) (Groombridge *et al.*, 2013) o el Sistema de Introducción Unicelular de Alta Sensibilidad de Glass Expansion, mediante los cuales se han registrado eficiencias de transporte de alrededor del 70 % para células de levadura (Álvarez-Fernández García *et al.*, 2020) y del 55 % para células cancerosas humanas (Corte-Rodríguez *et al.*, 2019).

En cuanto al procesamiento y tratamiento de datos, primeramente, es necesario discriminar las señales correspondientes a eventos de células con respecto a aquellas debidas a la línea base, que corresponden a la señal de fondo y las especies disueltas (**Figura 14**). Para ello, se aplica un algoritmo iterativo desarrollado por Pace *et al.*, que consiste en seleccionar las señales cuya intensidad sea tres veces superior a la desviación estándar (3σ) de todo el conjunto de datos por encima de la media, rechazándose las inferiores (Pace *et al.*, 2011). A partir de la intensidad de los eventos seleccionados se puede calcular la masa del elemento acumulada en cada célula se determina aplicando la **ecuación (3)**:

$$\mathrm{m_c} = \frac{\eta \cdot \mathrm{F} \cdot \mathrm{t} \cdot \mathrm{I}}{\mathrm{b}} \qquad \textbf{(3)}$$

donde m_c es la masa del elemento de interés en la célula individual, η es la eficiencia de transporte de la solución patrón, F es el caudal de la muestra, t es el *dwell time*, I es la señal neta de intensidad del evento y b es la pendiente de la curva de calibración externa.

A partir de los datos obtenidos de la masa acumulada por célula individual se puede obtener un perfil completo sobre el número de células que acumulan concentraciones determinadas de los elementos a los que se han expuesto, pudiendo considerar así la variabilidad en el comportamiento de las distintas células que componen una población celular.

IV. OBJETIVOS

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Uno de los principales objetivos de la presente Tesis Doctoral es la identificación de biomoléculas y especies de selenio y mercurio presentes en pescados, y sus productos derivados y procesados, mediante técnicas basadas en espectrometría de masas, con el fin de evaluar su seguridad y calidad en relación con los elementos objeto de estudio. Las muestras seleccionadas se han dividido en dos grupos: muestras de pescado, productos derivados y procesados adquiridas en supermercados, y muestras involucradas en tres cadenas de producción de productos procesados, considerando tanto las materias primas como los productos intermedios y finales. El otro objetivo general de esta Tesis se centra en la evaluación del efecto protector de distintas especies de selenio frente a la neurotoxicidad inducida por el mercurio empleando la línea celular de neuroblastoma humano (SH-SY5Y).

Para la consecución de dichos objetivos generales se han planteado los siguientes objetivos específicos:

1. Evaluación de la relación molar Se:Hg y del parámetro HBV_{Se}, en base a los contenidos totales de selenio y mercurio determinados mediante ICP-MS y DMA, respectivamente, en músculo de pescado (atún, pez espada, salmón de piscifactoría y salmón rosado salvaje), productos derivados (huevas de lumpo, de trucha y de salmón rosado salvaje) y productos procesados (palitos de cangrejo, gulas, tallarines de salmón y de bacalao). Los parámetros de relación molar Se:Hg y HBV_{Se} permitirán establecer la seguridad del consumo de los productos anteriormente mencionados en relación a la ingesta de mercurio.

2. Determinación de la distribución de selenio en las distintas fracciones proteicas de músculo de pescado (sarcoplasmáticas, miofibrilares, álcali-solubles y solubles en Tris-HCl) mediante su extracción secuencial y posterior medida por SEC-ICP-MS. Identificación y cuantificación de las especies de selenio presentes en los extractos proteicos mediante HPLC-ICP-MS y HPLC-ESI-MS/MS.

3. Evaluación de la distribución de las especies de mercurio ($MeHg^+$ y Hg^{2+}) presentes en pescados, productos derivados y procesados, mediante HPLC-ICP-MS.

4. Seguimiento de los niveles de selenio y mercurio y especies de selenio, y estabilidad de las especies de selenio a lo largo de las etapas involucradas en tres cadenas de producción de productos procesados basados en surimi: palitos de cangrejo, gulas y tallarines de salmón. La consecución de este objetivo específico implicó los siguientes estudios:

   4.1 Determinación de los contenidos totales de selenio y mercurio, mediante ICP-MS y DMA, respectivamente, así como las relaciones molares Se:Hg y el parámetro HBV_{Se} en todos los eslabones de las cadenas de producción, desde las materias primas hasta los productos finales comercializados, pasando por los productos intermedios.

   4.2 Evaluación de las posibles transformaciones de las especies de selenio durante el proceso de producción de los productos procesados basados en surimi mediante extracción de las mismas y posterior análisis por HPLC-ICP-MS y HPLC-ESI-MS/MS.

5. Determinación de la bioaccesibilidad de selenio, mercurio y especies de selenio en las materias primas y los productos finales considerados de las cadenas de producción, mediante la aplicación de un modelo de digestión gastrointestinal *in vitro*. Evaluación de la citotoxicidad de los extractos gastrointestinales empleando la línea celular Caco-2.

6. Desarrollo de un método de análisis mediante HPLC-ESI-MS/MS, previa optimización de las condiciones de extracción, para la identificación y cuantificación simultánea de selenoneína y ergotioneína en pescados, productos derivados y procesados.

7. Estudio del efecto protector de distintas especies de selenio frente a la citotoxicidad inducida por el $MeHg^+$ en la línea celular de neuroblastoma humano SH-SY5Y. La consecución de este objetivo específico implicó los siguientes estudios:

   7.1 Evaluación de la citotoxicidad inducida por el $MeHg^+$ en ausencia y coexposición con especies de selenio (SeMet, SeMeSeCys y SeNPs) en la línea celular SH-SY5Y, mediante ensayos MTT.

7.2 Determinación de la acumulación de mercurio a nivel de células individuales en presencia de distintas especies de selenio (SeMet y SeMeSeCys), utilizando la técnica SC-ICP-MS.

V. OBJECTIVES

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One of the main objectives of this Doctoral Thesis is the identification of selenium and mercury-containing biomolecules and selenium and mercury species present in fish, fish-derived and processed products, by means of mass spectrometry-based techniques in order to correctly assess their safety and quality in relation to the elements under study. For this purpose, two types of samples were evaluated: those acquired in local supermarkets as final products, and those involved in three production lines of fish-processed products, considering raw materials, intermediate and final products. Additionally, another main objective focuses on the evaluation of the protective effect of different selenium species against mercury-induced neurotoxicity using the human neuroblastoma cell line (SH-SY5Y).

To achieve these main objectives the following specific objectives have been established:

1. Evaluation of Se:Hg molar ratio and HBV_{Se} parameter, based on total selenium and mercury contents determined by ICP-MS and DMA, respectively, in muscle of fish (tuna, swordfish, farmed salmon and wild salmon), fish-derived products (lumpfish roe, trout roe and wild salmon roe) and processed products (crab sticks, surimi-derived elvers, salmon noodles and cod noodles). Both parameters, Se:Hg molar ratio and HBV_{Se}, are expected to provide a more realistic information about the risk of the fish and fish-based products consumption due to the presence of mercury.

2. Distribution of selenium and mercury bound to proteins found in fish muscle (sarcoplasmic, myofibrillar, alkali-soluble and Tris-HCl soluble proteins) by means of sequential extraction followed by SECP-ICP-MS analysis. Identification and quantification of selenium species in the protein extracts by HPLC-ICP-MS and HPLC-ESI-MS/MS.

3. Evaluation of the distribution of mercury species ($MeHg^+$ and Hg^{2+}) present in fish, fish-derived and processed products by HPLC-ICP-MS.

4. Tracking of selenium, mercury and selenium species levels, and selenium species integrity along the process steps of three production lines of surimi-based products: crab sticks, surimi-derived elvers and salmon noodles. To achieve this goal, the following studies were carried out:

4.1 Determination of total selenium and mercury contents by ICP-MS and DMA, respectively, as well as Se:Hg molar ratio and the HBV_{Se} parameter in all steps of the production lines, from raw materials through intermediate products to finished products marketed.

4.2 Evaluation of the potential selenium species transformations during the elaboration of surimi-based products, by means of species extraction and subsequent HPLC-ICP-MS and HPLC-ESI-MS/MS analysis.

5. Determination of the bioaccessibility of selenium, mercury and selenium species in the raw materials and final products from the production lines, by applying an *in vitro* gastrointestinal digestion model. Evaluation of the cytotoxicity of gastrointestinal extracts using the Caco-2 cell line.

6. Development of a method of analysis based on HPLC-ESI-MS/MS, after optimization of the extraction conditions, for the simultaneous identification and quantification of selenoneine and ergothioneine in fish, fish-derived and processed products.

7. Study of the protective effect of different selenium species against $MeHg^+$-induced cytotoxicity in the human neuroblastoma cell line SH-SY5Y. For the achievement of this specific objective the following studies were developed:

7.1 Evaluation of $MeHg^+$-induced cytotoxicity alone or in co-exposure with selenium species (SeMet, SeMeSeCys and SeNPs) in SH-SY5Y cells by MTT assays.

7.2 Determination of mercury accumulation at single cell level in the presence of different selenium species (SeMet and SeMeSeCys) using SC-ICP-MS technique.

VI. PARTE EXPERIMENTAL

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1. Identificación y cuantificación de especies de selenio y mercurio presentes en pescados de consumo habitual, productos derivados y procesados. Evaluación de la bioaccesibilidad y toxicidad mediante modelos *in vitro* y la línea celular Caco-2

El pescado posee un elevado valor nutricional, ya que constituye una importante fuente de vitaminas, minerales, elementos traza esenciales y proteínas para el ser humano.

Entre los elementos traza esenciales presentes de forma natural en el pescado se encuentra el selenio, cuya esencialidad está principalmente relacionada con su incorporación a las proteínas como el 21º aminoácido, SeCys. Asimismo, es un agente antioxidante y, como tal, participa en numerosas reacciones metabólicas en los seres vivos. Sin embargo, el selenio puede llegar a ser también un elemento tóxico dependiendo de la forma química en la que se encuentre, así como de su concentración. En general, las especies inorgánicas de selenio (Se (IV) y Se (VI)) son más tóxicas que las especies orgánicas (SeMet, SeCys, y SeMeSeCys), siendo el Se (VI) el de mayor toxicidad. En lo que respecta a los pescados, la forma química predominante es la SeMet, si bien es cierto que otras formas orgánicas como la SeMeSeCys también han sido determinadas en dicha matriz.

Además, en el pescado también nos encontramos con la presencia de mercurio, cuyo significado biológico se limita únicamente a su toxicidad. Puede estar presente de forma natural y acumularse en cantidades elevadas en ciertas especies de peces, sobre todo en aquellas que se encuentran en la parte superior de la cadena trófica, es decir, en especies depredadoras. En este caso, su toxicidad también varía en función de la especie, siendo el $MeHg^+$ la forma química más tóxica y la que se bioacumula y biomagnifica debido a su liposolubilidad. Generalmente, esta es la especie mayoritaria de mercurio presente en el músculo de los pescados, y el resto del mercurio total lo constituye el Hg^{2+}. Sin embargo, no existe un factor de conversión fijo que pueda correlacionar la cantidad de $MeHg^+$ presente en los peces con su contenido de mercurio total, ya que depende en gran medida del nivel trófico en el que se encuentren, del tamaño y de la edad del organismo acuático. Además, la estabilidad de las especies de mercurio difiere, ya que el $MeHg^+$ permanece más tiempo en un organismo que el Hg^{2+}. Por lo tanto, en los peces depredadores, el $MeHg^+$ puede llegar a representar hasta el 90 % del contenido total de mercurio. Por el contrario, los peces situados en eslabones más bajos de la cadena trófica presentan un menor contenido relativo de $MeHg^+$, en ocasiones inferior al 50 % del total.

Por tanto, debido a la diferencia de esencialidad/toxicidad de las especies de selenio y de mercurio, función de su forma química y de su dosis administrada, los estudios de especiación en pescados son de especial relevancia.

El selenio y el mercurio se encuentran en el pescado unidos a biomoléculas, como son las proteínas. Por lo tanto, estos elementos suelen identificarse unidos a proteínas y otras biomoléculas de interés. En este sentido, la gran mayoría de estudios existentes se basan en la determinación de selenio y mercurio en un solo tipo de proteínas: las proteínas solubles extraídas con Tris-HCl. Sin embargo, las proteínas del tejido muscular pueden dividirse en diferentes tipos: sarcoplásmicas (mioglobina, globulina, etc.), miofibrilares (actina, miosina, tropomiosina, etc.) y álcali-solubles (colágeno, elastina, etc.). Cada tipo de proteínas tiene diferente solubilidad y función: las proteínas sarcoplasmáticas son solubles en disoluciones salinas de baja fuerza iónica y su principal función está relacionada con el metabolismo celular; las proteínas miofibrilares son solubles en disoluciones salinas de alta fuerza iónica y su principal función está relacionada con la actividad muscular; y, finalmente, las proteínas álcali-solubles son insolubles en soluciones salinas tanto de alta como de baja fuerza iónica y su principal función está relacionada con el tejido conectivo.

Por lo anteriormente mencionado, el primer trabajo (**Artículo 1**) titulado **“Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS”** (***Talanta 237 (2022) 122922***) se centró en el estudio de la distribución de selenio y mercurio en distintas fracciones proteicas obtenidas a partir del músculo de pescados (parte comestible) frecuentemente consumidos, como son el atún (*Thunnus*), el pez espada (*Xiphias gladius*), el salmón de piscifactoría (*Salmo salar*) y el salmón rosado salvaje (*Oncorhynchus gorbuscha*), incluyendo aquellas proteínas menos estudiadas como son las proteínas sarcoplasmáticas, miofibrilares y álcali-solubles.

Se eligieron el atún y el pez espada entre la variedad de pescados comerciales en España debido a su considerable tamaño y naturaleza depredadora. Estas especies tienden a acumular niveles relativamente altos de selenio y mercurio debido al proceso de biomagnificación que tiene lugar. Además, se incluyeron los dos tipos de salmones para investigar posibles disparidades entre pescados criados en cautividad y aquellos capturados en su entorno natural.

Para ello, una vez determinados los contenidos totales de selenio y mercurio en el músculo de los pescados, se llevó a cabo el estudio de la especiación de selenio tanto en proteínas solubles en Tris-HCl, que son las más comúnmente investigadas en la literatura científica, como en los tres tipos de proteínas menos estudiadas (sarcoplasmáticas, miofibrilares y álcali-solubles) del músculo de los pescados, mediante HPLC-ICP-MS, y posterior confirmación por HPLC-ESI-MS/MS. La especie de selenio que se identificó en los hidrolizados enzimáticos de las fracciones proteicas de todos los pescados analizados fue la SeMeSeCys. Además, en el atún, también se identificó la SeMet en los hidrolizados enzimáticos de las proteínas solubles en Tris-HCl. Finalmente, se evaluaron los perfiles proteicos en los extractos correspondientes a las proteínas sarcoplasmáticas, miofibrilares y álcali-solubles empleando la técnica SEC-UV/Vis y, posteriormente, para el estudio de las biomoléculas con selenio y/o mercurio en estos mismos extractos, se utilizó el acoplamiento SEC-ICP-MS. Estos análisis evidenciaron que los perfiles proteicos eran distintos en función del tipo de pescado analizado, incluso en aquellos que tenían contenidos totales de selenio y mercurio parecidos. De forma general, se observó que el selenio y el mercurio estaban principalmente unidos a proteínas de unos 2-12 kDa y de hasta unos 574 kDa, respectivamente. Sin embargo, también se encontraron ambos analitos unidos a proteínas de igual masa molecular, lo que podría indicar una posible interacción entre estos dos elementos. Cabe destacar que es la primera vez que se ha estudiado la distribución de selenio y mercurio en los extractos proteicos correspondientes a las proteínas sarcoplasmáticas, miofibrilares y álcali-solubles obtenidos a partir del músculo de pescado.

Por otro lado, los productos derivados del pescado o de productos procesados fabricados a base de este constituyen formas innovadoras y alternativas de introducir la ingesta de pescado en nuestra dieta, habiendo aumentado su consumo de forma considerable en los últimos años. La versatilidad que nos ofrecen para la preparación de distintos platos, el poco tiempo de elaboración necesario y, en ocasiones, el menor coste económico, son algunas de las ventajas a destacar que explican su elevada aceptación entre los consumidores. Sin embargo, estos tipos de productos están menos estudiados en lo que respecta a los contenidos de selenio y mercurio, y sus especies, siendo un aspecto clave tanto para conocer su valor nutricional como para evaluar su seguridad alimentaria. Algunas de las herramientas empleadas para este fin son el cálculo de las relaciones molares Se:Hg y del parámetro *Selenium Health Benefit Value* o HBV_{Se} , índice que representa la cantidad de selenio que queda disponible una vez que se produce su interacción con el mercurio. Valores de relaciones molares Se:Hg mayores que la unidad y valores positivos de HBV_{Se} indican que el consumo del pescado aparentemente no conlleva riesgos de toxicidad en lo que respecta al mercurio.

En este sentido, en el segundo trabajo (**Artículo 2**), titulado **"Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of non-traditional food products derived from fish" (*Food Chemistry 435 (2024) 137544*)**, se calcularon las relaciones molares Se:Hg y el índice HBV_{Se} a partir de los contenidos totales de selenio y mercurio, determinados mediante ICP-MS y DMA, respectivamente, tanto en productos derivados del pescado, huevas de lumpo (*Cyclopterus lumpus)*, huevas de trucha (*Oncorhynchus mykiss)* y huevas de salmón rosado salvaje (*Oncorhynchus gorbuscha*), como en productos procesados fabricados a base de pescado, en concreto con base en surimi de pescado como son: palitos de cangrejo, gulas, tallarines de bacalao y tallarines de salmón. Asimismo, también se escogieron el atún (*Thunnus*), el pez espada (*Xiphias gladius*), el salmón de piscifactoría (*Salmo salar*) y el salmón rosado salvaje (*Oncorhynchus gorbuscha*) para realizar una comparación entre los productos derivados del pescado y pescados de consumo habitual. Asimismo, dada la importancia de conocer la forma química en la que se encuentran estos elementos, también se llevaron a cabo estudios de especiación de selenio y mercurio mediante HPLC-ICP-MS y HPLC-ESI-MS/MS.

Las relaciones molares Se:Hg y el índice HBV_{Se} proporcionaron valores mayores que 1 y positivos, respectivamente, en todos los casos, lo que sugiere que el selenio está presente en exceso molar con respecto al mercurio y, por tanto, suficientemente biodisponible para realizar las correspondientes funciones biológicas en el organismo. Como consecuencia, los beneficios obtenidos a través del consumo de pescado y de sus productos derivados y procesados compensarían el riesgo asociado a la presencia de mercurio. Por otro lado, las especies de selenio SeMeSeCys y SeMet fueron identificadas en todas las muestras analizadas, y la especie de mercurio $MeHg^+$ fue detectada como forma mayoritaria, no solamente en pescados, sino también en los productos procesados, aunque su contenido relativo con respecto al mercurio total varió en función del alimento considerado.

Además de conocer los niveles de selenio y mercurio, y sus especies, en los productos procesados de pescado, también es indispensable evaluar si durante el proceso de fabricación de estos se mantienen las concentraciones de selenio y mercurio, así como la integridad de sus especies en estos productos en comparación con las materias primas empleadas para su elaboración, que son generalmente músculo de pescados que tienen una menor salida en el mercado. Para ello, es importante tener en cuenta los principales eslabones de la cadena de producción de los productos procesados considerados, como son las materias primas, los productos intermedios y el producto final que se comercializa. Otro aspecto clave que es necesario estudiar, tanto en las materias primas como en los productos finales, y que es incluso más importante que los contenidos totales, es la bioaccesibilidad de ambos elementos y sus especies, la cual se suele estudiar aplicando modelos de digestión gastrointestinal *in vitro* y empleando cultivos celulares, como la línea celular de adenocarcinoma colorrectal humano, Caco-2. Hacer este tipo de seguimiento a lo largo de la cadena de producción, y posteriores estudios de biodisponibilidad, permite obtener una información más realista de la calidad nutricional del alimento.

En el tercer trabajo (**Artículo 3**), titulado **“Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBV_{Se} index, bioaccessibility and Caco-2 cells toxicity” (*enviado a la revista Food Research International*)**, se aborda el seguimiento de los niveles de mercurio y selenio y sus especies, así como de su bioaccesibilidad en tres cadenas de producción de productos procesados derivados de pescado basados en surimi: palitos de cangrejo, gulas y tallarines de salmón, cuyos procesos de elaboración se describen a continuación. Cabe destacar que la totalidad de las muestras (materias primas, productos intermedios y productos finales) fue suministrada directamente por las empresas colaboradoras en esta investigación: Angulas Aguinaga y Grupo Nueva Pescanova.

El punto de partida para la fabricación de este tipo de productos es la elaboración del surimi, que implica la realización de sucesivas etapas de lavado, y que concentran las proteínas presentes en el músculo de los pescados utilizados como materias primas. A continuación, se mezclan con otros ingredientes, y mediante un proceso de amasado se le da la forma deseada, obteniéndose los productos intermedios que, una vez pasteurizados, dan lugar a los productos finales. Las diferencias entre los distintos productos finales estudiados se basan fundamentalmente en la coloración y la forma que adquieren. A los palitos de cangrejo se les confiere un tono anaranjado, y a las gulas se les da la forma característica, imitando a las angulas. En el caso de los tallarines, tras obtener el surimi y mezclarlo con otros ingredientes (almidón, clara de huevo, aromas, sal y agua), se lleva a cabo una etapa de cocción a 90 °C para posteriormente darle la forma deseada en forma de tiras. Por todo esto es importante hacer un seguimiento en la cadena de producción, ya que se dan procesos que pueden afectar a los contenidos y formas químicas del selenio y mercurio.

En este contexto, se determinaron los contenidos totales de selenio y mercurio tanto en los productos finales como en las materias primas de partida y en los productos intermedios anteriormente mencionados, mediante ICP-MS y DMA, respectivamente, calculándose las relaciones molares Se:Hg y el parámetro HBV_{Se}. Además, se evaluaron las principales especies de selenio presentes en cada etapa de los procesos de producción, mediante HPLC-ICP-MS y HPLC-ESI-MS/MS, con el fin de hacer un seguimiento de las posibles transformaciones que se pueden dar en las especies de selenio a lo largo de la cadena de producción. Seguidamente, se investigó la bioaccesibilidad de los contenidos totales de selenio y mercurio, así como de las especies de selenio presentes en las muestras, mediante un modelo *in vitro* que simula las condiciones fisiológicas de la digestión gastrointestinal humana. Finalmente, se evaluó la influencia de los extractos gastrointestinales (fracción potencialmente biodisponible para su posterior absorción por las células epiteliales del intestino) en la viabilidad celular de la línea Caco-2 mediante el ensayo MTT.

En todos los casos se obtuvieron resultados favorables con respecto a las relaciones molares Se:Hg y el parámetro HBV_{Se}, siendo superiores a 1 y positivos, respectivamente, lo que sugiere que los productos con base de surimi con respecto a su contenido en mercurio son seguros. Además, se constató la presencia de las especies SeMeSeCys y SeMet en todas las muestras, concluyendo, por tanto, que el proceso de elaboración de los productos procesados derivados del pescado no parece afectar a la integridad de las especies de selenio identificadas, manteniéndose desde las materias primas hasta los productos finales. Por otro lado, se obtuvieron bioaccesibilidades del 20-39 % para el selenio y del 8-37 % para el mercurio, y se evidenció la presencia de las especies SeMeSeCys y SeMet en los extractos gastrointestinales, lo que sugiere que son estables durante el proceso de digestión *in vitro*. Por último, no se observó citotoxicidad tras la exposición de células Caco-2 a los extractos gastrointestinales de los pescados y productos estudiados.

Adicionalmente, en los últimos años, el interés por el selenio en pescados se ha centrado en la identificación de la selenoneína, siendo considerada la especie mayoritaria de selenio presente en animales marinos. Aunque se ha incrementado el número de estudios sobre la identificación de la selenoneína en peces, principalmente en peces depredadores, así como sobre su función metabólica en el ser humano una vez ingerida, estas investigaciones son todavía limitadas. Se ha demostrado que juega un importante papel en la detoxificación del $MeHg^+$, ya que promueve su desmetilación previa formación de los compuestos insolubles estables de HgSe.

Otro compuesto que se considera químicamente análogo de la selenoneína es la ergotioneína, que presenta azufre en vez de selenio en su estructura, la cual ha sido fundamentalmente estudiada en hongos, donde se produce, así como en alimentos de origen vegetal y en carnes. Este compuesto tiene una relación directa con la selenoneína, ya que esta se transporta a través de las membranas celulares por el transportador de ergotioneína, OCTN1, que está involucrado en el transporte de los complejos formados entre la selenoneína y el $MeHg^+$. Sin embargo, a pesar de la implicación de estos dos compuestos en los procesos de detoxificación de $MeHg^+$, la información existente acerca de los niveles de ergotioneína en pescados y en alimentos de origen marino es muy escasa. Hasta la fecha, muy pocos estudios han abordado el análisis simultáneo de estos dos potentes antioxidantes en alimentos, especialmente en pescados, y no se dispone de información acerca de su posible presencia en productos derivados del pescado o productos procesados fabricados a partir del pescado.

A este respecto, en el cuarto trabajo (**Artículo 4**) titulado **"Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products" (*enviado a la revista Journal of Agricultural and Food Chemistry*)** se evaluó la presencia conjunta de selenoneína y ergotioneína en pescados (atún (*Thunnus)*, pez espada (*Xiphias gladius)*, salmón de piscifactoría (*Salmo salar)*, y salmón rosado salvaje (*Oncorhynchus gorbuscha*)), productos derivados del pescado (huevas de lumpo (*Cyclopterus lumpus)*, huevas de trucha (*Oncorhynchus mykiss)* y huevas de salmón rosado salvaje (*Oncorhynchus gorbuscha*)) y productos procesados fabricados a partir del pescado (palitos de cangrejo, gulas, tallarines de bacalao y tallarines de salmón) mediante HPLC-ESI-MS/MS. Para ello, se estudiaron diferentes métodos de extracción y se optimizaron las condiciones cromatográficas de HPLC y de espectrometría de masas. Finalmente, también se investigó la presencia de biomoléculas que contienen selenio mediante SEC-ICP-MS.

Las mejores eficiencias de extracción se obtuvieron empleando una extracción acuosa, con acetato o formiato de amonio, asistida mediante el empleo de bolas de vidrio o la sonda de ultrasonidos. Este proceso de extracción permitió detectar mediante HPLC-ESI-MS/MS la presencia de selenoneína y ergotioneína, de forma simultánea, en el músculo del atún y del pez espada, lo que sugiere que, al menos en este tipo de pescados depredadores, ambos compuestos pueden coexistir. Sin embargo, en el salmón y en las huevas de los pescados considerados solo se evidenció la presencia de ergotioneína. Finalmente, ninguno de estos dos potentes antioxidantes se identificó en los productos derivados del pescado estudiados. Estos resultados podrían contribuir a elucidar la interacción entre la selenoneína y la ergotioneína, así como su papel en los mecanismos de detoxificación del mercurio, lo que es de especial relevancia en productos procedentes del mar.

Por otro lado, se observó que la mayor parte del selenio existente en la fracción soluble del músculo de los pescados, productos derivados y procesados analizados se encontraba unido a biomoléculas de bajo peso molecular, formando selenocompuestos, entre los cuales se encuentran las selenoproteínas y otros compuestos como la selenoneína.

ARTÍCULO 1:

Análisis de la distribución de Se y Hg en biomoléculas y especiación de Se en fracciones proteicas poco estudiadas de músculo de pescados frecuentemente consumidos mediante SEC-UV-ICP-MS y HPLC-ESI-MS/MS

Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS

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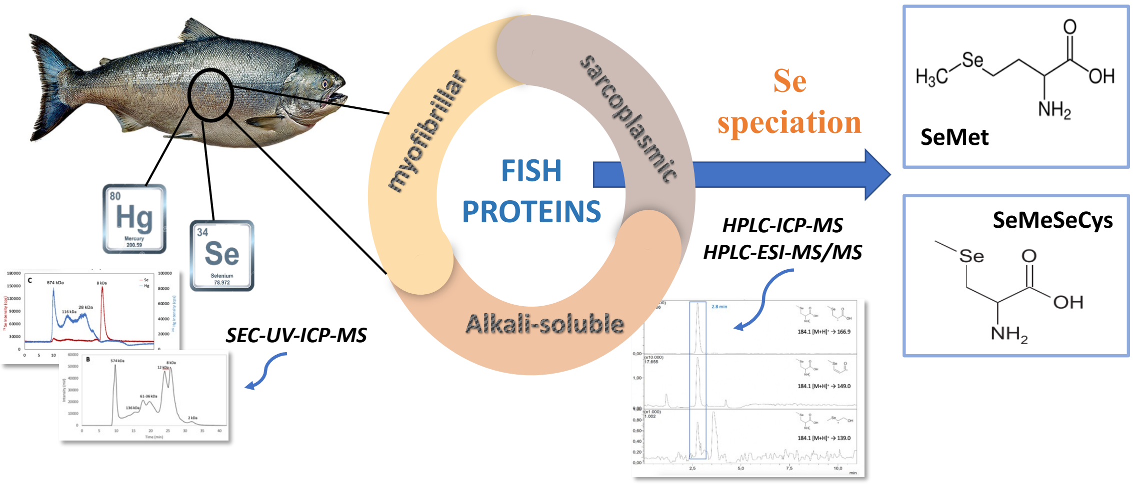

Los resultados de este trabajo se presentaron en las siguientes reuniones científicas:

- **RSEQ Symposium 2021**. *Analysis of Se and Hg biomolecules distribution in poorly studied protein fractions of muscle tissues of highly consumed fishes*. Online, septiembre de 2021. Tipo de comunicación: póster.
- **9ème édition de Spectr'atom**. *Distribution de biomolécules avec Se et Hg et spéciation du Se dans différentes fractions protéiques extraites des poissons par HPLC-UV-ICP-MS et HPLC-ESI-MS/MS*. Pau, Francia, mayo de 2022. Tipo de comunicación: póster.
- **XXIII Reunión de la Sociedad Española de Química Analítica**. *Análisis de Se y Hg en fracciones proteicas extraídas del músculo de pescados de consumo habitual. Evaluación de su unión a biomoléculas e interacción*. Oviedo, España, julio de 2022. Tipo de comunicación: póster.
- **Jornada de Especiación de la XXIII Reunión de la Sociedad Española de Química Analítica**. *Estudio de biomoléculas y especies con Se en fracciones proteicas (sarcoplasmáticas, miofibrilares y álcali-solubles) de pescados de consumo frecuente mediante HPLC-UV-ICP-MS y HPLC-ESI-MS/MS*. Oviedo, España, julio de 2022. Tipo de comunicación: oral.

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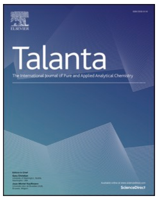

Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS

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ABSTRACT

Distribution of Se and Hg in sarcoplasmic, myofibrillar and alkali-soluble protein fractions extracted from muscle tissue of tuna, swordfish and salmon (wild vs. farmed) is investigated for the first time. SEC-UV-ICP-MS analyses revealed that Se and Hg are mostly bound to proteins of 2–12 kDa and up to 574 kDa, respectively. Moreover, Se and Hg appeared associated to proteins of same molecular weight, evidencing that Se–Hg interaction may occur at the level of the fish tissue evaluated. Important differences were found between farmed and wild salmon, suggesting the effect of the type of feed and growing conditions on Se and Hg content and their distribution through protein fractions. Finally, Se speciation studies performed by HPLC-ICP-MS and confirmed by HPLC-ESI-MS/MS showed SeMeSeCys as the only Se specie found in soluble, sarcoplasmic, myofibrillar and alkali-soluble proteins of all fishes analysed, except in soluble proteins extracted from tuna, where SeMet was also identified.

1. Introduction

Selenium (Se) is an essential micronutrient for human and animal health with important antioxidant properties. However, it can be essential or toxic depending on its chemical form, its bioavailability, as well as the ingested dose [1]. Inorganic forms such as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are known to be more toxic than organic compounds, in which selenium is mainly present as selenoamino acids. For instance, selenomethionine (SeMet) and selenocysteine (SeCys) are the most bioavailable forms of selenium ingested for humans through the consumption of plants and animal sources such as meat, seafood or fish [2]. On the other hand, the biological significance of mercury (Hg) is limited to its toxicity, which also depends on its chemical form. In particular, organic mercury compounds, for instance methylmercury (CH_3Hg^+), are bioaccumulating and biomagnifying pollutants [3], reaching levels of $\mu g \cdot g^{-1}$ in predatory fishes. Among mercury adverse effects described in bibliography are sensory and motor deficiency, loss of vision, paralysis, coma, congenital deformities affecting foetuses and, finally, death [4].

Fish is an important source of high-quality proteins in the human diet and also provides many other important nutrients. Fishes can accumulate in their muscle tissues a vast number of elements, such as selenium and mercury. Selenium is mainly present as amino acids such as SeMet and SeCys and consequently mostly incorporated into proteins [5]. Mercury, for its part, is mainly present in fish as CH_3Hg^+ [6], being also the most toxic form of this element. It is predominantly bound to thiol groups, due to their high affinity for mercury compounds ($k_a = 10^{39}$) [7].

It has long been described that selenium protects against mercury toxicity. In case of fish, numerous studies have shown that the presence of high selenium levels could protect these fish species from mercury toxicity [8,9]. Although a Se–Hg antagonism effect is generally observed, additive or even synergistic effects of mercury and selenium have also been reported in the literature [10]. On the whole, the net effect is dependent on the relative concentrations of both elements, their bioavailability, the type of tissue or organ tested, as well as the size and the age of the fish [11,12]. Therefore, studies based on the evaluation of the presence of both selenium and mercury in fish samples and how they interact become an essential issue to properly assess the risk-benefit of fish consumption because of mercury toxic effects.

Selenium and mercury are usually present and found in fishes binding biomolecules, such as proteins. That is the reason why both

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elements are commonly identified within proteins or other biomolecules of great interest. Up to now, the vast majority of the studies performed are based on the determination of both selenium and mercury in only one kind of proteins: soluble proteins [13–15]. Nevertheless, according to Hashimoto et al. [16], fish proteins in muscle tissue can be divided into different types: sarcoplasmic (myoglobin, globulin …), myofibrillar (actin, myosin, tropomyosin …) and alkali-soluble and stromal (collagen, elastin …). Each type of proteins has different solubility and function: sarcoplasmic proteins are soluble in saline solutions of low ionic strength and their main function is related to the cellular metabolism; myofibrillar proteins are soluble in saline solutions of high ionic strength and their main function is related to the muscle activity; and, finally, alkali-soluble and stromal proteins are insoluble in saline solution of both low and high ionic strength and they are related to the connective tissue [17]. In this sense, there is a lack of information regarding selenium and mercury distribution in this specific type of proteins. Consequently, studies related to the determination, transformation and distribution of both mercury and selenium species within fish proteins might contribute to a more in-depth understanding of the effects of Se–Hg interaction and it might open new insights on the risks and benefits of consuming fish and fish products.

Therefore, the aim of this paper is to determine the total content and the distribution of selenium and mercury species in three poorly studied protein fractions (sarcoplasmic, myofibrillar and alkali-soluble) from muscle tissue of highly consumed fishes such as tuna, swordfish, farmed salmon and wild salmon. For this purpose, size-exclusion chromatography coupled to ultraviolet–visible (UV-VIS) and to inductively coupled plasma mass spectrometry (ICP-MS) detectors was employed to study Se and Hg-containing biomolecules in each protein fraction. Moreover, Se speciation analyses were also carried out in soluble proteins as well as in the sarcoplasmic, myofibrillar and alkali-soluble protein fraction of the same fishes by high performance liquid chromatography coupled to ICP-MS (HPLC-ICP-MS) and by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS).

2. Material and methods

2.1. Determination of the total Se content in muscle tissue of fish by ICP-MS

Fish fillets of highly consumed fish species such as tuna (*Thunnus*), swordfish (*Xiphias gladius*), farmed salmon (*Salmo salar*) and wild salmon (*Oncorhynchus gorbuscha*, from northwest Pacific) were acquired in a local supermarket and stored correctly at −20 °C until analysis. With the aim of having a representative composite sample for the fishes analysed, fish fillets were obtained from 10 individuals of tuna, swordfish, farmed and wild salmon.

The total Se content in fresh fish fillets was performed by using inductively coupled plasma mass spectrometry (ICP-MS) after acid digestion in a 1600W MSP microwave oven from CEM (Stallings, NC). For this purpose, about 1 g of each fresh fish fillet sample (3 replicates) was digested with 6 mL of a mixture (5:1) of 65% v/v nitric acid (Merck, Madrid, Spain) and 30% v/v hydrogen peroxide (Panreac, Barcelona, Spain) and submitted to a microwave heating program consisting of a 20-min ramp to 130 °C and a 10-min hold time. Once cooled until room temperature, the solutions were appropriately diluted with deionized water (18.2 MΩ cm, from a Milli-Q® water purification system; Millipore, Bedford, MA) to a final volume of 10 mL and filtered through a 0.22 μm syringe Nylon filter (Scharlab, Barcelona, Spain) prior analyses. Total Se content was determined with an Agilent 7700× Series ICP-MS. The operational conditions for ICP-MS measurements have been listed in Table 1. Se quantification was performed by external calibration with the isotope ^{78}Se. The accuracy of the method was further evaluated by the analysis of Certified Reference Materials from mussel tissue.

Table 1
Operating instrumental conditions ICP-MS.

ICP-MS parameters	
RF Power (W)	1550 W
Plasma gas flow rate	15 mL min^{-1}
Ar auxiliary flow rate	1 mL min^{-1}
Carrier gas flow rate	1 mL min^{-1}
Nebulizer	Conikal
Spray Chamber	Scott
Acquisition mode	Continuous
Isotopes monitored	^{78}Se, ^{202}Hg
HPLC parameters	
Analytical column	Superdex 200 (10–600 kDa) (10 × 300 mm, 8.6 μm)
Mobile phase	50 mmol L^{-1} Tris-HCl, 0.1 mmol L^{-1} KCl (pH 6.8)
Mode	Isocratic
Flow rate	0.8 mL min^{-1}
Injection volume	100 μL
Run time	45 min
Analytical column	Phenomenex Kinetex EVO-C18 (150 × 3 mm, 5 μm)
Mobile phase	0.1% formic acid, 0.5% MeOH (pH 3.2)
Mode	Isocratic
Flow rate	0.5 mL min^{-1}
Injection volume	20 μL
Run time	10 min

2.2. Determination of the total Hg content in muscle tissue of fish by DMA

Total Hg determination in fresh fish fillet (tuna, swordfish, farmed salmon and wild salmon samples) was carried out by using a Direct Mercury Analyzer (DMA-80 evo; Milestone, Italy). Determination is based on sample thermal decomposition, amalgamation and electrothermal atomization of mercury. The sample (without applying any previous treatment) is deposited in a boat of quartz and passes to an oven in which combustion occurs at 73 °C. The sample is then dried and the resulting mercury is released as vapour. The O_2 from a synthetic air bullet acts as carrier gas driving mercury vapour through the catalyst tube, where impurities are filtered and all oxidation states of mercury are reduced to elemental mercury (Hg^0), at a temperature of 565 °C. Finally, elemental mercury is caught in a gold and platinum amalgamator, which is heated to 650 °C and releases mercury, which is detected and quantified by atomic absorption at 253.65 nm. Around 50 mg of fresh fish fillet (tuna, swordfish, farmed salmon and wild salmon samples) were weighted on quartz sample boats and directly analysed by DMA (3 replicates). Mercury quantification was performed by external calibration, composed by three different analytical curves, one for each "absorbance cell". The instrument has three detection cells with different optical paths for readings within different concentration ranges: from 0.2 to 5 ng, from 5 to 20 ng and to from 20 to 200 ng of Hg. The accuracy of the method was further evaluated by the analysis of Certified Reference Materials from oyster and mussel tissues. Quantification limit (LOQ = 0.033 μg kg^{-1}) was calculated by analysing 10 replicates of a blank and applying 10*SD criteria.

2.3. Protein extraction procedures

Protein extraction was carried out by following the procedure described by Solari [16], in which three types of proteins (sarcoplasmic, myofibrillar and alkali-soluble) from muscle tissue of fish were extracted and separated by their differential solubility in different buffer solutions.

2.3.1. Sarcoplasmic proteins

15 g of each fish fillet (tuna, swordfish, farmed salmon and wild salmon, 3 replicates) were homogenized in a blender (Saivod® blender BW500) with 90 mL of 0.05 mol L^{-1} phosphate buffer pH 7.5 and 600 μL of protease inhibitor cocktail (Sigma-Aldrich, Spain) and subsequently centrifuged at 10000 rpm during 15 min at 4 °C (Eppendorf 5804 F34-6-

38 centrifuge, Hamburg, Germany). The resulting supernatant is the sarcoplasmic protein fraction and the remaining solid phase was subsequently submitted to the next extraction procedure in order to obtain myofibrillar protein.

2.3.2. *Myofibrillar proteins*

The residue obtained from the previous extraction of sarcoplasmic proteins was homogenized in a blender with 90 mL of 0.05 mol L^{-1} phosphate buffer, 0.45 mol L^{-1} KCl (Riedel-de Häen, Germany) pH 7.5 and 600 μL of protease inhibitor cocktail and subsequently centrifuged at 10000 rpm during 15 min at 4 °C. The resulting supernatant is the myofibrillar protein fraction and the remaining solid phase was subsequently submitted to the next extraction procedure to get the alkali-soluble protein fraction.

2.3.3. *Alkali-soluble proteins*

The final residue obtained from myofibrillar proteins extraction procedure was homogenized in a blender with 100 mL of 0.1 mol L^{-1} NaOH (Merck, Germany) pH 13.2 and 600 μL of protease inhibitor cocktail. The mixture was incubated overnight with shaking at room temperature. Subsequently, it was centrifuged at 10000 rpm during 15 min at 4 °C. The resulting supernatant is the alkali-soluble protein fraction.

2.4. *Determination of Se and Hg content in sarcoplasmic, myofibrillar and alkali-soluble protein fractions*

2 mL of each protein extract (sarcoplasmic, myofibrillar and alkali-soluble) were digested with 6 mL of a mixture (5:1) of 65% v/v nitric acid and 30% v/v hydrogen peroxide in an analytical microwave oven. With regards to sarcoplasmic and alkali-soluble proteins, the solutions were then diluted to a final volume of 10 mL or 25 mL in case of myofibrillar protein and filtered through a 0.22 μm syringe Nylon filter.

Se quantification by ICP-MS was performed by using three different external calibrations with the isotope ^{78}Se, one for each extraction buffer, because of matrix effects. The operational conditions for ICP-MS are given in Table 1. Quantification limit (LOQ = 0.006 mg kg^{-1}) was calculated by analysing 10 replicates of a blank solution and applying 10*SD criteria.

For total Hg determination, 200 μL of each protein fraction (sarcoplasmic, myofibrillar and alkali-soluble) were added on quartz sample boats and directly analysed without any pre-treatment by DMA as it was already described in Section 2.2.

2.5. *Study of Se and Hg-containing biomolecules and protein profile by size-exclusion chromatography coupled to UV–Vis and ICP-MS detectors*

Se and Hg-containing biomolecules in protein fractions (sarcoplasmic, myofibrillar and alkali-soluble proteins) from muscle tissue of fish species were detected by size-exclusion chromatography coupled to UV–Vis and ICP-MS detectors (SEC-UV-Vis and SEC-ICP-MS). A size-exclusion column, Superdex 200 (10–600 kDa) (GE Healthcare Life Sciences, UK) coupled to an UV–Vis spectrophotometer connected to a PU-2089 HPLC pump (JASCO, Tokyo, Japan), was firstly employed to study protein profile in the protein extracts. The UV–Vis detector was set to 280 nm. Operational conditions are listed in Table 1. Protein standard solutions of known molecular weight (5 mg mL^{-1} thyroglobulin (669 kDa); 0.3 mg mL^{-1} apoferritin (443 kDa); 8 mg mL^{-1} albumin (67 kDa); 2 mg mL^{-1} carbonic anhydrase (30 kDa) and 1.5 mg mL^{-1} cytochrome C (12.4 kDa)) were used to calibrate the size-exclusion column with nominal molecular-weight ranges. Moreover, in a second step, Superdex 200 column was also coupled to an Agilent ICP-MS (Model 7700×, Agilent, Tokyo, Japan) applying the same experimental conditions (Table 1).

2.6. *Determination of selenium species in muscle tissue of fish by HPLC-ICP-MS*

Selenium species in samples were determined in the soluble protein fraction as well as in the three protein fractions of all the fishes by enzymatic hydrolysis followed by HPLC-ICP-MS analyses.

Regarding soluble proteins, about 0.1 g of each fresh fish fillet samples (tuna, swordfish, farmed salmon and wild salmon; 3 replicates of each) was treated with 5 mL of 30 mmol L^{-1} tris-(hydroxymetyl)-aminomethane (Tris) at pH 7.5 (adjusted with 0.05 mol L^{-1} HCl) and 20 mg of the non-specific protease *Streptomyces griseus* (Protease type XIV; Sigma-Aldrich, Madrid, Spain). The mixture was incubated for 24 h at 37 °C in a thermostatic bath (Termotronic II from P Selecta, Spain). Samples were centrifuged at 10000 rpm during 15 min at 4 °C, and supernatants were filtered through a 0.22 μm syringe Nylon filters before analysis. Total Se content in the obtained extract was measured by ICP-MS after performing a microwave acid digestion as described in Section 2.1.

Se species were also determined in the three protein fractions (sarcoplasmic, myofibrillar and alkali-soluble). Sarcoplasmic, myofibrillar and alkali-soluble proteins from muscle tissue of fish were extracted as it was described in Section 2.3, but this time without using protease inhibitor cocktail. Then, 33 mg of the non-specific protease *Streptomyces griseus* (Protease type XIV) were added to 1 mL of each protein fraction. It is mandatory to adjust pH with HCl of alkali-soluble protein fraction from 13.2 to 7.5 because the optimum activity of the protease is at pH 7–8. The mixture was incubated for 24 h at 37 °C in a thermostatic bath, being then centrifuged at 10000 rpm during 15 min at 4 °C, and supernatants were filtered through a 0.22 μm syringe Nylon filters before analysis.

Selenium species analysis were carried out by HPLC-ICP-MS using a Phenomenex Kinetex EVO C18 (150 × 3.0 mm, 5 μm) with a reversed phase mechanism. The experimental conditions are listed in Table 1. Se species identification was performed by matching the retention times with those of standard solutions: selenomethylseleno-L-cysteine (SeMeSeCys), D-L-selenocystine ($SeCys_2$), seleno-L-methionine (SeMet), Se (IV) (Na_2SeO_3) and Se (VI) (Na_2SeO_4), all from Sigma-Aldrich (Madrid, Spain). Standard stock solutions of 1000 mg L^{-1} were prepared in acidic conditions and stored at 4 °C and working solutions were freshly prepared daily by dilution before use. The recovery of the column was calculated by comparing the total Se concentration of a solution containing a mixture of SeMet, SeMeSeCys, $SeCys_2$ Se (IV) and Se (VI) measured by ICP-MS to the concentration resulting of the sum of selenium compounds from speciation analysis of the same solution. The column recovery achieved was 92%.

2.7. *HPLC-ESI-MS/MS analyses*

In order to confirm the identity of the Se species previously detected by HPLC-ICP-MS, analyses by HPLC-ESI-MS/MS were carried out. Once again, a Phenomenex Kinetex EVO C18 column was employed because of the compatibility of its mobile phase with the HPLC-ESI-MS/MS system. The targeted Se species were analysed by means of a Shimadzu LC-MS-8030 triple quadrupole system (Shimadzu Scientific Instrument, Columbia, MD, USA) equipped with a Nexera LC-30AD solvent delivery unit, a Nexera SIL 30AC autosampler with temperature-controlled tray and a CTO-20AC column oven. The analyses were performed at room temperature, using a mixture of 0.1% formic acid aqueous solution at pH 3.2 and methanol as mobile phase, the flow rate was 0.50 mL min^{-1} and the injection volume 20 μL. Mass analysis was performed in positive electrospray ionization (ESI) mode. High purity nitrogen was used as nebulizing (1.5 L min^{-1}) and drying (15.0 L min^{-1}) gas. Furthermore, argon gas (230 kPa) was employed in the collision cell for collision-induced dissociation applying a collision energy voltage between 10 and 33 eV. Ionization voltage for ESI was set at 4.5 kV, the interface current was fixed at 2.4 μA and the detector voltage at 2.18 kV.

Full-scan spectra and MS/MS spectra were acquired to obtain the available transitions for each Se species identified by ICP-MS. $[M+H]^+$ was selected as precursor ion and the detection was carried out in multiple reaction monitoring (MRM) mode, with a dwell time of 100 ms, by monitoring three selective transitions for each analyte.

3. Results and discussion

3.1. Total Se and Hg content in muscle tissue of fish species

Total Se content in fresh fish fillets (tuna, swordfish, farmed salmon and wild salmon) was determined by ICP-MS, after acid digestion in a microwave oven, as it was described in Section 2.1. Total Hg content in fresh fish fillet samples was carried out with DMA, which provides concentration levels of mercury without any sample pre-treatment (described in Section 2.2).

Validation of the results for selenium and mercury determinations were achieved by the analysis of Certified Reference Materials from the Institute for Reference Materials and Measurements. Oyster (BCR-710) and mussel (ERM-CE 278k) tissues were employed for Hg determinations, which certified for Hg (0.26 ± 0.05) mg kg^{-1} and (0.073 ± 0.007) $mgkg^{-1}$, respectively. For Se determinations, mussel tissue (ERM-CE 278k) was employed, which certified for Se (1.62 ± 0.12) $mgkg^{-1}$. The methods used for the Se and Hg determinations were considered accurate since no significant differences were found at 95% confidence level between the certified values and the experimental ones. In case of Hg the experimental values were (0.30 ± 0.04) $mgkg^{-1}$ and (0.079 ± 0.009) $mgkg^{-1}$, for oyster and mussel tissues, respectively. In case of Se, it was (1.60 ± 0.15) $mgkg^{-1}$.

Se and Hg values found in tuna, swordfish, farmed salmon and wild salmon are detailed in Table 2.

According to the results in Table 2, concentrations of Se and Hg vary from 0.68 to 2.2 mg kg^{-1} and from 0.011 to 1.45 mg kg^{-1}, respectively, being tuna and swordfish the fish species with the highest content of both elements, probably due to their biomagnification processes through food chain [3]. Moreover, ANOVA analysis evidenced statistically significant differences ($P < 0.05$) between Se and Hg values for farmed salmon and wild salmon. These variations could be explained by differences in the kind of feeding and growing conditions, in captivity or free. The feed in fish farming is highly controlled and comes from a variety of sources whereas the diet of wild salmon is usually composed of small fishes and crustaceans [17].

Mercury contents found in the fish samples studied are in consonance with the mercury levels allowed by European Food Safety Authority (EFSA) [18]. It has been widely reported that predatory fishes, such as shark, swordfish and marlin usually have high concentrations of Hg, which may exceed 1 mg kg^{-1} (wet weight). Fresh tuna often contains Hg concentrations between 0.1 and 1.5 mg kg^{-1} (wet weight), whereas salmon present lower Hg concentrations (<0.1 mg kg^{-1} wet weight). However, other factors such as gender, tissue or organ, kind of feed, age and size of the fish, region and specific ecosystem characteristics also have an impact on Hg concentration [19]. Concerning Se, an Adequate Intake (AI) of 70 μg·day^{-1} of this element for adults was set by the EFSA [20].

Table 2
Total Se and Hg content found in fish samples measured by ICP-MS and DMA, respectively.

Sample	[Se] (mg·kg^{-1})	[Hg] (mg·kg^{-1})
Tuna	2.2 ± 0.1	0.7 ± 0.1
Swordfish	2.2 ± 0.2	1.45 ± 0.04
Farmed salmon	0.92 ± 0.05	0.011 ± 0.001
Wild salmon	0.68 ± 0.05	0.048 ± 0.002

Results are referred to wet weight. Results expressed as mean ± standard deviation (SD); n = 3 for each of the fish species.

3.2. Total Se and Hg content in protein fractions from muscle tissue of fish

As it has been previously stated, selenium in fish is mainly found associated with proteins, therefore Se and Hg content in protein fractions were also determined. For this purpose, three types of fish proteins, sarcoplasmic, myofibrillar and alkali-soluble, were considered. Each type of proteins were extracted from fresh fish fillet samples (tuna, swordfish, farmed salmon and wild salmon) and separated according to their solubilities in different buffers (Section 2.3). Values of total Se and Hg contents in the resulting proteins fractions were analysed by ICP-MS and DMA, respectively, and the results obtained are compiled in Fig. S1.

Although Se total content in tuna and swordfish is very similar (Table 2), differences on its distribution in the protein fractions are observed. In case of tuna, the highest Se content is found in sarcoplasmic proteins (41 ± 5% total Se content) and the lowest in myofibrillar proteins (17 ± 2% total Se content), whereas in swordfish most of selenium was associated to myofibrillar proteins (94 ± 11% of total Se) with a minor content in sarcoplasmic proteins (7 ± 2% of total Se). Related to salmon species, not only differences in total Se content were detected, but also in Se distribution of the protein fractions studied. In this sense, the highest Se concentration in proteins extracted from farmed salmon was observed in myofibrillar fraction (77 ± 9% total Se content), followed by alkali-soluble and sarcoplasmic proteins, with similar amount of Se (18 ± 1 and 16 ± 3 total Se content, respectively). Nonetheless, concerning wild salmon, alkali-soluble fraction is the one with the highest Se content (72 ± 8% total Se content), followed by sarcoplasmic (26 ± 6% total Se content) and, finally, myofibrillar proteins (9 ± 2% total Se content).

Mercury in tuna and swordfish is mainly distributed in alkali-soluble fractions (29 ± 4 and 46 ± 7% total Hg content, respectively) followed by sarcoplasmic (14 ± 2 and 16 ± 2% total Hg content, respectively) and myofibrillar proteins (9 ± 1 and 14 ± 1% total Hg content, respectively), where similar Hg levels were detected. Interestingly, the highest percentage of Hg with respect to the total Hg concentration was found in myofibrillar and alkali-soluble protein fractions extracted from farmed salmon samples (70 ± 1 and 56 ± 5% total Hg content, respectively). In addition, it is also noticeable that farmed salmon Hg distribution pattern is completely opposite to that of wild salmon. For wild salmon, myofibrillar proteins exhibited the lowest concentration of Hg (16 ± 2% total Hg content) with the highest amount of mercury associated to sarcoplasmic proteins (37 ± 4% total Hg content). The results obtained evidenced that Hg and Se are mainly bound to proteins, as it is demonstrated in Se and Hg mass balance calculations compiled in Table S1. Furthermore, owing to the fact that Se and Hg were both found in the same kind of protein fractions, it could be hypothesized that Se–Hg interaction may occur in muscle tissues of the fish species tested. Se:Hg molar ratios for total Se and Hg amount in fish tissues have been estimated, being >1 for all the fishes analysed.

3.3. Determining Se and Hg-containing biomolecules and protein profiles by size-exclusion chromatography (SEC) coupled to UV–Vis and ICP-MS detectors

Once the total content of Se and Hg was determined in sarcoplasmic, myofibrillar and alkali-soluble protein fractions, each protein extract was also analysed by SEC-UV-Vis, as it has been already described in Section 2.5. The resulting UV–Vis chromatographic profiles obtained with a Superdex 200 column are shown in Fig. 1.

The resulting chromatographic profiles were similar, regardless of the fish analysed. In particular, sarcoplasmic proteins in all fish samples (Fig. 1 A, D, G, and J) show chromatographic peaks corresponding to relatively low molecular weight proteins that are distributed in three different ranges of molecular weights: 2–6, 7–13 and 48–64 kDa. Myofibrillar (Fig. 1 B, E, H and K) and alkali-soluble proteins (Fig. 1C, F, I and L) show a similar pattern although there were also peaks at the high molecular weight zone of the chromatogram (574–585 kDa).

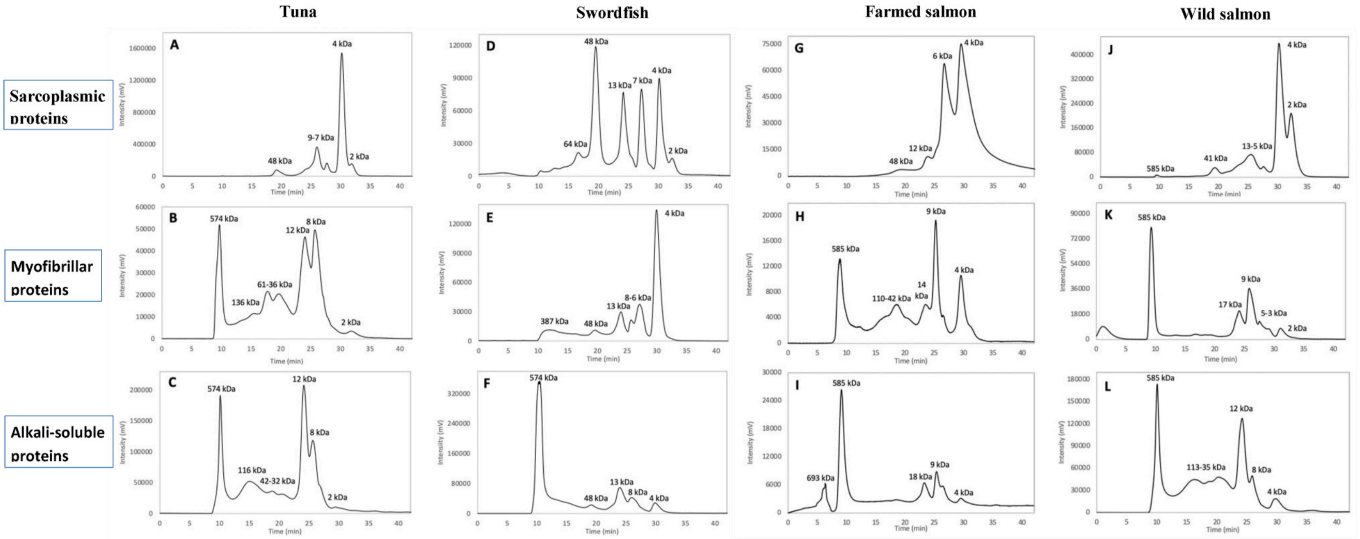

Fig. 1. Chromatographic profiles obtained by SEC-UV-Vis. A, B and C referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of tuna, respectively. D, E and F referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of swordfish, respectively. G, H and I referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of farmed salmon, respectively. Finally, J, K and L referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of wild salmon, respectively.

Despite all the similarities appreciated, tuna and swordfish show some differences. The main difference was found in their sarcoplasmic protein profile (Fig. 1 A and D). This variation could be attributed to differences in fat content between the two fish species. Tuna is usually classified as a bluefish or fatty fish (>5% of fat content) while swordfish as semi-fat (2–5% of fat content). One important protein included in sarcoplasmic proteins group is myoglobin, which is responsible of the oxygen transport in the muscle. Contrary as it takes place in swordfish, myoglobin constitutes about 80% of all the haemoproteins present in tuna and is responsible of its dark reddish colour [16,21]. All these facts could justify the differences observed in sarcoplasmic protein profiles between tuna and swordfish.

Afterwards, the determination of Se and Hg-containing biomolecules in the protein fractions extracted from fish fillets was carried out by size-exclusion chromatography (Superdex 200) coupled to ICP-MS detector (described in Section 2.5). The chromatographic profiles obtained are depicted in Fig. 2.

Se and Hg-containing biomolecules profiles are very different in all fish samples. Se (red lines) seems to be bound to biomolecules of about 8 kDa for all the protein fractions (sarcoplasmic, myofibrillar and alkali-

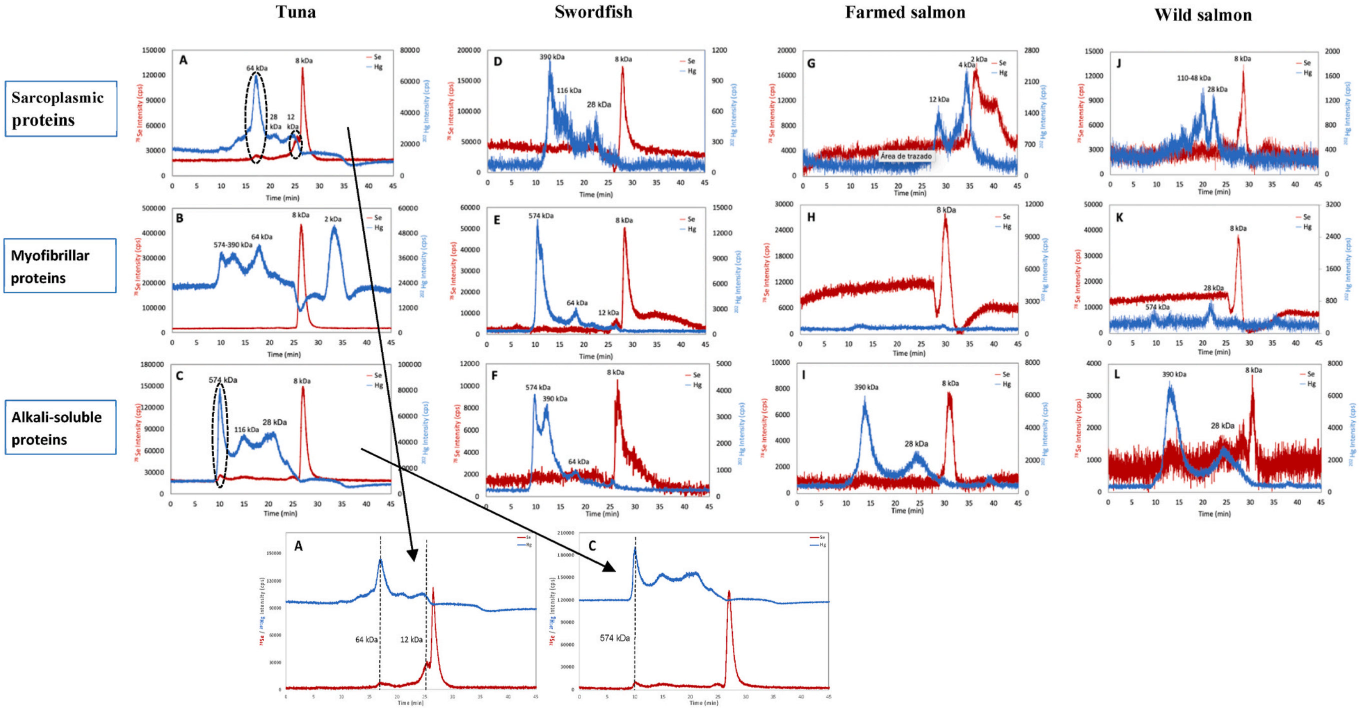

Fig. 2. Se (red lines) and Hg (blue lines)-containing biomolecules in fish protein fractions obtained by SEC-ICP-MS. A, B and C referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of tuna, respectively. D, E and F referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of swordfish, respectively. G, H and I referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of farmed salmon, respectively. Finally, J, K and L referred to sarcoplasmic, myofibrillar and alkali-soluble proteins of wild salmon, respectively. Discontinuous circled lines indicate a possible co-existence of Se and Hg.

soluble) and fish species (tuna, swordfish, farmed salmon and wild salmon) evaluated.

With respect to mercury (blue lines in Fig. 2) this element seems to be bound to biomolecules with molecular weights in the high molecular weight zone of the chromatogram (up to 390–574 kDa). This fact is particularly noticeable in case of myofibrillar (Fig. 2 B, E, H and K) and alkali-soluble (Fig. 2C, F, I and L) protein fractions, with the exception of myofibrillar protein extracts from farmed salmon and wild salmon. In farmed and wild salmon and regarding to myofibrillar extracts, a smaller number of mercury-containing peaks or even none peak were detected, which might indicate that there is likely no Hg-containing biomolecules in the molecular weight range studied. On the other hand, Hg in sarcoplasmic proteins (Fig. 2 A, D, G and J) seems to be bound to biomolecules with a lower molecular weight that those found in myofibrillar and alkali-soluble fractions, being in the range of 12–120 kDa except for swordfish (Fig. 2 D) in which proteins up to 390 kDa were detected.

Moreover, similarities between farmed salmon and wild salmon biomolecules profiles could be also noticed despite differences between Se and Hg total content in myofibrillar, sarcoplasmic and alkali-soluble fractions described in Section 3.2. Therefore, it can be concluded that although differences in Se and Hg total content determined in protein fractions (myofibrillar, sarcoplasmic and alkali-soluble) could be found between wild and farmed salmon, the biomolecules to which these elements are bound to in both fish samples seem to be in the same molecular weight range (Fig. 2 G, H, I and Fig. 2 J, K, L). Therefore, the differences between both salmon species related to their kind of feeding or their way of growing might affect the total Se and Hg content and their distribution through protein fraction but not their Se and Hg-containing biomolecules profiles.

Finally, it is worthwhile mentioning that Se and Hg were found at same retention times ranges. Therefore, both elements might be present in proteins with similar molecular weight. In particular, sarcoplasmic proteins of about 64 and 12 kDa (Fig. 2 A) and alkali-soluble proteins of about 574 kDa (Fig. 2C) in case of tuna, both highlighted with a circle in Fig. 2. This fact may indicate a possible Se–Hg interaction. However, this statement requires of further analysis to be totally confirmed due to the low resolution of the size exclusion chromatographic column used. It is well-known the high affinity of Hg for Se-containing biomolecules. For many years, it was widely believed that the presence of Hg in fish, in its organic form methylmercury (CH_3Hg^+), had been mainly associated to the lipidic content of the fish [22]. Nonetheless, this theory has been modified due to later studies which postulate that methylmercury is present in the muscle tissue of fish (its edible part) owing to its predominant union with the thiol groups and also with Se-containing proteins [6].

3.4. *Selenium speciation in soluble proteins of fish fillet samples. Analysis by HPLC-ICP-MS and HPLC-ESI-MS/MS*

Apart from determining Se and Hg-containing biomolecules and protein profiles, selenium speciation studies were also performed in order to determine the form in which selenium could be present in the fish species studied. For this purpose, enzymatic hydrolysis with protease was applied to the soluble protein extracts previously obtained from all the fishes following the procedure already described in Section 2.6. Total Se content in the obtained extracted was measured in advance by ICP-MS and results were as follows: (51 ± 4), (43 ± 1), (84 ± 6) y (35 ± 1) % for tuna, swordfish, farmed salmon and wild salmon, respectively. Moreover, enzymatic hydrolysis was also applied to the three protein fractions (sarcoplasmic, myofibrillar and alkali-soluble) extracted from their muscle tissues.

Once Se species in fishes and protein fractions were extracted, their detection was performed by HPLC-ICP-MS using a reversed phase column (EVO-C18), owing to the compatibility of its mobile phase with the subsequent HPLC-ESI-MS/MS analyses. Fig. S2 shows, as an example, the chromatogram of Se species corresponding to tuna, in which SeMet and SeMeSeCys were identified by comparing their retention time (3.8 and 2.8 min, respectively) with those of the standards and by spiking experiments with 50 μg L^{-1} of each Se specie. Same analysis for swordfish, farmed salmon and wild salmon brought to light that SeMeSeCys was the only Se specie found (data not shown). Concerning sarcoplasmic, myofibrillar and alkali-soluble proteins the only Se specie found was SeMeSeCys (as an example: Fig. S3 A, B and C), being SeMet not detected on this kind of protein extracts in any fish tested.

Although HPLC-ICP-MS is a sensitive technique to detect Se species, their identification by using Se species standards or spiking experiments are not entirely reliable [23]. Therefore, confirmatory analysis employing HPLC-ESI-MS/MS are mandatory in speciation studies.

For this purpose, reversed phase column (EVO-C18) was also used for HPLC-ESI-MS/MS analysis in order to confirm the identity of the Se species determined by HPLC-ICP-MS with the same chromatography column. A bibliographic search [23–26] of the main fragmentations of our Se species of interest, SeMet and SeMeSeCys, as well as the adequate HPLC-ESI-MS/MS conditions (Section 2.7), allowed us to confirm the results obtained by HPLC-ICP-MS. The three transitions selected for SeMet with the precursor ion *m/z* 198.0 were as follows: MRM *m/z* 198.0-153.0 (loss of CO and NH_3), MRM *m/z* 198.0-135.0 (loss of CO, NH_3 and H_2O) and MRM *m/z* 198.0-109.0 (formation of $CH_3SeCH_2^+$). For its part, the transitions measured for SeMeSeCys were also three with the precursor ion *m/z* 184.1: MRM *m/z* 184.1-166.9 (loss of NH_3), MRM *m/z* 184.1-149.0 (loss of NH_3 and H_2O) and MRM *m/z* 184.1-139.0 (loss of NH_3 and CO). Chromatograms of enzymatic hydrolysis of tuna, swordfish, farmed salmon and wild salmon are compiled in Fig. 3 (A, B, C, D and E), where the peaks highlighted in blue correspond to the transitions mentioned above.

Similarly, Fig. 4 (A, B and C) show, as an example, chromatograms of enzymatic hydrolysis in different protein fractions extracted from tuna: sarcoplasmic, myofibrillar and alkali-soluble proteins. Similar results were obtained regarding the other fishes tested (data not shown). Therefore, the results of the Se species found by HPLC-ICP-MS for tuna (SeMet, in case of soluble proteins, and SeMeSeCys), swordfish (SeMeSeCys), farmed salmon (SeMeSeCys) and wild salmon (SeMeSeCys) have been corroborated.

Contrary to the extensive existing bibliography about Se accumulation and Se speciation in plants, similar information concerning fish is really limited. In this sense, studies focus on Se speciation and its biotransformation in tissues and fish muscles show that SeMet is the main Se specie identified [13,27]. Moreover, other species like SeCys or SeMeSeCys have been also detected for instance in swordfish [28,29]. However, in none of these studies, LC-MS/MS were employed as a confirmatory technique, being indispensable to accurately identify selenium species.

To our knowledge, it is the first time LC-MS/MS technique is used to conduct Se speciation studies in fish fillet of tuna, swordfish, farmed salmon and wild salmon. For years, HPLC-ICP-MS coupling was the most applied technique to carry out Se speciation studies in fish species. Nonetheless, although HPLC-ICP-MS is a powerful sensible technique for elemental Se analysis, it is not entirely reliable when performing Se species determination [23]. As it has been previously mentioned, Se is mostly bound to proteins. However, when speciation studies were performed in soluble proteins of fish fillets, SeMeSeCys was the only Se specie found for all fishes tested except tuna, where SeMet was also detected. Furthermore, only SeMeSeCys could be found when applied enzymatic hydrolysis to other type of proteins such as sarcoplasmic, myofibrillar and alkali-soluble proteins. One of the most important data of this work is the detection of SeMet and SeMeSeCys in fish tissues. These selenium species have been mainly detected in plant and animal tissues but in less extend in fish tissues. Other studies reported in bibliography have shown the presence of SeMet and SeMeSeCys in the muscle tissue of fish species such as trout, swordfish or salmon [14,27, 29]. For instance, Vicente et al. [29] described that SeMet and

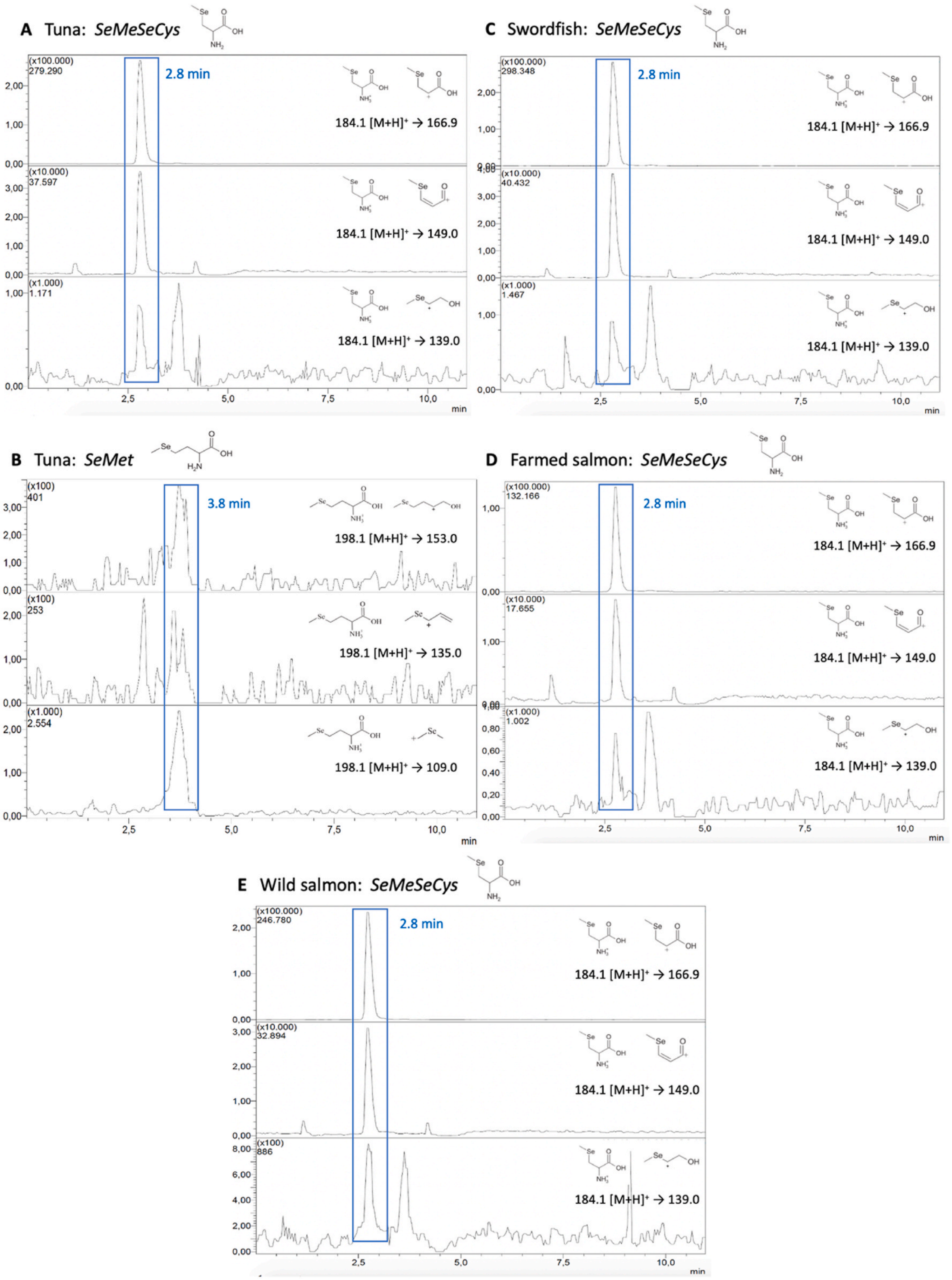

Fig. 3. Chromatographic profiles obtained by reversed phase HPLC-ESI-MS/MS of Se species found in fishes: SeMeSeCys and SeMet for tuna (A and B, respectively) and SeMeSeCys for swordfish (C), farmed salmon (D) and wild salmon (E).

SeMeSeCys are the major selenium species found in muscle tissue of swordfish. Moreover, Sele et al. [14] also determined SeMeSeCys in muscle of salmon fed with diets supplemented with organic Se. Selenium enters in marine environments mainly as inorganic selenium through erosion of rocks and soils as well as because of industrial activities and, afterwards, it is transformed and biomagnified through food webs [31]. Indeed, it seems that phytoplankton is a key factor in transforming inorganic selenium into Se-containing amino acids, being crucial for accumulation and magnification selenium processes [31]. In this sense, Orr et al. [30] indicate that Se is introduced in form of organoselenides into aquatic food webs mainly by being present in aquatic plants and bacteria. Once Se is accumulated by plants in the form of oxyanion (selenite and selenate), it is transformed into selenoamino acids and subsequently incorporated into proteins. Therefore, it might be thought

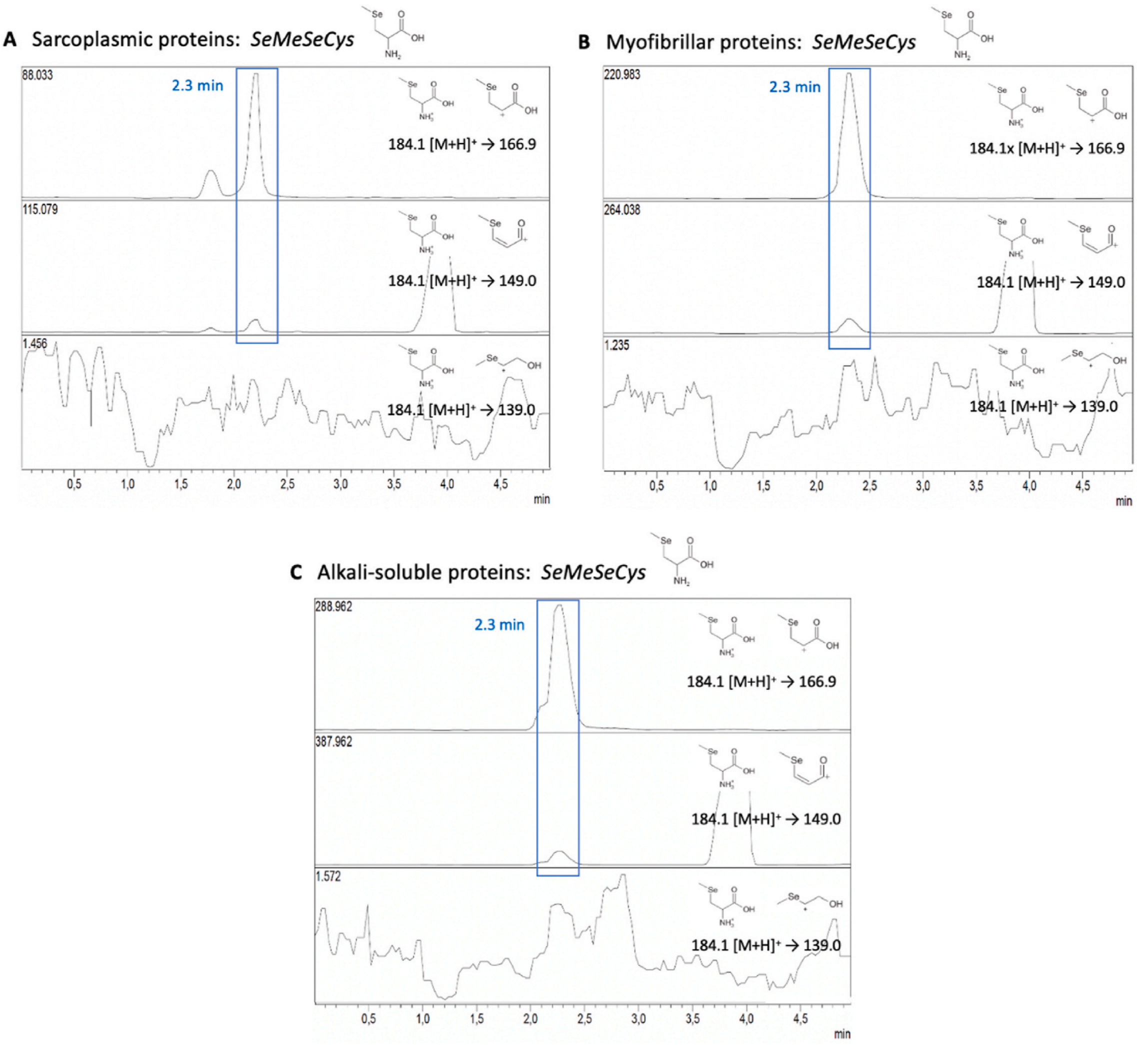

Fig. 4. Chromatographic profiles obtained by reversed phase HPLC-ESI-MS/MS of Se species found in different protein fractions extracted from tuna: sarcoplasmic (A), myofibrillar (B) and alkali-soluble (C) proteins.

that the presence of SeMet and SeMeSeCys in fish tissues can derived from the pathway of selenium in environmental waters through the food web which involves biotransformation of Se and biomagnification processes.

4. Conclusions

Determination of total content and distribution of selenium and mercury in three poorly studied protein fractions (sarcoplasmic, myofibrillar and alkali-soluble) from muscle tissue of highly consumed fishes has been successfully carried out for the first time. Results evidenced that fish samples with similar total Se content had Se distribution patterns in protein fractions completely different, being these dissimilarities more noticeable between farmed and wild salmon samples. On the contrary, Hg distribution patterns in protein fractions analysed were similar in tuna and swordfish but completely different in case of farmed and wild salmon samples. Moreover, SEC-UV-ICP-MS analyses brought to light that Hg seems to be bound to higher molecular weight proteins (up to around 574 kDa) than Se (2–12 kDa), being Se and Hg-containing biomolecules profiles very different in all fish samples except for wild and farmed salmon fish samples. Differences between both salmon species related to their kind of feeding or the way of growing might affect the total Se and Hg content and their distribution through protein fractions alike, but not their Se and Hg-containing biomolecules profiles. Furthermore, it could be thought that Se–Hg interaction may occur since Se and Hg were detected in proteins with same molecular weight in tuna (sarcoplasmic and alkali-soluble). Finally, Se species studies by HPLC-ICP-MS and subsequent confirmation by HPLC-ESI-MS/MS after enzymatic hydrolysis in soluble protein fraction revealed that SeMeSeCys was the unique Se specie found in all the fishes, with the exception of tuna, in which SeMet has been also identified. However, in sarcoplasmic, myofibrillar and alkali-soluble proteins only SeMeSeCys could be found. It is also worth mentioning that this is the first time that selenium speciation studies in highly consumed fish samples were performed by using both HPLC-ICP-MS and HPLC-ESI-MS/MS.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122922.

Credit author statement

Tamara Fernández-Bautista: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing original draft, Writing-review and editing; **Beatriz Gómez-Gómez**: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing-original draft, Writing-review and editing; **Roberto Palacín-García**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation; **Emma Gracia-Lor**: Conceptualization, Investigation, Methodology, Supervision; **Teresa Pérez-Corona**: Conceptualization, Data curation, Investigation, Methodology, Resources, Supervision, Validation, Writing-review and editing; **Yolanda Madrid**: Conceptualization, Founding acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Writing-review and editing.

References

[1] C. Thiry, A. Ruttens, L. Pussemier, Y.J. Schneider, An in vitro investigation of species-dependent intestinal transport of selenium and the impact of this process on selenium bioavailability, Br. J. Nutr. 109 (2012) 2126–2134, https://doi.org/10.1017/S0007114512004412.

[2] P.D. Whanger, Selenocompounds in plants and animals and their biological significance, J. Am. Coll. Nutr. 21 (2002) 223–232, https://doi.org/10.1080/07315724.2002.10719214.

[3] J. Burger, K.F. Gaines, C.S. Boring, W.L. Stephens, J. Snodgrass, M. Gochfeld, Mercury and selenium in fish from the Savannah River: species, trophic level, and locational differences, Environ. Res. 87 (2001) 108–118, https://doi.org/10.1006/enrs.2001.4294.

[4] WHO (World Health Organization), Mercury in Health Care, 2005. http://www.who.int/water_sanitation_health/medicalwaste/mercurypolpap230506.pdf.

[5] D.M. Janz, Selenium, Fish Physiol. 31 (2012) 327–374, https://doi.org/10.1016/S1546-5098(11)31007-2.

[6] A. Giacomino, A. Ruo Redda, R. Caligiuri, P. Inaudi, S. Squadrone, M.C. Abete, O. Abollino, S. Morandi, E. Conca, M. Malandrino, Development of an easy portable procedure for on-site determination of mercury and methylmercury, Food Chem. (2020) 128347, https://doi.org/10.1016/j.foodchem.2020.128347.

[7] N.V.C. Ralston, L.J. Raymond, Mercury's neurotoxicity is characterized by its disruption of selenium biochemistry, Biochim. Biophys. Acta Gen. Subj. 1862 (2018) 2405–2416, https://doi.org/10.1016/j.bbagen.2018.05.009.

[8] M.A. Friedman, L.R. Eaton, W.H. Carter, Protective effects of freeze dried swordfish on methylmercury chloride toxicity in rats, Bull. Environ. Contam. Toxicol. 19 (1978) 436–443, https://doi.org/10.1007/BF01685823.

[9] L. Jin, L. Liang, G. Jiang, Y. Xu, Methylmercury, total mercury and total selenium in four common freshwater fish species from Ya-Er Lake, China, Environ. Geochem. Health 28 (2006) 401–407, https://doi.org/10.1007/s10653-005-9038-5.

[10] M.A.K. Khan, F. Wang, Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism, Environ. Toxicol. Chem. 28 (2009) 1567–1577, https://doi.org/10.1897/08-375.1.

[11] N.V.C. Ralston, L.J. Raymond, Dietary selenium's protective effects against methylmercury toxicity, Toxicology 278 (2010) 112–123, https://doi.org/10.1016/j.tox.2010.06.004.

[12] J. Burger, C. Jeitner, M. Donio, T. Pittfield, M. Gochfeld, Mercury and selenium levels, and selenium: mercury molar ratios of brain, muscle and other tissues in bluefish (Pomatomus saltatrix) from New Jersey, USA, Sci. Total Environ. 443 (2013) 278–286, https://doi.org/10.1016/j.scitotenv.2012.10.040.

[13] Z. Pedrero, Y. Madrid, C. Cámara, E. Schram, J.B. Luten, I. Feldmann, L. Waentig, H. Hayen, N. Jakubowski, Screening of selenium containing proteins in the Tris-buffer soluble fraction of African catfish (Clarias gariepinus) fillets by laser ablation-ICP-MS after SDS-PAGE and electroblotting onto membranes, J. Anal. At. Spectrom. 24 (2009) 775–784, https://doi.org/10.1039/b818367h.

[14] V. Sele, R. Ørnsrud, J.J. Sloth, M.H.G. Berntssen, H. Amlund, Selenium and selenium species in feeds and muscle tissue of Atlantic salmon, J. Trace Elem. Med. Biol. 47 (2018) 124–133, https://doi.org/10.1016/j.jtemb.2018.02.005.

[15] M.A. Bryszewska, A. Måge, Determination of selenium and its compounds in marine organisms, J. Trace Elem. Med. Biol. 29 (2015) 91–98, https://doi.org/10.1016/j.jtemb.2014.10.004.

[16] K. Hashimoto, S. Watabe, M. Kono, K. Shiro, Muscle protein composition of sardine and mackerel, Bull. Japanese Soc. Sci. Fish. 45 (1979) 1435–1441, https://doi.org/10.2331/suisan.45.1435.

[17] Solari, Variaciones en la composición proteica del músculo de Colossoma macropomun (Cuvier, 1818) (Characiformes: Characidae), provenientes de criaderos durante su almacenamiento en frío. http://www.cybertesis.edu.pe/sisbib/2006/solari_gf/pdf/solari_gf.pdf, 2006.

[18] M.D. Renkawitz, T.F. Sheehan, Feeding ecology of early marine phase Atlantic salmon Salmo salar post-smolts, J. Fish. Biol. 79 (2011) 356–373, https://doi.org/10.1111/j.1095-8649.2011.03020.x.

[19] EFSA, Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food, EFSA J 10 (2012), https://doi.org/10.2903/j.efsa.2012.2985.

[20] M. Djermanovic, I. Baralic, S. Pejic, Total mercury levels in commercial fish in market of the Republic of Srpska, Bosnia and Herzegovina, Biol. Trace Elem. Res. 194 (2019) 545–551, https://doi.org/10.1007/s12011-019-01805-4.

[21] EFSA, Scientific opinion on dietary reference values for selenium, EFSA J 12 (2014) 1–67, https://doi.org/10.2903/j.efsa.2014.3846.

[22] S. Watabe, G.C. Hwang, M. Nakaya, X.F. Guo, Y. Okamoto, Fast skeletal myosin isoforms in thermally acclimated carp, J. Biochem. 111 (1992) 113–122, https://doi.org/10.1093/oxfordjournals.jbchem.a123706.

[23] H.H. Harris, I.J. Pickering, G.N. George, The chemical form of mercury in fish, Science 301 (2003) 1203, https://doi.org/10.1126/science.1085941.

[24] G. Moreno-Martín, J. Sanz-Landaluze, M.E. León-González, Y. Madrid, Insights into the accumulation and transformation of Ch-SeNPs by Raphanus sativus and Brassica juncea: effect on essential elements uptake, Sci. Total Environ. 725 (2020), https://doi.org/10.1016/j.scitotenv.2020.138453.

[25] F. Gosetti, P. Frascarolo, S. Polati, C. Medana, V. Gianotti, P. Palma, R. Aigotti, C. Baiocchi, M.C. Gennaro, Speciation of selenium in diet supplements by HPLC-MS/MS methods, Food Chem. 105 (2007) 1738–1747, https://doi.org/10.1016/j.foodchem.2007.04.072.

[26] E. Dumont, Y. Ogra, F. Vanhaecke, K.T. Suzuki, R. Cornelis, Liquid chromatography-mass spectrometry (LC-MS): a powerful combination for selenium speciation in garlic (Allium sativum), Anal. Bioanal. Chem. 384 (2006) 1196–1206, https://doi.org/10.1007/s00216-005-0272-6.

[27] J. Zembrzuska, H. Matusiewicz, H. Polkowska-Motrenko, E. Chajduk, Simultaneous quantitation and identification of organic and inorganic selenium in diet supplements by liquid chromatography with tandem mass spectrometry, Food Chem. 142 (2014) 178–187, https://doi.org/10.1016/j.foodchem.2013.05.004.

[28] R. Jagtap, W. Maher, F. Krikowa, M.J. Ellwood, S. Foster, Measurement of selenomethionine and selenocysteine in fish tissues using HPLC-ICP-MS, Microchem. J. 128 (2016) 248–257, https://doi.org/10.1016/j.microc.2016.04.021.

[29] D. Vicente-Zurdo, B. Gómez-Gómez, M.T. Pérez-Corona, Y. Madrid, Impact of fish growing conditions and cooking methods on selenium species in swordfish and salmon fillets, J. Food Compos. Anal. 83 (2019) 103275, https://doi.org/10.1016/j.jfca.2019.103275.

[30] P.L. Orr, K.R. Guiguer, C.K. Russel, Food chain transfer of selenium in lentic and lotic habitats of a western Canadian watershed, Ecotoxicol. Environ. Saf. 63 (2006) 175–188, https://doi.org/10.1016/j.ecoenv.2005.09.004.

[31] D. Schlenk, G. Batley, C. King, J. Stauber, M. Adams, S. Simpson, W. Maher, J. T. Oris, Effects of light on microalgae concentrations and selenium uptake in bivalves exposed to selenium-amended sediments, Arch. Environ. Contam. Toxicol. 53 (2007) 365–370, https://doi.org/10.1007/s00244-006-0248-3.

Supplementary Material

Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS

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Table S1. Se and Hg mass balances for protein extractions (sum of sarcoplasmic, myofibrillar and alkali-soluble fractions) from each fish.

Sample	% Se	% Hg
Tuna	84	52
Swordfish	126	76
Farmed salmon	111	141
Wild salmon	107	86

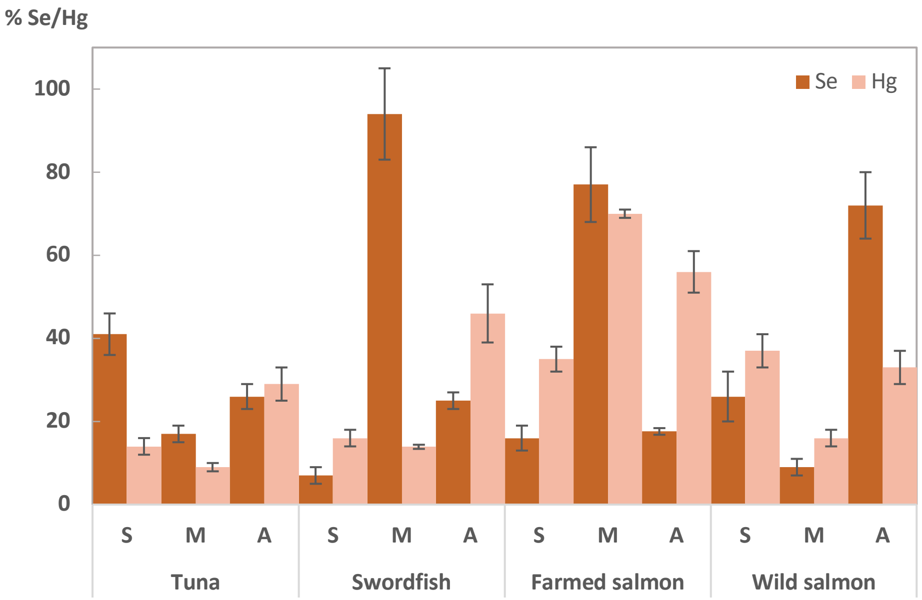


Figure S1. Distribution of Se and Hg content in protein extracts. Percentages were calculated with respect to the total selenium found. The error bars represent standard deviation of means (n = 3). S referred to sarcoplasmic proteins, M to myofibrillar proteins and A to alkali-soluble proteins.

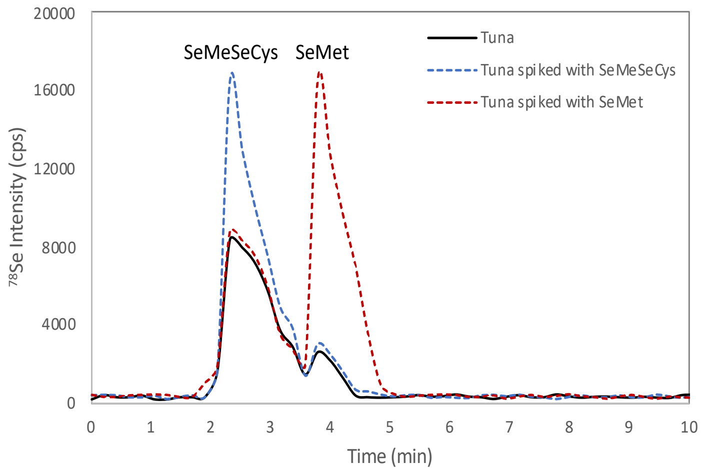


Figure S2. Chromatographic profile obtained by reversed-phase HPLC-ICP-MS from enzymatic hydrolysis of tuna (in black). Discontinuous lines refers to spiking experiments of the sample with 50 µg·L^{-1} of SeMeSeCys (in blue) and 50 µg·L^{-1} of SeMet (in red).

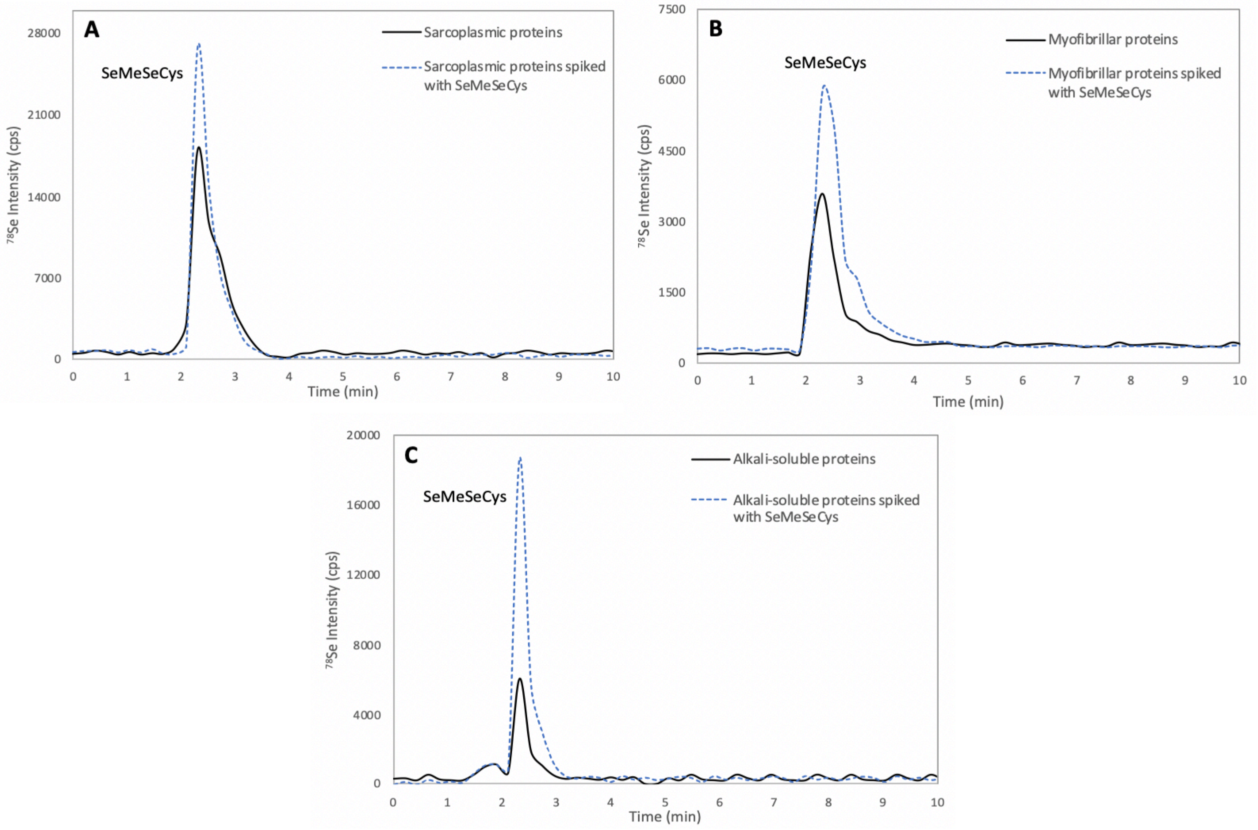


Figure S3. Chromatographic profile obtained by reversed-phase HPLC-ICP-MS from enzymatic hydrolysis of different protein fractions extracted from tuna: sarcoplasmic (A), myofibrillar (B) and alkali-soluble (C) proteins (in black). Discontinuous lines refer to spiking experiments of the sample with 50 µg·L^{-1} of SeMeSeCys (in blue).

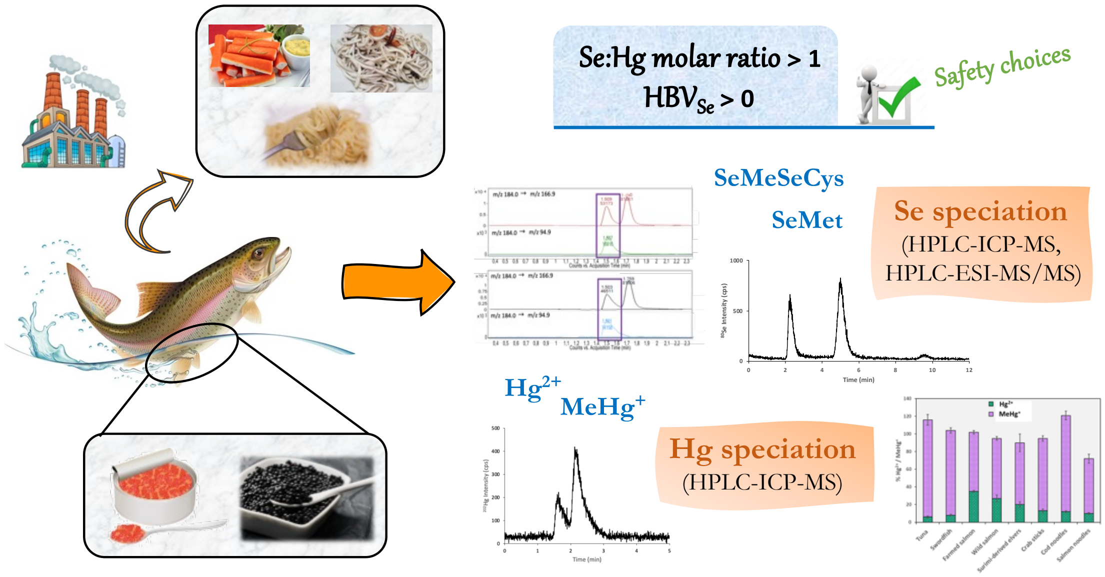
ARTÍCULO 2:

Selenium Health Benefit Values **y estudios de especiación de Hg y Se para evaluar la calidad y seguridad de especies de pescado frecuentemente consumidas y productos derivados del pescado**

Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of highly consumed fish species and fish-derived products

Tamara Fernández-Bautista, Beatriz Gómez-Gómez, Emma Gracia-Lor, Teresa Pérez-Corona, Yolanda Madrid

Food Chemistry 435 (2024) 137544. Factor de impacto: 8.8 (D1, Q1)



Los resultados de este trabajo se presentaron en las siguientes reuniones científicas:

- **European Winter Conference on Plasma Spectrochemistry**. *Determination of Se and Hg species and HgSe particles found in fish and derived products by HPLC-ICP-MS and spICP-MS.* Ljubljana, Eslovenia, enero de 2023. Tipo de comunicación: oral.

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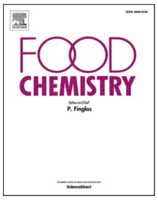

Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of highly consumed fish species and fish-derived products

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ABSTRACT

Nowadays, there has been an increase in the consumption of fish-derived products that constitute alternative ways of introducing fish into the diet, such as fish roe and products made from fish (crab sticks and surimi-derived products). However, there is no data available considering selenium and mercury total contents along with speciation studies in these kinds of samples, which are mandatory for a proper safety and quality assessment. In this study, Selenium Health Benefit Values (HBV_{Se}) in fish and fish-derived products were evaluated, resulting in positive in all cases. Selenium speciation studies performed by HPLC-ICP-MS and confirmed by HPLC-ESI-MS/MS revealed that SeMet and SeMeSeCys are present in fish and fish-derived products. Finally, Hg speciation studies show that the percentage of Hg^{2+} increases in fishes lower in the food chain as well as in fish-derived products.

1. Introduction

Consumer habits are changing as society's needs change, leading to the introduction of new products. Nowadays, there has been an increase in the consumption of fish-derived products that constitute alternative ways of introducing fish into the diet. Among the main advantages of eating fish-derived products versatility in meals, less preparation time and, in some cases, less cost of money can be highlighted. For instance, noodles made of fish or even crab sticks represent alternative modes of eating fish, being also more attractive for children. Fish-derived products might represent a sustainable trade because this kind of food is usually made from fish that have less outlet in the market, although they still have high nutritional value. Fish roe are also products for consumption derived directly from fish, having essential nutritional components for humans.

Mercury (Hg) is highly accumulated in muscle tissue of some fish species and it has been recognized as a major public health concern by World Health Organization (WHO (World Health Organization), 2005, 2008). The biological significance of Hg is limited to its toxicity, which is strongly dependent on both its ingested dose and chemical form. Methylated form of Hg, methylmercury (MeHg), constitutes a potent neurotoxicant and the consumption of fish and fish-derived products is the main source of MeHg exposure for humans. Hg is emitted to the environment from both natural (geothermal activities or releasement from rocks and sediments) and anthropogenic activities (mining, combustion of fossil fuels, metal productions, to name but a few) (EFSA, 2012). However, Hg is in continuous movement through its biogeochemical cycle that integrates the atmosphere, the ocean and the land (Selin, 2009). In the atmosphere, the most abundant form of this element is Hg (0) which oxides to Hg (II), which is deposited in lake and ocean surfaces. Hg (II) can suffer different transformations since it can be reduced to Hg (0), methylated or absorbed onto particles. Conversion of Hg (II) to MeHg is mediated by bacteria and is influenced by several factors such as microbial activity, concentration of bioavailable Hg, redox potential, pH, temperature, etc. MeHg is then bioaccumulated through the tropic chain, reaching high concentration levels in predator fishes that can entail a risk for human consumption (Burger et al., 2001).

Selenium (Se) is a strong antioxidant and an essential micronutrient for humans. It is also present in fish as two of the 22 primary amino acids: selenocysteine (SeCys) and selenomethionine (SeMet) (Raymond & Ralston, 2020). The protective effects of Se against Hg toxicity have been widely evidenced (Gajdosechova et al., 2016; Ralston, Ralston,

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Blackwell & Raymond, 2008; Ralston, Ralston & Raymond, 2016; Suzuki, Kondo, Akiyama & Ogra, 2022). Although the complete mechanisms of Se-Hg interaction are still unknown, it is quite clear that toxicity of MeHg mainly occurs through the alteration of brain Se metabolism (Ralston, Kaneko & Raymond, 2019). When Se-Hg interaction occurs due to the high affinity Hg has for Se, Hg is thought to sequester Se, forming MeHg-SeCys complexes that block Se bioavaility (hence selenoenzymes and antioxidant activities), forming insoluble Hg selenide compounds (Raymond & Ralston, 2020) or even promoting MeHg demethylation and redistribution of Hg to less sensitive organs (Azad et al., 2019). Therefore, Se bioavaility after this interaction is the key factor to assess safety consumption guidances for fish. Se:Hg molar ratio in fish has to be greater than 1, thus after Se sequestration by Hg, molar excess of Se is bioavailable for developing the corresponding biological functions in an organism, for instance, Se-dependent enzyme synthesis and activities (Gochfeld & Burger, 2021).

Additionally, in order to conduct a correct evaluation of the safety of these products regarding Hg and Se, speciation analyses are of special relevance. Chemical speciation helps to determine the molecular form or species in which the metal/metalloid is present in the product and thus becomes crucial for understanding toxicity, bioavailability, and environmental fate and transport. It is widely accepted that the toxicity and essentiality of these elements are highly dependent on their chemical forms. In this sense, to our knowledge, there are no data available considering Se and Hg species in fish-derived products.

Based on that, the main objective of this study is to determine Se and Hg contents, Se:Hg molar ratio, Se and Hg species and Selenium Health Benefit Values in fish-derived and ultra-processed seafood products as well as in traditionally consumed fish in order to assess their safety related to Hg intake and Se protective effect.

2. Materials and methods

2.1. Samples

The samples included in this work were fish fillets (edible parts) of highly consumed fish species and fish-derived products, all of them acquired in a local supermarket in Madrid (Spain) and stored correctly at −20 °C until analysis.

Fish species were tuna (*Thunnus thynnus*), swordfish (*Xiphias gladius*), farmed salmon (*Salmo salar*) and wild salmon (*Oncorhynchus gorbuscha*, from northwest Pacific). Fish fillets were obtained from 10 individuals of each specie.

On the other hand, fish-derived products, fish roe and ultra-processed seafood products made from fish were considered. In particular, three different edible fish roe such as lumpfish roe (from *Cyclopterus lumpus*), trout roe (from *Oncorhynchus mykiss*) and wild salmon roe (from *Oncorhynchus gorbuscha*) were part of this study. Concerning ultra-processed products made from fish, crab sticks, surimi-derived elvers, cod noodles and salmon noodles were selected for this study.

All the analysis performed in this study were in wet weight (ww).

2.2. Determination of the total Se content in muscle tissue of fish and in fish-derived products by ICP-MS

The total Se content in fresh fish fillets and fish-derived products was performed by using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., Santa Clara, CA, USA) after acid digestion in a 1600 W MSP microwave oven from CEM (Stallings, NC, USA). For this purpose, about 1 g of each fresh fish fillet or fish-derived product samples (3 replicates) was digested with 6 mL of a mixture (5:1) of 65 % v/v nitric acid (Merck, Madrid, Spain) and 30 % v/v hydrogen peroxide (Panreac, Barcelona, Spain) and submitted to a microwave heating program consisting of a 20-min ramp to 130 °C and a 10-min hold time. Blank solutions (3 replicates) were also performed. Once cooled until room temperature, the solutions were appropriately diluted with deionized water (18.2 MΩ cm, from a Milli-Q® water purification system; Millipore, Bedford, MA, USA) to a final volume of 10 mL and filtered through a 0.22 μm syringe Nylon filter (Scharlab, Barcelona, Spain) prior analyses. Total Se content was determined by ICP-MS, being the operational conditions listed in Table S1 of Supplementary Material. Se quantification was performed by external calibration with the isotope ^{80}Se. A Certified Reference Material (ERM-CE 278 k, from mussel) from the Institute for Reference Materials was analyzed to validate the accuracy of the results obtained. Certified value is (1.62 ± 0.12) mg kg^{-1} for total Se and experimental value obtained was (1.64 ± 0.14) mg kg^{-1}. The limit of detection (LOD) was 0.002 mg kg^{-1}. The limit of quantification (LOQ) was calculated according to International Union of Pure and Applied Chemistry (IUPAC) (IUPAC, 1978). The LOQ was obtained as $10\sigma/S$, where σ is the standard deviation of 10 measurements of the blank solution and *S* is the slope of the line equation for external calibration curve. For total Se, the LOQ was 0.006 mg kg^{-1}.

2.3. Determination of the total Hg content in muscle tissue of fish and in fish-derived products by DMA

Total Hg determination in fresh fish fillet and fish-derived products was carried out by using a Direct Mercury Analyzer (DMA-80 evo; Milestone, Sorisole, Italy). Around 50–150 mg of fresh fish fillet or fish-derived product samples (3 replicates) were weighed on quartz sample boats and directly analyzed by DMA. The accuracy of the method was further evaluated by the analysis of Certified Reference Materials from oyster (BCR-710) and mussel (ERM-CE 278 k) tissues, which certified for total Hg (0.26 ± 0.05) mg kg^{-1} and (0.073 ± 0.007) mg kg^{-1}, respectively. Experimental values obtained were (0.29 ± 0.05) mg kg^{-1} and (0.075 ± 0.009) mg kg^{-1}, respectively. The limit of quantification (LOQ) calculated as described in the section above according to IUPAC rules (IUPAC, 1978) was 0.033 μg kg^{-1}.

2.4. Se:Hg molar ratios and HBV$_{Se}$

It is well known that Se ameliorates Hg toxicity and it is therefore convenient to know Se:Hg molar ratios as well as HBV$_{Se}$, since these values are more informative than just presenting separately total contents of both elements. Both indexes become crucial in order to correctly assess seafood safety concerning Se and Hg ingestion. The use of these values makes also easier identifying healthy seafood choices. Therefore, once Se and Hg total concentrations were determined, Se:Hg molar ratio and Selenium Health Benefit Values (HBV$_{Se}$) were calculated for each sample from the average concentrations of both elements.

Se:Hg molar ratio was calculated according to Eq. (1) (Burger & Gochfeld, 2011; Ralston et al., 2008; Ulusoy, Mol, Karakulah & Kahraman, 2019):

$$Se : Hg\ molar\ ratio = \frac{mmol\ Se\ kg^{-1}\ ww}{mmol\ Hg\ kg^{-1}\ ww} \tag{1}$$

Concerning HBV$_{Se}$, it is an index that shows how much Se remains available once part of it has been sequestered by Hg through a Se-Hg interaction. HBV$_{Se}$ was determined by applying Eq. (2) (Ralston et al., 2019):

$$HBV_{Se} = \frac{Se - Hg}{Se}\ (Se + Hg) \tag{2}$$

Se = selenium content in molar concentration (mmol Se kg^{-1}).

Hg = mercury content in molar concentration (mmol Hg kg^{-1}).

Positive values of HBV$_{Se}$ indicate that the consumption of the fish/fish-derived product does not apparently entail risks derived from Hg toxicity. However, negative values indicate potential risks of Hg for consumers.

Table 1

Fragmentations and collision energies optimized for Se species analyzed by HPLC-ESI-MS/MS.

Compound	Precursor ion (m/z) [M + H$^+$]	Product ion Q (m/z)	CE(eV)	Product ion I (m/z)	CE(eV)
Selenomethylseleno-L-cysteine(SeMeSeCys)	184.0	166.9	2	94.9	26
D-L-selenocystine (SeCys$_2$)	336.9	247.8	10	88.0	26
Seleno-L-methionine(SeMet)	198.0	180.9	2	56.1	22
Selenomethionine Se-oxide (SeMetO)	214.0	102.0	6	56.1	26

Q: quantification transition, I: confirmation transition.

2.5. Determination of Se species extracted from muscle tissue of fish and fish-derived products by HPLC-ICP-MS and confirmed by HPLC-ESI-MS/MS

Se species in samples were determined in the soluble protein fraction extracted from fish-derived products by enzymatic hydrolysis followed by HPLC-ICP-MS analyses. About 0.5 g of each sample (3 replicates of each) were treated with 5 mL of 30 mM tris-(hydroxymetyl)-aminomethane (Tris; Sigma-Aldrich, Madrid, Spain) at pH 7.5 (adjusted with 0.05 M HCl) and 20 mg of the non-specific protease *Streptomyces griseus* (Protease type XIV; Sigma-Aldrich, Madrid, Spain). The mixture was incubated for 24 h at 37 °C in a thermostatic bath (Termotronic II from P Selecta, Barcelona, Spain). Samples were centrifuged at 10000 rpm for 15 min at 4 °C (Eppendorf 5804 F34-6–38 centrifuge, Hamburg, Germany) and supernatants were filtered through a 0.22 µm syringe Nylon filters before analysis. Total Se content in the obtained extracts was also measured by ICP-MS after performing a microwave acid digestion as described in Section 2.2.

The separation of Se species was accomplished on a Hamilton PRP-X100 column (250x4.1 mm, 10 µm), with an anion exchange mechanism, and detected by ICP-MS. The experimental conditions are listed in Table S1 of Supplementary Material. Se species identification was performed by matching the retention times with those of standard solutions: selenomethylseleno-L-cysteine (SeMeSeCys), D-L-selenocystine (SeCys$_2$), seleno-L-methionine (SeMet), Se (IV) (Na_2SeO_3) and Se (VI) (Na_2SeO_4), all from Sigma-Aldrich (Spain). Standard stock solutions (>1000 mg L^{-1}) were prepared in acidic conditions (inorganic Se species, Se (IV) and Se (VI), in 2 % v/v HNO_3, SeMet in 0.1 M HCl and SeMeSeCys in 3 % v/v HCl) and stored at 4 °C. From these solutions, working solutions less concentrated were freshly prepared daily by dilution, before each analysis. From working solutions, calibration solutions were prepared by dilution with Milli-Q® water. Matrix effects were checked by performing standard addition calibration curves for each sample and comparing their slopes to that of the external calibration curve by means of a Student́s *t*-test ($\alpha = 0.05$).

In order to confirm the identity of the Se species previously detected by HPLC-ICP-MS, analyses by HPLC-ESI-MS/MS were carried out. Chromatographic separation was carried out using a Phenomenex Kinetex EVO C18 column (150x3 mm, 5 µm), due to its compatibility with the HPLC-ESI-MS/MS system. The targeted Se species were analyzed by means of an Agilent G6410B Triple Quadrupole LC/MS System (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with an Agilent 1200 Series G1312 binary pump, an Agilent 1200 Series G1322A degasser, an Agilent 1200 Series G1329A standard autosampler with temperature-controlled tray and an Agilent 1200 Series G1316A column oven.

The injection volume onto the column was 5 µL and the mobile phase consisted of A (95 %): MilliQ® water with 0.1 % formic acid (Scharlab, Barcelona, Spain) and B (5 %): acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) with 0.1 % formic acid. The elution mode was isocratic with a flow rate of 0.5 mL min^{-1}.

Analyses were done in positive electrospray ionization (ESI) mode using the Multiple Reaction Monitoring (MRM) mode at various fragmentor voltages (from 60 to 110). The ion source settings were as follows: gas temperature, 200 °C; drying gas flow, 10 L min^{-1}; nebulizing gas pressure, 35 psi; and capillary voltage, 200 V. Dwell times of 50 ms were selected for all the transitions. Masshunter Data Acquisition B.04.01 and Masshunter Qualitative Analysis B.07.00 were employed for data acquisition and processing. Selected transitions and collision energies (CE) for Se species are detailed in Table 1.

2.6. Determination of Hg species extracted from muscle tissue of fish and fish-derived products by HPLC-ICP-MS

Determination of Hg species extracted from the muscle tissue of fish and from fish derived-products was carried out by extraction with a L-cysteine·HCl·H_2O extraction solution followed by HPLC-ICP-MS analysis.

Extractions were carried out according to the procedure of Hight & Cheng (Hight & Cheng, 2006) with slight modifications. Portions of 0.5 or 2 g of each sample (3 replicates) previously homogenized were weighted in glass vials and 5 or 10 mL, respectively, of 1 % (w/v) L-cysteine·HCl·H_2O (Sigma-Aldrich, Madrid, Spain) extraction solution was added. Vials were shaken vigorously for a few seconds and subsequently heated at 60 °C for 2 h. Once vials were cooled at room temperature, they were centrifuged at 5000 rpm for 20 min. Supernatants were separated and introduced to the HPLC-ICP-MS system with the appropriate dilution.

Separation of Hg species (Hg^{2+}, CH_3Hg^+ and $CH_3CH_2Hg^+$) was accomplished using a reversed-phase column (Phenomenex Kinetex EVO C18 column, 150x3 mm, 5 µm). Operational conditions are listed in Table S1 of Supplementary Material. External calibration was performed with a series of standard solutions: mercury (II) chloride (Hg^{2+}), methylmercury (II) chloride (CH_3Hg^+) and ethylmercury chloride ($CH_3CH_2Hg^+$), all from Sigma-Aldrich (Madrid, Spain). Moreover, standard addition calibration method and internal standard calibration method with Bi^{3+} (Sigma-Aldrich, Madrid, Spain) were also tested to evaluate matrix effects and to compensate for possible inaccuracies throughout the experimental procedure, respectively. Bi^{3+} was chosen as the internal standard due to its similar affinity to SH-group, chromatographic behaviour and because ^{209}Bi isotope is close to ^{202}Hg and ^{201}Hg isotopes (Santoyo, Figueroa, Wrobel & Wrobel, 2009). For recovery experiments, each Hg species (Hg^{2+}, CH_3Hg^+ or $CH_3CH_2Hg^+$) was added to the fresh tissue (30 µg L^{-1}) before starting the extraction process. Standard stock solutions of 2000 mg L^{-1} were prepared in MeOH (CH_3Hg^+ and $CH_3CH_2Hg^+$) or in 0.1 % v/v HCl (Hg^{2+}) and stored at 4 °C. From these solutions, working solutions less concentrated were freshly prepared daily by dilution, before each analysis. From working solutions, calibration solutions were prepared by dilution with Milli-Q® water. In order to validate the results obtained, BCR-710 (from oyster), a Certified Reference Material from the Institute for Reference Materials was analyzed, following the entire extraction process and subsequent HPLC-ICP-MS detection. The certified concentration for $MeHg^+$ in BCR-710 is 0.115 mg kg^{-1} with an uncertainty of 0.000018 mg kg^{-1} and the experimental value obtained was 0.119 ± 0.008 mg kg^{-1}. The limits of quantification (LOQ) calculated according to IUPAC (IUPAC, 1978) were as follows: LOQ (Hg^{2+}) = 0.02 mg kg^{-1} and LOQ ($MeHg^+$) = 0.002 mg kg^{-1}.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was performed to detect possible differences in Se and Hg total contents and species as well as in Se:Hg molar ratio and HBV_{Se} among the different samples analysed. A significance level of $p\text{-value} < 0.05$ was adopted for all comparisons. Statistical analysis of data was carried out using Statgraphics Centurion XIX (Statgraphics Technologies Inc., The Plains, VA, USA).

3. Results and discussion

3.1. Total Se and Hg content in muscle tissue of fish species and in fish-derived products. Se:Hg molar ratios and HBV_{Se}

Total Se content in fresh fish fillets and in fish-derived products was carried out by ICP-MS after acid digestion in a microwave oven, as it was described in Section 2.2. Moreover, total Hg content in same samples was determined without any sample pre-treatment by DMA (described in Section 2.3).

Total Se and Hg contents in fresh fish fillets and fish-derived products are gathered in Table 2 in which it is possible to observe that Se and Hg concentrations vary from 0.74 to 4.4 mg kg^{-1} and from 0.0030 to 0.75 mg kg^{-1}, respectively. As it was expected, tuna and swordfish present high Se concentration and high Hg levels (Table 2), since they are fish species higher up in the food chain. Biomagnification processes are the main responsible for this fact, which has been widely reported in the literature (Azad et al., 2019; Ralston et al., 2016, 2019; Ribeiro et al., 2022). Regarding farmed and wild salmon, they show slight differences in Hg concentration and a more noticeable difference in Se content (Table 2), likely due to differences in the diets they are given and environments in which they are grown (Fernández-Bautista et al., 2022). Furthermore, fish-derived products, which are increasingly consumed, have relatively elevated concentrations of Se (> 0.74 mg kg^{-1}) and, when it comes to fish roe, low levels of Hg (among 0.0030–0.0048 mg kg^{-1}). Zhang *et al.* (Zhang et al., 2020) claimed that muscle tissue is thought to be a storage organ for Hg, while roe might be an important source of Se, which is consistent with our results.

ANOVA analysis was carried out for studying differences between Se and Hg concentrations found in fishes and fish-derived products, as well as for elucidating independently if there were differences in Se or Hg levels among the fishes and fish-derived products selected for this study. The most relevant results obtained evidenced statistically significant differences ($p\text{-value} < 0.05$) in Hg values for farmed and wild salmon which can be attributed to their different origin, way of living and source of nutrients (Colombo & Mazal, 2020; Fernández-Bautista et al., 2022; Vicente-Zurdo, Gómez-Gómez, Pérez-Corona & Madrid, 2019). Moreover, statistically significant differences between Se and Hg concentrations have also been evaluated by ANOVA test and results did not show statistically significant differences ($p\text{-value} < 0.05$) in Se and Hg contents for any sample (Table 2).

European Food Safety Authority (EFSA, 2012) has informed about the average of Hg levels found in fish, although they are highly dependent not only on the trophic level, but also on other factors such as gender, organ, age, size or even kind of feed of the fish. Our results are in consonance with these levels: predator fishes (in which swordfish is included) normally have high Hg concentrations, up to 1 mg kg^{-1} ww, tuna presents Hg concentrations ranging between 0.1 and 1.5 mg kg^{-1} ww and salmon species contains lower concentrations, which are < 0.1 mg kg^{-1} ww.

Once Se and Hg total concentrations were determined, Se:Hg molar ratio and Selenium Health Benefit Value (HBV_{Se}) were calculated for each sample following Eqs. (1) and (2) described in Section 2.4. Results are shown in Table 2 and Figure S1 of Supplementary Material, where it can be observed that Se:Hg molar ratios were greater than 1 for all fishes and fish-derived products considered in this study, which indicates that Se is present in molar excess of Hg content.

Additionally, Figure S1 of Supplementary Material represents graphical comparisons between Hg and Se contents and HBV_{Se}, respectively. Positive values of HBV_{Se} were obtained for all fish and fish-derived products (Table 2). The highest HBV_{Se} can be observed for fish roe, especially for wild salmon roe followed by trout roe since they presented high content of Se but low Hg levels. Conversely, although tuna exhibited one of the highest total Hg concentrations, HBV_{Se} value remains positive due to its relatively high Se content, which is high enough to satisfy biological functions after Hg sequestration according to this index.

Therefore, moderate consumption of these fish and fish-derived products seems not to have negative effects on human health related to Hg consumption. In particular, fish roe have the highest values of Se:Hg ratio and HBV_{Se}, due to its low amount of total Hg content. Hence, these products derived from fish seem to be good choices for consumption. Until now, little information was available on this kind of seafood concerning Se and Hg. Nevertheless, positive Se:Hg molar ratio and HBV_{Se} have been widely reported in the literature concerning fish species, being in agreement with our study. Ordiano-Flores *et al.* (Ordiano-Flores, Rosíles-Martínez & Galván-Magaña, 2012) reported a Se:Hg molar ratio of 10.29 ($HBV_{Se} = 64.54$) for yellowfin tuna. Moreover, Olmedo *et al.* (Olmedo et al., 2013) show Se:Hg molar ratios of 3.06, 2.32 and > 100 for tuna, swordfish and farmed salmon, respectively, leading to 21, 13 and > 1000 the HBV_{Se} calculated. Cabañero *et al.* (Cabañero, Madrid & Cámara, 2007) also found favorable Se:Hg molar ratios of 4.9 and 2.0 for tuna and swordfish, respectively.

3.2. Se speciation in soluble proteins of fish fillets and fish-derived products. Analysis by HPLC-ICP-MS and HPLC-ESI-MS/MS

Extraction of Se species present in fish and fish-derived products was carried out by enzymatic hydrolysis with protease following the procedure described in Section 2.5. Detection of Se species was accomplished by HPLC-ICP-MS, using an anion exchange column.

Table 2
The mean Se and Hg concentrations (mg kg^{-1} ww) and Se:Hg molar ratios.

Fish/Fish-derived product	[Se] (mg kg^{-1} ww)	[Hg] (mg kg^{-1} ww)	Se:Hg molar ratio	HBV_{Se}
Tuna	$^{a}1.6 \pm 0.1$	$^{a}0.75 \pm 0.03$	5.44	0.0196
Swordfish	$^{b}1.4 \pm 0.2$	$^{b}0.23 \pm 0.04$	15.91	0.0174
Farmed salmon	$^{c}1.03 \pm 0.07$	$^{c}0.022 \pm 0.002$	131.00	0.0131
Wild salmon	$^{c}0.74 \pm 0.08$	$^{d}0.026 \pm 0.003$	72.31	0.0094
Surimi-derived elvers	$^{d}0.75 \pm 0.05$	$^{d}0.026 \pm 0.001$	73.08	0.0095
Crab sticks	$^{d}0.78 \pm 0.08$	$^{d}0.028 \pm 0.009$	70.71	0.0099
Lumpfish roe	$^{e}1.3 \pm 0.2$	$^{e}0.0048 \pm 0.0003$	800.00	0.0160
Wild salmon roe	$^{f}4.4 \pm 0.4$	$^{f}0.0030 \pm 0.0003$	4280.00	0.0642
Trout roe	$^{g}1.9 \pm 0.3$	$^{g}0.0036 \pm 0.0007$	1200.00	0.0240
Cod noodles	$^{h}0.74 \pm 0.03$	$^{h}0.047 \pm 0.009$	39.17	0.0094
Salmon noodles	$^{h}0.79 \pm 0.02$	$^{h}0.054 \pm 0.013$	37.11	0.0100

Results are expressed as mean ± standard deviation (SD); n = 3. Different superscript letters in values indicate significant differences ($p\text{-value} < 0.05$) for each analyte (Se or Hg) between fish and fish-derived products.

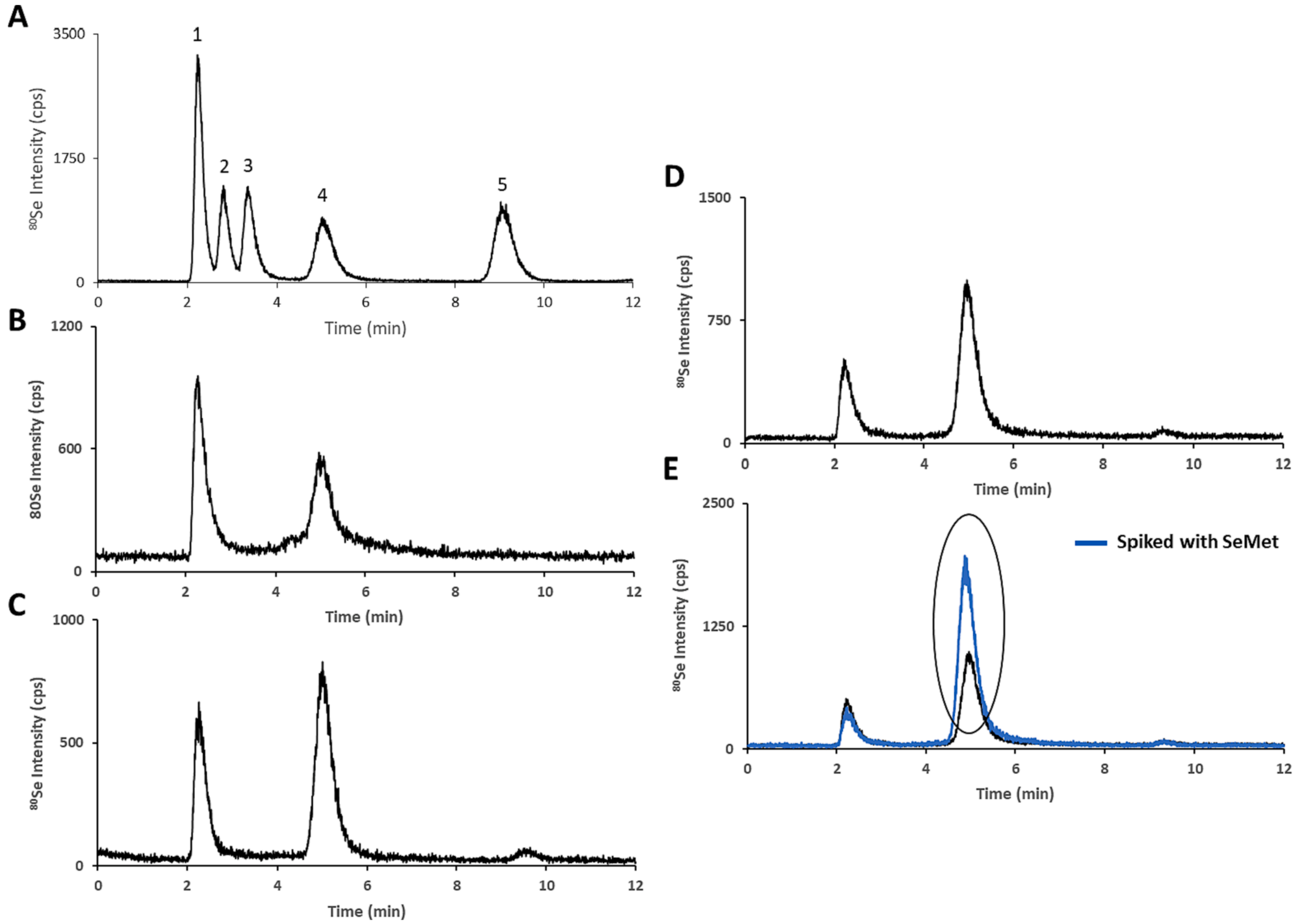

Fig. 1. Chromatographic profiles obtained by anion exchange HPLC-ICP-MS of (A) standard solution containing 10 µg L^{-1} of (1) SeCys$_2$, (2) SeMeSeCys, (3) Se (IV), (4) SeMet and (5) Se (VI), (B) surimi-derived elvers, (C) salmon noodles, (D) cod noodles and (E) cod noodles spiked with 10 µg L^{-1} of SeMet.

Previously, experiments were carried out to study whether the extraction procedure was quantitative for Se species. Percentages of Se extracted with respect to the total Se content were as follows: 101 ± 7, 108 ± 15, 99 ± 13, 84 ± 13, 77 ± 3, 93 ± 20 and 88 ± 7 % for crab sticks, surimi-derived elvers, lumpfish, salmon and trout roe, cod noodles and salmon noodles, respectively. It can be said that quantitative extraction has been achieved for all fish-derived products analyzed in this study.

Fig. 1 shows some examples of chromatographic profiles obtained by HPLC-ICP-MS in which the separation and detection of the Se species of interest can be observed. The retention times of the Se species analyzed are the following: SeCys$_2$ (RT = 2.270 min), SeMeSeCys (RT = 2.826 min), Se (IV) (RT = 3.453 min), SeMet (RT = 4.881 min) and Se (VI) (RT = 9.741 min), respectively.

Chromatograms of all the samples analyzed (crab sticks, surimi-derived elvers, lumpfish, salmon and trout roe, cod noodles and salmon noodles) show two main peaks, eluting at around 2.5 and 4.86 min, respectively. Comparing the retention time to that of the standard solution and confirmed by spiking experiments (Fig. 1 D and E), the second peak corresponds to SeMet. However, the identity of the first Se-containing peak cannot be elucidated by using exclusively this technique, because in the first part of the chromatogram and due to the complexity of the matrix of the samples, chromatographic peaks were not well resolved. To overcome this problem, HPLC-ESI-MS/MS analysis were carried out (described in Section 2.5) and results evidenced the presence of SeMeSeCys in all samples. Figure S2 of Supplementary Material displays an example of the identification of SeMeSeCys in lumpfish roe and crab sticks.

Table 3
SeMeSeCys and SeMet concentrations (mg kg^{-1} ww) found in fish-derived products by HPLC-ICP-MS.

Fish-derived product	[SeMeSeCys] (mg kg^{-1} ww)	[SeMet] (mg kg^{-1} ww)
Surimi-derived elvers	^{a}0.15 ± 0.02	^{a}0.39 ± 0.05
Crab sticks	^{b}0.13 ± 0.01	^{a}0.38 ± 0.04
Lumpfish roe	^{c}0.29 ± 0.03	^{b}0.52 ± 0.06
Wild salmon roe	^{d}0.69 ± 0.03	^{c}2.40 ± 0.07
Trout roe	^{e}0.40 ± 0.05	^{d}0.99 ± 0.02
Cod noodles	^{f}0.14 ± 0.03	^{e}0.32 ± 0.06
Salmon noodles	^{f}0.14 ± 0.02	^{e}0.31 ± 0.04

Results are expressed as mean ± standard deviation (SD); n = 3. Different superscript letters in values indicate significant differences (p-value < 0.05) for each Se species between fish-derived products.

Similarly to our previous work concerning Se speciation in fish samples (Fernández-Bautista et al., 2022), SeMet and SeMeSeCys were identified in all fish-derived products analyzed.

For quantification purposes, matrix effects were checked by performing standard addition calibration curves for each sample and comparing their slopes to that of the external calibration curve using a Student́s *t*-test (α = 0.05). Results evidenced that there were not significant differences between the slopes of the two calibration curves (data not shown), concluding that there is no matrix effect for any of the samples studied (fish and fish-derived products). Therefore, external calibration method was employed for quantifying Se species in each sample. Table 3 shows SeMet and SeMeSeCys contents found in fish roe and in products made from fish, being SeMet more abundant than SeMeSeCys. Regarding products made from fish (crab sticks, surimi-derived elvers, cod noodles and salmon noodles), they show similar Se species. However, fish roe (from lumpfish, salmon and trout) evidenced statistically significant differences (ANOVA test, p-value < 0.05) in their SeMeSeCys but especially SeMet content.

SeMet and SeMeSeCys are also the main Se species present in muscle tissues of highly consumed fishes (Fernández-Bautista et al., 2022; Sele, Ørnsrud, Sloth, Berntssen & Amlund, 2018; Vicente-Zurdo et al., 2019; Zembrzuska, Matusiewicz, Polkowska-Motrenko & Chajduk, 2014), which are the raw material for products made from fish.

Crab sticks and surimi-derived elvers are made from the raw material of the following fish: walleye pollock (*Gadus chalcogrammus*) and from North Pacific hake (*Merluccius productus*). The elaboration process for crab sticks and surimi-derived elvers is the same and consists of repeated washing processes with water that concentrates the fish proteins, which have gelling properties, allowing the elaboration of these products. Differences are based on the staining and the shape they acquire, since the crab sticks are made with an orange tone and in the case of surimi-derived elvers they are shaped like elvers. Regarding cod and salmon noodles, the elaboration process is similar to that of crab sticks and surimi-derived elvers with an additional step. Once proteins are concentrated, they are mixed and kneaded with other ingredients such as water, salt, starch, scents and egg white. Then, a cooking process is carried out at 90 °C where defatting and formation of spaghetti or noodle-like strips are carried out. Raw materials are also different, cod noodles are made from North Pacific hake (*Merluccius productus*) and shallow-water Cape hake (*Merluccius capensis/paradoxus*), whereas salmon noodles are made from farmed salmon (*Salmo salar*) and North Pacific hake (*Merluccius productus*).

Therefore, it can be concluded that the same Se species (SeMet and SeMeSeCys) have been identified in fish and in fish-derived products although the elaboration process of fish-derived products could affect their stability. Nevertheless, for instance, SeMet and SeMeSeCys were identified in salmon noodles as well as previously in farmed and wild salmon (Fernández-Bautista et al., 2022), which constitute part of the raw material employed in the elaboration process of this fish-derived product.

3.3. Hg speciation by HPLC-ICP-MS in fish species and fish-derived products

Hg speciation studies were also carried out in fish and fish-derived products to investigate how Hg is distributed regarding to its species. For this purpose, extraction of Hg species with 1 % (w/v) L-cysteine·HCl·H_2O was accomplished, followed by detection by HPLC-ICP-MS, using a reversed-phase chromatographic mechanism (Section 2.6).

Fig. 2 displays the chromatographic profile for Hg standard species (Hg^{2+}, $MeHg^{+}$ and $EtHg^{+}$) separation obtained by HPLC-ICP-MS as well as the chromatograms obtained for tuna, wild salmon and crab sticks, as examples, in which Hg^{2+} and $MeHg^{+}$ were identified. These Hg species were the only ones found in the remaining samples of fish and fish-derived products except for fish roe.

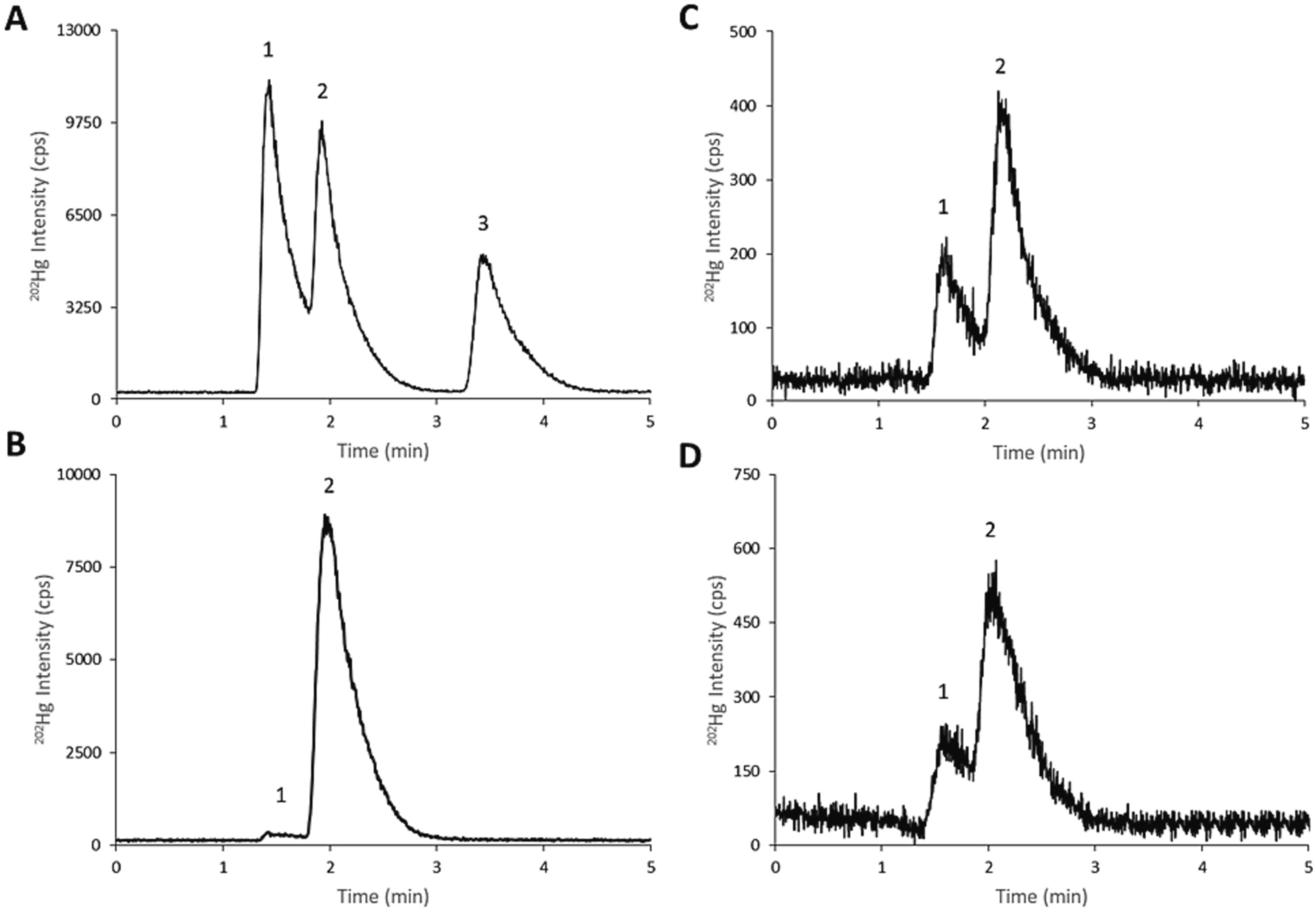

Fig. 2. Chromatographic profiles obtained by reversed-phase HPLC-ICP-MS of (A) standard solution containing 50 µg L^{-1} of Hg^{2+}, $MeHg^{+}$ and $EtHg^{+}$, (B) tuna, (C) wild salmon and (D) crab sticks extractions. Numbers 1, 2 and 3 on top of the peaks are referred to Hg^{2+} (RT = 1.418 min), $MeHg^{+}$ (RT = 1.906 min) and $EtHg^{+}$ (RT = 3.442 min), respectively.

Table 4

Hg^{2+} and $MeHg^{+}$ concentrations (mg kg^{-1} ww) and distribution in percentage of Hg^{2+} and $MeHg^{+}$ present in fish and fish-derived products. Sum of Hg^{2+} and $MeHg^{+}$ was also expressed in the Table as % values.

Fish/Fish-derived product	[Hg^{2+}] (mg kg^{-1} ww)	[$MeHg^{+}$] (mg kg^{-1} ww)	Hg^{2+} (%)	$MeHg^{+}$ (%)	∑ Hg (%)
Tuna	0.043 ± 0.002	0.819 ± 0.005	6 ± 1	110 ± 6	116 ± 9
Swordfish	0.017 ± 0.001	0.196 ± 0.006	8 ± 1	96 ± 3	104 ± 3
Farmed salmon	0.0077 ± 0.0002	0.0147 ± 0.0005	35 ± 1	67 ± 2	102 ± 2
Wild salmon	0.008 ± 0.002	0.018 ± 0.002	27 ± 4	68 ± 2	95 ± 6
Surimi-derived elvers	0.005 ± 0.001	0.018 ± 0.003	20 ± 3	70 ± 10	90 ± 12
Crab sticks	0.004 ± 0.001	0.023 ± 0.001	13 ± 2	82 ± 3	95 ± 2
Cod noodles	0.006 ± 0.001	0.051 ± 0.002	12 ± 1	109 ± 5	121 ± 4
Salmon noodles	0.005 ± 0.001	0.033 ± 0.003	10 ± 1	62 ± 5	71 ± 5

Results are expressed as mean ± standard deviation (SD); n = 3.

Some authors claimed that EtHg is not stable during the extraction process (Hight & Cheng, 2006; Qvarnström, Lambertsson, Havarinasab, Hultman & Frech, 2003), being partially de-alkylated producing inorganic Hg. Experiments were carried out in order to confirm this statement with the samples of this study. Three replicates of each fish and three replicates of each fish-derived product were fortified with 30 μg L^{-1} of EtHg before starting the extraction process. The other three replicates of each fish/fish-derived product without fortification were subjected to the extraction process at the same time and samples were analyzed under the same conditions. Inorganic Hg peak areas obtained for each group of samples (fortified *vs.* non-fortified) were different, leading to approximately 3 times more concentration of inorganic Hg in the samples fortified with EtHg than in those that had not been fortified. Thus, it can be affirmed that the peak appearing at the retention time of inorganic Hg could correspond to the sum of both the inorganic Hg originally present in the sample and that coming from the dealkylation of other compounds during the extraction process such as EtHg. Although this EtHg transformation process has been studied, this Hg species was not present in any sample analyzed in this study (peaks at the retention time of EtHg were not detected) and, what is more, the presence of this compound in seafood in general has not been reported in the literature yet.

MeHg was stable during the extraction process but the analysis of the extracted samples by HPLC-ICP-MS must be accomplished the same day, just after the extraction process. If not, MeHg can be de-alkylated during storage, producing inorganic Hg (Hight & Cheng, 2006).

For quantification purposes, external calibration, standard addition and internal standard calibration method with Bi^{3+} were tested. Firstly, the effect of using an internal standard compared to the external standard approach was evaluated. In this regard, the employment of Bi^{3+} as an internal standard did not improve the precision and accuracy of results (data not shown). However, when external calibration was compared to that of standard addition, Studentś *t*-test ($\alpha = 0.05$) showed significant differences between the slopes of the two calibration curves (data not shown), which evidenced the existence of matrix effects in all samples studied (fish and fish-derived products). Therefore, standard addition calibration method was employed for quantifying Hg species in each sample.

Furthermore, spiking experiments were carried out in order to evaluate Hg^{2+} and $MeHg^{+}$ extraction efficiencies due to the complexity of the fish/fish-derived products matrix. Recoveries obtained from spiking experiments with 30 μg L^{-1} of each species (Hg^{2+} and $MeHg^{+}$) are compiled in Table S2 of Supplementary Material, in which quantitative results can be seen for the majority of samples taking part in this study. However, fewer recoveries were obtained in the case of salmon (wild and farmed) and in the case of surimi-derived elvers, especially when it comes to Hg^{2+} (46–57 %). Hg^{2+} and $MeHg^{+}$ contents determined in non-fortified samples were corrected by applying the corresponding percentage of recovery in each case.

Table 4 gathers Hg^{2+} and $MeHg^{+}$ concentrations determined after speciation studies by HPLC-ICP-MS, as well as the percentage of each Hg species accounted for total Hg content found in fish and fish-derived products. Concentrations and percentages of Hg^{2+} and $MeHg^{+}$ were calculated considering recoveries of each compound for each sample. Based on the values collected in Table 4 and Figure S3 of Supplementary Material, it can be concluded that $MeHg^{+}$ is the major Hg species in fish and fish derived products, as it was expected. Moreover, data of the sum of Hg species content in percentage (∑ Hg, %) (Table 4) between Hg species for all samples indicates that quantitative results were achieved. However, in the case of fish roe, no Hg species were identified likely due to the low Hg total concentration found in lumpfish, wild salmon and trout roe (Table 2).

Forsyth *et al.* (Forsyth, Casey, Dabeka & McKenzie, 2004) reported that there is not a fixed conversion factor that could correlate the amount of $MeHg^{+}$ with the total Hg content of fish, being highly dependent on the trophic level, the size and the age. In predator fishes, $MeHg^{+}$ can represent up to 90 % of the total Hg content. EFSA (EFSA, 2012) also reported that fish lower in the food chain, present lower content of total Hg and $MeHg^{+}$. Although it is quite variable, $MeHg^{+}$ can represent below approximately 50 % of total Hg in these fish. The stability of Hg species differs, $MeHg^{+}$ is thought to remain for more time in an organism than inorganic Hg (Forsyth et al., 2004; Park & Zheng, 2012; Porvari, 2003). Therefore, predator and aged fishes will have more Hg as $MeHg^{+}$ form. Results obtained in this study are in consonance with this statement, having tuna and swordfish contents of $MeHg^{+}$ of 110 ± 6 and 96 ± 3 %, respectively (Table 4, Figure S3 of Supplementary Material). In turn, farmed and wild salmon are the fishes in which the levels of inorganic Hg with respect to the total Hg content are the highest (Table 4, Figure S3 of Supplementary Material). By the same token, fish-derived products such as crab sticks, surimi-derived elvers, cod noodles and salmon noodles, will be expected to have relatively less amount of Hg in the form of $MeHg^{+}$, because these products are principally made from non-predator fishes, such as walleye pollock, North Pacific hake, farmed salmon and shallow-water Cape hake.

4. Conclusions

Hg and Se content, Se:Hg molar ratio and Selenium Health Benefit Values (HBV_{Se}) have been studied in seven fish-derived and ultra-processed products and compared with four fish species traditionally consumed. In this regard, relatively high total Se contents and low Hg levels have been found in the fish-derived products considered, especially when it comes to fish roe. Furthermore, HBV_{Se} were positive in all cases, being Se present in molar excess with respect to Hg and, therefore, bioavailable enough to carry out the corresponding biological functions in the organisms. On the other hand, SeMet and SeMeSeCys were the main Se species found in all the samples analysed, while $MeHg^{+}$ was the major form of Hg found in fish, but also in fish-derived products. However, the relative contents of both species with respect to the total Hg content vary depending on the sample. Therefore, Se and Hg species have been detected in fish-derived products even though they are subjected to an elaboration process that could have affected the integrity of the Se and Hg species. To our knowledge, this is the first time that HBV_{Se} have been calculated and Hg and Se speciation studies have been carried out on products derived from fish such as fish roe and ultra-processed fish products being crucial for assuring their quality and safety. Future investigations will be focused on the assessment of the Se and Hg species bioavailability in fish-derived products of special concern on the context of human nutrition.

Author contributions

All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Tamara Fernández-Bautista: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Beatriz Gómez-Gómez:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Emma Gracia-Lor:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Teresa Pérez-Corona:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Yolanda Madrid:** Conceptualization, Investigation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137544.

References

Azad, A. M., Frantzen, S., Bank, M. S., Nilsen, B. M., Duinker, A., Madsen, L., & Maage, A. (2019). Effects of geography and species variation on selenium and mercury molar ratios in Northeast Atlantic marine fish communities. *Science of the Total Environment, 652*, 1482–1496. https://doi.org/10.1016/j.scitotenv.2018.10.405

Burger, J., Gaines, K. F., Boring, C. S., Stephens, W. L., Snodgrass, J., & Gochfeld, M. (2001). Mercury and selenium in fish from the Savannah River: Species, trophic level, and locational differences. *Environmental Research, 87*(2), 108–118. https://doi.org/10.1006/enrs.2001.4294

Burger, J., & Gochfeld, M. (2011). Mercury and selenium levels in 19 species of saltwater fish from New Jersey as a function of species, size, and season. *Science of the Total Environment, 409*(8), 1418–1429. https://doi.org/10.1016/j.scitotenv.2010.12.034

Cabañero, A. I., Madrid, Y., & Cámara, C. (2007). Mercury-selenium species ratio in representative fish samples and their bioaccessibility by an in vitro digestion method. *Biological Trace Element Research, 119*(3), 195–211. https://doi.org/10.1007/s12011-007-8007-5

Colombo, S. M., & Mazal, X. (2020). Investigation of the nutritional composition of different types of salmon available to Canadian consumers. *Journal of Agriculture and Food Research, 2*(June), Article 100056. https://doi.org/10.1016/j.jafr.2020.100056

Efsa. (2012). Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal, 10*(12). https://doi.org/10.2903/j.efsa.2012.2985

Fernández-Bautista, T., Gómez-Gómez, B., Palacín-García, R., Gracia-Lor, E., Pérez-Corona, T., & Madrid, Y. (2022). Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS. *Talanta, 237*(October 2021). https://doi.org/10.1016/j.talanta.2021.122922

Forsyth, D. S., Casey, V., Dabeka, R. W., & McKenzie, A. (2004). Methylmercury levels in predatory fish species marketed in Canada. *Food Additives and Contaminants, 21*(9), 849–856. https://doi.org/10.1080/02652030400004259

Gajdosechova, Z., Lawan, M. M., Urgast, D. S., Raab, A., Scheckel, K. G., Lombi, E., … Krupp, E. M. (2016). In vivo formation of natural HgSe nanoparticles in the liver and brain of pilot whales. *Scientific Reports, 6*(October). https://doi.org/10.1038/srep34361

Gochfeld, M., & Burger, J. (2021). Mercury interactions with selenium and sulfur and the relevance of the Se: Hg molar ratio to fish consumption advice. *Environmental Science and Pollution Research, 28*(15), 18407–18420. https://doi.org/10.1007/s11356-021-12361-7

Hight, S. C., & Cheng, J. (2006). Determination of methylmercury and estimation of total mercury in seafood using high performance liquid chromatography (HPLC) and inductively coupled plasma-mass spectrometry (ICP-MS): Method development and validation. *Analytica Chimica Acta, 567*(2), 160–172. https://doi.org/10.1016/j.aca.2006.03.048

Iupac. (1978). International Union of Pure and Applied Chemistry. (1978). Nomenclature, symbols, units and their usage in spectrochemical analysis-III. Analytical flame spectroscopy and associated non-flame procedures. *Spectrochimica Acta Part B, 33*, 247–269.

Olmedo, P., Hernández, A. F., Pla, A., Femia, P., Navas-Acien, A., & Gil, F. (2013). Determination of essential elements (copper, manganese, selenium and zinc) in fish and shellfish samples. Risk and nutritional assessment and mercury-selenium balance. *Food and Chemical Toxicology, 62*, 299–307. https://doi.org/10.1016/j.fct.2013.08.076

Ordiano-Flores, A., Rosíles-Martínez, R., & Galván-Magaña, F. (2012). Biomagnification of mercury and its antagonistic interaction with selenium in yellowfin tuna Thunnus albacares in the trophic web of Baja California Sur, Mexico. *Ecotoxicology and Environmental Safety, 86*, 182–187. https://doi.org/10.1016/j.ecoenv.2012.09.014

Park, J. D., & Zheng, W. (2012). Human exposure and health effects of inorganic and elemental mercury. *Journal of Preventive Medicine and Public Health, 45*(6), 344–352. https://doi.org/10.3961/jpmph.2012.45.6.344

Porvari, P. (2003). Sources and fate of mercury in aquatic ecosystems. *Monographs of the Boreal Environment Research, 23*, 1–53. https://helda.helsinki.fi/bitstream/handle/10138/39340/BERMon_23.pdf?sequence=1.

Qvarnström, J., Lambertsson, L., Havarinasab, S., Hultman, P., & Frech, W. (2003). Determination of methylmercury, ethylmercury, and inorganic mercury in mouse tissues, following administration of thimerosal, by species-specific isotope dilution GC-inductively coupled plasma-MS. *Analytical Chemistry, 75*(16), 4120–4124. https://doi.org/10.1021/ac0342370

Ralston, N. V. C., Kaneko, J. J., & Raymond, L. J. (2019). Selenium health benefit values provide a reliable index of seafood benefits vs. risks. *Journal of Trace Elements in Medicine and Biology, 55*(May), 50–57. https://doi.org/10.1016/j.jtemb.2019.05.009

Ralston, N. V. C., Ralston, C. R., Blackwell, J. L., & Raymond, L. J. (2008). Dietary and tissue selenium in relation to methylmercury toxicity. *Neurotoxicology, 29*(5), 802–811. https://doi.org/10.1016/j.neuro.2008.07.007

Ralston, N. V. C., Ralston, C. R., & Raymond, L. J. (2016). Selenium Health Benefit Values: Updated Criteria for Mercury Risk Assessments. *Biological Trace Element Research, 171*(2), 262–269. https://doi.org/10.1007/s12011-015-0516-z

Raymond, L. J., & Ralston, N. V. C. (2020). Mercury: Selenium interactions and health implications. *Neurotoxicology, 81*(1), 294–299. https://doi.org/10.1016/j.neuro.2020.09.020

Ribeiro, M., Zephyr, N., Silva, J. A. L., Danion, M., Guérin, T., Castanheira, I., Leufroy, A., & Jitaru, P. (2022). Assessment of the mercury-selenium antagonism in rainbow trout fish. *Chemosphere*, *286*(April 2021). https://doi.org/10.1016/j.chemosphere.2021.131749.

Santoyo, M. M., Figueroa, J. A. L., Wrobel, K., & Wrobel, K. (2009). Analytical speciation of mercury in fish tissues by reversed phase liquid chromatography-inductively coupled plasma mass spectrometry with Bi3+ as internal standard. *Talanta, 79*(3), 706–711. https://doi.org/10.1016/j.talanta.2009.04.057

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H. G., & Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *Journal of Trace Elements in Medicine and Biology, 47*(May 2017), 124–133. https://doi.org/10.1016/j.jtemb.2018.02.005

Selin, N. E. (2009). Global biogeochemical cycling of mercury: A review. *Annual Review of Environment and Resources, 34*, 43–63. https://doi.org/10.1146/annurev.environ.051308.084314

Suzuki, Y., Kondo, M., Akiyama, H., & Ogra, Y. (2022). Presence of nano-sized mercury-containing particles in seafoods, and an estimate of dietary exposure. *Environmental Pollution, 307*(March), Article 119555. https://doi.org/10.1016/j.envpol.2022.119555

Ulusoy, Ş., Mol, S., Karakulak, F. S., & Kahraman, A. E. (2019). Selenium-Mercury Balance in Commercial Fish Species from the Turkish Waters. *Biological Trace Element Research, 191*(1), 207–213. https://doi.org/10.1007/s12011-018-1609-2

Vicente-Zurdo, D., Gómez-Gómez, B., Pérez-Corona, M. T., & Madrid, Y. (2019). Impact of fish growing conditions and cooking methods on selenium species in swordfish and salmon fillets. *Journal of Food Composition and Analysis, 83*(August), Article 103275. https://doi.org/10.1016/j.jfca.2019.103275

WHO (World Health Organization). (2005). *Mercury in health care*. http://www.who.int/water_sanitation_health/medicalwaste/mercurypolpap230506.pdf.

WHO (World Health Organization). (2008). *Guidance for identifying populations at risk from mercury exposure*.

Zembrzuska, J., Matusiewicz, H., Polkowska-Motrenko, H., & Chajduk, E. (2014). Simultaneous quantitation and identification of organic and inorganic selenium in diet supplements by liquid chromatography with tandem mass spectrometry. *Food Chemistry, 142*, 178–187. https://doi.org/10.1016/j.foodchem.2013.05.004

Zhang, H., Guo, C., Feng, H., Shen, Y., Wang, Y., Zeng, T., & Song, S. (2020). Total mercury, methylmercury, and selenium in aquatic products from coastal cities of China: Distribution characteristics and risk assessment. *Science of the Total Environment, 739*, Article 140034. https://doi.org/10.1016/j.scitotenv.2020.140034

Supplementary Material

Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of highly consumed fish species and fish-derived products.

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Table S1. ICP-MS and HPLC operating instrumental conditions.

ICP-MS parameters	
RF Power	1550 W
Plasma gas flow rate	15 mL min^{-1}
Auxiliary Ar flow rate	1 mL min^{-1}
Carrier gas flow rate	1 mL min^{-1}
H_2 flow rate	6 mL min^{-1}
Sample introduction	Meinhard nebulizer
Sample and skimmer cones	Nickel
Spray Chamber	Scott
Acquisition mode	Spectrum analysis
Isotopes monitored	^{78}Se, ^{80}Se, ^{82}Se
HPLC-ICP-MS conditions (Se speciation analysis)	
Analytical column	Hamilton PRP-X100 (250 ×4.1 mm,10 µm)
Mobile phase	10 mM citric acid (pH 5.0), 2 % MeOH
Mode	Isocratic
Flow rate	1 mL min^{-1}
Injection volume	100 µL
Run time	12 min
Acquisition mode	Time-resolved analysis
Isotopes monitored	^{78}Se, ^{80}Se, ^{82}Se
HPLC-ICP-MS conditions (Hg speciation analysis)	
Analytical column	Phenomenex Kinetex EVO-C18 (150 x 3 mm, 5 µm)
Mobile phase	0.1 % L-cysteine, 0.1 % L-cysteine·HCl·H_2O (pH 2.5)
Mode	Isocratic
Flow rate	0.5 mL min^{-1}
Injection volume	20 µL
Run time	5 min
Acquisition mode	Time-resolved analysis
Isotopes monitored	^{201}Hg, ^{202}Hg, ^{209}Bi

Table S2. Recoveries (%) obtained from spiking experiments with 30 µg L^{-1} of each specie (Hg^{2+} and $MeHg^{+}$).

Fish/Fish-derived product	Recovery Hg^{2+} (%)	Recovery $MeHg^{+}$ (%)
Tuna	90 ± 6	93 ± 9
Swordfish	101 ± 4	103 ± 5
Farmed salmon	52 ± 4	64 ± 1
Wild salmon	46 ± 3	66 ± 1
Surimi-derived elvers	57 ± 1	68 ± 1
Crab sticks	72 ± 2	69 ± 1
Lumpfish roe	73 ± 1	74 ± 1
Wild salmon roe	70 ± 1	76 ± 1
Trout roe	75 ± 2	72 ± 3
Cod noodles	72 ± 2	69 ± 3
Salmon noodles	65 ± 5	70 ± 7

Results are referred to wet weight. Results are expressed as mean ± standard deviation (SD); n= 3 for each fish/fish-derived product.

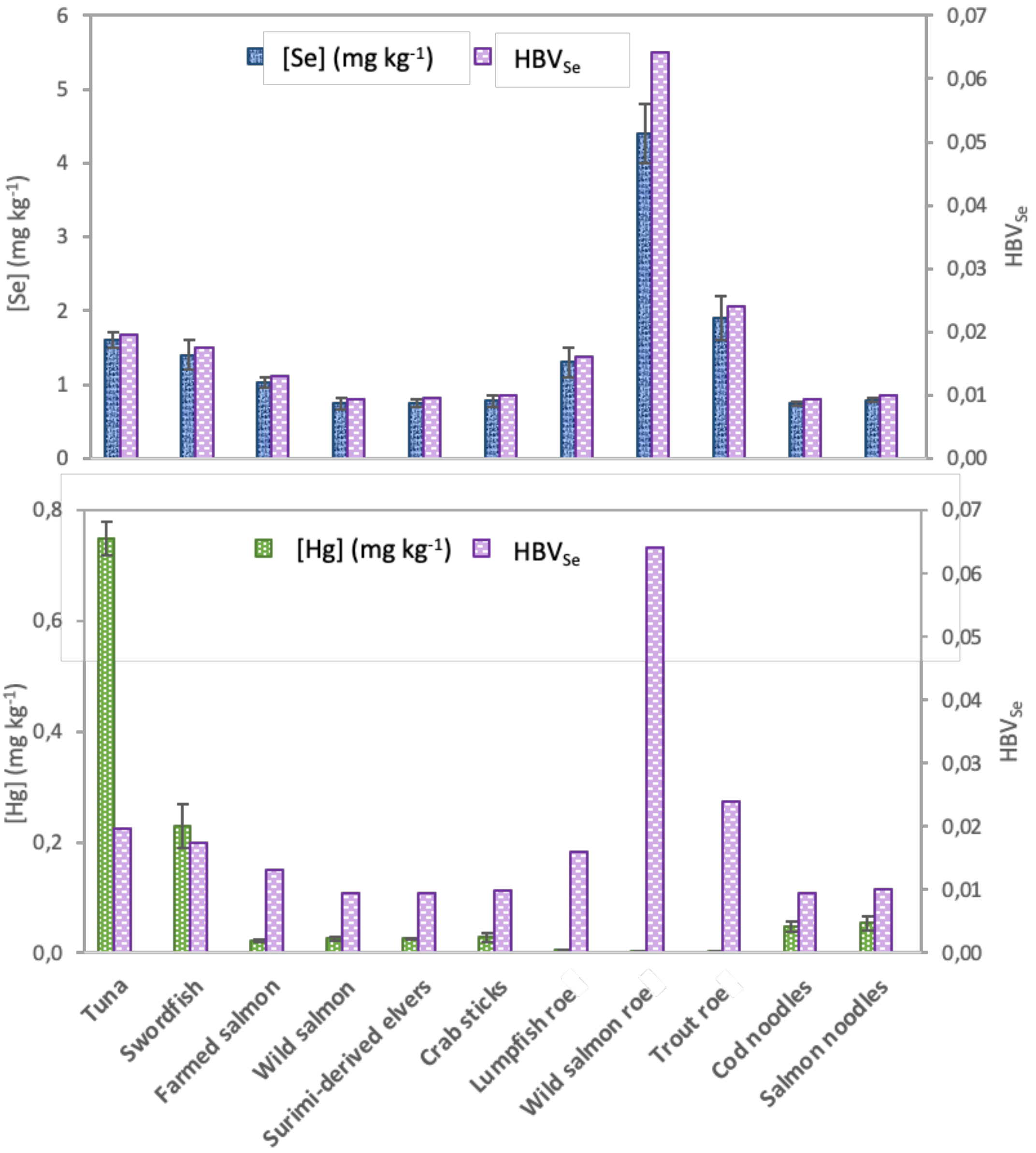


Figure S1. Means of total selenium and mercury contents (left Y axis) and HBV_{Se} (right Y axis) in fish and fish-derived products samples. Error bars in total Hg and Se contents represent standard deviations (n=3). HBV_{Se} were calculated from mean values.

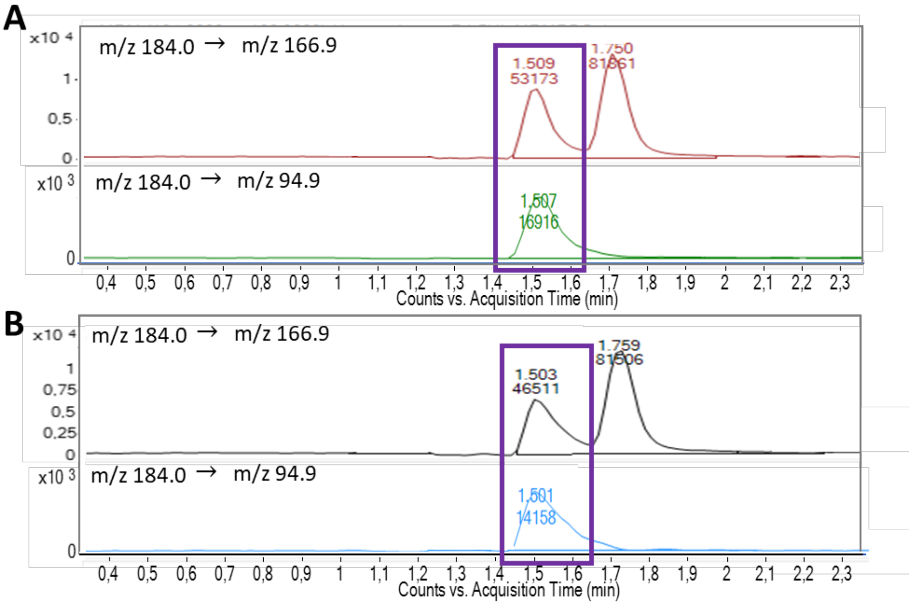


Figure S2. HPLC-ESI-MS/MS chromatograms of the identification of SeMeSeCys in (A) lumpfish roe and (B) crab sticks.

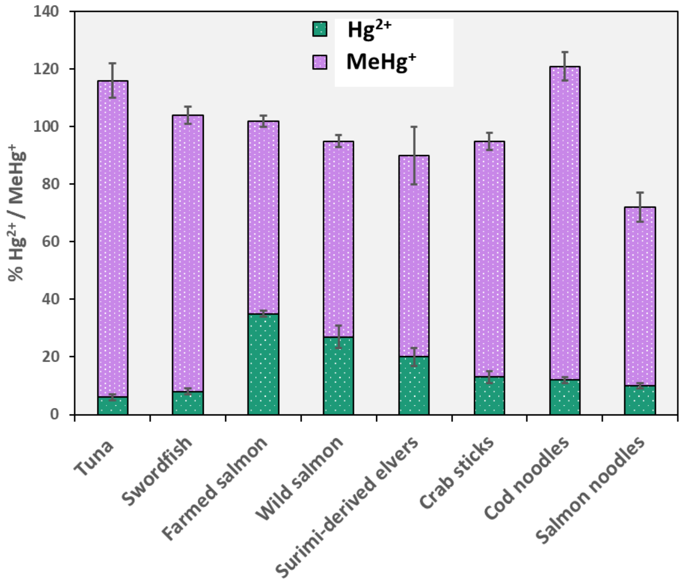


Figure S3. Distribution of the concentration of the species (Hg^{2+} and $MeHg^{+}$) detected by HPLC-ICP-MS in fish and fish-derived products.

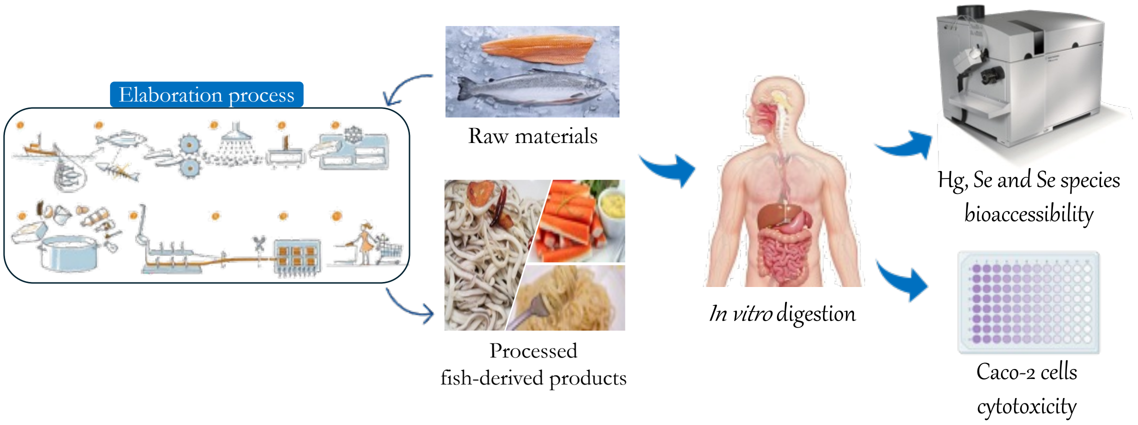
ARTÍCULO 3:

Se y Hg en productos procesados derivados del pescado y sus materias primas: presencia, relación molar Se:Hg, índice HBV_{Se}, bioaccesibilidad y toxicidad en las células Caco-2

Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBV_{Se} index, bioaccessibility and Caco-2 cells toxicity

Tamara Fernández-Bautista, Beatriz Gómez-Gómez, Emma Gracia-Lor, Teresa Pérez-Corona, Yolanda Madrid

Enviado a Food Research International. Factor de impacto: 8.1 (Q1)



Los resultados de este trabajo se presentaron en las siguientes reuniones científicas:

- **$10^{\text{ème}}$ édition de Spectr'atom**. *Surveillance des changements dans les contenus totaux en Se et Hg et leurs espèces au cours de l'élaboration des produits transformés dérivés du poisson.* Pau, Francia, junio de 2024. Tipo de comunicación: póster.
- **Jornada de Especiación de la XXIV Reunión de la Sociedad Española de Química Analítica**. *Determinación de Hg, Se y especies de Se en productos procesados derivados de pescados y sus materias primas. Estudios de bioaccesibilidad y toxicidad mediante ensayos in vitro*. Zaragoza, España, julio de 2024. Tipo de comunicación: oral.

Food Research International

Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBVSe index, bioaccessibility and Caco-2 cells toxicity

--Manuscript Draft--

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Article Type:	Research Paper
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Abstract:	Processing conditions applied during food production could affect food component contents and bioaccessibility. Here, possible changes in Hg and Se total and species contents and bioaccessibility have been tracked in each stage of the production chain of processed fish-derived products. Therefore, Se:Hg molar ratio and Selenium Health Benefit Value (HBVSe) were calculated for final products and raw materials, resulting favorable in all cases, suggesting the safety of surimi-based products regarding mercury. Speciation studies revealed the presence of SeMeSeCys and SeMet in all samples. Thus, the integrity of the selenium species seems to be maintained. Moreover, in vitro gastrointestinal digestion model evidenced that Se bioaccessibility ranged between 20-39% for all samples, while in case of Hg was between 8-37%. Additionaly, SeMeSeCys and SeMet were also identified in the gastrointestinal extracts. Finally, no cytotoxicity was observed after exposure of Caco-2 cells to the gastrointestinal extracts.

Highlights:

- $HBV_{Se} > 0$ were obtained for raw materials and processed fish-derived products
- SeMet and SeMeSeCys were identified in all raw materials and final products
- Elaboration processes of products might not affect the integrity of the Se species
- Hg, Se and Se species bioaccessibilities were assessed using an *in vitro* model
- Caco-2 cell viability did not decreased after exposure to bioaccesible fraction

Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBV$_{Se}$ index, bioaccessibility and Caco-2 cells toxicity

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ABSTRACT

Processing conditions applied during food production could affect food component contents and bioaccessibility. Here, possible changes in Hg and Se total and species contents and bioaccessibility have been tracked in each stage of the production chain of processed fish-derived products. Therefore, Se:Hg molar ratio and Selenium Health Benefit Value (HBV_{Se}) were calculated for final products and raw materials, resulting favorable in all cases, suggesting the safety of surimi-based products regarding mercury. Speciation studies revealed the presence of SeMeSeCys and SeMet in all samples. Thus, the integrity of the selenium species seems to be maintained. Moreover, *in vitro* gastrointestinal digestion model evidenced that Se bioaccessibility ranged between 20-39 % for all samples, while in case of Hg was between 8-37 %. Additionaly, SeMeSeCys and SeMet were also identified in the gastrointestinal extracts. Finally, no cytotoxicity was observed after exposure of Caco-2 cells to the gastrointestinal extracts.

Keywords: Fish-derived products, Selenium Health Benefit Value, Se speciation, HPLC-ICP-MS, *in vitro* gastrointestinal digestion, Caco-2 cells

1. Introduction

Sustainability becomes a crucial factor when advising public to increase seafood intake. Although aquatic food production has shifted from primarily relying on wild fish capture to the cultivation of farmed species, the global issue of overexploitation of wild fish stocks affects numerous seafood species (Bogard, Farmery, Little, Fulton & Cook, 2019; FAO, 2020), which have led to the exploitation of other fish species with a lower commercial value, for instance North Pacific hake (*Merluccius productus*), walleye pollock (*Gadus chalcogrammus*), blue whiting (*Micromesistius poutassou*), Pacific whiting (*Merluccius productus*) or Atlantic Horse Mackerel (*Trachurus trachurus*). These fish with low output in the market are being utilized as raw materials for the development of semi-industrial ready-to-eat products or processed fish-derived products based on surimi (a versatile, mild-tasting seafood ingredient made of minced and washed fish mixed with starch, egg white, salt, and flavorings). These imitation seafood products are designed to be affordable, attractive, versatile, and delicious foods tailored to meet the nutritional and textural needs (absence of bones and easy to chew), specially for children. Moreover, these fish-based products present added nutritional value as contribute to the uptake of omega-3-fatty acids.

Although a risk-benefit analysis suggests that usually the benefits linked to fish consumption outweigh the associated risks (Azad *et al*., 2019; Cabañero, Madrid & Cámara, 2007; Fernández-Bautista, Gómez-Gómez, Gracia-Lor, Pérez-Corona & Madrid, 2024; Ferraris *et al*., 2021; Olmedo *et al*., 2013; Ordiano-Flores, Rosíles-Martínez & Galván-Magaña, 2012), a thorough characterization of the chemical 'fingerprint' of each fish species becomes essential for a comprehensive evaluation encompassing both nutritional value and food safety. This is even more important when it comes to less explore products such as processed seafood products, in which the different technological process (e.g., pasteurization, sterilization, etc.) and processing conditions (e.g., temperature, pressure, time, etc.) applied during food production could affect food nutrient content and bioaccessibility as well as food safety (Capozzi *et al.*, 2021).

Fish and seafood are an important source of mercury (Hg) exposure for population, being methylmercury ($MeHg^+$) able to bioaccumulate along the trophic chain, resulting in elevated concentrations in predatory fish, posing a potential risk to human consumption (Burger *et al.*, 2001). Selenium (Se), an essential micronutrient, has long shown protective effects against Hg toxicity through the Se-Hg interaction (Ralston, Ralston, Blackwell & Raymond, 2008; Ralston, Ralston & Raymond, 2016; Suzuki, Kondo, Akiyama & Ogra, 2022). Although the mechanisms of this interaction are not yet fully elucidated, it is well stablished that Se is able to interact with $MeHg^+$ promoting its demethylation to form insoluble HgSe and Hg(S, Se) particles (Raymond and Ralston, 2020; Suzuki *et al.*, 2022), possibly via forming selenocysteinate ($Hg(Sec)_4$) complexes before interaction with selenoprotein P and selenoneine (Azad *et al.*, 2019; Gajdosechova *et al.*, 2016; Manceau, Gaillot, Glatzel, Cherel & Bustamante, 2021; Yamashita *et al.*, 2013). The key factor to evaluate the results of this interaction and thus the risks associated with Hg through fish consumption is the Se:Hg molar ratio and Selenium Heath Benefit Value (HBV_{Se}). Both indexes provide information of the amount of the Se available for developing its essential biological functions once part of it has been sequestered by Hg through the Se-Hg interaction (Burger & Gochfeld, 2011; Gochfeld & Burger, 2021; Ralston *et al.*, 2008). Moreover, a correct assessment of the safety of these fish-products related to their contents on Se and Hg implies to determine the chemical forms in which these analytes are present in the products as well as the bioaccessibility. Bioaccessibility refers to the fraction of the total amount of substance that is potentially available for absorption in the small intestine (Rodrigues *et al.*, 2022). *In vitro* gastrointestinal models in which the human digestive system is simulated are widely used to study the bioaccessibility of different compounds (Etcheverry, Grusak & Fleige, 2012). Lately, the human colorectal adenocarcinoma Caco-2 cell line, that closely mimic human intestinal epithelial cells, has shown as a powerful tool to evaluate the risk of toxins to humans (Akter *et al.*, 2022). Consequently, bioaccessibility studies performed by using *in vitro* methods such as the simulated gastrointestinal digestion combined with Caco2-cells cytotoxicity of the resulting intestinal fractions become of relevance in order to get a more accurately evaluation of the exposure to the target elements which is essential for evaluating nutritional quality and safety of food products.

In this research, Se:Hg molar ratio, HBV_{Se} determination and Se speciation studies are first reported in processed fish-derived commercialized products and in the fish employed as raw materials in order to track possible changes during the food processing chain with respect to Se and Hg. Most importantly, an *in vitro* gastrointestinal digestion procedure was applied to the samples to evaluate the bioaccessible total Hg and Se and Se species. Finally, the cytotoxicity of the bioaccessible fraction was also evaluated by using the Caco-2 cell line.

2. Materials and Methods

2.1 Fish raw materials and processed fish-derived products from three different fish production lines

This study focuses on three processed fish-derived products commonly found in local supermarkets of Spain, namely crab sticks, surimi-derived elvers and salmon noodles, coming from three different production lines. Figure 1 shows the stages involved in these fish production lines and the samples obtained at each step. Hence, samples from raw materials, intermediate products and final products were all considered for performing this study. These samples were kindly provided by companies specialized on the manufacture of fish-derived products and stored correctly at -20 °C until analysis.

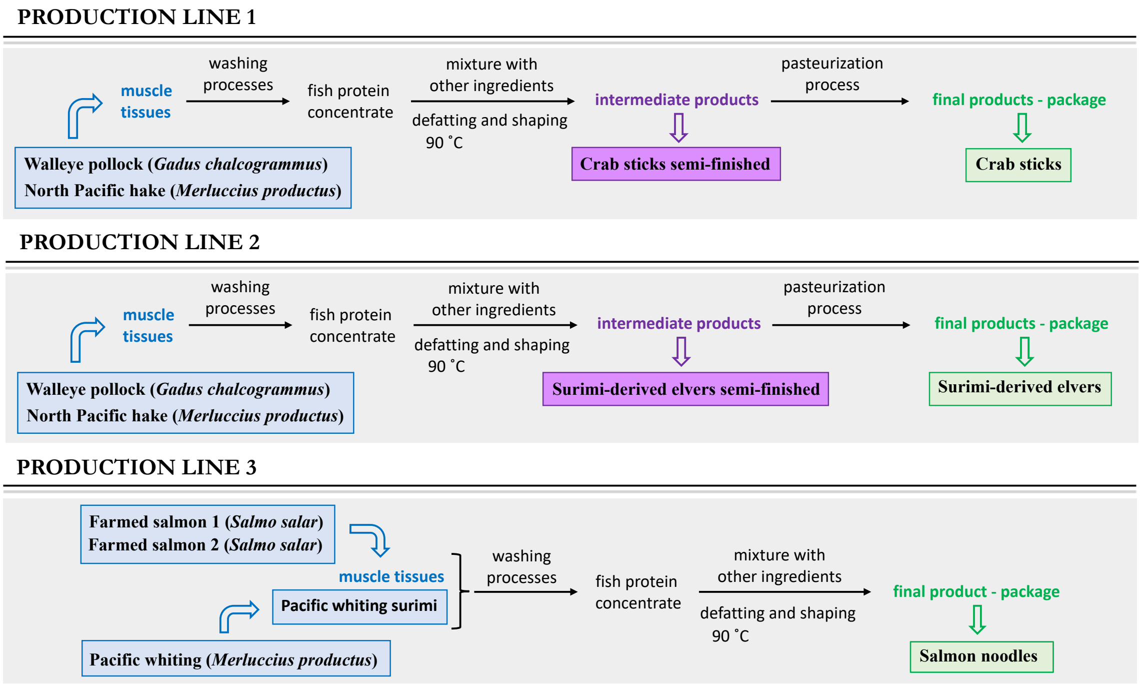

Figure 1. Schematic of the processed fish-derived products production lines. The samples considered in this study are squared in blue (raw materials), purple (intermediate products) and green (final products).

The elaboration process of the three fish-derived commercial products consists of performing successive washing processes of fish muscle raw materials to obtain a concentrate of fish proteins (myofibrillar proteins) with gelling properties. In case of the first and the second production lines, the mixing of the fish protein concentrate with other ingredients, and subsequent shaping, produces intermediate products (crab sticks semi-finished and surimi-derived elvers semi-finished), which led to the final products (crab sticks and surimi-derived elvers) after applying a pasteurization process. The main difference between crab sticks and surimi-derived elvers is that the former is dyed orange while the latter is shaped to get a characteristic shape imitating elvers. Nevertheless, and according to the information given by the manufactures, the raw materials employed: muscle tissues of walleye pollock (*Gadus chalcogrammus*) and North Pacific hake (*Merluccius productus*) are the same for both fish-derived products.

In the third production line, the raw materials employed are muscle tissues of two farmed salmon (*Salmo salar*) with different feedings and muscle tissue of Pacific whiting (*Merluccius productus*). From the latter, Pacific whiting surimi is elaborated and afterwards, and by following an elaboration process like the first and second production lines, the final product (salmon noodles) is obtained.

2.2 Determination of total Se and Hg content by ICP-MS and DMA, respectively

The total Se content in samples was determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., Santa Clara, CA, USA) after acid digestion in a 1600 W MSP microwave oven from CEM (Stallings, NC, USA). For this purpose, about 1 g of each raw material or processed fish-derived product (3 replicates) was digested with 6 mL of a mixture (5:1) of 65 % v/v nitric acid (Merck, Madrid, Spain) and 30 % v/v hydrogen peroxide (Panreac, Barcelona, Spain) and submitted to a microwave heating program consisting of a 20-min ramp to 130 ºC and a 10-min hold time. Once cooled until room temperature, the digests were appropriately diluted with deionized water (18.2 MΩ cm, from a Milli-Q® water purification system; Millipore, Bedford, MA) to a final volume of 10 mL and filtered through a 0.22 µm syringe Nylon filter (Scharlab, Barcelona, Spain) prior analyses. Blank solutions (3 replicates) were also performed.

Total Se content in the digests was measured by ICP-MS by monitoring ^{78}Se, ^{80}Se and ^{82}Se isotopes under continuous acquisition mode. The operational conditions for ICP-MS measurements are as follows: RF power of 1550W, sample depth of 8 mm, plasma gas flow rate of 15.0 L min^{-1}, auxiliary gas flow rate of 1.0 L min^{-1}, conikal nebulizer with a flow rate of 1.0 L min^{-1} and H_2 is used in the collision-reaction cell at a flow rate of 6.0 L min^{-1}. Se quantification was performed by external calibration with the isotope ^{80}Se.

The accuracy of the results obtained was further evaluated by the analysis of a Certified Reference Material (ERM-CE 278k, from mussel) from the Institute for Reference Materials. No statistically significant differences were found for total Se between the certified value 1.62 ± 0.12 mg kg^{-1} and the experimental one 1.65 ± 0.14 mg kg^{-1}. The limit of quantification (LOQ) was calculated according to International Union of Pure and Applied Chemistry (IUPAC) (IUPAC, 1978). The LOQ was obtained as $10\sigma/S$, where σ is the standard deviation of 10 measurements of the blank solution and S is the slope of the line equation for external calibration curve. For total Se, the LOQ was 0.006 mg kg^{-1}.

The total Hg content in samples was determined by using a Direct Mercury Analyzer (DMA-80 evo; Milestone, Italy). Around 50-150 mg of each sample were weighed on quartz boats and directly analyzed by DMA. The accuracy of the method was further evaluated by the analysis of Certified Reference Materials from oyster (BCR-710) and mussel (ERM-CE 278k) tissues, which certified for total Hg 0.26 ± 0.05 mg kg^{-1} and 0.073 ± 0.007 mg kg^{-1}, respectively. Experimental values obtained were 0.28 ± 0.04 mg kg^{-1} and 0.075 ± 0.008 mg kg^{-1}, respectively. The LOQ calculated according to IUPAC rules (IUPAC, 1978) was 0.033 μg kg^{-1}.

2.3 Se:Hg molar ratio and Selenium Health Benefit Values (HBV_{Se})

According to International Bioindicators Roundtable (Ralston, 2008), Se:Hg molar ratio is needed for the risk assessment of fish consumption related to the toxic effects of Hg exposure. Moreover, the calculation of Se:Hg molar ratio is crucial not only for the correct assessment of seafood safety concerning Se and Hg ingestion, but also for making easier the identification of healthy seafood choices.

Se:Hg molar ratio can be calculated according to Equation (1) (Burger & Gochfeld, 2011; Ralston *et al.*, 2008; Ulusoy, Mol, Karakulak & Kahraman, 2019):

$$\text{Se:Hg molar ratio} = \frac{\text{mmol Se kg}^{-1}\text{ ww}}{\text{mmol Hg kg}^{-1}\text{ ww}} \quad (1)$$

In addition, HBV_{Se} is an index developed by the US Environmental Protection Agency also related to the fish consumption safety that indicates how much Se remains available once part of it has been sequestered by Hg through the Se-Hg interaction (Ralston, 2008). HBV_{Se} was determined by applying Equation (2) (Ralston, Kaneko & Raymond, 2019):

$$\text{HBV}_{\text{Se}} = \frac{\text{Se}-\text{Hg}}{\text{Se}}\,(\text{Se} + \text{Hg}) \quad (2)$$

Se = selenium content in molar concentration (mmol Se kg^{-1})

Hg = mercury content in molar concentration (mmol Hg kg^{-1})

Positive values of HBV_{Se} indicate that the consumption of the fish does not apparently entail risks and show Se-dependent health benefits. However, negative values indicate potential health risks for consumers.

2.4 Se species identification and quantification by HPLC-ICP-MS and HPLC-ESI-MS/MS

Se speciation in raw materials and in processed fish-derived products was performed by high-performance liquid chromatography coupled to ICP-MS (HPLC-ICP-MS) and by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) after enzymatic hydrolysis.

For this purpose, about 0.5 g of sample (3 replicates) were treated with 5 mL of 30 mM tris-(hydroxymetyl)-aminomethane (Tris; Sigma-Aldrich, Madrid, Spain) at pH 7.5 (adjusted with 0.05 mol L^{-1} HCl) and 20 mg of the non-specific protease *Streptomyces griseus* (Protease type XIV; Sigma-Aldrich, Madrid, Spain) and incubated for 24 h at 37 °C in a thermostatic bath (Termotronic II from P Selecta, Barcelona, Spain). Afterwards, samples were centrifuged at 10000 rpm for 15 min at 4 °C (Eppendorf 5804 F34-6-38 centrifuge, Hamburg, Germany), filtered through a 0.22 μm syringe Nylon filters and analyzed.

Total Se content in the obtained extracts was also measured by ICP-MS after performing a microwave acid digestion as described in section 2.2.

The separation of Se species was performed on a Hamilton PRP-X100 column (250×4.1 mm, 10 μm), with an anion exchange mechanism, and detected by ICP-MS. The HPLC unit consisted of a PU-2089 HPLC pump (JASCO, Tokyo, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne Rohner Park, CA, USA) and with an injection loop of 100 μL. The mobile phase for the chromatographic separation consisted of 10 mM citric acid at pH 5.0 adjusted with ammonia and 2 % methanol (Scharlab, Barcelona, Spain). The elution mode was isocratic with a flow rate of 1 mL min^{-1}. After separation, Se species were identified by matching the retention times with those of standard solutions: selenomethylseleno-L-cysteine (SeMeSeCys), D-L-selenocystine ($SeCys_2$), seleno-L-methionine (SeMet), Se (IV) (Na_2SeO_3) and Se (VI) (Na_2SeO_4), all from Sigma-Aldrich (Madrid, Spain). Standard stock solutions (> 1000 mg L^{-1}) were prepared in acidic conditions (inorganic Se species, Se (IV) and Se (VI), in 2 % v/v HNO_3, SeMet in 0.1 mol L^{-1} HCl and SeMeSeCys in 3 % v/v HCl) and stored at 4 °C. From these solutions, working solutions less concentrated were freshly prepared daily by dilution before each analysis. From working solutions, calibration solutions were prepared by dilution with Milli-Q® water. Matrix effects were checked by performing standard addition calibration curves for each sample and comparing their slopes to that of the external calibration curve by means of a Student´s t-test ($\alpha = 0.05$).

In order to confirm the identity of the Se species previously detected by HPLC-ICP-MS, analyses by HPLC-ESI-MS/MS were accomplished. Chromatographic separation was carried out using a Phenomenex Kinetex EVO C18 column (150x3 mm, 5 μm), due to its compatibility with the HPLC-ESI-MS/MS system. The targeted Se species were analyzed by means of an Agilent G6410B Triple Quadrupole LC/MS System (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with an Agilent 1200 Series G1312 binary pump, an Agilent 1200 Series G1322A degasser, an Agilent 1200 Series G1329A standard autosampler with temperature-controlled tray and an Agilent 1200 Series G1316A column oven. The injection volume onto the column was 5 µL and the mobile phase consisted of A (95 %): MilliQ water with 0.1% formic acid (Scharlab, Barcelona, Spain) and B (5 %): acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) with 0.1 % formic acid. The elution mode was isocratic with a flow rate of 0.5 mL min^{-1}.

Analyses were done in positive electrospray ionization (ESI) mode using the Multiple Reaction Monitoring (MRM) mode at various fragmentor voltages (from 60 to 110). The ion source settings were as follows: gas temperature, 200 °C; drying gas flow, 10 L min^{-1}; nebulizing gas pressure, 35 psi; and capillary voltage, 200 V. Dwell times of 50 ms were selected for all the transitions. Masshunter Data Acquisition B.04.01 and Masshunter Qualitative Analysis B.07.00 were employed for data acquisition and processing. Selected transitions and collision energies (CE) for Se species are detailed in Table 1.

Table 1. Fragmentations and collision energies optimized for Se species analyzed by HPLC-ESI-MS/MS.

Compound	Precursor ion [M+H$^+$] (m/z)	Product ion Q (m/z)	CE (eV)	Product ion I (m/z)	CE (eV)
Selenomethylseleno-L-cysteine (SeMeSeCys)	184.0	166.9	2	94.9	26
D-L-selenocystine (SeCys$_2$)	336.9	247.8	10	88.0	26
Seleno-L-methionine (SeMet)	198.0	180.9	2	56.1	22
Selenomethionine Se-oxide (SeMetO)	214.0	74.1	8	56.1	26

Q: quantification transition, I: confirmation transition

2.5 Se and Hg bioaccessibility by using an in vitro gastrointestinal digestion method

The *in vitro* method that simulates the physiological conditions that take place during human gastrointestinal digestion employed was based on that described by Gómez-Gómez *et al.* (Gómez-Gómez, Pérez-Corona & Madrid, 2016) with slight modifications. Briefly, about 5 g of the fish raw materials selected (walleye pollock, North Pacific hake and farmed salmon 1)/processed fish-derived products (crab sticks, surimi-derived elvers and salmon noodles) were placed into 50-mL polystyrene tubes and 10 mL of 1.7 mg mL^{-1} α-amylase solution (artificial saliva) were added. The mixture was heated at 37 °C in an incubator coupled to an orbital-rocking platform shaker (Incubator Genie™, from Scientific Industries, Inc., New York, NY, USA) (rocking–horizontal shaking at 70 rpm) for 15 min. Afterwards, 15 mL of 12 mg mL^{-1} pepsin in 0.15 M NaCl (gastric solution) were added and once being the pH adjusted to 1.8 with HCl, the mixture was incubated at 37 °C at 70 rpm (rocking–horizontal shaking) for 120 min. Gastric phase was stopped by adjusting the pH to 6.8 with a saturated sodium bicarbonate solution. Finally, 10 mL of 3 mg mL^{-1} pancreatin and 0.3 mg mL^{-1} bile salts in 0.15 M NaCl (intestinal solution) were added and the mixture was incubated at 37 °C at 70 rpm (rocking–horizontal shaking) for 120 min. Once the *in vitro* digestion process was finished, samples were centrifuged at 11000 rpm for 15 min. The procedure was performed in triplicate and blanks of the gastrointestinal digestion were prepared in parallel. Supernatants were kept for further total Se and Hg content determinations and Se speciation.

Total Se content was determined by ICP-MS after acid digestion in a microwave oven, as described in section 2.2. Total Hg content was also determined in the digests by ICP-MS by monitoring ^{201}Hg and ^{202}Hg under continuous acquisition mode. The operational conditions for ICP-MS measurements were similar to those already described in section

2.2, with the exception of the use of H_2 in the collision-reaction cell, being in this case in no gas mode.

On the other hand, the amount of SeMet and SeMeSeCys in the bioaccesible fraction was determined by quantifying the content of these two Se-species in the extracts resulting from the *in vitro* gastrointestinal digestion process. In this case, pH of extracts was adjusted to 7.5 before performing enzymatic extraction of Se species, since the optimum activity of the protease is at pH 7–8. Then about 20 mg of the non-specific protease *Streptomyces griseus* (Protease type XIV) were added to 1 mL of each sample and the mixture was subsequently incubated for 24 h at 37 ˚C in a thermostatic bath. Once centrifuged at 11000 rpm for 15 min at 4 ˚C, supernatants were filtered through a 0.22 µm syringe Nylon filters. Separation, identification, and quantification of Se species were performed by HPLC-ICP-MS and HPLC-ESI-MS/MS as described in section 2.4.

The percentages of total Se, total Hg and Se species bioaccessibilities were calculated following the Equation (3):

$$\text{Bioaccessibility (\%)} = \frac{\text{[Element/Compound in bioaccessible fraction]}}{\text{[Element/Compound in sample]}} \text{ x } 100 \qquad (3)$$

2.6 Culture of Caco-2 cells

Human Caco-2 cell line was purchased from the American Type Culture Collection (ATCC) and stored in 90 % FBS (ThermoFisher Scientific, Madrid, Spain) and 10 % DMSO (Sigma-Aldrich, Madrid, Spain) at −130 ˚C in liquid nitrogen vapor until use. Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS) and 1 % penicillin/streptomycin (ThermoFisher Scientific, Madrid, Spain) and kept in an incubator at 37 ˚C with a humidified atmosphere of 5 % CO_2. For the subculture procedure, phosphate-buffered saline (PBS, from ThermoFisher Scientific, Madrid, Spain) at pH 7.4 and trypsin-EDTA (0.05 %) (ThermoFisher Scientific, Madrid, Spain) were employed.

2.7 Cell viability assay

MTT assay (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed to assess the cytotoxicity in Caco-2 cells of the intestinal extracts obtained from an *in vitro* gastrointestinal digestion method applied to raw materials and processed fish-derived products considered in this study (section 2.5).

Firstly, optimization of the number of cells seeded in 96-well plates was carried out. Densities of $6 \cdot 10^3$, $10 \cdot 10^3$, $15 \cdot 10^3$, $20 \cdot 10^3$ and $24 \cdot 10^3$ cells well^{-1} (five replicates of each condition) were seeded in 96-well plates for 24 h and cultured. Negative controls (DMEM supplemented with 10 % (v/v) FBS) were performed in parallel. Then, the culture medium was replaced by new and after 2 h of incubation at 37 ˚C, 20 μL of MTT solution (5 mg mL^{-1} in PBS) were added to each well and plates were incubated at 37 ˚C during 4 h. Afterwards, the culture medium was removed and 100 μL of dimethyl sulfoxide (DMSO, from Scharlab, Barcelona, Spain) was then added to dissolve the MTT formazan crystals formed. Finally, absorbance was measured with a microplate spectrophotometer (Multiskan SkyHigh, from ThermoFisher Scientific, Madrid, Spain) at a wavelength of 570 nm.

Secondly, optimization of the dilution of a mixture containing the digestive enzymes employed in the gastro-intestinal phase of the *in vitro* gastrointestinal digestion method (3 mg mL^{-1} pancreatin and 0.3 mg mL^{-1} bile salts in 0.15 M NaCl), considered as the blank, was performed. For this purpose, different dilutions were tested: 1/2, 1/3, 1/4, 1/5, 1/7 and 1/10 (five replicates of each condition). Negative (DMEM supplemented with 10 % (v/v) FBS) and positive controls (diluted cell culture without blank solution) were performed in parallel. Cell viability after 2 h of exposure was measured by the MTT reduction assay, as aforementioned.

Finally, with the optimum density of $24 \cdot 10^3$ cells well^{-1} and dilution 1/2 (stated in section 3.4), Caco-2 cells were exposed to the gastrointestinal extracts obtained from the *in vitro* gastrointestinal digestion method applied for raw materials and processed fish-derived products (section 2.7). For this purpose, a density of $24 \cdot 10^3$ cells well^{-1} was seeded in 96-well plates for 24 h and cultured. Then, the culture medium was removed, and cells were exposed to the gastrointestinal extracts diluted 1/2 (five replicates for each condition). Negative (DMEM supplemented with 10 % (v/v) FBS) and positive controls (diluted cell culture without blank solution) were performed in parallel. Cell viability after 2 h of exposure was measured by the MTT reduction assay, as aforementioned.

2.8 Statistical analysis

One-way analysis of variance (ANOVA) was applied as statistical tool to evaluate the results obtained. A significance level of $p\text{-value} < 0.05$ was adopted for all comparisons. Statistical analysis of data was carried out using Statgraphics Centurion XIX (Statgraphics Technologies Inc., The Plains, VA, USA).

3. Results and Discussion

3.1 Total Se and Hg contents in raw materials and processed fish-derived products. Se:Hg molar ratio and HBV$_{Se}$

Total Se and Hg contents were determined in raw materials and processed fish-derived products by ICP-MS and DMA, respectively, as described in section 2.2. Results obtained are displayed in Table 2.

Concerning to the concentration of Se, no statistically significant differences ($p\text{-value} < 0.05$) were observed between fish raw materials (North Pacific hake and walleye pollock) employed to prepare crab sticks and surimi-derived elvers, according to the fish production lines 1 and 2, respectively. Regarding the semi-finished and finished products, results evidenced similar total Se contents (p-value < 0.05) between the two stages of the same product (semi-finished and finished). Crab sticks (final product) did not show statistically significant differences (p-value < 0.05) in its total Se content with respect to the raw materials employed in its fabrication, unlike surimi-derived elvers (final product) that do show statistically significant differences (p-value < 0.05) in its total Se content with respect to walleye pollock. With respect to Hg, North Pacific hake is the raw material that presents the highest total Hg content, differing significantly (p-value < 0.05) from the others. In addition, it can be observed that the final products (crab sticks and surimi-derived elvers) present less amount of total Hg than the raw material from which they have been manufactured.

Regarding the preparation of salmon noodles (fish production line 3) that implies the use of other fish species as raw materials, all of them, farmed salmon 1, farmed salmon 2 and Pacific whiting present statistically significant differences (p-value < 0.05) in its total Se content between them. In case of Hg, all the raw materials present different total Hg contents (p-value < 0.05), even both species of farmed salmon, which can be attributed to their different origin, developing and nutrition (Fernández-Bautista *et al.,* 2022). Similar Se and Hg contents to the raw materials were found in the salmon noodles, the slight differences found could be attributed to differences in the percentages of raw materials employed to produce the final product.

Percentage of Tolerable Weekly Intake (TWI) values of total Hg were evaluated in accordance with the European Food Safety Authority (EFSA) (EFSA, 2012), considering an average consumption of an amount of 200 g of fish, according to FAOSTAT data (https://www.fao.org/faostat/en/#data/FBS), by 70 kg adult body weight. TWI was calculated by applying Equation (4):

$$\text{TWI (\%)} = \frac{(\text{C x 0.2 kg})/70\text{ kg}}{\text{TWI}} \text{ x } 100 \qquad (4)$$

where C is the concentration of Hg (mg kg^{-1}) (Table 2), 0.2 kg is the average food portion, 70 kg is the body weight of an adult and TWI is the Tolerable Weekly Intake indicated by EFSA (EFSA, 2012), which is 1.6 µg kg^{-1} for $MeHg^+$. TWI value differs from $MeHg^+$ than for inorganic Hg. However, the main form in which Hg can be found in fish and in fish-derived products is $MeHg^+$ (Fernández-Bautista *et al.*, 2024; Forsyth, Casey, Dabeka & McKenzie, 2004; Zhang *et al.*, 2020).

TWI for fish (raw materials) and processed fish-derived products considered in this work varies from 2.75 to 36.07 % (Table S1 of Supplementary Material). Therefore, no sample exceeded TWI levels, showing the security of these samples in terms of total Hg content for the consumer.

Moreover, Se:Hg molar ratio and Selenium Health Benefit Value (HBV_{Se}) were also calculated for each sample following Eqs. (1) and (2) described in section 2.3. Results shown in Table 2 evidenced Se:Hg molar ratios greater than 1 in all cases, which indicates that Se is present in molar excess of Hg content, along with positive HBV_{Se}, which indicate that the Se content is high enough to satisfy the corresponding biological functions after Hg sequestration. It is worth mentioning that the final products of all production lines (crab sticks, surimi-derived elvers and salmon noodles) seem not to have negative effects on human health related to Hg consumption, regarding having favorable Se:Hg molar ratios and HBV_{Se}.

Table 2. Total Se and Hg contents found in raw materials and processed fish-derived measured by ICP-MS and DMA, respectively, and Se:Hg molar ratio and HBV_{Se} calculations.

PRODUCTION LINES 1 AND 2

Raw material/Processed fish-derived product	[Se] (mg kg^{-1})	[Hg] (mg kg^{-1})	Se:Hg molar ratio	HBV_{Se}
Walleye pollock	^{a}0.68 ± 0.03	^{a}0.025 ± 0.003	69	0.0086
North Pacific hake	^{ab}0.69 ± 0.05	^{b}0.039 ± 0.001	72	0.0090
Crab sticks semi-finished	^{b}0.87 ± 0.09	^{c}0.022 ± 0.001	100	0.0110
Crab sticks	^{ab}0.79 ± 0.09	^{c}0.0202 ± 0.0008	99	0.0100
Surimi-derived elvers semi-finished	^{ab}0.78 ± 0.07	^{d}0.0142 ± 0.0006	140	0.0099
Surimi-derived elvers	^{b}0.82 ± 0.09	^{e}0.0154 ± 0.0003	135	0.0104

PRODUCTION LINE 3

Raw material/Processed fish-derived product	[Se] (mg kg^{-1})	[Hg] (mg kg^{-1})	Se:Hg molar ratio	HBV_{Se}
Farmed salmon 1	^{c}0.41 ± 0.06	^{f}0.031 ± 0.001	34	0.0052
Farmed salmon 2	^{d}0.61 ± 0.01	^{g}0.0239 ± 0.0006	65	0.0077
Pacific whiting	^{b}0.89 ± 0.03	^{h}0.0471 ± 0.0009	48	0.0113
Pacific whiting surimi	^{b}0.82 ± 0.04	^{h}0.048 ± 0.003	43	0.0104
Salmon noodles	^{ab}0.72 ± 0.03	^{i}0.0345 ± 0.0008	53	0.0091

Results are referred to wet weight. Se and Hg contents are expressed as mean ± standard deviation (SD); n= 3 for each raw material/processed fish-derived product. Se:Hg molar ratios were calculated from mean values. Different superscript letters in values indicate statistically significant differences (p-value < 0.05) for each analyte (Se or Hg) between raw materials and processed fish-derived products.

3.2 Determination of Se species in raw materials and processed fish-derived products

Se speciation was carried out in raw materials and in processed fish-derived products by HPLC-ICP-MS and HPLC-ESI-MS/MS after enzymatic hydrolysis, as described in section 2.4.

Previously, total Se content in the soluble extracts was determined by ICP-MS to check quantitative extraction that in all cases was achieved (Table S2 of Supplementary Material).

Figure 2A shows the chromatogram profile obtained by HPLC-ICP-MS for the enzymatic extract obtained from surimi-derived elvers in which three main peaks could be observed at 2.29, 3.02 and 4.90 min. The same chromatogram profile was found in all samples considered in this study. The retention times obtained were compared to those of the standard solutions and confirmed by spiking experiments at a concentration of 30 µg L^{-1} of each Se species. The first Se-containing peak could be identified as $SeCys_2$ or SeMetO since both species coeluted when a PRP-X100 column is used. The second and third chromatographic peaks were identified as SeMeSeCys and SeMet, respectively. The presence of SeMet in the samples can lead to the formation of SeMetO, an analytical artifact produced during storage and sample treatment (Pedrero, Encinar, Madrid & Cámara, 2007). Due to the lack of SeMetO standard, it was synthesized by treating SeMet standard with hydrogen peroxide, according to the procedure described by Pedrero *et al.* (Pedrero *et al.*, 2007). It has been demonstrated that this chemical treatment leads to the formation of SeMetO. Consequently, both extracts from the enzymatic treatment and SeMet standard solution were submitted to H_2O_2 treatment. Due to the difficulties found in performing structural assignment of the Se-containing peaks derived from HPLC-ICP-MS, samples were also analysed by HPLC-ESI-MS/MS.

Figure 2 shows, as an example, the chromatographic profiles obtained by anion exchange HPLC-ICP-MS of the enzymatic extract from surimi-derived elvers (Figure 2A, B and C) in which the presence of SeMeSeCys and SeMet were corroborated by HPLC-ESI-MS/MS (Figure 2D, E, F and G), taking into account the retention time of the standards and analytes as well as the ion ratio for the selected fragment ions being monitored by the SRM transitions gathered in Table 1. Moreover, the first Se-containing peak was identified as SeMetO by the confirmatory analysis carried out by HPLC-ESI-MS/MS (Figure S1 of Supplementary Material).

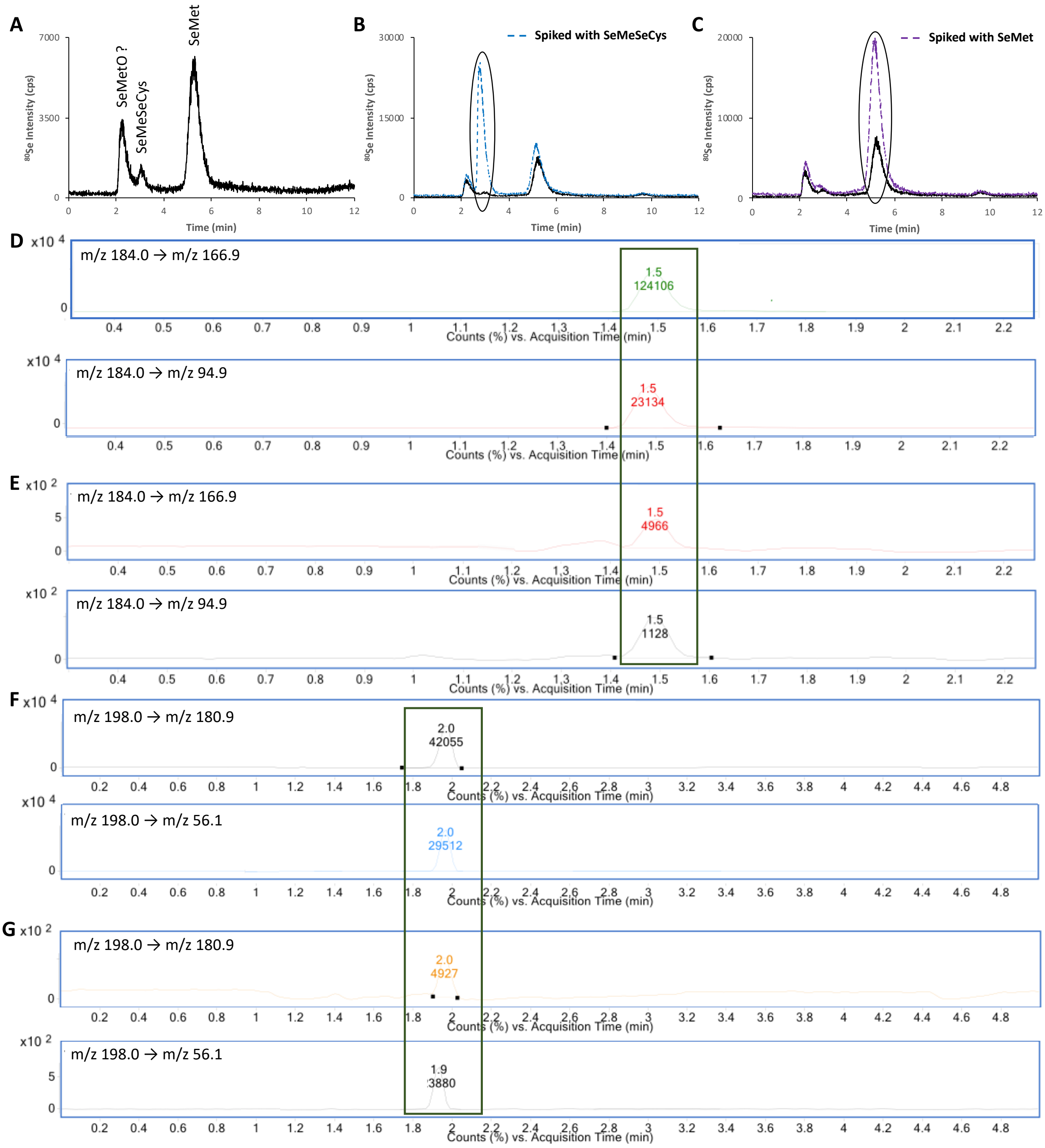

Figure 2. A, B, C: chromatographic profiles obtained by anion exchange HPLC-ICP-MS of surimi-derived elvers. The discontinuous lines in blue (**B**) and purple (**C**) correspond to the sample spiked with 30 µg L^{-1} of SeMeSeCys or SeMet, respectively. **D, E, F, G**: HPLC-ESI-MS/MS chromatograms of the identification of SeMeSeCys (**D, E**) and SeMet (**F, G**) in surimi-derived elvers. **D** and **F** correspond to the sample spiked with 700 µg L^{-1} of SeMeSeCys or SeMet, respectively. **E** and **G** correspond to the sample without spike.

For quantification purposes, an external calibration was employed since the samples did not present any matrix effect. The distribution of these species in percentage, calculated as the ratio of Se species concentration to the total Se content, is displayed in Table 3 for each sample.

Table 3. SeMet and SeMeSeCys quantification in raw materials and processed fish-derived products by HPLC-ICP-MS.

PRODUCTION LINES 1 AND 2				
Raw material/Processed fish-derived product	[SeMeSeCys] (mg kg^{-1})	% SeMeSeCys	[SeMet] (mg kg^{-1})	% SeMet
Walleye pollock	0.12 ± 0.04	17.6 ± 0.6	0.40 ± 0.04	58.8 ± 0.6
North Pacific hake	0.11 ± 0.03	15.9 ± 0.4	0.44 ± 0.02	64.5 ± 0.3
Crab sticks semi-finished	0.14 ± 0.01	16.1 ± 0.9	0.38 ± 0.01	43.7 ± 0.7
Crab sticks	0.15 ± 0.02	18.9 ± 0.9	0.35 ± 0.01	44.3 ± 0.9
Surimi-derived elvers semi-finished	0.13 ± 0.01	16.7 ± 0.1	0.36 ± 0.05	46.2 ± 0.7
Surimi-derived elvers	0.11 ± 0.03	13.4 ± 0.3	0.37 ± 0.07	43.9 ± 0.8

PRODUCTION LINE 3				
Raw material/Processed fish-derived product	[SeMeSeCys] (mg kg^{-1})	% SeMeSeCys	[SeMet] (mg kg^{-1})	% SeMet
Farmed salmon 1	0.09 ± 0.02	21.9 ± 0.3	0.23 ± 0.08	55.9 ± 0.5
Farmed salmon 2	0.11 ± 0.01	18.1 ± 0.2	0.30 ± 0.01	49.2 ± 0.2
Pacific whiting	0.18 ± 0.01	29.5 ± 0.4	0.35 ± 0.04	39.3 ± 0.4
Pacific whiting surimi	0.17 ± 0.03	20.7 ± 0.8	0.32 ± 0.07	40.2 ± 0.6
Salmon noodles	0.12 ± 0.02	16.6 ± 0.9	0.22 ± 0.01	30.6 ± 0.2

Results are referred to wet weight. SeMet and SeMeSeCys contents are expressed as mean ± standard deviation (SD); n= 3 for each raw material/processed fish-derived product.

As it can be observed in Table 3, SeMet is more abundant in samples than SeMeSeCys, being this fact true for all the raw materials and processed fish-derived products of the three production lines. In some cases, SeMet content is up to four times higher than SeMeSeCys content, for instance in North Pacific hake (production lines 1 and 2).

Concerning the first and the second production lines, when it comes to raw materials, walleye pollock and North Pacific hake, there is no statistically significant differences (p-value < 0.05) between their SeMet and SeMeSeCys contents. Regarding the intermediate and the final products (crab sticks and surimi-derived elvers), their Se species content has not been reduced with respect to that of their raw materials. Regarding the third production line, the relative Se species content is quite lower than in the first and second one, especially in case of SeMet.

Comparing the final products from the three production lines, the manufacturing process of the products does not affect the integrity of the Se species (SeMet and SeMeSeCys), but it does affect their original concentration (production line 3), which may be due to the fact that the amount of fish in these products only represents up to 50% of the total product ingredients.

Both Se species, SeMet and SeMeSeCys, have been identified as the major Se species in muscle tissues of highly consumed fishes. Fernández-Bautista *et al.* (Fernández-Bautista *et al.*, 2022) identified SeMet in the soluble proteins of tuna and SeMeSeCys in the soluble, sarcoplasmic, myofibrillar and alkali-soluble proteins of swordfish, farmed salmon and wild salmon. In addition, SeMeSeCys along with SeCys were detected in the muscle tissues of swordfish (Vicente-Zurdo *et al.*, 2019) and sand whiting (Jagtap, Maher, Krikowa, Ellwood & Foster*,* 2016). Moreover, SeMet was the major Se species found in muscle of Atlantic salmon fed with diets supplemented with organic or inorganic Se (Sele, Ørnsrud, Sloth, Berntssen, & Amlund, 2018).

3.3 Bioaccessible total Se and Hg and Se species in samples

Bioaccessibility, defined as the maximum fraction of a substance theoretically released from the food matrix in the gastrointestinal tract, and thus available for being absorbed in the intestine, could be considered as a marker of nutritional quality and safety. Hence, total Se and Hg bioaccessibilities and Se species distribution in the bioaccessible fraction were evaluated by applying an *in vitro* gastrointestinal digestion model in the raw materials and processed fish-derived products has been carried out as described in section

2.5. Percentages of bioaccessibility (Figure 3 and Table 4) in the intestinal extracts have been calculated by applying the Eq. (3) in section 2.5.

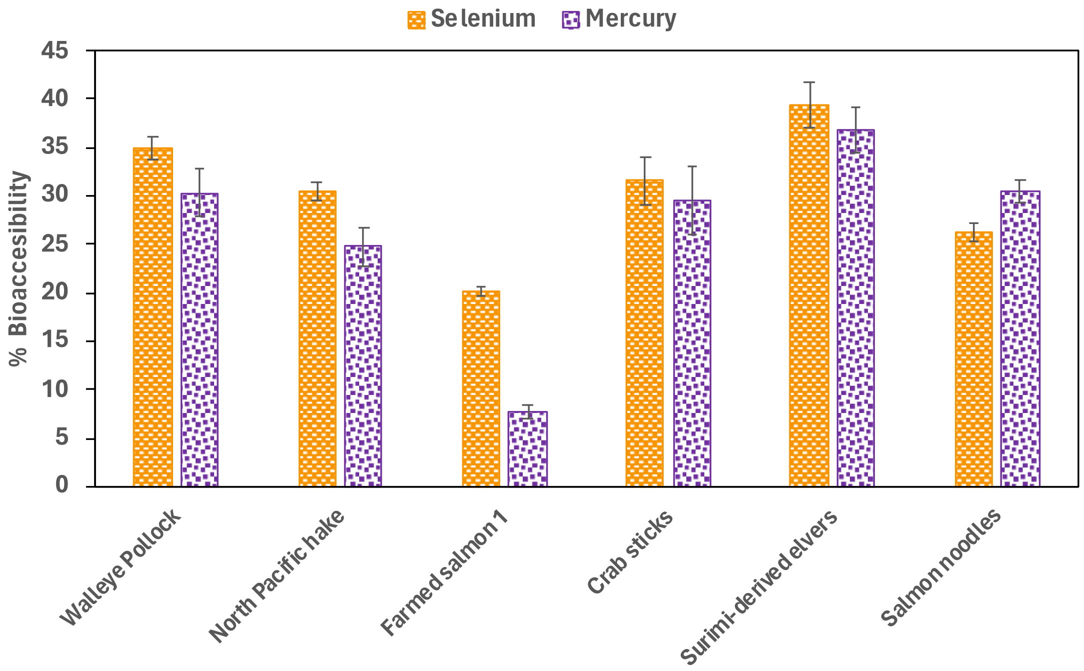

Figure 3. Bioaccessibility of total Se and Hg in raw materials and processed fish-derived products using an *in vitro* gastrointestinal digestion method.

In general, results concerning total Se bioaccessibility obtained for all samples in the simulated extracts of the *in vitro* gastrointestinal digestion method ranged between 20-39 %, while in case of Hg, there are more variability between samples, ranging between 8-37 % (Figure 3).

Although no significant differences in Se bioaccessibility values were obtained from raw materials (walleye pollock, North Pacific hake and farmed salmon 1) and those of final products (crab sticks, surimi-derived elvers and salmon noodles), it seems that surimi-derived elvers presented the highest percentages of bioaccessibility. This could be attributed to the elaboration process that implies defatting at 90 °C, shaping, mixing with other ingredients and finally applying a pasteurization process. The application of heat during the defatting stage induces the loss of water and the degradation of proteins and, thus favouring the release of bound Se which might increase Se bioaccessibility

(Cabañero, Madrid & Cámara, 2004). Therefore, in general, it can be concluded that the elaboration processes employed to obtain the final products from raw materials (muscle tissues of fresh fish) did not apparently negatively affect the bioaccessibility of total Se.

Regarding Hg, lower percentages of bioaccessibility were obtained than for Se in almost all cases, except for salmon noodles. Farmed salmon presents the lowest Se and Hg bioaccessibility values of all the analyzed samples.

In the bioaccessible fraction, the Se:Hg molar ratios (Table 4) are higher than one (46–389) and the HBV_{Se} parameter is positive (0.0027–0.0087) in all cases, and therefore, according to this information, none of the samples tested in this study should be considered a health risk related to Hg toxicity.

Other authors reported large variability in total Se bioaccessibility values found in seafood, which might be attributable to differences in the composition and matrix of the fish species evaluated, and in the experimental conditions of the *in vitro* gastrointestinal digestion method employed, such as pH, enzymes and salts or mode of shaking, for instance. In this sense, 70-96 % of Se bioaccessibility values were obtained for swordfish, 79-99 % for hake, 50-100 % for salmon, 17-91 % for blue whiting and 50 % for tuna (Cabañero *et al.*, 2004; 2007; Calatayud et al., 2012). In case of Hg, results of bioaccessibility reported in the literature are also very variable and scarce, for instance bioaccesibility values between 17-87 % were obtained for swordfish, 66-81 % for hake, 62-68 % for blue whiting and 9-19 % for tuna (Cabañero *et al.*, 2004; Calatayud et al., 2012; Torres-Escribano, Vélez & Montoro, 2010; Torres-Escribano, Ruiz, Barrios, Vélez & Montoro, 2011). Hg bioaccessibility results for fish have proven to be very variable to such an extent that even for the same fish species, swordfish, and employing the same analytical methodology, Torres-Escribano *et al.* (Torres-Escribano *et al.*, 2010) evidenced Hg bioaccessibilities ranging 38-83 %.

Table 4. Bioaccessibility of total Se, Hg and Se species in raw material and processed fish products using an *in vitro* gastrointestinal digestion method and Se:Hg molar ratio and HBV_{Se} parameters in bioaccessible fractions.

Raw material/Processed fish product	% Se bioaccessibility	% Hg bioaccessibility	Se:Hg bioaccessible molar ratio	HBV_{Se}	% SeMeSeCys in bioaccessible fraction	% SeMet in bioaccessible fraction
Walleye pollock	35 ± 1	30 ± 2	66	0.0030	12 ± 1	33 ± 4
North Pacific hake	31 ± 1	25 ± 2	46	0.0027	19 ± 3	41.9 ± 0.9
Farmed salmon 1	20.2 ± 0.5	7.8 ± 0.7	176	0.0087	17.2 ± 0.8	38.2 ± 0.8
Crab sticks	32 ± 3	30 ± 3	173	0.0041	10.2 ± 0.9	19.3 ± 0.9
Surimi-derived elvers	39 ± 2	37 ± 2	137	0.0034	9.4 ± 0.3	17.3 ± 0.9
Salmon noodles	26.3 ± 0.9	31 ± 1	389	0.0052	8.4 ± 0.9	16.1 ± 0.8

Results are expressed as mean ± standard deviation (SD); n= 3 for each raw material/processed fish-derived product.

Concerning Se species bioaccessibility, speciation studies by HPLC-ICP-MS and confirmed by HPLC-ESI-MS/MS were also carried out in the gastrointestinal extracts of raw materials and processed fish-derived products, since it is the final step which might reveal the Se species available for their absorption in the intestine and thus quite relevant to investigate whether the integrity of the Se species found in the samples is maintained once digested.

In all gastrointestinal extracts analyzed, the same Se species found in samples (Table 3), SeMeSeCys and SeMet, were identified and quantified and results obtained are displayed in Table 4 and some chromatograms in Figure S2 of Supplementary Material.

Similarly to what occurred in raw materials and fish derived products, SeMet was the most abundant Se specie in all gastrointestinal extracts. Raw materials present higher SeMet contents than their respective final products: walleye pollock and North Pacific hake (33 ± 4 % and 41.9 ± 0.9, respectively) with respect to crab sticks and surimi-derived elvers (19.3 ± 0.9 and 17.3 ± 0.9 %, respectively) and farmed salmon 1 (38.2 ± 0.8 %) with respect to salmon noodles (16.1 ± 0.8 %). According to literature, SeMet is the form of Se most rapidly absorbed by the body, being the most stable way to store it (Lavu, Van De Wiele, Pratti, Tack & Du Laing, 2016).

When it comes to SeMeSeCys, lower percentages were obtained and the comparison between raw materials and final products follows the same pattern as in the case of SeMet, the former present more relatively SeMeSeCys contents than the latter. In this sense, walleye pollock and North Pacific hake (12 ± 1 and 19 ± 3 %, respectively) with respect to crab sticks and surimi-derived elvers (10.2 ± 0.9 and 9.4 ± 0.3 %, respectively) and farmed salmon 1 (17.2 ± 0.8 %) with respect to salmon noodles (8.4 ± 0.9 %).

Cabañero *et al.* (Cabañero *et al.*, 2004; 2007) also identified the Se species SeMet in the gastrointestinal digests extracted from swordfish, sardine and tuna with bioaccessibility percentages range between 14-19 %, which are similar to that obtained in this study. Nevertheless, to the best of the authors' knowledge, it is the first time that bioaccessibility studies were carried out on products derived from fish, such as processed fish-derived products. Tracking Se and Hg bioaccessibility from raw materials to these new products available on the market is quite relevant from a nutritional point of view and safety assessment.

3.4 Influence of intestinal extracts in Caco-2 cells viability

Once the bioccessibility of Se and Hg has been studied in different fish species and processed fish-derived products, the cytotoxicity of gastrointestinal extracts on Caco-2 cell culture, employed as an *in vitro* intestinal barrier model, has been assessed by MTT assay.

Before evaluating the influence of gastrointestinal extracts in Caco-2 cells viability, cell number employed in MTT assay was optimized and the effect of blanks from the gastrointestinal phase which contained the mixture of enzymes used (3 mg mL^{-1}

pancreatin and 0.3 mg mL^{-1} bile salts in 0.15 M NaCl) was evaluated on cell viability, as described in section 2.7.

Concerning the number of cells, different cell number were seeded into a P96 wells and MTT procedure was performed without adding the intestinal extracts and absorbance values were measured at 570 nm after the incubation. Cell density of $24\cdot10^3$ cells $well^{-1}$ was selected as optimal since absorbance values were around 1, presenting statistically significant differences (p-value < 0.05) with respect to the absorbance obtained in case of $20\cdot10^3$ cells $well^{-1}$, which was the closest one (Table S3 of Supplementary Material).

On the other hand, before carrying out the cytotoxicity test, it is mandatory to check the effect of the mixtures of enzymes used during the *in vitro* digestion process on Caco-2 cell viability since it may hamper to obtain accurate conclusions. For this purpose, the effect of different dilutions of enzymes mixture on cell viability were evaluated. According to the results displayed in Table S4 of Supplementary Material, all the dilutions tested caused a reduction of nearly the 30 % of cell viability without no statistically significant differences (p-value < 0.05) between any of the values of cell viability obtained. Therefore, dilution ½ was selected for applying it to the gastrointestinal extracts from the raw materials and processed fish-derived products in the further MTT assay to simulate the most realistic situation and to avoid inaccurate results due to the effect on enzyme mixtures in cell viability of Caco-2 cell culture.

Under these optimal conditions, intestinal extracts diluted 1/2 were exposed to Caco-2 cells and results of cell viabilities obtained after MTT assay are displayed in Figure 4 and in Table S5 of Supplementary Material.

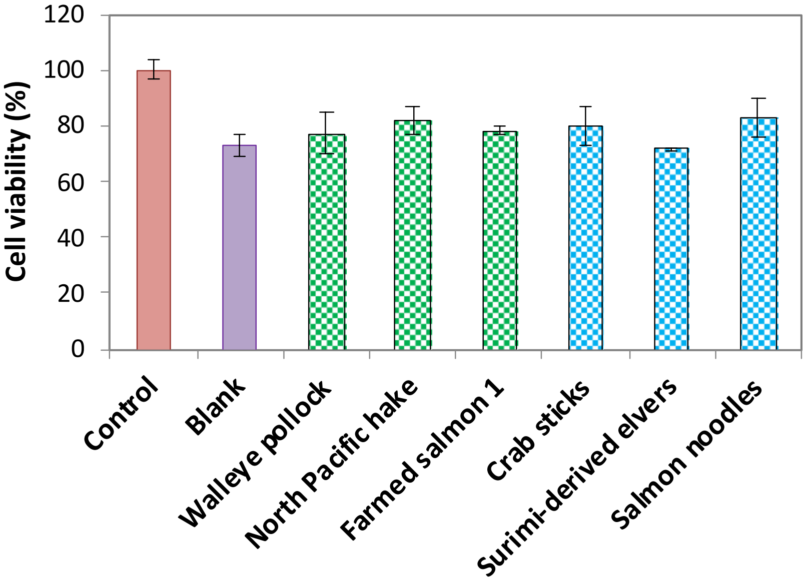

Figure 4. Cell viability of Caco-2 cells to gastrointestinal extracts exposure by MTT assay. Error bars represent standard deviation (n=5).

All the samples show similar cell viability percentage with respect to the blank of the gastrointestinal phase (73 ± 4 %). In general, it can be stated that 2 h of exposition of Caco-2 cells to the gastrointestinal extracts from samples did not result in a decrease in cell viability. Therefore, it seems that the bioaccessible fraction obtained from either the fish samples or the processed fish-derived products does not cause any effect on Caco-2 cells suggesting the absence of toxicity of the components of the intestinal extracts (including Se and Hg) in any of the sample analysed. To the best of our knowledge, the use of an *in vitro* gastrointestinal digestion combined with Caco2-cells cytotoxicity of the intestinal fraction by using a MTT colorimetric assay has been applied for the first time in processed fish products.

4. Conclusions

Three processed fish-derived products marketed in Spain, based on surimi, have been studied along with their corresponding raw materials (muscle tissues of fresh fish). Positive values of HBV_{Se} were obtained for all samples, with no apparent negative effects on human health related to Hg consumption. Moreover, Se speciation studies carried out evidenced SeMet as the major Se species found, followed by SeMeSeCys, not only in the fish employed as raw materials but also in the processed fish-derived products, which means that the integrity of the Se species has not been affected by the elaboration process. Moreover, an *in vitr*o gastrointestinal digestion method was carried out for assessing Se and Hg bioaccessibility. Results showed relatively low bioaccessibilities for all samples, ranging 20-39 % and 8-37 %, for total Se and Hg, respectively. The presence of SeMet and SeMeSeCys in the bioaccessible fraction was also confirmed. Finally, the cytotoxicity of the bioaccessible fraction was also evaluated by using the Caco-2 cell line, Cell viability was not significatively affected, showing all the samples similar cell viability percentage with respect to the blank of the gastrointestinal phase (73 ± 4 %). This is the first time that Se and Hg contents and their bioaccessibility have been monitored at all stages of the production chain involved in the manufacture of surimi-based products which is essential to ensure food safety and provide transparent and reliable information to consumers.

Credit authorship contribution statement

T. Fernández-Bautista: conceptualization, formal analysis, data curation, investigation, methodology, writing-original draft, writing-review & editing; **B. Gómez-Gómez**: conceptualization, formal analysis, investigation, supervision, writing-original draft, writing-review & editing; **E. Gracia-Lor**: conceptualization, investigation, supervision, writing-review & editing; **T. Pérez-Corona**: conceptualization, investigation, supervision, writing-review & editing; **Y. Madrid**: conceptualization, investigation, supervision, writing-review & editing, funding acquisition.

Author contributions

All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Akter, S., Addepalli, R., Netzel, M., Tinggi, U., Fletcher, M., Sultanbawa, Y. & Osborne, S. (2022). In Vitro Bioaccessibility and Intestinal Absorption of Selected Bioactive Compounds in Terminalia Ferdinandiana. *Frontiers in Nutrition, 8* (January), 1–11. https://doi.org/10.3389/fnut.2021.818195.

Azad, A. M., Frantzen, S., Bank, M. S., Nilsen, B. M., Duinker, A., Madsen, L., & Maage, A. (2019). Effects of geography and species variation on selenium and mercury molar ratios in Northeast Atlantic marine fish communities. *Science of the Total Environment*, *652*, 1482–1496. https://doi.org/10.1016/j.scitotenv.2018.10.405

Bogard, J. R., Farmery, A. K., Little, D. C., Fulton, E. A., & Cook, M. (2019). Will fish be part of future healthy and sustainable diets? *The Lancet Planetary Health*, *3*(4), e159–e160. https://doi.org/10.1016/S2542-5196(19)30018-X

Burger, J., Gaines, K. F., Boring, C. S., Stephens, W. L., Snodgrass, J., & Gochfeld, M. (2001). Mercury and selenium in fish from the Savannah River: Species, trophic level, and locational differences. *Environmental Research*, *87*(2), 108–118. https://doi.org/10.1006/enrs.2001.4294

Burger, J., & Gochfeld, M. (2011). Mercury and selenium levels in 19 species of saltwater fish from New Jersey as a function of species, size, and season. *Science of the Total Environment*, *409*(8), 1418–1429. https://doi.org/10.1016/j.scitotenv.2010.12.034

Cabañero, A. I., Madrid, Y., & Cámara, C. (2004). Selenium and mercury bioaccessibility in fish samples: An in vitro digestion method. *Analytica Chimica Acta, 526(1)*, 51–61. https://doi.org/10.1016/j.aca.2004.09.039

Cabañero, A. I., Madrid, Y., & Cámara, C. (2007). Mercury-selenium species ratio in representative fish samples and their bioaccessibility by an in vitro digestion method. *Biological Trace Element Research*, *119*(3), 195–211. https://doi.org/10.1007/s12011-007-8007-5

Calatayud, M., Devesa, V., Virseda, J. R., Barberá, R., Montoro, R., & Vélez, D. (2012). Mercury and selenium in fish and shellfish: Occurrence, bioaccessibility and uptake by Caco-2 cells. *Food and Chemical Toxicology, 50*(8), 2696–2702. https://doi.org/10.1016/j.fct.2012.05.028

Capozzi, F., Magkos, F., Fava, F., Milani, G. P., Agostoni, C., Astrup, A., & Saguy, I. S. (2021) A Multidisciplinary Perspective of Ultra-Processed Foods and Associated Food Processing Technologies: A View of the Sustainable Road Ahead. *Nutrients, 13*(11). https://doi.org/10.3390/nu13113948.

EFSA. (2012). Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal*, *10*(12). https://doi.org/10.2903/j.efsa.2012.2985

Etcheverry, P., Grusak, M. A. & Fleige, L. E. (2012). Application of in Vitro Bioaccessibility and Bioavailability Methods for Calcium, Carotenoids, Folate, Iron, Magnesium, Polyphenols, Zinc, and Vitamins B 6, B 12, D, and E. *Frontiers in Physiology, 3* (August), 1–22. https://doi.org/10.3389/fphys.2012.00317

FAO. (2020). The State of World Fisheries and Aquaculture. In *Sustainability in action*. https://doi.org/10.4060/ca9229en

Fernández-Bautista, T., Gómez-Gómez, B., Gracia-Lor, E., Pérez-Corona, T., & Madrid, Y. (2024). Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of highly consumed fish species and fish-derived products. *Food Chemistry*, *435*(137544). https://doi.org/10.1016/j.foodchem.2023.137544

Fernández-Bautista, T., Gómez-Gómez, B., Palacín-García, R., Gracia-Lor, E., Pérez-Corona, T., & Madrid, Y. (2022). Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS. *Talanta*, *237*(October 2021). https://doi.org/10.1016/j.talanta.2021.122922

Ferraris, F., Iacoponi, F., Raggi, A., Baldi, F., Fretigny, M., Mantovani, A., & Cubadda, F. (2021). Essential and toxic elements in sustainable and underutilized seafood species and derived semi-industrial ready-to-eat products. *Food and Chemical Toxicology*, *154*, 112331. https://doi.org/10.1016/j.fct.2021.112331

Forsyth, D. S., Casey, V., Dabeka, R. W., & McKenzie, A. (2004). Methylmercury levels in predatory fish species marketed in Canada. *Food Additives and Contaminants*, *21*(9), 849–856. https://doi.org/10.1080/02652030400004259

Gajdosechova, Z., Lawan, M. M., Urgast, D. S., Raab, A., Scheckel, K. G., Lombi, E., Kopittke, P. M., Loeschner, K., Larsen, E. H., Woods, G., Brownlow, A., Read, F. L., Feldmann, J., & Krupp, E. M. (2016). In vivo formation of natural HgSe nanoparticles in the liver and brain of pilot whales. *Scientific Reports*, *6*(October). https://doi.org/10.1038/srep34361

Gochfeld, M., & Burger, J. (2021). Mercury interactions with selenium and sulfur and the relevance of the Se:Hg molar ratio to fish consumption advice. *Environmental Science and Pollution Research*, *28*(15), 18407–18420. https://doi.org/10.1007/s11356-021-12361-7

Gómez-Gómez, B., Pérez-Corona, M. T., & Madrid, Y. (2016). Availability of zinc from infant formula by in vitro methods (solubility and dialyzability) and size-exclusion chromatography coupled to inductively coupled plasma-mass spectrometry. *Journal of Dairy Science*, *99*(12), 9405–9414. https://doi.org/10.3168/jds.2016-11405

IUPAC. (1978). International Union of Pure and Applied Chemistry. (1978). Nomenclature, symbols, units and their usage in spectrochemical analysis-III. Analytical flame spectroscopy and associated non-flame procedures. *Spectrochimica Acta Part B*, *33*, 247–269.

Jagtap, R., Maher, W., Krikowa, F., Ellwood, M. J., & Foster, S. (2016). Measurement of selenomethionine and selenocysteine in fish tissues using HPLC-ICP-MS. *Microchemical Journal*, *128*, 248–257. https://doi.org/10.1016/j.microc.2016.04.021

Lavu, R. V. S., Van De Wiele, T., Pratti, V. L., Tack, F. & Du Laing, G. (2016). Selenium Bioaccessibility in Stomach, Small Intestine and Colon: Comparison between Pure Se Compounds, Se-Enriched Food Crops and Food Supplements. *Food Chemistry, 197*, 382–387. https://doi.org/10.1016/j.foodchem.2015.08.001.

Manceau, A., Gaillot, A. C., Glatzel, P., Cherel, Y., & Bustamante, P. (2021). In Vivo Formation of HgSe Nanoparticles and Hg-Tetraselenolate Complex from Methylmercury in Seabirds-Implications for the Hg-Se Antagonism. *Environmental Science and Technology*, *55*(3), 1515–1526. https://doi.org/10.1021/acs.est.0c06269

Olmedo, P., Hernández, A. F., Pla, A., Femia, P., Navas-Acien, A., & Gil, F. (2013). Determination of essential elements (copper, manganese, selenium and zinc) in fish and shellfish samples. Risk and nutritional assessment and mercury-selenium balance. *Food and Chemical Toxicology*, *62*, 299–307. https://doi.org/10.1016/j.fct.2013.08.076

Ordiano-Flores, A., Rosíles-Martínez, R., & Galván-Magaña, F. (2012). Biomagnification of mercury and its antagonistic interaction with selenium in yellowfin tuna Thunnus albacares in the trophic web of Baja California Sur, Mexico. *Ecotoxicology and Environmental Safety*, *86*, 182–187. https://doi.org/10.1016/j.ecoenv.2012.09.014

Pedrero, Z., Encinar, J. R., Madrid, Y. & Cámara, C. (2007). Identification of Selenium Species in Selenium-Enriched Lens Esculenta Plants by Using Two-Dimensional Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry and [^{77}Se]Selenomethionine Selenium Oxide Spikes. *Journal of Chromatogaphy. A, 1139* (2), 247–253. https://doi.org/10.1016/j.chroma.2006.11.031.

Ralston, N. V. C. (2008). Selenium health benefit values as seafood safety criteria. *EcoHealth*, *5*(4), 442–455. https://doi.org/10.1007/s10393-008-0202-0

Ralston, N. V. C., Kaneko, J. J., & Raymond, L. J. (2019). Selenium health benefit values provide a reliable index of seafood benefits vs. risks. *Journal of Trace Elements in Medicine and Biology*, *55*(May), 50–57. https://doi.org/10.1016/j.jtemb.2019.05.009

Raymond, L. J., & Ralston, N. V. C. (2020). Mercury: selenium interactions and health implications. *NeuroToxicology*, *81*(1), 294–299. https://doi.org/10.1016/j.neuro.2020.09.020

Ralston, N. V. C., Ralston, C. R., Blackwell, J. L., & Raymond, L. J. (2008). Dietary and tissue selenium in relation to methylmercury toxicity. *NeuroToxicology*, *29*(5), 802–811. https://doi.org/10.1016/j.neuro.2008.07.007

Ralston, N. V. C., Ralston, C. R., & Raymond, L. J. (2016). Selenium Health Benefit Values: Updated Criteria for Mercury Risk Assessments. *Biological Trace Element Research*, *171*(2), 262–269. https://doi.org/10.1007/s12011-015-0516-z

Rodrigues, D. B., Marques, M. C., Hacke, A., Loubet Filho, P. S., Cazarin, C. B. B. & Mariutti, L. R. B. (2022). Trust Your Gut: Bioavailability and Bioaccessibility of Dietary Compounds. *Current Research in Food Science, 5*, 228–233. https://doi.org/10.1016/j.crfs.2022.01.002.

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H. G., & Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *Journal of Trace Elements in Medicine and Biology*, *47*(May 2017), 124–133. https://doi.org/10.1016/j.jtemb.2018.02.005

Suzuki, Y., Kondo, M., Akiyama, H., & Ogra, Y. (2022). Presence of nano-sized mercury-containing particles in seafoods, and an estimate of dietary exposure. *Environmental Pollution*, *307*(March), 119555. https://doi.org/10.1016/j.envpol.2022.119555

Torres-Escribano, S., Ruiz, A., Barrios, L., Vélez, D. & Montoro, R. (2011). Influence of Mercury Bioaccessibility on Exposure Assessment Associated with Consumption of Cooked Predatory Fish in Spain. *Journal of the Science of Food and Agriculture, 91* (6), 981–986. https://doi.org/10.1002/jsfa.4241.

Torres-Escribano, S., Vélez, D. & Montoro, R. (2010). Mercury and Methylmercury Bioaccessibility in Swordfish. *Food Additives and Contaminants - Part A, 27* (3), 327–337. https://doi.org/10.1080/19440040903365272.

Ulusoy, Ş., Mol, S., Karakulak, F. S., & Kahraman, A. E. (2019). Selenium-Mercury Balance in Commercial Fish Species from the Turkish Waters. *Biological Trace Element Research*, *191*(1), 207–213. https://doi.org/10.1007/s12011-018-1609-2

Vicente-Zurdo, D., Gómez-Gómez, B., Pérez-Corona, M. T., & Madrid, Y. (2019). Impact of fish growing conditions and cooking methods on selenium species in swordfish and salmon fillets. *Journal of Food Composition and Analysis*, *83*(August), 103275. https://doi.org/10.1016/j.jfca.2019.103275

Yamashita, M., Yamashita, Y., Suzuki, T., Kani, Y., Mizusawa, N., Imamura, S., Takemoto, K., Hara, T., Hossain, M. A., Yabu, T., & Touhata, K. (2013). Selenoneine, a Novel Selenium-Containing Compound, Mediates Detoxification Mechanisms against Methylmercury Accumulation and Toxicity in Zebrafish Embryo. *Marine Biotechnology*, *15*(5), 559–570. https://doi.org/10.1007/s10126-013-9508-1

Zhang, H., Guo, C., Feng, H., Shen, Y., Wang, Y., Zeng, T., & Song, S. (2020). Total mercury, methylmercury, and selenium in aquatic products from coastal cities of China: Distribution characteristics and risk assessment. *Science of the Total Environment*, *739*, 140034. https://doi.org/10.1016/j.scitotenv.2020.140034

Electronic Supplementary Information

Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBV$_{Se}$ index, bioaccessibility and Caco-2 cells toxicity

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Table S1. Tolerable weekly intake (TWI, %) values for Hg contents in analyzed samples.

PRODUCTION LINES 1 AND 2		PRODUCTION LINE 3	
Raw material/Processed fish-derived product	TWI (%)	Raw material/Processed fish-derived product	TWI (%)
Walleye pollock	4.46	Farmed salmon 1	5.54
North Pacific hake	6.96	Farmed salmon 2	4.27
Crab sticks semi-finished	3.93	Pacific whiting	8.41
Crab sticks	36.07	Pacific whiting surimi	8.57
Surimi-derived elvers semi-finished	25.36	Salmon noodles	6.16
Surimi-derived elvers	2.95		

Table S2. Percentages of Se extracted in the soluble protein fraction from raw materials and processed fish-derived products, with respect to their total Se content.

PRODUCTION LINES 1 AND 2		PRODUCTION LINE 3	
Raw material/Processed fish-derived product	Se extracted (%)	Raw material/Processed fish-derived product	Se extracted (%)
Walleye pollock	99 ± 6	Farmed salmon 1	93 ± 9
North Pacific hake	87 ± 3	Farmed salmon 2	101 ± 2
Crab sticks semi-finished	102 ± 9	Pacific whiting	98 ± 3
Crab sticks	101 ± 7	Pacific whiting surimi	91 ± 6
Surimi-derived elvers semi-finished	98 ± 7	Salmon noodles	88 ± 7
Surimi-derived elvers	108 ± 15		

Results are expressed as mean ± standard deviation (SD); n= 3.

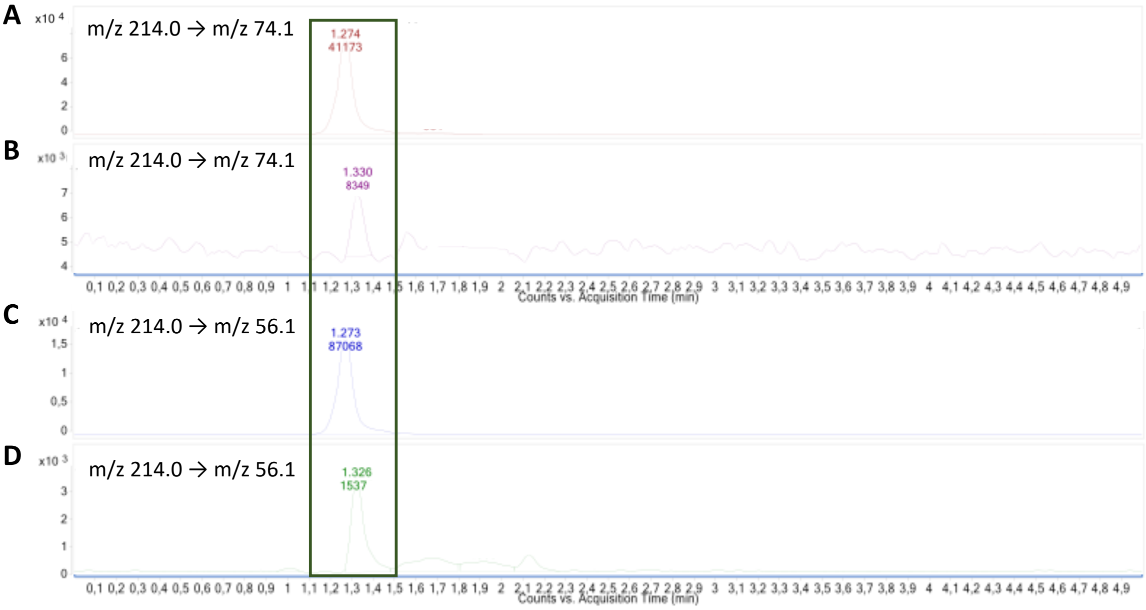

Figure S1. HPLC-ESI-MS/MS chromatograms of the identification of SeMetO in surimi-derived elvers. **A** and **C** correspond to the standard SeMetO chemically synthesized in the laboratory and **B** and **D** correspond to the sample.

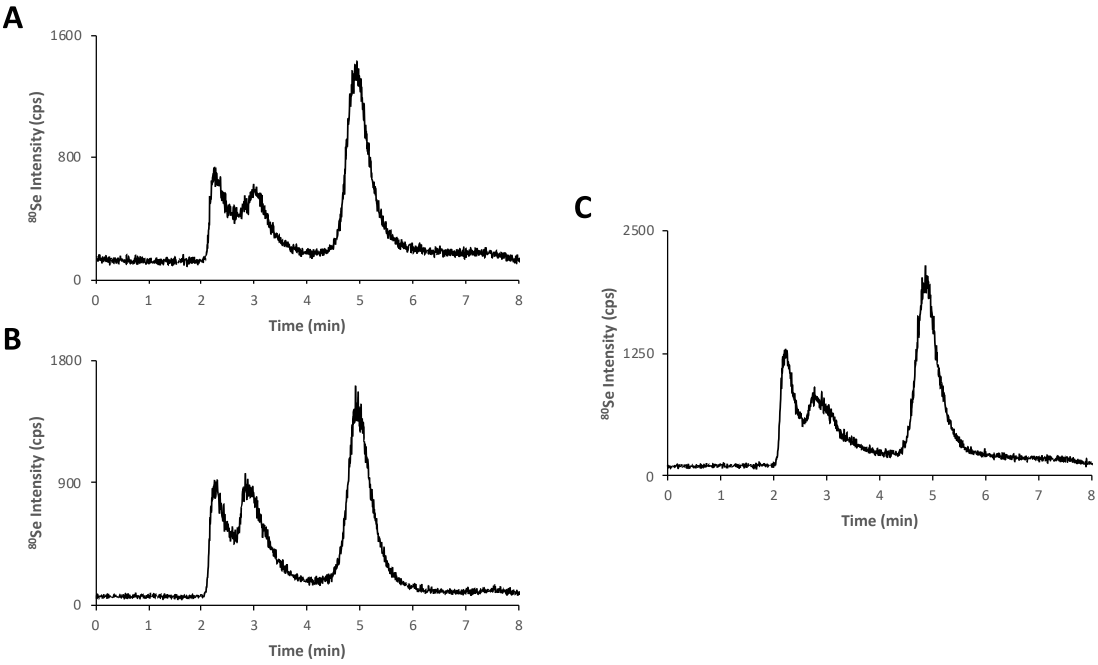

Figure S2. Chromatographic profiles obtained by anion exchange HPLC-ICP-MS of the bioaccessible fraction obtained from walleye pollock (**A**), North Pacific hake (**B**) and crab sticks (**C**).

Table S3. Optimization of the cell number seeded in p96-well plates.

Density $\cdot 10^3$ (cells well^{-1})	Cell viability (%)
6	33 ± 1
10	53 ± 4
15	72 ± 4
2	83 ± 1
24	96.8 ± 0.7

Results are expressed as mean ± standard deviation (SD); n= 5.

Table S4. Optimization of the dilution of the blank of the gastro-intestinal phase.

Dilution	Cell viability (%)
1/2	73 ± 4
1/3	70 ± 4
1/4	68 ± 3
1/5	69 ± 4
1/7	72 ± 3
1/10	74 ± 3

Results are expressed as mean ± standard deviation (SD); n= 5.

Table S5. Cell viability of Caco-2 cells to gastro-intestinal exposure by MTT assay.

Sample	Cell viability (%)
Walleye pollock	77 ± 8
North Pacific hake	82 ± 5
Farmed salmon 1	78 ± 1
Crab sticks	80 ± 7
Surimi-derived elvers	72 ± 1
Salmon noodles	83 ± 7

Results are expressed as mean ± standard deviation (SD); n= 5.

ARTÍCULO 4:

Investigación de la presencia de selenoneína, ergotioneína y biomoléculas con selenio en pescados y productos comerciales derivados de pescados

Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products

Tamara Fernández-Bautista, Beatriz Gómez-Gómez, Emma Gracia-Lor, Teresa Pérez-Corona, Yolanda Madrid

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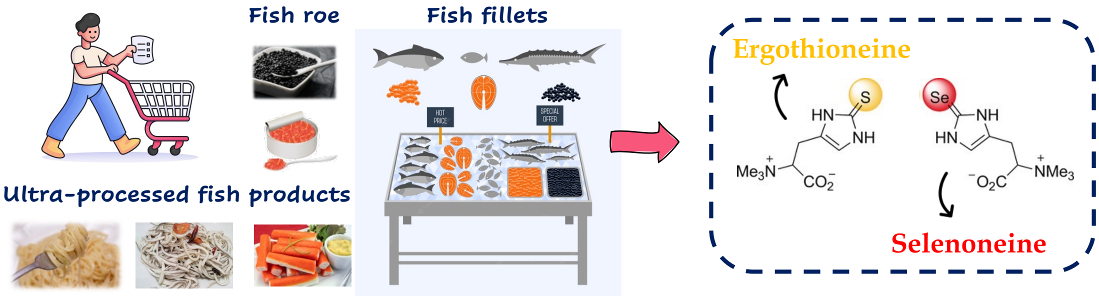

Los resultados de este trabajo se presentaron en las siguientes reuniones científicas:

- **European Winter Conference on Plasma Spectrochemistry**. *Identification of selenoneine and ergothioneine in highly consumed fish and derived products.* Ljubljana, Eslovenia, enero de 2023. Tipo de comunicación: póster.

Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products

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Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products

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ABSTRACT

Selenoneine and ergothioneine have been determined simultaneously for the first time in the muscle of highly consumed fish and in fish-derived products. The extraction methods were optimized, and their subsequent analysis was performed by HPLC-ESI-MS/MS. Best extraction efficiency was attained by performing a simple aqueous extraction containing ammonium acetate or ammonium formate as extractants. Selenoneine and ergothioneine were simultaneously detected in tuna and swordfish muscle tissues, while in fish roe and in farmed salmon only ergothioneine was detected. Moreover, the presence of their respective methylated forms was also considered. Additionally, Se-containing biomolecules profiles from water soluble proteins were obtained by SEC-ICP-MS analysis. Results revealed similar profiles for fish and fish roe whereas for ultra-processed fish products, Se seems to be associated to low molecular weight biomolecules. The results included in this work may contribute to a better understanding of the relations between selenoneine and ergothioneine in fishery products as well as their fate and potential modifications of Se-containing biomolecules profiles during fish processing.

Keywords: selenoneine, ergothioneine, selenium biomolecules, fish-derived products, HPLC-ESI-MS/MS, HPLC-ICP-MS.

1. Introduction

Selenium is an essential element as it takes part of selenoproteins as the selenoamino acid selenocysteine. Selenoproteins have a key role in human health as they are fundamental in numerous metabolic reactions in living organisms (1). In the last years, interest on selenium has also been focused on the presence of the strong antioxidant selenoneine (2-selenyl-N_α, N_α, N_α-tri-methyl-L-histidine, the Se analog of ergothioneine). Selenoneine can bind hemoglobin and myoglobin to protect them from ion auto-oxidation and it also reacts with radicals through free-radical detoxification mechanisms, decreasing the reactive oxygen radicals formation, thus inhibiting carcinogenesis, aging, cancer, cardiovascular disease, neurodegeneration and immunological dysfunction, among others (2). Moreover, its antagonist effect on methylmercury ($MeHg^+$) has been demonstrated since selenoneine reacts with $MeHg^+$ and regulates its detoxification and its metabolism, leading to a decrease in mercury toxicity (3).

Ergothioneine (2-thiol-L-histidine-betaine) is a natural occurring amino acid that cannot be synthetized by animals, but for bacteria and fungi. However, it can be assimilated through dietary intake by vertebrates and accumulated in certain organs, tissues and cells. In this respect, the ergothioneine ingested can be transferred to the whole body through the OCTN1, which is an ergothioneine specific transporter (4). OCTN1 also plays an important role in selenoneine incorporation and excretion and is involved in transporting selenoneine-$MeHg^+$ complex which allows the decrease of $MeHg^+$ toxicity (3). Similarly to selenoneine, ergothioneine also presents strong antioxidant capacity. However, it has been reported differences in the auto-oxidative behavior of these two compounds since although ergothioneine might present greater ability to prevent oxidative damage, selenoneine presents more radical scavenging activity (2,5). Moreover, the antioxidant potential of ergothioneine together with its anti-inflammatory properties make this compound promising for preventing some chronic diseases related to ageing (6).

In recent years, investigation of selenoneine in fish, mainly in predator fishes, has been successfully developed, although more in-depth studies are still needed to further evaluate its properties and functions. On the other hand, ergothioneine has been mainly studied in mushrooms since it is produced mainly by fungus, as well as in plant-based food and meat (6). However, limited knowledge exists regarding ergothioneine contents in seafood, being only reported to have been detected in trout (7). Furthermore, to date, few studies have addressed the simultaneous analysis of these two potent antioxidants in food, especially in fish products and there is not data available about their presence in fish-derived products.

Nowadays there has been an increase in the consumption of new products derived from fish, such as fish roe, or ultra-processed fish-based food, such as crab sticks, surimi-derived elvers, cod and salmon noodles. Those fish-derived products constitute innovative ways of eating fish. However, the incorporation of different ingredients and the processes applied for manufacturing the final products could alter the occurrence of Se-containing biomolecules, selenoneine or its sulfur analogue, ergothioneine (8), with respect to the raw materials.

Therefore, in the present study, the co-existence of selenoneine and ergothioneine in four fish species and seven fish-derived products have been investigated by using HPLC-ESI-MS/MS. For this purpose, different extraction methods were tested and HPLC and mass spectrometry conditions were optimized. Additionally, Se-containing biomolecules profiles from water soluble proteins obtained by SEC-ICP-MS analysis for all the fish and fish-derived products were considered.

2. Material and Methods

2.1 Samples

Four fish species (tuna, *Thunnus thynnus*, swordfish, *Xiphias gladius*, farmed salmon, *Salmo salar,* and wild salmon, *Oncorhynchus gorbuscha*, from northwest Pacific) along with three edible fish roe (lumpfish roe, from *Cyclopterus lumpus*, trout roe, from *Oncorhynchus mykiss,* and wild salmon roe, from *Oncorhynchus gorbuscha*) and products made from fish (crab sticks, surimi-derived elvers, cod noodles and salmon noodles) are the samples considered in this work. All of them were acquired in a local supermarket and stored correctly at -20 °C until analysis.

Regarding ultra-processed fish products, raw materials were different: crab sticks and surimi-derived elvers are made from walleye pollock (*Gadus chalcogrammus*) and from North Pacific hake (*Merluccius productus*), cod noodles are made from North Pacific hake (*Merluccius productus*) and shallow-water Cape hake (*Merluccius capensis/paradoxus*), and salmon noodles are made from farmed salmon (*Salmo salar*) and North Pacific hake (*Merluccius productus*). Their elaboration process is similar and consists of performing successive washing processes to get a concentrate of fish proteins with gelling properties, allowing the elaboration of these products. The main difference between crab sticks and surimi-derived elvers is that the former is dyed orange while the latter is shaped to get a characteristic shape imitating elvers. Cod and salmon noodles elaboration process has an additional step of mixing and kneading the protein concentrate with other ingredients such as water, salt, starch, scents and egg white. Finally, the mixture is submitted to a cooking process at 90 ºC for defatting and forming the spaghetti or noodle-like strips.

2.2 Determination of the total Se content in muscle tissue of fish and in fish-derived products by ICP-MS

Total Se determination in fresh fish fillets and fish-derived products was performed by using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies Inc., USA) after acid digestion in a microwave oven (1600W MSP, CEM, USA). About 1 g of each fresh fish fillet or fish-derived product sample (3 replicates) was digested with a mixture (5:1) of 65 % v/v nitric acid (Merck, Spain) and 30 % v/v hydrogen peroxide (Panreac, Spain). The mixture was submitted to a microwave heating program consisting of a 20-min ramp to 130 °C and a 10-min hold time. After digestion, the solutions were appropriately diluted with deionized water (18.2 MΩ cm, from a Milli-Q® water purification system; Millipore, USA) to a final volume of 10 mL, filtered through a 0.22 μm syringe Nylon filter (Scharlab, Spain) and analyzed.

Total Se content in the digests was measured by ICP-MS by monitoring ^{78}Se, ^{80}Se and ^{82}Se isotopes under continuous acquisition mode. The operational conditions for ICP-MS measurements are as follows: RF power of 1550W, sample depth of 8 mm, plasma gas flow rate of 15.0 L min^{-1}, auxiliary gas flow rate of 1.0 L min^{-1}, conikal nebulizer with a flow rate of 1.0 L min^{-1} and H_2 is used in the collision-reaction cell at a flow rate of 6.0 L min^{-1}. Se quantification was performed by external calibration with the isotope ^{80}Se. A Certified Reference Material (ERM-CE 278k, from mussel) from the Institute for Reference Materials was analyzed to validate the accuracy of the results obtained. No statistically significant differences were found for total Se between the certified value 1.62 ± 0.12 mg kg^{-1} and the experimental one 1.65 ± 0.13 mg kg^{-1}. The limit of quantification (LOQ) was calculated according to International Union of Pure and Applied Chemistry (IUPAC) (9). The LOQ was obtained as $10\sigma/S$, where σ is the standard deviation of 10 measurements of the blank solution and S is the slope of the line equation for external calibration curve. For total Se, the LOQ was 0.006 mg kg^{-1}.

2.3 Optimization of Se extraction from muscle tissue of fish and fish-derived products

In order to maximize the total amount of Se extracted from muscle tissue of fish and fish-derived products, several factors have been optimized such as optimal sample mass, the type of extractant and its concentration used for the extraction process (deionized water, ammonium acetate, ammonium formate, sodium phosphate, potassium citrate and tris-(hydroxymethyl)-aminomethane (Tris), at 100 or 200 mmol L^{-1}) and the type of methodology employed for performing the extraction (ultrasound probe or glass beads with the combination of vortex and ultrasonic bath). Tuna muscle tissue has been selected as a representative matrix for performing the optimization of the extraction procedure. The extraction efficiency was evaluated by comparing the results obtained with those provided by ICP-MS after performing a microwave assisted acid digestion with HNO_3/H_2O_2 5:1.

2.4 Screening of Se-containing biomolecules in soluble protein fractions by size-exclusion chromatography coupled to ICP-MS detector (SEC-ICP-MS)

The water-soluble protein fraction was extracted from muscle tissue of fish species and from fish-derived products using ammonium acetate as extractant after optimizing the extraction conditions described in section 2.3. In summary, 1 g of fresh fish fillet or fish-derived product was treated with 2.0 mL of 100 mmol L^{-1} ammonium acetate at pH 7.4, 0.9 g of glassbeads were added and samples were homogenized during 60 s with the combination of vortex (3000 rpm) and ultrasonic bath (720 W, 3.1 % amplitude). The obtained extracts were then centrifuged at 11000 rpm during 15 min at 4 ºC (Eppendorf 5804 F34-6-38 centrifuge, Germany).

Se-containing biomolecules in the soluble protein fractions from muscle tissue of fish species and from fish-derived products were detected by size-exclusion chromatography coupled to ICP-MS detector (SEC-ICP-MS). Two size-exclusion columns, Superdex 200 (10–600 kDa) and Superdex Peptide (0.1-7 kDa) (GE Healthcare Life Sciences, UK), were employed, having different molecular weight fractionation ranges. Operational conditions are the following for both columns: the injection volume onto the column was 100 µL and the mobile phase consisted of 100 mM ammonium acetate pH 7.4. The elution mode was isocratic with a flow rate of 0.7 mL min^{-1}. The acquisition mode was in time-resolved analysis with a run time of 50 min, monitoring ^{78}Se, ^{80}Se and ^{82}Se isotopes.

Mass calibrations curves for both SEC were performed by coupling to an UV-Vis spectrophotometer connected to a PU-2089 HPLC pump (JASCO, Japan). For this purpose, the UV-Vis detector was set to 280 nm and the following peptides and proteins standard solutions were employed: 5 mg mL^{-1} thyroglobulin (669 kDa), 0.3 mg mL^{-1} apoferritin (443 kDa), 8 mg mL^{-1} albumin (67 kDa), 2 mg mL^{-1} carbonic anhydrase (30 kDa), 1.5 mg mL^{-1} cytochrome C (12.4 kDa), 2 mg mL^{-1} aprotinin (6.5 kDa), 0.1 mg mL^{-1} vitamin B12 (1.36 kDa) and 1 mg mL^{-1} glycine (75 Da) (Sigma-Aldrich, Spain).

2.5 Extraction procedure and determination of selenoneine and ergothioneine by HPLC-ESI-MS/MS

Optimal extraction conditions for total Se (Section 2.3) were applied as extraction methods for selenoneine and ergothioneine analysis. In this sense, two different extracting agents were used: ammonium acetate and ammonium formate. When using ammonium acetate, the extraction procedure is as follows: 1 g of fresh fish fillet or fish-derived product was treated with 2.0 mL of 100 mmol L^{-1} ammonium acetate at pH 7.4, 0.9 g of glassbeads were added and samples were homogenized during 60 s with the combination of vortex (3000 rpm) and ultrasonic bath (720 W, 3.1 % amplitude). When using ammonium formate, the extraction procedure is similar except for the homogenization step that in this case is carried out with the ultrasound probe (20 % amplitude) for 120 s. Afterwards, samples were centrifuged at 11000 rpm during 20 min, supernatants were then diluted 1:2 with acetonitrile and the precipitated proteins were separated by centrifugation (11000 rpm during 10 minutes at 4 ºC). Supernatants were evaporated to dryness using a dry-heating block and N_2 flow and the resulting dry residues were re-dissolved in 200 μL of mobile phase before performing HPLC-ESI-MS/MS analysis.

The analyses were carried out by high-performance liquid chromatography using an Agilent 1200 HPLC equipped with a G1312A binary pump, a G1329A degasser, a G1329A automatic thermostatic injector and a G1316A thermostatic module column, coupled to a triple quadrupole mass spectrometer (Agilent G6410B Triple Quadrupole LC/MS System, Agilent Technologies Inc., USA).

The compounds were separated using a Phenomenex Kinetex EVO C18 column (150x3 mm, 5 μm), which was kept at 30 ºC. The mobile phase consisted of eluent A (MilliQ® water with 0.1 % formic acid) and eluent B (acetonitrile with 0.1 % formic acid). The elution mode was isocratic (95% A – 5% B) with a flow rate of 0.5 mL min^{-1} and the injection volume onto the column was 5 μL.

Full-scan spectra and MS/MS spectra were obtained from infusion of individual standard solutions of synthesized selenoneine (kindly provided by Prof. Florian Peter Seebeck, from University of Basel, Switzerland [10]) and L-(+)-ergothioneine (purity ≥ 98.0 %, purchased from Sigma-Aldrich, Madrid, Spain), using an electrospray ionization (ESI) source. The ion source settings were as follows: gas temperature, 200 °C; drying gas flow, 10 L min^{-1}; nebulizing gas pressure, 35 psi; and capillary voltage, 2000 V. $[M+H]^+$ was selected as the precursor ion and the detection was carried out in multiple reaction monitoring (MRM) mode by monitoring the two most sensitive transitions for each compound. For both compounds the most abundant fragment, corresponding to $[M+H-CO_2-N(CH_3)_3]^+$, was selected for quantification and $[M+H-CO_2]^+$ for confirmation. Selected transitions, fragmentors, collision energies (CE) and retention times for selenoneine and ergothioneine are detailed in Table S1 in the Supplementary Material.

Masshunter Data Acquisition B.04.01 and Masshunter Quantitative Analysis B.07.00 were employed for data acquisition and processing.

The HPLC-MS/MS method was evaluated in terms of linearity, LOD and LOQ. Linearity was assessed using a calibration curve for each compound (selenoneine and ergothioneine) with three replicates for each point, with standards in solvent at concentrations ranging from 2 to 500 μg L^{-1}. The LODs and LOQs were estimated considering the analyte concentration providing a signal to noise (S/N) ratio of 3 and of 10, respectively. Recovery and global matrix effects were determined with individual spiking experiments at concentrations of 30 μg L^{-1} for both compounds.

Methylated compounds of selenoneine (Se-methylselenoneine) and ergothioneine (S-methylergothioneine) were also analyzed. A bibliographic search (11) of the main fragments of both compounds allowed us to detect them in multiple reaction monitoring (MRM) mode. The two most selective transitions for Se-methylselenoneine with the precursor ion m/z 292.1 were monitored: MRM m/z 292.1-189.0 (loss of CO_2 and $N(CH_3)_3$) and MRM m/z 292.1-174.0 (loss of CO_2, $N(CH_3)_3$ and CH_3). For S-methylergothioneine, with the precursor ion m/z 244.1, MRM m/z 244.1-200.1 (loss of CO_2) and MRM m/z 244.1-141.0 (loss of $N(CH_3)_3$) were monitored. These compounds were also analyzed by hybrid quadrupole time-of-flight (QTOF) mass spectrometry (G6530A Accurate Mass Q-TOF, Agilent, USA) combined with HPLC using an Agilent 1200 HPLC equipped with a G1311A quaternary pump, a G1322A degasser, a G1367B automatic thermostatic injector and a G1316A thermostatic module column. For the chromatographic separation, the same column and mobile phase composition than in the analysis performed by QqQ were employed. Masshunter Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 SP2 were used.

3. Results and Discussion

3.1 Optimization of selenoneine extraction from fish samples based on Se total contents

In this work the following parameters affecting extraction efficiency have been considered: type of extracting agent and concentration, sample mass and extraction methodology. Extraction efficiency has been calculated based on the total Se content extracted.

Selenoneine has been previously reported to be extracted in aqueous solutions (12–14). Therefore, six different aqueous extractant solutions at two concentrations, 100 and 200 mmol L^{-1}, were initially studied: deionized water, ammonium acetate, ammonium formate, sodium phosphate, potassium citrate and tris-(hydroxymethyl)-aminomethane (Tris). Thus, 1.5 g of tuna muscle was treated with 2.0 mL of the corresponding extractant solution at pH 7.4. The extraction process was mechanically assisted with glass beads (0.9 g) and the homogenization was carried out with the combination of vortex (3000 rpm) and ultrasonic bath (720 W, 3.1 % amplitude) for 1 min each. Percentages of total Se found based on ICP-MS analysis in the soluble fraction were above 40% for all the extractants tested (data not shown), obtaining the highest efficiency values when 100 mmol L^{-1} ammonium acetate and ammonium formate were used, leading to 64 ± 2 % and 47 ± 2 %, respectively. The percentages of Se extracted were calculated comparing the total Se content in the extracts with total Se content determined in muscle tissue of fish and fish derived product before applying the extraction process by ICP-MS after acid digestion (Table S2).

To improve extraction efficiency, the amount of sample was also modified within the range 0.5-1.5 g. Percentages of Se extraction efficiency were higher when 1 g of tuna muscle samples was treated with 2.0 mL of 100 mmol L^{-1} ammonium acetate and ammonium formate, resulting in 82 ± 1 % and 60 ± 7 %, respectively. Finally, the extraction time and process were optimized to further improve the percentages of Se extracted and to achieve quantitative results with the selected extractants (100 mmol L^{-1} ammonium acetate and 100 mmol L^{-1} ammonium formate). In this sense, two extraction processes were studied: one combining glass beads as mechanical assistance with vortex (3000 rpm) and ultrasonic bath (720 W, 3.1 % amplitude) for homogenization and the other one based on the use of the ultrasound probe (20 % amplitude). In addition, the extraction times tested for both were 60 and 120 s. In case of 100 mmol L^{-1} ammonium acetate, the highest percentage of Se extracted was obtained when using glass beads combined with vortex and ultrasonic bath during 60 s, leading to an extraction efficiency of 106 ± 3 %. On the other hand, the best result for 100 mmol L^{-1} ammonium formate was obtained when the ultrasound probe was used during 120 s, achieving 104 ± 5 % of Se extracted. Therefore, the optimal conditions were:

- 1 g of fresh fish fillet or fish-derived product was treated with 2.0 mL of 100 mmol L^{-1} ammonium acetate at pH 7.4, 0.9 g of glassbeads were added and samples were homogenized during 60 s with the combination of vortex (3000 rpm) and ultrasonic bath (720 W, 3.1 % amplitude).
- 1 g of fresh fish fillet or fish-derived product was treated with 2.0 mL of 100 mmol L^{-1} ammonium formate at pH 7.4 and samples were homogenized during 120 s with the ultrasound probe (20 % amplitude).

3.2 Screening of Se-containing biomolecules in soluble protein fractions by SEC-ICP-MS

Once the extraction procedure was optimized, SEC-ICP-MS was firstly used as a screening tool to investigate the presence of Se-containing compound of low molecular weights in the aqueous extracts obtained. Selenoneine might be expected to appear in the low molecular weight range as reported by El Hanafi *et al.* (12). For this purpose, two different columns with different molecular weight fractionation ranges were considered: Superdex 200 (10–600 kDa) and Superdex Peptide 0.1-7 kDa), as it was described in Section 2.4. In Figure 1 and Figure 2 chromatographic profiles obtained by Superdex 200 and Superdex Peptide coupled to ICP-MS for all the fishes and fish-derived products are shown, respectively. The resulting chromatographic profiles obtained by Superdex 200 column (10–600 kDa) in Figure 1 show Se binding to different biomolecules of different molecular weight. In general, for all fish and fish-derived products it can be appreciated that Se in the soluble protein fraction is mainly bound to low molecular weight compounds (< 10 kDa) since the presence of two main major Se fraction could be observed between 27-40 min.

Furthermore, in case of fish species and fish roe samples Se containing biomolecules of higher molecular weight (between 10-60 kDa and up to 550 kDa) were also identified. Indeed, the main differences between samples were found in soluble protein fractions of fish roe (Figure 1I-K) in which Se seems to be bound to higher molecular weight biomolecules up to 550 kDa. In addition, Se is also bound in fish roe to a variety of different molecular weight compounds in both the middle (15-70 kDa) and lower part (< 10 kDa) of the molecular weight range covered by Superdex 200 column.

On the other hand, comparing fish (Figure 1A-D) and ultra-processed fish products (Figure 1E-H) chromatograms, similar pattern can be observed with two main peaks eluting at around 30 and 40 min, corresponding to an estimated molecular weight of 10 kDa and less than 10 kDa, respectively. Furthermore, another chromatographic peak eluting approximately at 18 min is observed in fish muscle samples (Figure 1A-D) corresponding to biomolecules up to 60-70 kDa. In addition, differences in chromatographic profiles between farmed and wild salmon can be appreciated in Figure 1C and D. In soluble proteins extracted from wild salmon, chromatographic peaks corresponding to Se bound compounds of molecular weights at around 15, 60 and 70 kDa gain importance with respect to farmed salmon, in which most of the Se is bound to lower molecular weight biomolecules (< 10 kDa). Finally, products made from fish (Figure 1E-H), such as crab sticks, surimi-derived elvers, cod and salmon noodles, show similar chromatographic profiles, being the intensity of the peak corresponding to Se bound biomolecules of around 10 kDa the main difference between them.

To further investigate the biomolecules containing Se in the extracts, a Superdex Peptide (0.1-7 kDa) column was subsequently employed since Se in the soluble protein fractions extracted was mostly found bound to low molecular weight compounds (< 10 kDa) for the vast majority of the fish and fish-derived products analyzed. Figure 2 shows the chromatograms obtained for all the samples analyzed in which 3 peaks at 10, 23 and 40 min with different intensities can be observed, except for cod and salmon noodles in which only one peak at 40 min appeared. Those peaks correspond to Se-binding biomolecules of 7, 0.5-1 and 0.1 kDa, respectively (Figure 2). Concerning fish samples, differences can be appreciated between farmed and wild salmon as described before for Superdex 200 results. In this case, the main difference lies in the medium molecular weight region covered by Superdex Peptide column, since two peaks were observed in case of wild salmon which correspond to biomolecules associated with Se of around 1 and 0.7 kDa (Figure 2D), whereas these peaks were not identified in farmed salmon chromatograms (Figure 2C). Likewise, similar profiles were obtained for the different varieties of fish roe (Figure 2I-K), where the only difference among then might reside in the presence of Se binding biomolecules below 10 kDa in case of lumpfish roe and probably also in trout roe. Finally, it is important to highlight that in ultra-processed derived products (Figure 2E-H) Se seems to be associated with biomolecules of less than 10 kDa, although in case of products based on surimi (surimi-derived elvers and crab sticks) biomolecules around 0.7 kDa were also observed (Figure 2E and F). One of the main drawbacks of SEC is its low resolution that makes difficult to reach a proper separation of selenocompounds in order to correctly identify the Se-species. However, the results obtained by SEC-ICP-MS revealed that most Se is present in the soluble fraction as compounds within the LMW range. Moreover, the information given by this technique might be of relevance when the quality and nutritional value of non-traditional food products are evaluated. The comparison of Se-binding biomolecules in fish fillets, fish roe and ultra-processed fish products might be a helpful tool to evaluate the influence of fish processing on selenocompounds. Nevertheless, distribution of Se-containing biomolecules has never been investigated in fish-derived products such as fish roe nor in ultra-processed fish products, which is quite interesting not only because of the high nutritional value that selenocompounds have in food, but also because of the lack of information about the chemical form of Se in these non-traditional products derived from fish.

3.2 Determination of selenoneine and ergothioneine by HPLC-ESI-MS/MS in fish and fish derived products

The presence of selenoneine and ergothioneine was evaluated by HPLC-MS/MS in the extracts from four fish species (tuna, swordfish, farmed salmon, and wild salmon), three edible fish roe (lumpfish roe, trout roe and wild salmon roe) and four ultra-processed products made from fish (crab sticks, surimi-derived elvers, cod noodles and salmon noodles). The extraction procedure applied for ergothioneine was the same one optimized for selenoneine since selenoneine is the selenium-analogue of ergothioneine and thus presents similar properties and chemical behaviour (13).

As it was expected, a similar chromatographic behavior was evidenced between selenoneine and ergothioneine standards, being their retention times very close from each other: 1.4 and 1.3 min, respectively. However, their simultaneous determination was achieved due to detection of different product ions from dissociation of the compounds, as it can be seen in the collision-induced dissociation mass spectra (ESI-MS/MS) in Figure 3. Thus, the MRM transitions selected for each analyte were selective enough.

Concerning the validation of the HPLC-MS/MS methodology, LOD and LOQ obtained for selenoneine were 0.0011 and 0.0035 $\mu g\ g^{-1}$, respectively. LOD and LOQ obtained for ergothioneine were 0.0009 and 0.0030 $\mu g\ g^{-1}$, respectively. Both compounds showed satisfactory linearity in the range from 2 to 500 $\mu g\ L^{-1}$, being the coefficients of determinations obtained, R^2, above 0.999 (Table 1).

Recovery studies were performed by spiking samples with known concentrations (30 $\mu g\ L^{-1}$) of selenoneine and ergothioneine standard solutions. Recoveries yielded satisfactory for both compounds in all samples, being between 76 and 93 %, with standard deviations between 4 and 12 % (data not shown). Therefore, no matrix effects were expected derived from a possible matrix-induced ion suppression.

Concerning the samples analyzed, the chromatograms obtained from the analysis of extracts from tuna, swordfish, farmed salmon, lumpfish roe, wild salmon roe and trout roe showed a peak at 1.3 min corresponding to ergothioneine. However, a peak appearing at 1.4 min corresponding to selenoneine was only detected in tuna and swordfish. Figure 4 shows an example of an HPLC-ESI-MS/MS chromatogram for the identification of each compound in two different samples with their selective MRM transitions. Confirmation of positive findings was based on the accomplishment of the ion ratio and retention time as regards the reference standard, that were within the tolerances established by current guidelines (15). Moreover, it is important to highlight that neither selenoneine nor ergothioneine were identified in the ultra-processed fish products analyzed (crab sticks, surimi-derived elvers, cod noodles and salmon noodles). It is possible that maybe selenoneine and ergothioneine were also not present in the fish employed as raw materials to produce these ultra-processed fish products.

On the other hand, results from quantification of ergothioneine and selenoneine in the extracts from fish and in fish-derived products with two different extraction methods are compiled in Table 2. According to that, the highest amount of ergothioneine was found in tuna, followed by wild salmon roe and trout roe. Concerning salmon, ergothioneine seems to be present only in the muscle of farmed salmon, since it has not been detected in wild salmon. The non-detectability of selenoneine in salmon was also reported by other authors (16). Ergothioneine is not present either in products made from fish, such as crabs sticks, surimi-derived elvers and cod and salmon noodles, suggesting than the elaboration process of these products induces losses of this compound.

Regarding selenoneine, results evidenced its presence in the muscle of tuna and swordfish. Nevertheless, this selenocompound was not detected either in salmon (farmed and wild), fish roe or fish-derived products. To our knowledge, it is the first time that the presence of selenoneine is investigated in fish roe. Although elevated total Se contents in fish roe (4.4 ± 0.4 mg kg^{-1} in wild salmon roe) have been reported in this investigation (Table S2), selenoneine was not detected. Therefore, although it is reported that fish roe is a good source of selenium, it might appear to be accumulated in form of selenoamino acids rather than as selenoneine.

In view of results compiled in Table 2, the co-existence of selenoneine and ergothioneine has been detected only in tuna and swordfish, which are predatory fishes. However, further studies are needed in order to elucidate the differences or similarities between these two compounds regarding their uptake from food. Ergothioneine and selenoneine are probably assimilated by fish through diet since they are only synthetized by bacteria and fungi, not by plants or animals (3, 17). Moreover, selenoneine can be biologically synthetized from ergothioneine in erythrocytes and other animal cells (2), but also from inorganic and organic forms of selenium (18).

Once ingested, methylated compounds (Se-methylselenoneine and S-methylergothioneine) are thought to be formed by methyltransferase enzymes present in liver or kidney of organisms (19). S-methylergothioneine has similar redox properties to those of its non-methylated compound, whereas Se-methylselenoneine, contrary to selenoneine, is not inclined to cause S-adenosyl methionine (SAM) degradation (11). However, its presence in fish has only been reported in tuna muscle (13) and not in other fish species or in ultra-processed fish-derived products. Therefore, in the current study the presence of these methylated compound has been studied in the fish and fish-derived products by HPLC-ESI-MS/MS (QqQ) applying the conditions described in Section 2.5. In this respect, the two MRM transitions monitored for S-methylergothioneine were observed in all samples containing ergothioneine: tuna, swordfish, farmed salmon, lumpfish roe, wild salmon roe and trout roe; while Se-methylselenoneine was only identified in tuna. As the reference standards of both compounds were not available, in order to confirm the presence of these compounds, the extracts were also analyzed by LC/MS TOF. However, when performing the extraction ion chromatograms (XICs) at the masses of S-methylergothioneine and Se-methylselenoneine, any peak was observed (Figure S1). This might be due to the lower sensitivity in full-scan mode of the QTOF in comparison with the excellent sensitivity and selectivity achieved in MRM using a triple quadrupole analyzer. Therefore, further studies are needed in order to confirm the occurrence of these selenium and sulfur metabolites in food. However, the presence of Se-methylselenoneine has been detected mainly in urine as a detoxification mechanism of selenoneine, which is methylated in liver and kidney and then excreted through the urine via a unique metabolic pathway (20).

Finally, regarding the extraction process, in all cases, contents of selenoneine and ergothioneine are higher when using the extraction process with ammonium acetate, although there are not statistically significant differences (ANOVA analysis, $p\text{-value} < 0.05$) between the contents of these compounds when using both extraction methods (100 mmol L^{-1} ammonium acetate at pH 7.4 and glassbeads or 100 mmol L^{-1} ammonium formate and ultrasonic probe), respectively. It is important to mention that there are very few methods reported in the literature that provide an extraction method that allows the isolation of selenoneine and ergothioneine simultaneously. In case of selenoneine, the extraction has been usually performed in aqueous solutions by using ultrasonic energy, in some cases, for different edible fish species (16), seabird muscle and organs (12), beluga skin (21) or the liver of sea turtles (14). Likewise, Ey *et al.* (8) described the extraction of ergothioneine from food by employing perchloric acid and ultrasonic bath during 10 min. More recently, ergothioneine extraction in muscle, intestine and liver tissues of salmonoids has been carried out by using MeOH and zirconium oxide beads during 40 min (22). On the other hand, selenoneine and ergothioneine have been simultaneously analyzed in yeast extracts. In this case two purification steps employing different chromatographic columns before analysis (23) were required. Moreover, Kroepfl *et al.* (6) determined simultaneously selenoneine and ergothioneine from human blood cells in which the extraction process was based on the procedure applied for cell lysis. Therefore, it seems that the extraction methods suggested in this work based on the use of 100 mmol L^{-1} ammonium acetate at pH 7.4 and glassbeads or 100 mmol L^{-1} ammonium formate at pH 7.4 and ultrasonic probe constitute good alternatives for the simultaneous determination of selenoneine and ergothioneine in seafood samples and open new perspectives to be used in other types of matrices.

4. Abbreviations

CE: collision energy

ESI: electrospray ionization

ESI-MS/MS: collision-induced dissociation mass spectra

HPLC: high performance liquid chromatography

HPLC-ESI-MS/MS: high performance liquid chromatography-electrospray tandem mass spectrometry

HPLC-MS/MS: high performance liquid chromatography tandem mass spectrometry

ICP-MS: inductively coupled plasma mass spectrometry

IUPAC: International Union of Pure and Applied Chemistry

LC/MS TOF: liquid chromatography–mass spectrometry with time-of-flight

LOQ: limit of quantification

OCTN1: organic cation/carnitine transporter 1

$MeHg^+$: methylmercury

MRM: multiple reaction monitoring

MS/MS: tandem mass spectrometry

SAM: S-adenosyl methionine

SEC-ICP-MS: size-exclusion chromatography coupled to inductively coupled plasma mass spectrometry

Tris: tris-(hydroxymethyl)-aminomethane

UV-Vis: ultraviolet-visible

XICs: extraction ion chromatograms

Credit authorship contribution statement

T. Fernández-Bautista: conceptualization, formal analysis, investigation, methodology, roles/writing-original draft, writing-review & editing; **B. Gómez-Gómez**: conceptualization, investigation, supervision, roles/writing-original draft, writing-review & editing; **E. Gracia-Lor**: conceptualization, investigation, supervision, writing-review & editing; **T. Pérez-Corona**:

conceptualization, investigation, supervision, writing-review & editing; **Y. Madrid**: conceptualization, investigation, supervision, writing-review & editing, funding acquisition.

Author contributions

All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

(1) Gochfeld, M.; Burger, J. Mercury interactions with selenium and sulfur and the relevance of the Se:Hg molar ratio to fish consumption advice. *Environ. Sci. Pollut. Res.* 2021, *28*, 18407–18420. https://doi.org/10.1007/s11356-021-12361-7.

(2) Yamashita, Y. Discovery of the strong antioxidant selenoneine in tuna and selenium redox metabolism. *World J. Biol. Chem.* 2010, *1*, 144. https://doi.org/10.4331/wjbc.v1.i5.144.

(3) Yamashita, M.; Yamashita, Y.; Suzuki, T.; Kani, Y.; Mizusawa, N.; Imamura, S.; Takemoto, K.; Hara, T.; Hossain, M.A.; Yabu, T.; Touhata, K. Selenoneine, a Novel Selenium-Containing Compound, Mediates Detoxification Mechanisms against Methylmercury Accumulation and Toxicity in Zebrafish Embryo. *Mar. Biotechnol.* 2013, *15*, 559–570. https://doi.org/10.1007/s10126-013-9508-1.

(4) Xiong, K.; Xue, S.; Guo, H.; Dai, Y.; Ji, C.; Dong, L.; Zhang, S. Ergothioneine: new functional factor in fermented foods. *Crit. Rev. Food Sci. Nutr.* 2023, *0*, 1–12. https://doi.org/10.1080/10408398.2023.2185766.

(5) Kroepfl, N.; Francesconi, K.A.; Schwerdtle, T.; Kuehnelt, D. Selenoneine and

ergothioneine in human blood cells determined simultaneously by HPLC/ICP-QQQ-MS. *J. Anal. At. Spectrom.* 2019, *34*, 127–134. https://doi.org/10.1039/c8ja00276b.

(6) Beelman, R.B.; Phillips, A.T.; Richie, J.P.; Ba, D.M.; Duiker, S.W.; Kalaras, M.D. Health consequences of improving the content of ergothioneine in the food supply. *FEBS Lett.* 2022, *596*, 1231–1240. https://doi.org/10.1002/1873-3468.14268.

(7) Ey, J.; Schömig, E.; Taubert, D. Dietary sources and antioxidant effects of ergothioneine. *J. Agric. Food Chem.* 2007, *55*, 6466–6474. https://doi.org/10.1021/jf071328f.

(8) Raymond, L.J.; Ralston, N.V.C. Mercury: selenium interactions and health implications. *Neurotoxicology*. 2020, *81*, 294–299. https://doi.org/10.1016/j.neuro.2020.09.020.

(9) IUPAC, International Union of Pure and Applied Chemistry. Nomenclature, symbols, units and their usage in spectrochemical analysis-III. Analytical flame spectroscopy and associated non-flame procedures. *Spectrochim. Acta Part B*. 1978, *33*, 247–269.

(10) Lim, D., Gründemann, D., Seebeck, F. P. Total Synthesis and Functional Characterization of Selenoneine. *Angewandte Chemie - International Edition*. 2019, *58*, 15026–15030. https://doi.org/10.1002/anie.201908967

(11) Klein, M.; Ouerdane, L.; Bueno, M.; Pannier, F. Identification in human urine and blood of a novel selenium metabolite, Se-methylselenoneine, a potential biomarker of metabolization in mammals of the naturally occurring selenoneine, by HPLC coupled to electrospray hybrid linear ion trap-orbital ion trap. *Metallomics*. 2011, *3*, 513–520. https://doi.org/10.1039/c0mt00060d.

(12) El Hanafi, K.; Z. Pedrero, Z.; L. Ouerdane, L.; C. Marchán Moreno, C.; S. Queipo-Abad, S.; M. Bueno, M.; F. Pannier, F.; W.T. Corns, W.T.; Y. Cherel, Y.; P. Bustamante, P.; D. Amouroux, D. First Time Identification of Selenoneine in Seabirds and Its Potential Role in Mercury Detoxification. *Environ. Sci. Technol.* 2022. https://doi.org/10.1021/acs.est.1c04966.

(13) N. Kroepfl, N.; K.B. Jensen, K.B.; K.A. Francesconi, K.A.; Kuehnelt, D. Human excretory products of selenium are natural constituents of marine fish muscle. *Anal. Bioanal. Chem.* 2015, *407,* 7713–7719. https://doi.org/10.1007/s00216-015-8936-3.

(14) Y. Anan, Y.; K. Ishiwata, K.; N. Suzuki, N.; S. Tanabe, S.; Ogra, Y. Speciation and identification of low molecular weight selenium compounds in the liver of sea turtles. *J. Anal. At. Spectrom.* 2011, *26*, 80–85. https://doi.org/10.1039/c0ja00031k.

(15) T. Pihlstrom, T.; A.R. Fernández-Alba, A.R.; M. Gamón, M.; Amate, C.F.; M.E. Poulsen, M.E.; R. Lippold, R.; M. Anastassiades, M. Analytical quality control and method validation procedures for pestice residues analysis in food and feed. *Sante/11813/2017*. 2018, 42. https://www.eurl-pesticides.eu/userfiles/file/EurlALL/SANTE_11813_2017-fin.pdf.

(16) Y. Yamashita, Y.; H. Amlund, H.; T. Suzuki, T.; T. Hara, T.; M.A. Hossain, M.A.; T.

Yabu, T.; K. Touhata, K.; Yamashita, M. Selenoneine, total selenium, and total mercury content in the muscle of fishes. *Fish. Sci.* 2011, *77,* 679–686. https://doi.org/10.1007/s12562-011-0360-9.

(17) Nguyen, T.H.; A. Giri, A.; T. Ohshima, T. A rapid HPLC post-column reaction analysis for the quantification of ergothioneine in edible mushrooms and in animals fed a diet supplemented with extracts from the processing waste of cultivated mushrooms. *Food Chem.* 2012, *133*, 585–591. https://doi.org/10.1016/j.foodchem.2012.01.061.

(18) M. Yamashita, M.; Y. Yamashita, Y.; T. Ando, T.; J. Wakamiya, J.; Akiba, S. Identification and determination of selenoneine, 2-Selenyl-N α, N α, N α -Trimethyl-l-histidine, as the major organic selenium in blood cells in a fish-eating population on remote Japanese Islands. *Biol. Trace Elem. Res.* 2013, *156,* 36–44. https://doi.org/10.1007/s12011-013-9846-x.

(19) Ohta, Y.; K.T. Suzuki, K.T. Methylation and demethylation of intermediates selenide and methylselenol in the metabolism of selenium. *Toxicol. Appl. Pharmacol.* 2008, *226,* 169–177. https://doi.org/10.1016/j.taap.2007.09.011.

(20) Seko, T.; Uchida, H.; Sato, Y.; Imamura, S.; Ishihara, K.; Yamashita, Y.; Yamashita, M. Selenoneine Is Methylated in the Bodies of Mice and Then Excreted in Urine as Se-Methylselenoneine. *Biol. Trace Elem. Res.* 2023, No. 0123456789. https://doi.org/10.1007/s12011-023-03936-1.

(21) A. Achouba, A.; P. Dumas, P.; N. Ouellet, N.; M. Little, M.; M. Lemire, M.; Ayotte, P. Selenoneine is a major selenium species in beluga skin and red blood cells of Inuit from Nunavik. *Chemosphere.* 2019, *229,* 549–558. https://doi.org/10.1016/j.chemosphere.2019.04.191.

(22) Kitsanayanyong, L., Ishikawa, Y., Ohshima, T. Putative ergothioneine transporter of salmonids as a functional tool for ergothioneine supplementation. *Aquaculture.* 2022, *547,* 737496. https://doi.org/10.1016/j.aquaculture.2021.737496

(23) T. Seko, T.; H. Uchida, H.; Y. Yamashita, Y.; Yamashita, M. Novel method for separating selenoneine reduced monomer and ergothioneine from fission yeast extracts. *Sep. Purif. Technol.* 2021, *254,* 117607. https://doi.org/10.1016/j.seppur.2020.117607.

Tables

Table 1. Calibration parameters, LOD and LOQ for selenoneine and ergothioneine.

Compound	Molecular ion (m/z) [M+H]+	Transition ion I (m/z)	Linear range (µg L^{-1})	LOD (µg g^{-1})	LOQ (µg g^{-1})	Calibration equation $y = ax + b$		
						a	b	R^2
Selenoneine	278	175	1-500	0.0011	0.0035	925.5	5119.0	0.9991
Ergothioneine	230	127	1-500	0.0009	0.0030	1321.9	7789.8	0.9996

Table 2. Ergothioneine and selenoneine contents in the soluble fraction extracted from fish and fish-derived products using two different extraction procedures.

Fish/ fish-derived product	**[Ergothioneine] (µg g^{-1})**	
	Extractant solution (extraction procedure)	
	Ammonium acetate (1)	**Ammonium formate (2)**
Tuna	3.5 ± 0.2	3.4 ± 0.3
Swordfish	1.1 ± 0.4	0.80 ± 0.07
Farmed salmon	0.03 ± 0.01	< LOQ
Lumpfish roe	0.082 ± 0.004	0.03 ± 0.02
Wild salmon roe	3.3 ± 0.3	3.16 ± 0.08
Trout roe	2.7 ± 0.5	1.63 ± 0.06

Fish/ fish-derived product	**[Selenoneine] (µg g^{-1})**	
	Extractant solution (extraction procedure)	
	Ammonium acetate (1)	**Ammonium formate (2)**
Tuna	0.306 ± 0.006	0.290 ± 0.09
Swordfish	0.14 ± 0.02	0.114 ± 0.004

Results are expressed as mean ± standard deviation (SD); n= 3 for each fish/fish-derived product.

Figure captions

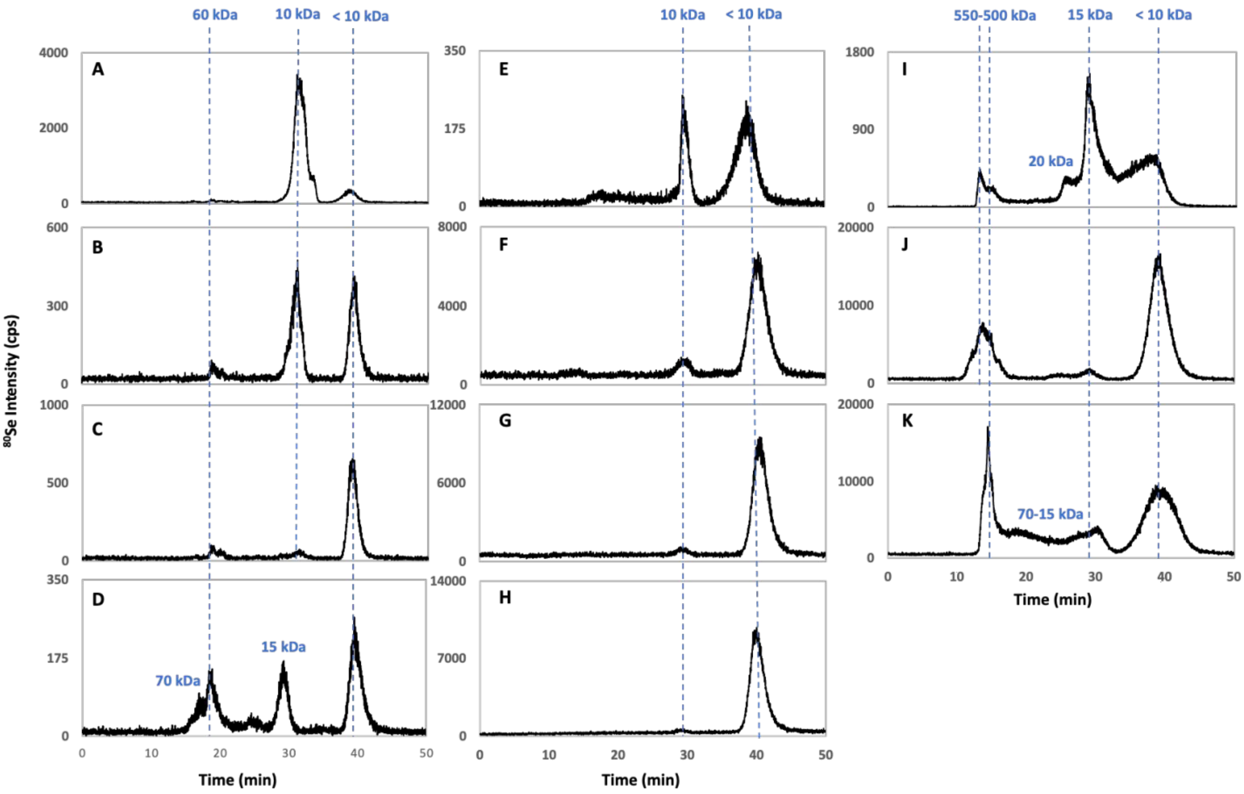

Figure 1. Chromatographic profiles obtained by SEC-ICP-MS using Superdex 200 column for all fishes and fish-derived products: (A) tuna, (B) swordfish, (C) farmed salmon, (D) wild salmon, (E) surimi-derived elvers, (F) crab sticks, (G) cod noodles, (H) salmon noodles, (I) wild salmon roe, (J) lump roe and (K) trout roe.

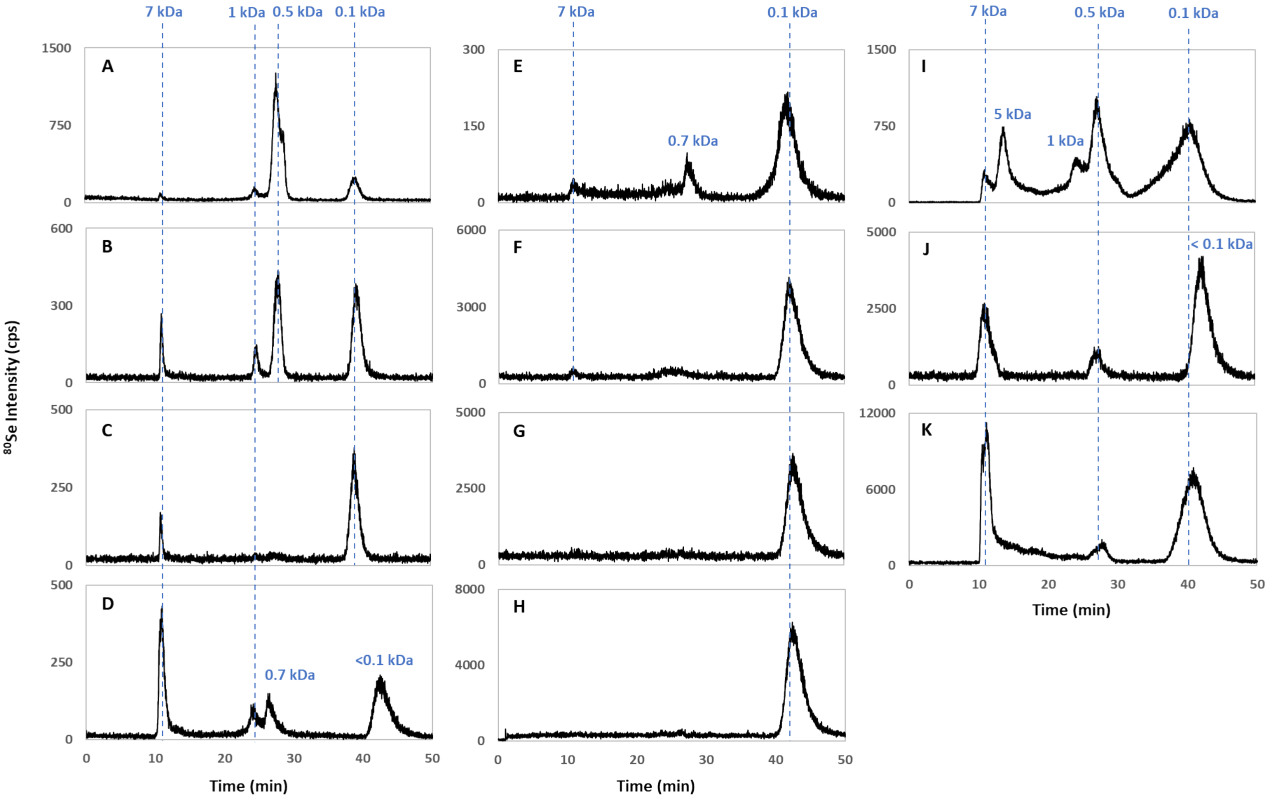

Figure 2. Chromatographic profiles obtained by SEC-ICP-MS using Superdex Peptide column for all fishes and fish-derived products: (A) tuna, (B) swordfish, (C) farmed salmon, (D) wild salmon, (E) surimi-derived elvers, (F) crab sticks, (G) cod noodles, (H) salmon noodles, (I) wild salmon roe, (J) lump roe and (K) trout roe.

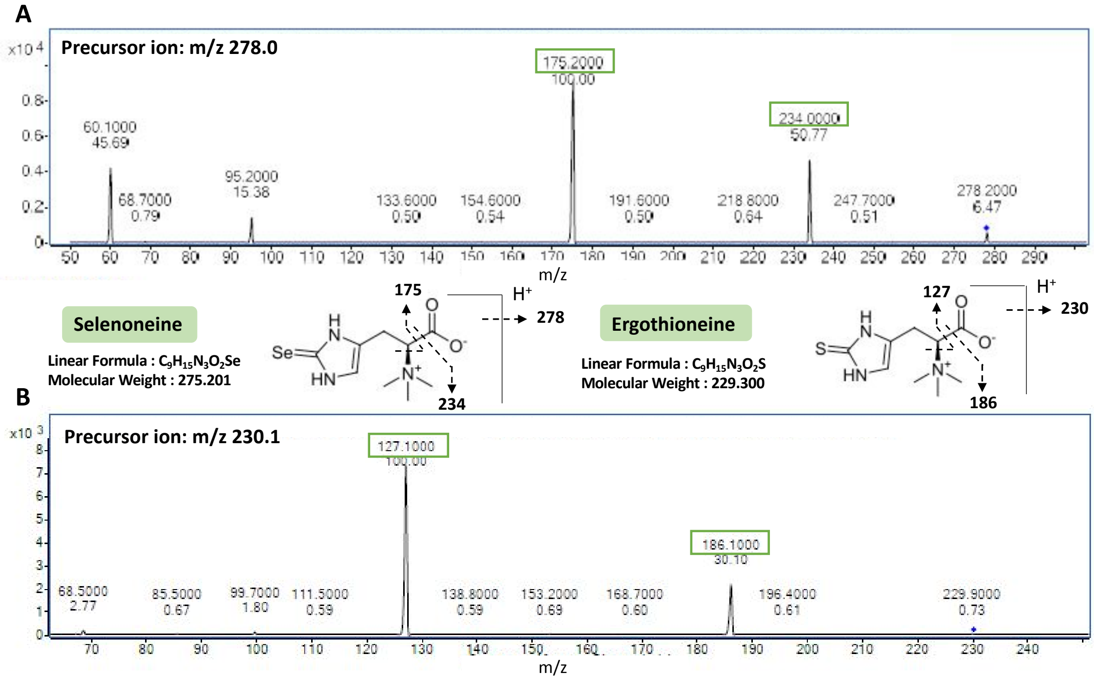

Figure 3. Collision-induced dissociation mass spectra (ESI-MS/MS) of (A) selenoneine and (B) ergothioneine standards. The most abundant product ions (quantifier and qualifier used for quantification and confirmation, respectively) are framed in green.

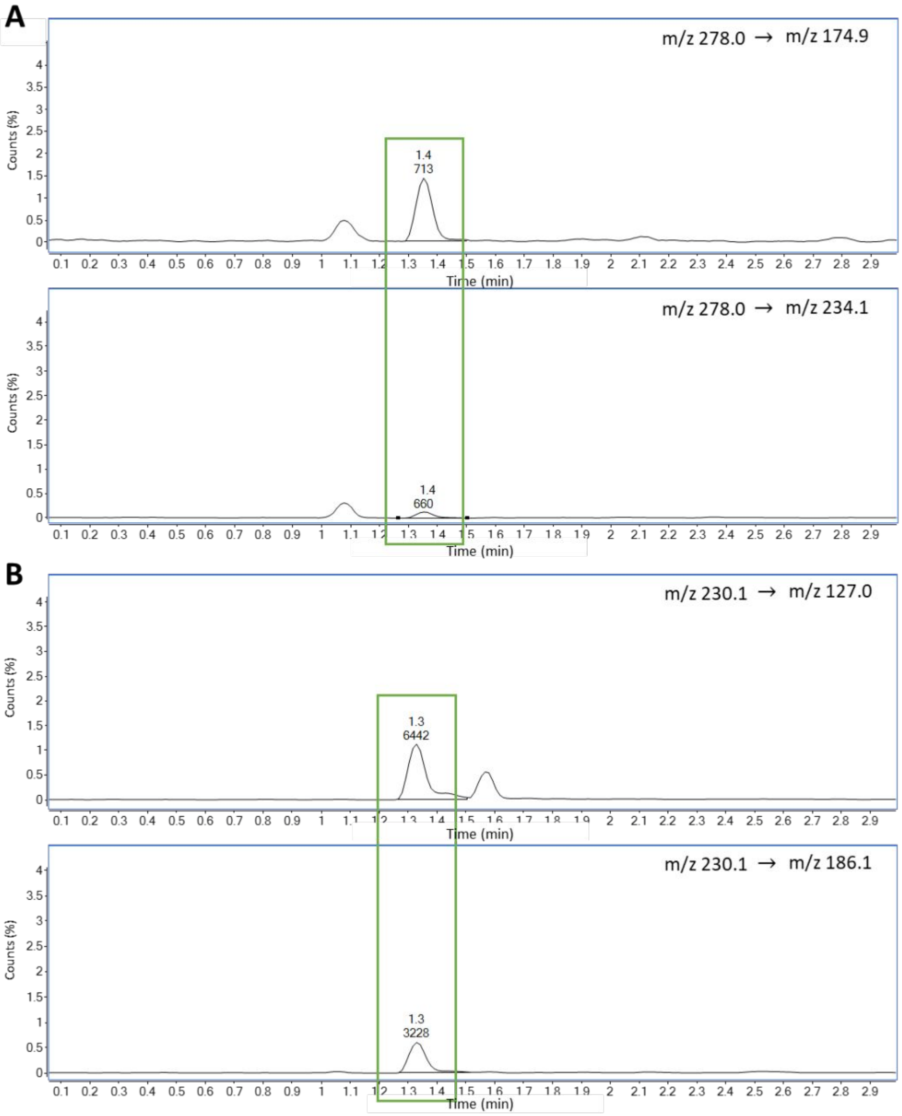

Figure 4. HPLC-ESI-MS/MS chromatograms of the identification of (A) selenoneine in tuna muscle (B) ergothioneine in swordfish muscle, using in both cases ammonium acetate as extractant.

Electronic Supplementary Information

Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products

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Table S1. Experimental MS/MS conditions for selenoneine and ergothioneine.

Compound	RT (min)	$[M+H]^+$ m/z	Product Ion I			Product Ion II		
			m/z	Frag (V)	CE (V)	m/z	Frag (V)	CE (V)
Selenoneine	1.4	278.0	174.9	100	17	234.1	100	9
Ergothioneine	1.3	230.1	127.0	110	14	186.1	110	6

RT: retention time; I: quantification transition; II: confirmation transition; Frag: fragmentor; CE: collision energy.

Table S2. Total Se content found in muscle tissues of fish and in fish-derived products by ICP-MS.

Fish/Fish-derived product	[Se] (mg kg^{-1})
Tuna	1.6 ± 0.1
Swordfish	1.4 ± 0.2
Farmed salmon	1.03 ± 0.07
Wild salmon	0.74 ± 0.08
Surimi-derived elvers	0.75 ± 0.05
Crab sticks	0.78 ± 0.08
Lumpfish roe	1.3 ± 0.2
Wild salmon roe	4.4 ± 0.4
Trout roe	1.9 ± 0.3
Cod noodles	0.74 ± 0.03
Salmon noodles	0.79 ± 0.02

Results are referred to wet weight and they are expressed as mean ± standard deviation (SD); n= 3 for each fish/fish-derived product.

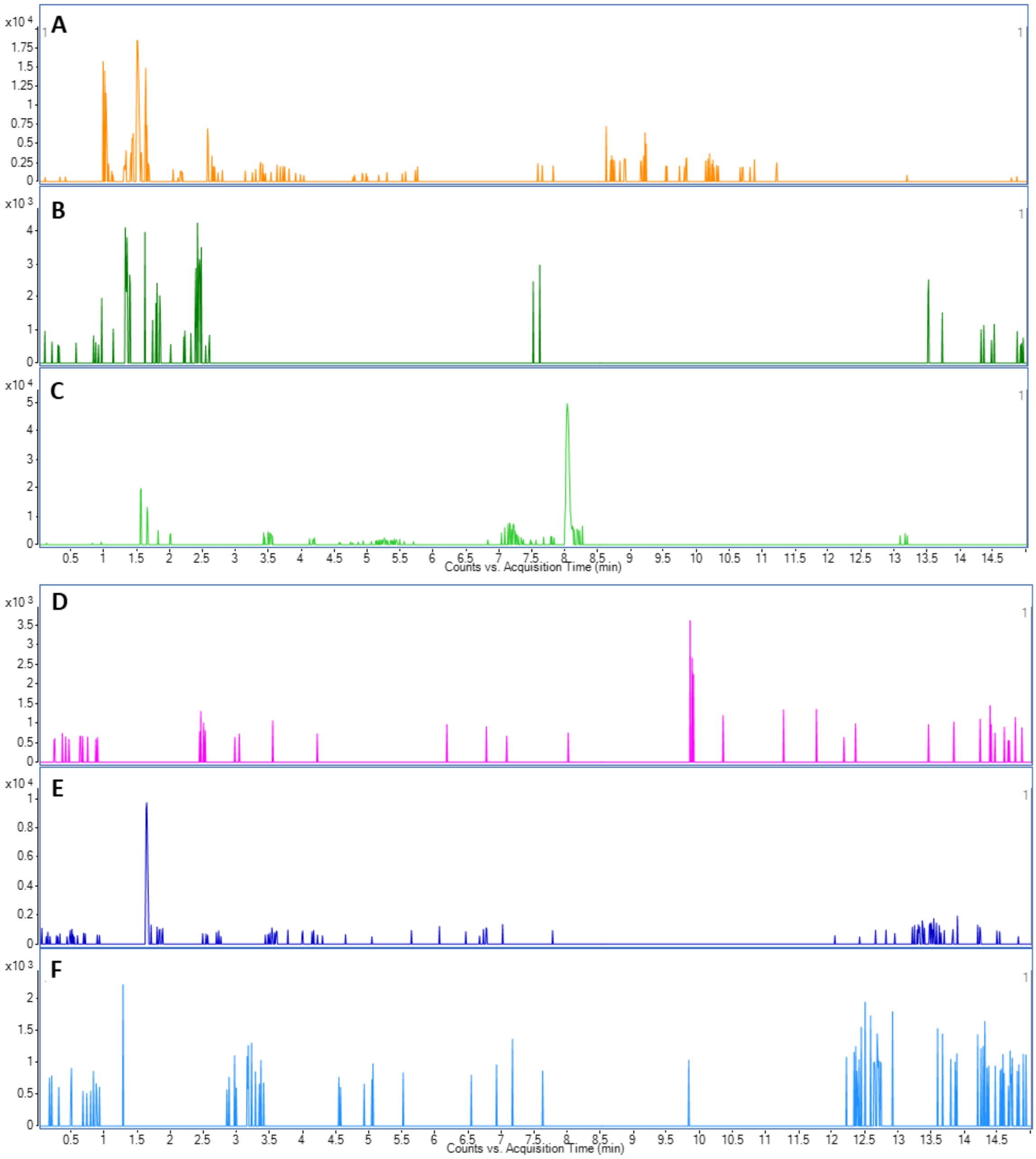

Figure S1. Extraction ion chromatograms (XICs) obtained by LC/MS TOF at the masses of S-methylergothioneine (A, B and C) and Se-methylselenoneine (D, E and F) for tuna (A, D), swordfish (B, E) and farmed salmon (C, F).

2. Aplicación de técnicas (bio)analíticas para la evaluación del efecto protector del selenio frente a la neurotoxicidad inducida por el mercurio

El mercurio, al igual que otros metales pesados con presencia medioambiental como el arsénico o el cadmio, presentan una elevada neurotoxicidad. Por otro lado, se ha descrito en la literatura científica que el selenio desarrolla un papel fundamental en la regulación del correcto funcionamiento cerebral. Asimismo, como ya se ha comentado en la introducción, este elemento ejerce un efecto protector frente a la toxicidad de mercurio. Es por ello por lo que cada vez hay un mayor número de estudios en los que el selenio se propone como agente neuroprotector frente a contaminantes tóxicos ambientales, como pueden ser los citados anteriormente. Sin embargo, este tipo de estudios requiere investigar las posibles interacciones entre el elemento protector y los elementos neurotóxicos, haciéndose imprescindible el empleo de una gran variedad de técnicas. Entre estas, el ICP-MS constituye una herramienta indispensable, y su uso en modo de células individuales o *single-cell* ICP-MS (SC-ICP-MS) puede ser de especial relevancia en los estudios de neurotoxicidad, ya que permite evaluar dichas interacciones a nivel de célula individual y, por tanto, tener en cuenta la variabilidad dentro de las poblaciones celulares. Dicha información se pierde cuando se considera que todas las células de un mismo cultivo se comportan de la misma forma frente a un agente externo.

Asimismo, en los estudios de neurotoxicidad es imprescindible el uso de líneas celulares de fenotipo neuronal como modelos *in vitro,* que suponen una primera aproximación para poder elucidar si un compuesto tiene efecto neuroprotector o neurotóxico. Los modelos *in vitro* que se pueden emplear para este tipo de estudios son cultivos neuronales primarios o líneas celulares inmortalizadas. Entre estas últimas, la línea celular de neuroblastoma humano catecolaminérgico (SH-SY5Y) es una de las más comúnmente empleadas debido a que son poblaciones celulares de origen neuronal, relativamente sencillas de mantener en cultivo, pueden ser replicadas fácilmente, ofrecen una amplia versatilidad en cuanto a exposición a neurotoxinas o agentes químicos y, además, suelen ofrecer resultados reproducibles.

A este respecto, en el quinto trabajo (**Artículo 5**) titulado **"Single-cell ICP-MS for evaluating the Se-protective effect against MeHg$^+$-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y)"** (***Analytical and Bioanalytical Chemistry (2024)***

***416:2749–2759*)** se describe por primera vez el efecto protector de distintas especies de selenio frente a la citotoxicidad inducida por el $MeHg^+$ en la línea celular de neuroblastoma humano (SH-SY5Y) mediante la evaluación de la acumulación de mercurio a nivel de células individuales en presencia de distintas especies de selenio utilizando la técnica SC-ICP-MS.

En primer lugar, se determinó la concentración letal del $MeHg^+$ para el 50 % de la población (LC_{50}) mediante el ensayo MTT, que resultó ser de 3 µM. Seguidamente, mediante el mismo ensayo se evaluó la influencia de distintas especies orgánicas de selenio (SeMet y SeMeSeCys) y de nanopartículas de selenio (SeNPs) (comerciales estabilizadas con citrato y sintetizadas recubiertas con quitosano) en la citotoxicidad inducida por el $MeHg^+$ y a diferentes concentraciones. La SeMet y la SeMeSeCys exhibieron un efecto protector frente a la muerte celular inducida por el $MeHg^+$, especialmente a altas concentraciones del tóxico (LC_{50}). Además, las SeNPs recubiertas con quitosano mostraron una mayor protección comparadas con las citrato-SeNPs cuando se expusieron junto con el $MeHg^+$ en los cultivos celulares.

Asimismo, es importante evaluar la acumulación de mercurio en los cultivos celulares tratados con las especies de selenio seleccionadas en el estudio anterior. Para ello, las células se expusieron a diferentes situaciones: con $MeHg^+$, con $MeHg^+$ y SeMet, y con $MeHg^+$ y SeMeSeCys, para determinar el porcentaje de acumulación medio de mercurio por parte de las mismas mediante ICP-MS. Seguidamente, para cuantificar el contenido de mercurio acumulado por cada célula individual, y constatar así diferencias dentro de una misma población celular debido a la heterogeneidad que presentan, se desarrolló un método de análisis por SC-ICP-MS. Para ello se optimizaron previamente las condiciones de fijación de las células con paraformaldehído (PFA). En este sentido, la mayor eficiencia de nebulización se obtuvo cuando se empleó PFA al 4 %, resultando ser del (93 ± 6) %. Los resultados obtenidos evidenciaron una distribución heterogénea en la acumulación de mercurio por parte de la línea celular SH-SY5Y. Por otro lado, la coexposición de las especies de selenio junto con el $MeHg^+$ tuvo como consecuencia una reducción en la cantidad máxima de mercurio acumulada por célula individual, llegándose a disminuir a la mitad (de 60 fg Hg $célula^{-1}$ a 30 fg Hg $célula^{-1}$) cuando SeMeSeCys estaba presente, así como una disminución en el porcentaje de células que acumulan las cantidades más elevadas de $MeHg^+$. Sin embargo, las cantidades medias de acumulación de mercurio fueron similares en los tres casos de exposición evaluados, justificando, por tanto, y debido a la heterogeneidad celular, la necesidad del empleo de la técnica SC-ICP-MS en estudios de neurotoxicidad.

Además de frente al mercurio, el selenio ejerce un efecto protector frente a otros contaminantes ambientales como son el arsénico o el cadmio, como se ha comentado anteriormente.

Debido al aumento de la esperanza de vida humana, la incidencia de las enfermedades neurodegenerativas ha aumentado con los años. Además, el creciente impacto de la contaminación ambiental en diferentes zonas geográficas también podría repercutir negativamente en el desarrollo de estas afecciones. El uso de técnicas (bio)analíticas empleadas junto con ensayos *in vitro* permite una correcta y necesaria evaluación de aspectos clave para los estudios neurotoxicológicos relacionados con la concentración de exposición a los tóxicos, la absorción, la acumulación en tejidos, la biotransformación, la biodisponibilidad o la excreción de estos. Todos estos aspectos son fundamentales para contribuir al diagnóstico y al desarrollo de los tratamientos adecuados para estas enfermedades neurológicas que, según las previsiones, afectarán a 152 millones de personas en 2050.

En este sentido, el desarrollo de técnicas (bio)analíticas para la evaluación del papel de los metales/metaloides directamente implicados en las enfermedades neurodegenerativas se ha incrementado en los últimos años. Es por ello que, en el sexto y último trabajo presentado en esta Tesis doctoral (**Artículo 6**), titulado **“(Bio)analytical approaches for investigating the role of selenium in preventing neurological disorders and neurotoxicity induced by environmental neurotoxicants: As, Cd and Hg” (*Trends in Analytical Chemistry 174 (2024) 117661*)**, basándonos en la bibliografía existente, se pretende presentar las perspectivas actuales y futuras de los distintos enfoques (bio)analíticos para la evaluación del papel que desempeña el selenio, incluidas las SeNPs, en la prevención de enfermedades neurodegenerativas y los efectos neurotóxicos inducidos por el arsénico, el cadmio y el mercurio.

ARTÍCULO 5:

ICP-MS en modo de detección de células individuales para la evaluación del efecto protector del Se frente a la neurotoxicidad inducida por el MeHg$^+$ en la línea celular de neuroblastoma humano (SH-SY5Y)

Single-cell ICP-MS for evaluating the Se-protective effect against MeHg$^+$-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y)

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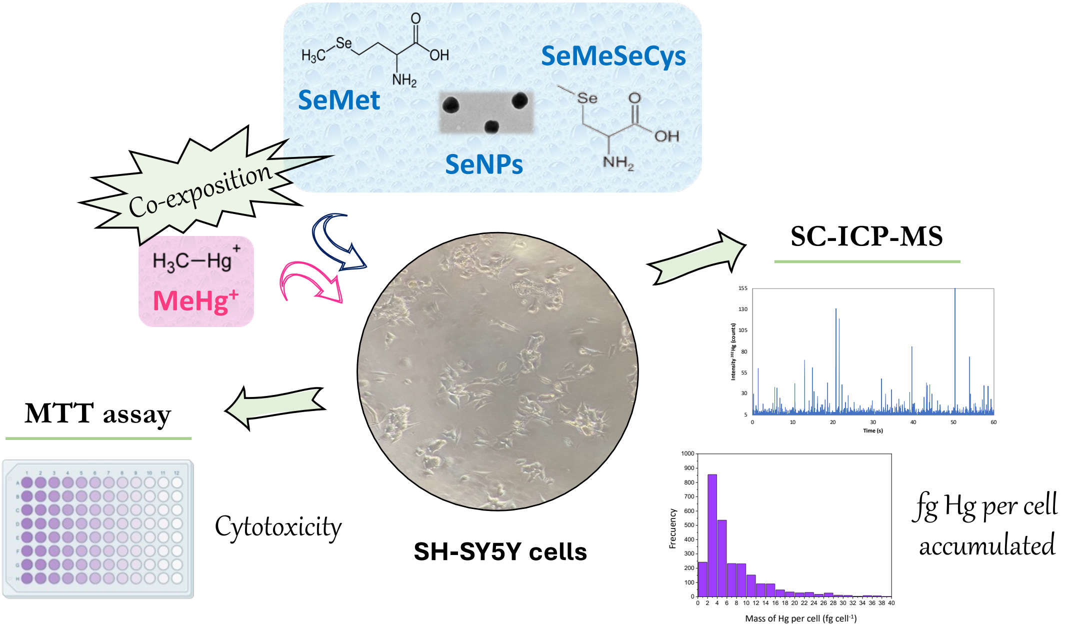

- **XXIV Reunión de la Sociedad Española de Química Analítica**. *Single-cell ICP-MS como herramienta analítica en estudios de neurotoxicidad. Evaluación del efecto protector de distintas especies de selenio frente a la toxicidad del mercurio en células de neuroblastoma humano (SH-SY5Y)*. Zaragoza, España, julio de 2024. Tipo de comunicación: oral.

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RESEARCH PAPER

Single-cell ICP-MS for evaluating the Se-protective effect against $MeHg^+$-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y)

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Abstract
The protective effect of selenium (Se) against Hg-induced neurotoxicity has been widely investigated; however, the mechanisms behind this interaction have not been fully elucidated yet. In the current work, the role of Se against $MeHg^+$-induced cytotoxicity in the human neuroblastoma cell line (SH-SY5Y) is reported for the first time by tracking Hg uptake and accumulation at the single-cell level by inductively coupled plasma-mass spectrometry in single-cell mode (SC-ICP-MS). The influence of different Se species (SeMet, SeMeSeCys, citrate-SeNPs, and chitosan-SeNPs) on $MeHg^+$ cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. SeMet and SeMeSeCys exhibited protective effects against $MeHg^+$-induced cell death, particularly at high $MeHg^+$ concentrations (LC_{50}). In addition, chitosan-SeNPs showed greater protection compared to citrate-SeNPs when co-exposed with $MeHg^+$. Interestingly, SC-ICP-MS unveiled the heterogeneous distribution of Hg uptake by SH-SY5Y cells. Co-exposure of SeMet and SeMeSeCys with $MeHg^+$ led to a reduction of the amount of Hg accumulated per individual cell, which decreased the maximum level of Hg per cell by half (from 60 fg Hg $cell^{-1}$ to 30 fg Hg $cell^{-1}$) when SeMet was present, along with a decrease in the percentage of cells that accumulated the highest quantity of $MeHg^+$. All these data corroborate the protective role of Se against Hg toxicity at the cellular level.

Keywords Single-cell ICP-MS · Selenoamino acids · SeNPs · Methylmercury · SH-SY5Y · Cytotoxicity

Introduction

Mercury (Hg) is a global pollutant and a well-known neurotoxicant, being recognized as a chemical of major public health concern by the World Health Organization (WHO) [1, 2]. Hg exposure can cause brain damage, sensory and motor deficiency, paralysis, loss of vision, incoherent speech, delirium, coma, and even death [2]. Its toxicological characteristics are highly dependent not only on the exposed dose but also on its chemical form, being methylmercury ($MeHg^+$) the most toxic one. Hg(II) is converted to $MeHg^+$ by microorganisms, being highly influenced by factors such as microbial activity, redox potential, pH, temperature, or concentration of bioavailable Hg. $MeHg^+$ is then submitted to bioaccumulation and biomagnification processes in aquatic systems, leading to a potential threat to human health through seafood consumption [3–8]. Moreover, $MeHg^+$ is able to cross the blood-brain barrier (BBB) and the placenta by forming a cysteine conjugate to reach its main target: the brain [9].

The protective effect of selenium (Se) against Hg-induced toxicity has been widely investigated. Although the mechanisms of the Se-Hg interaction are not yet fully elucidated, the Se-protective effect is mainly attributed to the formation of stable Se-Hg complexes which reduce the availability of free Hg ions in the body, facilitating its excretion and decreasing its accumulation in tissues and organs, for instance, the brain [4, 10]. In addition, the outstanding antioxidant properties of Se help to counteract

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the oxidative stress and cellular damage induced by Hg through neutralization of harmful free radicals and reactive oxygen species (ROS). Interestingly, when the Se levels in an organism decrease, the remaining sources of Se will maintain its supplies in the brain up to 60%, being the brain the organ of preference [11, 12]. As a result of this, the selenoprotein synthesis continues to maintain brain metabolism and to prevent neurodegeneration [13].

A great variety of (bio)analytical techniques has been widely developed and applied for assessing Hg neurotoxicity along with the understanding of the corresponding Se-protective effect. In this sense, in vitro models such as immortalized cell lines derived from neuronal cells combined with flow cytometry, MTT, or LDH activity assays, among others, have been extensively employed [14–16]. For instance, human neuroblastoma SH-SY5Y cell line is indeed one of the most widely used for neurotoxicity studies, which can be used as immortalized human cell line or can be differentiated into a more relevant disease-specific phenotype by using differentiation agent such as retinoic acid (RA), 12-o-tetradecanoyl-phorbol-13-acetate (TPA), or dibutyryl cyclic AMP (dBcAMP) [15, 17–23]. This cell line has been widely employed for elucidating the role of Se as neuroprotective agent [15, 17, 21].

Moreover, extremely sensitive techniques capable of identifying and quantifying metal and metalloids at low levels become crucial for investigating trace elements as neurotoxicants. In this regard, inductively coupled plasma-mass spectrometry (ICP-MS) is by far the technique of choice [24–30]. Furthermore, ICP-MS in single-cell mode (SC-ICP-MS) is a powerful analytical technique that enables to accurately determine the uptake and accumulation of metal/metalloids that are potential neurotoxicant or neuroprotective agents at single-cell level, considering the huge variability that exists within cellular population [31]. Although SC-ICP-MS has been quite a lot used for bioanalysis purposes, its use in neurotoxicity studies is still scarce. Taking into account the cell-to-cell variation in terms of single-cell element accumulation, SC-ICP-MS could be a very useful analytical technique not yet fully explored in this area of research.

To the best of the author's knowledge, no previous study has reported on the Se-protective effect against $MeHg^+$-induced cytotoxicity in neuronal cell lines by tracking Hg accumulation at single-cell level using SC-ICP-MS. In consequence, here, it was hypothesized that Se has inhibitory properties on Hg uptake and accumulation in human neuroblastoma cell line (SH-SY5Y). To corroborate this hypothesis, undifferentiated SH-SY5Y cells have been co-exposed to different concentrations of $MeHg^+$ and Se species (SeMet, SeMeSeCys, and SeNPs) in order to carry out cell viability assays (MTT) and evaluate Hg uptake and accumulation by cells at single-cell level by SC-ICP-MS analysis.

Materials and methods

Culture of SH-SY5Y cells

Human neuroblastoma cell line SH-SY5Y (CRL-2266) was purchased from the American Type Culture Collection (ATCC) and stored in 90% FBS (ThermoFisher Scientific, Madrid, Spain) and 10% DMSO (Sigma-Aldrich, Madrid, Spain) at − 130 °C in liquid nitrogen vapor until use. Undifferentiated SH-SY5Y cells (Fig. S1) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (ThermoFisher Scientific, Madrid, Spain) and kept in an incubator at 37 °C with a humidified atmosphere of 5% CO_2. Due to the intrinsic characteristics of the assays performed in this study and to efficiently discard death cells in subculture passes, only adherent cells, which represented more than 95% of the total population, were considered. For the subculture procedure, phosphate-buffered saline (PBS) at pH 7.4 and trypsin–EDTA (0.05%) (ThermoFisher Scientific, Madrid, Spain) were employed.

Selenium nanoparticles

Two different kinds of Se nanoparticles have been employed in this study: citrate-stabilized Se nanoparticles (citrate-SeNPs) and chitosan-modified Se nanoparticles (Ch-SeNPs).

Citrate-stabilized SeNP standard suspension (citrate-SeNPs, 80 nm at 0.15 wt.% in water) was purchased from Sigma-Aldrich (Lyon, Francia).

The synthesis and characterization of Ch-SeNPs were performed according to previous published reports [32, 33], through chemical reduction of sodium selenite (0.054 M) (Sigma-Aldrich, Madrid, Spain) with ascorbic acid (0.23 M) (Sigma-Aldrich, Madrid, Spain) and using 0.1% chitosan (Sigma-Aldrich, Madrid, Spain) as coating agent. Once synthesized, Ch-SeNPs were purified through a dialysis process using a 12-kDa molecular weight cutoff (MWCO) membrane (Merck, Darmstadt, Germany). Ch-SeNPs were characterized by transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDS) using a JEPL JEM-1400Plus (JEOL Ltd, Tokyo, Japan) operating at 120 kV and equipped with an EDS microanalysis composition system (Oxford Inca).

MTT assay

MTT assay was performed in order to estimate the cytotoxicity of different concentrations of Hg and Se species over SH-SY5Y cells and to elucidate the protective effect of Se against Hg toxicity. Cells were seeded in 96-well plates at a density of $8\cdot10^3$ cells per well, approximately at 40% of confluence, and cultured for 24 h. Then, the culture medium was removed and cells were exposed to methylmercury (MeHg$^+$, 0.05–10 µM, from Sigma-Aldrich, Madrid, Spain) alone or in presence of seleno-L-methionine (SeMet, 25 and 50 µM, from Sigma-Aldrich, Madrid, Spain), selenomethylseleno-L-cysteine (SeMeSeCys, 25 and 50 µM, from Sigma-Aldrich, Madrid, Spain), citrate-SeNPs (25 and 50 µM), or Ch-SeNPs (25 and 50 µM). In a different experiment, cells were also incubated with citrate-SeNPs (25 and 50 µM) and Ch-SeNPs (25 and 50 µM) to evaluate separately the effect of these two types of Se nanoparticles. After 48 h of incubation, 20 µL of MTT solution (5 mg mL^{-1} in PBS, from ThermoFisher Scientific, Madrid, Spain) was added to each well and plates were incubated at 37 °C during 4 h. Afterwards, the culture medium was removed and 100 µL of dimethyl sulfoxide (DMSO, from Scharlab, Barcelona, Spain) was then added to dissolve the MTT formazan crystals formed. Finally, absorbance was measured with a microplate spectrophotometer (Multiskan SkyHigh, from ThermoFisher Scientific, Madrid, Spain) at a wavelength of 570 nm. Five replicates were performed for each condition and concentration. In addition, measurements of negative (DMEM supplemented with 10% (v/v) FBS) and positive controls (diluted cell culture without adding Hg or Se species) were performed in parallel. Cell viability was calculated as described below:

$$\text{Cell viability }(\%) = \frac{A_s - A_{c-}}{A_{c+} - A_{c-}} \times 100 \tag{1}$$

Being A_s, A_{c-}, and A_{c+} the absorbances obtained for the sample, the negative control, and the positive control, respectively. The average of the cell viability was compared with those obtained from the positive controls.

Cell mineralization for determining total Hg content by ICP-MS

SH-SY5Y cells were seeded in 60-mm-diameter dishes at a density of $4\cdot10^5$ cells per dish and cultured for 24 h. Then, the culture medium was removed, and cells were exposed to target compounds: MeHg$^+$ alone (1.5 µM, LC$_{20}$), MeHg$^+$ with SeMet (1.5 µM and 50 µM, respectively), and MeHg$^+$ with SeMeSeCys (1.5 µM and 50 µM, respectively) during 48 h. Afterwards, the culture medium was removed and cells were washed with PBS and trypsinized. Then, cells were centrifuged at 3000 rpm during 5 min, the culture medium was removed, and cell pellets underwent an acid digestion with 65% v/v HNO_3 Suprapur® (Merck, Madrid, Spain) and 30% v/v H_2O_2 (Panreac, Barcelona, Spain) (5:1) in a 1600-W MSP microwave oven from CEM (Stallings, NC, USA) at 130 °C for 20 min. Once cooled until room temperature, the solutions were appropriately diluted with deionized water (18.2 MΩ cm, from a Milli-Q® water purification system; Millipore, Bedford, MA, USA) up to a final volume of 5.0 mL. Culture medium and PBS removed from cells were also digested in the same conditions as the cells with the aim of performing mass balance calculations. Cells seeded without exposure to Se and Hg species were considered blanks and three replicates for each single condition were performed.

Digested samples were appropriately diluted and total Hg content was determined by an Agilent 7700 × Series ICP-MS (Agilent Technologies Inc., Santa Clara, CA, USA) by monitoring ^{201}Hg and ^{202}Hg isotopes under continuous acquisition mode. The operational conditions for ICP-MS measurements are as follows: RF power of 1550 W, sample depth of 8 mm, plasma gas flow rate of 15.0 L min^{-1}, auxiliary gas flow rate of 1.0 L min^{-1}, and Conikal nebulizer with a flow rate of 1.0 L min^{-1}. Hg quantification was performed by external calibration with the isotope ^{202}Hg. A certified reference material (ERM-CE 278 k, from mussel) from the Institute for Reference Materials was analyzed to validate the accuracy of the results obtained. No statistically significant differences (p-value < 0.05) were found for total Hg between the certified value (0.073 ± 0.007 mg kg^{-1}) and the experimental one (0.069 ± 0.009 mg kg^{-1}). The limit of quantification (LOQ) was calculated according to International Union of Pure and Applied Chemistry (IUPAC) [34]. The LOQ (0.057 µg kg^{-1}) was obtained as $10\sigma/S$, where σ is the standard deviation of 10 measurements of the blank solution and S is the slope of the line equation for external calibration curve.

SC-ICP-MS analysis

Single-cell ICP-MS analytical technique was employed in order to determine the amount of Hg accumulated at single-cell level in each condition of SH-SY5Y cells exposed to Hg and Se species.

Prior to SC-ICP-MS analysis (Agilent 7700 × Series ICP-MS), sample preparation procedure based on cell fixation was optimized using paraformaldehyde (PFA) (ThermoFisher Scientific, Madrid, Spain) at different concentrations, 1% or 4% (v/v). For this purpose, 10^6 cells suspended in 500 µL of PBS were added to each Eppendorf tube. Once centrifuged at 3000 rpm for 5 min, cells were resuspended in 500 µL of 1% or 4% (v/v) PFA and incubated at 4 °C overnight. Afterwards, cells were centrifuged to remove PFA and washed three times with PBS. Cells were then diluted until a concentration of $6\cdot10^4$ cells mL^{-1} prior to SC-ICP-MS analysis. For cell counting, a Neubauer hemocytometer (Sigma-Aldrich, Schnelldorf, Germany) was used under an optical microscope (Inverted Microscope, Leica DM IL LED, Leica Microsystems, Wetzlar, Germany) at tenfold magnification. Same treatment without PFA was performed for comparison purposes. Cell transport efficiency was evaluated by comparing the cell population before introducing the sample to the ICP-MS system to the number of cell signals obtained when monitoring cell-constitutive elements (^{55}Mn and ^{59}Co). Cell transport efficiencies obtained using fixated cell cultures were compared with those calculated from non-fixated cell cultures with the aim of selecting the most appropriate sample pre-treatment condition.

Once selected the best PFA cellular fixation condition (4%, stated in the "Quantitative cellular uptake of MeHg$^+$ by SH-SY5Y at single-cell level in presence of different Se species" section), SH-SY5Y cells were exposed to MeHg$^+$ alone (1.5 µM, LC$_{20}$), MeHg$^+$ combined with SeMet (1.5 µM and 50 µM, respectively), and MeHg$^+$ combined with SeMeSeCys (1.5 µM and 50 µM, respectively) during 48 h (three replicates of each one), as it was described in the "Cell mineralization for determining total Hg content by ICP-MS" section, following the same experimental procedure. Once the culture medium was removed, cells were washed with PBS, trypsinized, centrifuged, and resuspended in 500 µL of PBS in Eppendorf tubes. Once centrifuged at 3000 rpm for 5 min, cells were resuspended in 500 µL of 4% (v/v) PFA and incubated at 4 °C overnight. Afterwards, cells were centrifuged to remove PFA and washed three times with PBS. After centrifuging to remove the supernatant, the cells were resuspended in 250 µL of PBS. Cells were counted with a Neubauer hemocytometer under an optical microscope and appropriately diluted with PBS until a concentration of $6\cdot10^4$ cells mL^{-1}, prior to SC-ICP-MS analysis. Same treatment was also performed for a blank sample of cells.

Solutions and cell suspensions were introduced to the ICP-MS equipped with a Micromist nebulizer combined with a total consumption spray chamber (High Sensitivity Single-Cell Sample Introduction System for ICP-MS, Glass Expansion, Germany), at a flow rate of 10 µL min^{-1} using a syringe pump (model 100, Fisher Scientific, ThermoFisher Scientific, Spain) fitted with a 1-mL Hamilton syringe (Hamilton, Reno, USA). Measurements were acquired using time-resolved analysis (TRA) mode during 120 s with a dwell time of 3 ms and by monitoring the isotopes ^{202}Hg and the cell-constitutive elements (^{55}Mn and ^{59}Co), in separate runs since a simple quadrupole was used. Different mass scanning (^{202}Hg and ^{55}Mn) simultaneously by a single quadrupole is not possible because the acquisition time of transient signals originating from a single cell is too short to scan signals at different *m/z*. Final operating conditions are summarized in Table 1.

Transport efficiency of calibration standards was calculated daily by analyzing citrate-stabilized colloidal gold nanoparticles (AuNPs, nominal diameter 30 nm) reference material LGC5050, from LGC (Teddington, UK), at a concentration of 40 ng L^{-1}. Percentages of transport efficiency were always in the range of 60–90%.

In order to perform Hg mass quantification, a calibration curve of ionic Hg (standard from Sigma-Aldrich, Madrid, Spain) was prepared daily within a concentration range between 0 and 50 µg L^{-1}. The limit of detection (LOD) was found to be 0.937 fg Hg per cell.

The acquired data was further processed employing an iterative approach previously reported [35]. This procedure consisted in selecting the data points that were three times the standard deviation (3 s) above the mean and the iterative process is repeated until there were no data points higher than the value of mean plus 3σ, and that value was used as threshold for cell events. The mass of Hg in every cell was determined by applying Eq. (2).

Table 1 Operating parameters of ICP-MS for performing single-cell experiments

ICP-MS parameters	
RF Power	1550 W
Plasma gas flow rate	15.0 L min^{-1}
Carrier gas flow rate	0.70 L min^{-1}
Sheath gas flow rate	0.45 L min^{-1}
Nebulizer	Micromist
Spray chamber type	Total consumption, High Sensitivity Single-Cell Sample Introduction System for ICP-MS (Glass Expansion)
Sample flow rate	10 µL min^{-1}
Data acquisition parameters	
Acquisition mode	Time-resolved analysis
Dwell time	3 ms
Run time	120 s
Isotopes monitored	^{202}Hg, ^{55}Mn, ^{59}Co

$$m_c = \frac{\eta \cdot F \cdot t \cdot I}{b} \tag{2}$$

where m_c is the mass of element of interest in single cell, η is the transport efficiency for the liquid standards calculated using LGC5050 reference material, F is the sample flow rate, t is the integration time, I is the analyte count rate of Hg, and b is the slope of the calibration curve. The frequency distribution histograms of Hg uptake by each individual cell were plotted using Origin Pro 2021 software.

Finally, to validate SC-ICP-MS analysis, total Hg content determined as described in the "Cell mineralization for determining total Hg content by ICP-MS" section by ICP-MS was referred to the number of cells present in the samples and compared to the mean values of Hg contents obtained by SC-ICP-MS.

Statistical analysis

One-way analysis of variance (ANOVA) was applied as chemometric tool to evaluate the results obtained. A significance level of p-value < 0.05 was adopted for all comparisons. Statistical analysis of data was carried out using Statgraphics Centurion XIX (Statgraphics Technologies Inc., USA).

Results and discussion

Characterization of Ch-SeNPs

The protective effect of different Se species, including SeNPs, against MeHg$^+$ neurotoxicity has been evaluated in this work. For comparison purposes, two different kinds of SeNPs have been investigated in this study: commercialized citrate-stabilized Se nanoparticles (citrate-SeNPs spherical with an average size of 80 nm, according to the manufacturer) and in-house synthesized chitosan-modified Se nanoparticles (Ch-SeNPs). TEM micrographs shown in Fig. 1A evidenced the presence of spherical and dispersed Ch-SeNPs with an average size of 60 ± 15 nm synthesized as described in "Selenium nanoparticles" section. An EDS analysis (Fig. 1B) confirmed the presence of Se in the samples.

Cell viability by using MTT assay

MTT assay was performed for assessing MeHg$^+$ cytotoxicity in SH-SY5Y cells. SH-SY5Y has been selected as cell line model since it has been one of the most used cell lines for neurotoxicity studies being widely employed for investigating the role of Se as protective agent against different neurotoxic elements [15, 17, 21].

A lethal concentration for 50% of cell population (LC_{50}) was calculated by adjusting data to a logarithmic regression curve by applying Eq. (3).

$$y = a \cdot \ln x + b \tag{3}$$

Being a the slope, b the intercept, y the percentage of viable cells, and x the concentration of MeHg$^+$.

LC_{50} obtained for MeHg$^+$ was 3 µM. The cell viability assays for SH-SY5Y cells exposed to MeHg$^+$ were performed at two concentrations: 3 µM and 1.5 µM, being the LC_{50} and the LC_{20}, respectively. Moreover, the aim of these MTT assays was to evaluate whether the presence of Se species (SeMet, SeMeSeCys, citrate-SeNPs, and Ch-SeNPs) increases SH-SY5Y cell viability when combined with MeHg$^+$ in comparison to MeHg$^+$ alone. MTT assays allowed us to select the optimum Se concentration to be supplemented to alleviate the decrease in cell viability produced by MeHg$^+$. Cell viability of SeMet and SeMeSeCys was evaluated in a previous work in which cytotoxicity of SeMet and SeMeSeCys expressed as LC_{50} was found to be > 1000 µM for both species using SH-SY5Y cells. This

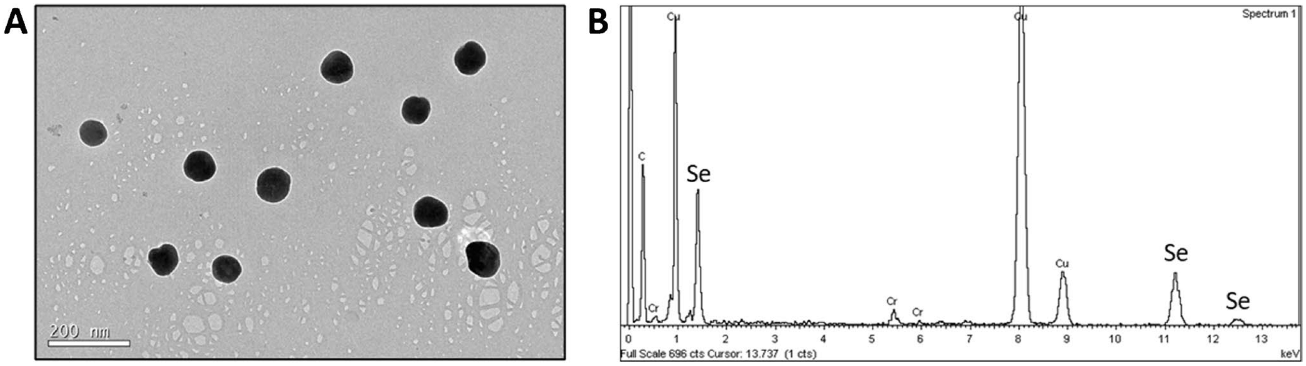

Fig. 1 TEM images (**A**) and EDS spectrum (**B**) of in-house synthesized Ch-SeNPs

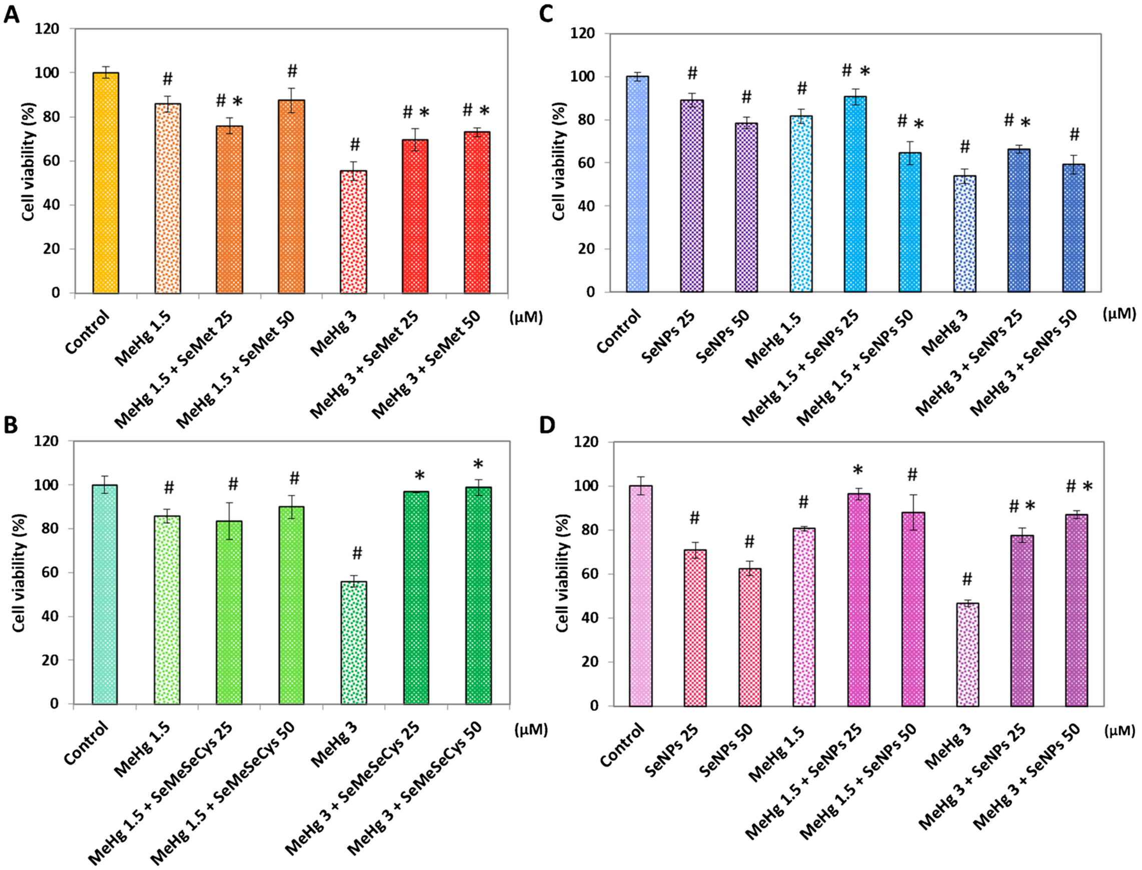

Fig. 2 Cell viability of SH-SY5Y neuroblastoma cell line to $MeHg^+$ and different Se species co-exposure by MTT assay: **A** $MeHg^+$ and SeMet; **B** $MeHg^+$ and SeMeSeCys; **C** $MeHg^+$ and citrate-SeNPs; **D** $MeHg^+$ and Ch-SeNPs. Error bars represent standard deviations ($n = 5$). * denotes p-value < 0.05, significantly difference from only $MeHg^+$-treated cells at the same concentration and # denotes p-value < 0.05, significantly difference from the control group

remarkably low toxicity is possibly due to the essential role of SeMet and SeMeSeCys in cell metabolism [17].

Results obtained are shown in Fig. 2 and in Table S1 of Supplementary Information. The supplementation of 3 µM $MeHg^+$ along with SeMet (25 or 50 µM) increases cell viability significantly (p-value < 0.05) (69 ± 5 and 73 ± 2%, respectively) compared to those exposed only to 3 µM $MeHg^+$ (55 ± 4%) (Fig. 2A). Similar positive results were obtained when SeMeSeCys was employed (Fig. 2B), evidencing significant increases ($p < 0.05$) in cell viability from 56 ± 3% to 97 ± 1% and 99 ± 4% when 25 or 50 µM SeMeSeCys were added, respectively. As it can be seen in both Fig. 2A and B, in the case of cells exposed to 1.5 µM $MeHg^+$, no significant increases (p-value < 0.05) of cell viability were observed for any condition, regardless of the concentration (25 or 50 µM) and the Se species (SeMet or SeMeSeCys) supplemented. Therefore, at high toxic $MeHg^+$ levels (LC_{50}), concentrations of SeMet and SeMeSeCys between 25 and 50 µM have a direct effect on cell viability, decreasing cell death induced by $MeHg^+$ toxicity. Best results were obtained when employed SeMeSeCys, not being dependent on the concentration tested, providing the highest cell viabilities, which are not significantly different from the control group (p-value < 0.05).

Regarding SeNPs, when both citrate-SeNPs and Ch-SeNPs at both concentrations, 25 and 50 µM were supplemented to the cells in absence of $MeHg^+$ provided significant statistical differences (p-value < 0.05) in cell viability with respect to the control group. A decrease from about 100% to 89 ± 3 and 78 ± 3% when cells were exposed to citrate-SeNPs at 25 and 50 µM (Fig. 2C), respectively, and to 71 ± 4 and 62 ± 3% when cells were exposed to Ch-SeNPs at 25 and 50 µM (Fig. 2D), respectively, was obtained. It seems that Ch-SeNPs are more toxic for SH-S5Y5 cells than citrate-SeNPs. Nevertheless, Ch-SeNPs show a better Se-protective effect against $MeHg^+$-induced toxicity than citrate-SeNPs when co-exposure with $MeHg^+$ at all concentrations tested. In this sense, the combination of 1.5 µM $MeHg^+$ with 25 µM Ch-SeNPs did not give statistically significant differences from the control group (p-value < 0.05), reaching 96 ± 3%. The presence of higher citrate-SeNPs and Ch-SeNPs concentrations (50 µM) in combination with 1.5 µM $MeHg^+$ did not provide greater improvements in cell viability. However, for those cells exposed to 3 µM MeHg$^+$ in combination with 25 µM citrate-SeNPs and 25 and 50 µM Ch-SeNPs, respectively, their viability increased significantly (p-value < 0.05), being the best results (87 ± 3%) obtained when 50 µM Ch-SeNPs were added to the culture medium.

This improvement in cell viability when Ch-SeNPs were used compared to citrate-SeNPs could be attributed to the coating agent as the mean size of the nanoparticles was quite similar. In addition, in a previous work, it has been observed that SH-SY5Y cells are able to internalize these Ch-SeNPs, being located in the cytosol and mitochondria and attached to the external surface of the membrane, as well as in the nuclei and nucleoli [17]. It is likely that this fact facilitates the protective effect of Ch-SeNPs against MeHg$^+$-induced toxicity. Additionally, Ch-SeNPs might interact in solution with the Hg added and therefore making more difficult its absorption. The results obtained evidence that selenoamino acids, especially SeMeSeCys, exhibit stronger protective effects against MeHg$^+$ toxicity in SH-SY5Y cells than Se in its nanoparticulate form. Therefore, SeMet and SeMeSeCys were chosen to perform further studies.

Evaluation of MeHg$^+$ accumulation by SH-SY5Y cells in presence of different Se species

To evaluate the accumulation of MeHg$^+$ as Hg within SH-SY5Y cells under different exposure conditions (1.5 µM MeHg$^+$ or 1.5 µM MeHg$^+$ + 50 µM SeMet/SeMeSeCys), the percentage of total Hg found in cell cultures was determined by ICP-MS after acid digestion as described in the "Cell mineralization for determining total Hg content by ICP-MS" section. The different exposure conditions were selected attending the results obtained from MTT assay ("Cell viability by using MTT assay" section).

To perform mass balance calculations, culture medium and PBS removed from cells were also digested and measured in the same conditions as cells. Results obtained compiled in Table 2 evidenced that only about 3% of MeHg$^+$ added to the culture medium was accumulated by cells, not observing statistically significant differences (p-value < 0.05) between conditions of cell exposure. Moreover, mass balance ($\sum$ Hg, %) was quantitative in all cases, as it can be seen in Table 2.

The accumulation of Hg by cells depends of several factors such as the cell line and the chemical Hg species employed, among others. For instance, the uptake of Hg by an embryonic and a tumor human cell line (HEK 293 and HeLa) exposed to Hg in the form of MeHg$^+$ and inorganic Hg at their LC$_{20}$ was assessed by Cid-Barrio et al. [36]. The authors evidenced notable differences in the total Hg contents accumulated by cells, not only between cell lines but also between Hg species. HeLa accumulates about 46 and 9% of Hg added when cells were exposed to MeHg$^+$ and inorganic Hg, respectively, whereas HEK 293 accumulates about 23 and 7% of Hg added when cells were exposed to MeHg$^+$ and inorganic Hg, respectively. In this work, only about 3% of Hg added in the form of MeHg$^+$ (also at LC$_{20}$) is accumulated by SH-SY5Y cells.

Quantitative cellular uptake of MeHg$^+$ by SH-SY5Y at single-cell level in presence of different Se species

The uptake of MeHg$^+$ by SH-SY5Y at single-cell level was assessed after 48 h of exposure to different MeHg$^+$ and Se species combinations at different concentrations by SC-ICP-MS.

Prior to SC-ICP-MS analysis, cell fixation was evaluated as a sample pre-treatment to ensure that the largest number of cells are nebulized and analyzed, improving the accuracy of the data obtained. For this purpose, PFA at different concentrations was added to the cell cultures and incubated overnight before SC-ICP-MS analysis ("SC-ICP-MS analysis" section). The effect of PFA was evaluated by means of cell transport efficiency (%) which is calculated by dividing the number of events detected by SC-ICP-MS (by monitoring ^{55}Mn isotope) by the number of cells counted by microscope.

Nebulizer transport efficiency reaches its highest value when 4% (v/v) PFA was employed, resulting in 93 ± 6%. When using 1% (v/v) PFA, the nebulizer transport efficiency was 55 ± 2%, and if no fixation is applied, only 10 ± 3% of SH-SY5Y cells introduced in the system were able to reach the plasma. These results show the need of performing a cellular fixation step when working with human cell lines due to their high fragility which it has been widely reported in the literature [37, 38].

Table 2 Hg accumulation percentages in SH-SY5Y cells and culture medium after exposure to MeHg$^+$ or MeHg$^+$ + SeMet/SeMeSeCys by ICP-MS (mean ± SD; $n = 3$)

Hg/Se exposure species and concentration	% Hg in cells	% Hg in culture medium and PBS	$\sum$ Hg (%)
1.5 µM MeHg$^+$	2.9 ± 0.4	92 ± 3	95 ± 3
1.5 µM MeHg$^+$ + 50 µM SeMet	3.6 ± 0.3	98 ± 4	101 ± 4
1.5 µM MeHg$^+$ + 50 µM SeMeSeCys	3.3 ± 0.4	98 ± 1	102 ± 2

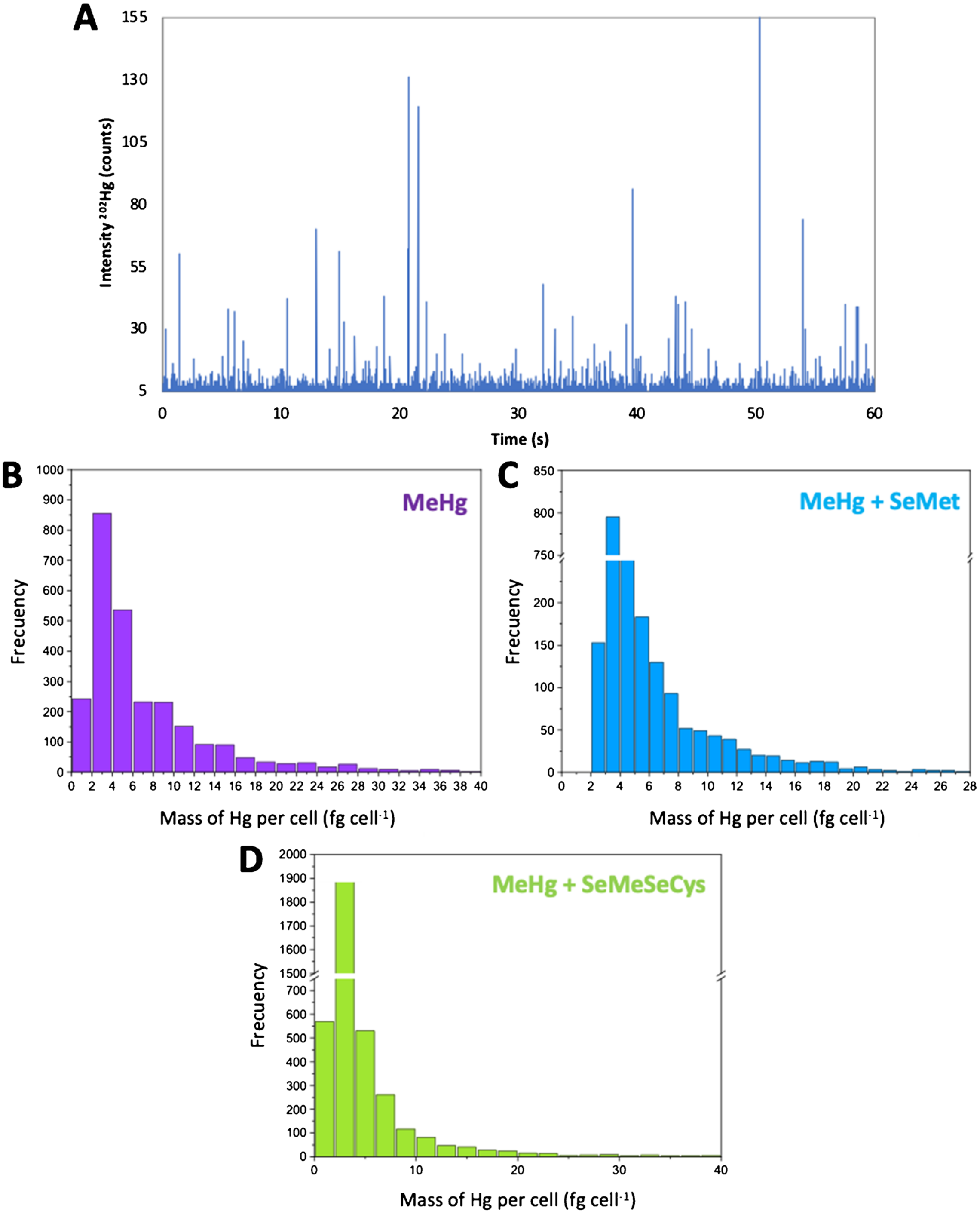

Fig. 3 Time-resolved scan of ^{202}Hg obtained for SH-SY5Y cells exposed to 1.5 µM MeHg$^+$ (**A**) and frequency histograms for SH-SY5Y cells incubated with (**B**) 1.5 µM MeHg$^+$; (**C**) 1.5 µM MeHg$^+$ + 50 µM SeMet, and (**D**) 1.5 µM MeHg$^+$ + 50 µM SeMeSeCys

As an example, Fig. 3A shows a time-resolved scan obtained by SC-ICP-MS analysis of SH-SY5Y cells exposed to 1.5 µM MeHg$^+$ in which several events with large differences in intensities could be observed above the baseline. In addition, mass distribution histograms showing Hg mass per individual cell (fg Hg per cell) at each exposure condition (1.5 µM MeHg$^+$ or 1.5 µM MeHg$^+$ + 50 µM SeMet/SeMeSeCys) are displayed in Fig. 3B–D.

The mass of Hg per event was calculated using 3-σ criterion to filtrate the data and after an external calibration with Hg standard measured under the same conditions than SC-ICP-MS analysis.

As it was expected due to differences in the intensity of the events, a heterogeneous distribution of Hg uptake can be appreciated in Fig. 3B–D for all cases.

When Se is added to the culture medium in co-exposure with $MeHg^+$, there is a higher number of cells that accumulate less amount of Hg compared to cells exposed only to $MeHg^+$ (Fig. 3B–D). For instance, around 76% of cells and 64% of cells accumulate up to 5 fg Hg cell^{-1} when 50 µM SeMeSeCys and 50 µM SeMet combined with 1.5 µM MeHg are supplemented, respectively, whereas this value drops to about 54% when only 1.5 µM $MeHg^+$ is present in the culture medium. Similar results were obtained when the amount of Hg uptake by cells is increased to 10 fg Hg cell^{-1}. The percentage of cells that accumulate up to 10 fg Hg cell^{-1} varies from 91 and 89% in case of cells co-treated with 50 µM SeMeSeCys and 50 µM SeMet and 1.5 µM $MeHg^+$, respectively, while 79% is obtained when cells are exposed to only 1.5 µM $MeHg^+$.

From these results, it can be highlighted the protective effect of SeMet and SeMeSeCys againts $MeHg^+$-induced toxicity in SH-SY5Y cells, not allowing them to accumulate as much Hg as they do when they are exposed to only $MeHg^+$. This protective effect can be explained by the interaction between Se and Hg in the culture medium, which leads to a more difficult internalization of Hg by cells and therefore decreasing its absorption.

This fact can also be appreciated in Table 3, in which minimum and maximum Hg accumulation levels per individual cell are shown. In the case of 1.5 µM $MeHg^+$ exposure and co-exposure of 1.5 µM $MeHg^+$ + 50 µM SeMeSeCys, some cells can accumulate up to 60 fg of Hg per cell. However, co-exposure of 1.5 µM $MeHg^+$ + 50 µM SeMet decreases the accumulation of Hg down to 30 fg of Hg per cell, being reduced the maximum from 60 fg Hg cell^{-1} when cells were exposed to only $MeHg^+$ to the half (30 fg Hg cell^{-1}) when co-exposure of $MeHg^+$ with SeMet. Therefore, it seems that the protective effect of Se against $MeHg^+$ cytotoxicity in SH-SY5Y might rely on decreasing the percentage of cells that accumulate high quantities of $MeHg^+$, as well as reducing the levels of $MeHg^+$ accumulated in cells.

It is important to highlight that these conclusions could be drawn only when using the SC-ICP-MS technique that takes into account the cell-to-cell variability whereas when employing acid digestion/ICP-MS no statistically significant differences (p-value < 0.05) were evidenced among the values of $MeHg^+$ (as Hg) accumulated by SH-SY5Y regardless of the different conditions of cell exposure carried out (Table 3).

Finally, for validation purposes, mean values of Hg accumulated determined by SC-ICP-MS were compared with those obtained when using ICP-MS after microwave-assisted acid digestion (Table 3). These results were calculated by dividing total Hg content obtained by ICP-MS analyses of digested cells treated with $MeHg^+$ or $MeHg^+$ + SeMet/SeMeSeCys by the number of cells present in the samples and counted by a Neubauer hemocytometer under an optical microscope. Differences in mean values of Hg levels found in the cell cultures between both methods can be observed in Table 3. In all cases, direct ICP-MS analyses after microwave digestion gave higher Hg concentrations (between 12.9 and 16.0 times higher) than SC-ICP-MS analyses, although all values were in the same order of magnitude. This fact has also been reported in the literature by other authors [38–41]. Indeed, Suárez-Oubiña et al. reported Ti and Ag contents up to 25 and 37 times higher, respectively, obtained by microwave-assisted acid digestion and ICP-MS measurements compared to SC-ICP-MS analyses for cell lines derived from aquaculture species exposed to citrate-TiO_2 NPs and PVP-Ag NPs [42].

Conclusions

The impact of different Se species such as SeMet, SeMeSeCys, citrate-SeNPs, and Ch-SeNPs on the cytotoxicity and accumulation of Hg in human neuroblastoma SH-SY5Y cells has been investigated using MTT cell toxicity assay and, for the first time, SC-ICP-MS. Results from MTT experiments evidenced that both SeMet and SeMeSeCys show a higher protective effect against $MeHg^+$-induced cell death at high $MeHg^+$ concentrations (LC_{50}) than SeNPs. Moreover, SC-ICP-MS analysis revealed that the co-exposure of SeMet and SeMeSeCys with $MeHg^+$ led to a reduction in the amount of the maximum mass of Hg accumulated per individual cell

Table 3 Hg levels in SH-SY5Y cells after exposure to $MeHg^+$ or $MeHg^+$ + SeMet/SeMeSeCys by SC-ICP-MS and after acid digestion (mean ± SD; $n = 3$)

Hg/Se exposure species and concentration	SC-ICP-MS			Digestion/ICP-MS	Ratio*
	fg Hg cell^{-1}			fg Hg cell^{-1}	
	Min	Max	Mean	Mean	
1.5 µM $MeHg^+$	1.65	59.04	5.8 ± 5.6	75 ± 9	12.9
1.5 µM $MeHg^+$ + 50 µM SeMet	1.70	56.04	5.0 ± 5.5	80 ± 8	16.0
1.5 µM $MeHg^+$ + 50 µM SeMeSeCys	2.98	28.08	5.6 ± 3.7	88 ± 11	15.7

*Ratio between the concentrations obtained by ICP-MS measurement after acid digestion and SC-ICP-MS measurement up to the half (from 60 fg Hg cell^{-1} to 30 fg Hg cell^{-1}) when SeMet is present, as well as a decrease in the percentage of cells that accumulate the highest quantities of MeHg$^+$. This can be explained by the interaction of Se with Hg in the culture medium, which leads to a more difficult internalization of Hg by cells and therefore decreasing its absorption. However, achieving these outcomes were only possible by applying SC-ICP-MS that allowed us to obtain information at single-cell level, since the results obtained through ICP-MS bulk analysis did not show differences among the values of MeHg$^+$ (as Hg) accumulated by SH-SY5Y regardless of the different conditions of cell exposure. These findings clearly demonstrate the importance of including analytical techniques based on elemental mass spectrometry such as SC-ICP-MS in neurotoxicology studies. The results included in this work might contribute to a better understanding of the potential neuroprotective role of the Se species against MeHg$^+$-induced toxicity at the cellular level, paving the way for further research in neuroprotection strategies against Hg-induced neurotoxicity.

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Declarations

Competing interests The authors declare no competing interests.

References

1. WHO (World Health Organization). Mercury in health care. 2005;2 . Available at https://www.who.int/docs/default-source/wash-documents/mercury-in-health-care.pdf?sfvrsn=11857b44_15.
2. WHO (World Health Organization). Guidance for identifying populations at risk from mercury exposure. Editor UNEP Chemical Branch. 2008;170. Available at: https://cdn.who.int/media/docs/defaultsource/chemical-safety/mercuryexposure.pdf?sfvrsn=e827b153_1&download=true.
3. Burger J, Gaines KF, Boring CS, Stephens WL, Snodgrass J, Gochfeld M. Mercury and selenium in fish from the Savannah River: species, trophic level, and locational differences. Environ Res. 2001;87:108–18. https://doi.org/10.1006/enrs.2001.4294.
4. Ralston NVC, Ralston CR, Raymond LJ. Selenium health benefit values: updated criteria for mercury risk assessments. Biol Trace Elem Res. 2016;171:262–9. https://doi.org/10.1007/s12011-015-0516-z.
5. Ralston NVC, Kaneko JJ, Raymond LJ. Selenium health benefit values provide a reliable index of seafood benefits vs. risks. J Trace Elem Med Biol. 2019;55:50–7. https://doi.org/10.1016/j.jtemb.2019.05.009.
6. Azad AM, Frantzen S, Bank MS, Nilsen BM, Duinker A, Madsen L, Maage A. Effects of geography and species variation on selenium and mercury molar ratios in Northeast Atlantic marine fish communities. Sci Total Environ. 2019;652:1482–96. https://doi.org/10.1016/j.scitotenv.2018.10.405.
7. Ribeiro M, Zephyr N, Silva JAL, Danion M, Guérin T, Castanheira I, Leufroy A, Jitaru P. Assessment of the mercury-selenium antagonism in rainbow trout fish. Chemosphere. 2022;286:(Pt2)131749. https://doi.org/10.1016/j.chemosphere.2021.131749.
8. Fernández-Bautista T, Gómez-Gómez B, Palacín-García R, Gracia-Lor E, Pérez-Corona T, Madrid Y. Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS. Talanta. 2022;237:122922. https://doi.org/10.1016/j.talanta.2021.122922.
9. Simmons-Willis TA, Koh AS, Clarkson TW, Ballatori N. Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. Biochem J. 2002;367:239–46. https://doi.org/10.1042/BJ20020841.
10. Khan MAK, Wang F. Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism. Environ Toxicol Chem. 2009;28:1567–77. https://doi.org/10.1897/08-375.1.
11. Ralston NVC, Raymond LJ. Mercury's neurotoxicity is characterized by its disruption of selenium biochemistry. Biochim Biophys Acta Gen Subj. 2018;1862:2405–16. https://doi.org/10.1016/j.bbagen.2018.05.009.
12. Raymond LJ, Ralston NVC. Mercury: selenium interactions and health implications. Neurotoxicology. 2020;81:294–9. https://doi.org/10.1016/j.neuro.2020.09.020.
13. Cardoso BR, Roberts BR, Bush AI, Hare DJ. Selenium, selenoproteins and neurodegenerative diseases. Metallomics. 2015;7:1213–28. https://doi.org/10.1039/c5mt00075k.
14. Li LX, Chu JH, Chen XW, Gao PC, Wang ZY, Liu C, Fan RF. Selenium ameliorates mercuric chloride-induced brain damage through activating BDNF/TrKB/PI3K/AKT and inhibiting NF-κB signaling pathways. J Inorg Biochem. 2022;229: 111716. https://doi.org/10.1016/j.jinorgbio.2022.111716.
15. Kim YJ, Chai YG, Ryu JC. Selenoprotein W as molecular target of methylmercury in human neuronal cells is down-regulated by GSH depletion. Biochem Biophys Res Commun. 2005;330:1095–102. https://doi.org/10.1016/j.bbrc.2005.03.080.
16. Hossain KFB, Rahman MM, Sikder MT, Hosokawa T, Saito T, Kurasaki M. Selenium modulates inorganic mercury induced cytotoxicity and intrinsic apoptosis in PC12 cells. Ecotoxicol Environ Saf. 2021;207: 111262. https://doi.org/10.1016/j.ecoenv.2020.111262.
17. Vicente-Zurdo D, Gómez-Gómez B, Romero-Sánchez I, Rosales-Conrado N, León-González ME, Madrid Y. Cytotoxicity, uptake and accumulation of selenium nanoparticles and other selenium species in neuroblastoma cell lines related to Alzheimer's disease by using cytotoxicity assays, TEM and single cell-ICP-MS. Anal Chim Acta. 2023;1249:340949. https://doi.org/10.1016/j.aca.2023.340949.
18. Heusinkveld HJ, Westerink RHS. Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity. Toxicol In Vitro. 2017;45:81–8. https://doi.org/10.1016/j.tiv.2017.07.030.
19. Sala G, Marinig D, Riva C, Arosio A, Stefanoni G, Brighina L, Formenti M, Alberghina L, Colangelo AM, Ferrarese C. Rotenone down-regulates HSPA8/hsc70 chaperone protein in vitro: a new possible toxic mechanism contributing to Parkinson's disease. Neurotoxicology. 2016;54:161–9. https://doi.org/10.1016/j.neuro.2016.04.018.
20. Faria J, Barbosa J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative study of the neurotoxicological effects

of tramadol and tapentadol in SH-SY5Y cells. Toxicology. 2016;359–360:1–10. https://doi.org/10.1016/j.tox.2016.06.010.

21. Branca JJV, Morucci G, Maresca M, Tenci B, Cascella R, Paternostro F, Ghelardini C, Gulisano M, Di Cesare Mannelli L, Pacini A. Selenium and zinc: two key players against cadmium-induced neuronal toxicity. Toxicol In Vitro. 2018;48:159–69. https://doi.org/10.1016/j.tiv.2018.01.007.
22. Sarkanen JR, Nykky J, Siikanen J, Selinummi J, Ylikomi T, Jalonen TO. Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. J Neurochem. 2007;102:1941–52. https://doi.org/10.1111/j.1471-4159.2007.04676.x.
23. Xicoy H, Wieringa B, Martens GJM. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. Mol Neurodegener. 2017;12:1–11. https://doi.org/10.1186/s13024-017-0149-0.
24. De Jesus JR, Arruda MAZ. Unravelling neurological disorders through metallomics-based approaches. Metallomics. 2020;12:1878–96. https://doi.org/10.1039/d0mt00234h.
25. Rembach A, Hare DJ, Lind M, Fowler CJ, Cherny RA, McLean C, Bush AI, Masters CL, Roberts BR. Decreased copper in alzheimer's disease brain is predominantly in the soluble extractable fraction. Int J Alzheimers Dis. 2013;623241. https://doi.org/10.1155/2013/623241.
26. Krebs N, Langkammer C, Goessler W, Ropele S, Fazekas F, Yen K, Scheurer E. Assessment of trace elements in human brain using inductively coupled plasma mass spectrometry. J Trace Elem Med Biol. 2014;28:1–7. https://doi.org/10.1016/j.jtemb.2013.09.006.
27. Dahlberg D, Ivanovic J, Mariussen E, Hassel B. High extracellular levels of potassium and trace metals in human brain abscess. Neurochem Int. 2015;82:28–32. https://doi.org/10.1016/j.neuint.2015.02.003.
28. Ramos P, Santos A, Pinto NR, Mendes R, Magalhães T, Almeida A. Anatomical regional differences in selenium levels in the human brain. Biol Trace Elem Res. 2015;163:89–96. https://doi.org/10.1007/s12011-014-0160-z.
29. Ramos P, Santos A, Pinto E, Pinto NR, Mendes R, Magalhães T, Almeida A. Alkali metals levels in the human brain tissue: anatomical region differences and age-related changes. J Trace Elem Med Biol. 2016;38:174–82. https://doi.org/10.1016/j.jtemb.2016.03.018.
30. Korvela M, Lind AL, Wetterhall M, Gordh T, Andersson M, Pettersson J. Quantification of 10 elements in human cerebrospinal fluid from chronic pain patients with and without spinal cord stimulation. J Trace Elem Med Biol. 2016;37:1–7. https://doi.org/10.1016/j.jtemb.2016.06.003.
31. Corte-Rodríguez M, Álvarez-Fernández R, García-Cancela P, Montes-Bayón M, Bettmer J. Single cell ICP-MS using on line sample introduction systems: Current developments and remaining challenges. TrAC -Trends Anal Chem. 2020;132:116042. https://doi.org/10.1016/j.trac.2020.116042.
32. Gomez-Gomez B, Perez-Corona MT, Madrid Y. Using single-particle ICP-MS for unravelling the effect of type of food on the physicochemical properties and gastrointestinal stability of ZnONPs released from packaging materials. Anal Chim Acta. 2020;1100:12–21. https://doi.org/10.1016/j.aca.2019.11.063.
33. Moreno-Martin G, Sanz-Landaluze J, León-González ME, Madrid Y. In vivo quantification of volatile organoselenium compounds released by bacteria exposed to selenium with HS-SPME-GC-MS. Effect of selenite and selenium nanoparticles. Talanta 2021;224. https://doi.org/10.1016/j.talanta.2020.121907.
34. IUPAC. International Union of Pure and Applied Chemistry. Nomenclature, symbols, units and their usage in spectrochemical analysis-III. Analytical flame spectroscopy and associated non-flame procedures. Spectrochim Acta Part B 1978;33:247–269.
35. Gomez-Gomez B, Corte-Rodríguez M, Perez-Corona MT, Bettmer J, Montes-Bayón M, Madrid Y. Combined single cell and single particle ICP-TQ-MS analysis to quantitatively evaluate the uptake and biotransformation of tellurium nanoparticles in bacteria. Anal Chim Acta. 2020;1128:116–28. https://doi.org/10.1016/j.aca.2020.06.058.
36. Cid-Barrio L, Bouzas-Ramos D, Salinas-Castillo A, Ogra Y, Encinar JR, Costa- Fernández JM. Quantitative assessment of cellular uptake and differential toxic effects of HgSe nanoparticles in human cells. J Anal At Spectrom. 2020;35:1979–88. https://doi.org/10.1039/d0ja00162g.
37. Liu T, Bolea-Fernandez E, Mangodt C, De Wever O, Vanhaecke F. Single-event tandem ICP-mass spectrometry for the quantification of chemotherapeutic drug-derived Pt and endogenous elements in individual human cells. Anal Chim Acta. 2021;1177:338797. https://doi.org/10.1016/j.aca.2021.338797.
38. Tanaka Y ki, Iida R, Takada S, Kubota T, Yamanaka M, Sugiyama N, Abdelnour Y, Ogra Y. Quantitative elemental analysis of a single cell by using inductively coupled plasma-mass spectrometry in fast time-resolved analysis mode. ChemBioChem 2020;21:3266–3272. https://doi.org/10.1002/cbic.202000358.
39. López-Serrano Oliver A, Baumgart S, Bremser W, Flemig S, Wittke D, Grützkau A, Luch A, Haase A, Jakubowski N. Quantification of silver nanoparticles taken up by single cells using inductively coupled plasma mass spectrometry in the single cell measurement mode. J Anal At Spectrom. 2018;33:1256–63. https://doi.org/10.1039/c7ja00395a.
40. Cao Y, Feng J, Tang L, Yu C, Mo G, Deng B. A highly efficient introduction system for single cell- ICP-MS and its application to detection of copper in single human red blood cells. Talanta. 2020;206: 120174. https://doi.org/10.1016/j.talanta.2019.120174.
41. Meyer S, López-Serrano A, Mitze H, Jakubowski N, Schwerdtle T. Single-cell analysis by ICP-MS/MS as a fast tool for cellular bioavailability studies of arsenite. Metallomics. 2018;10:73–6. https://doi.org/10.1039/c7mt00285h.
42. Suárez-Oubiña C, Herbello-Hermelo P, Mallo N, Vázquez M, Cabaleiro S, Pinheiro I, Rodríguez-Lorenzo L, Espiña B, Bermejo-Barrera P, Moreda-Piñeiro A. Single-cell ICP-MS for studying the association of inorganic nanoparticles with cell lines derived from aquaculture species. Anal Bioanal Chem. 2023;415:3399–3413. https://doi.org/10.1007/s00216-023-04723-6.

Electronic Supplementary Information

Single-cell ICP-MS for evaluating the Se-protective effect against MeHg$^+$-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y)

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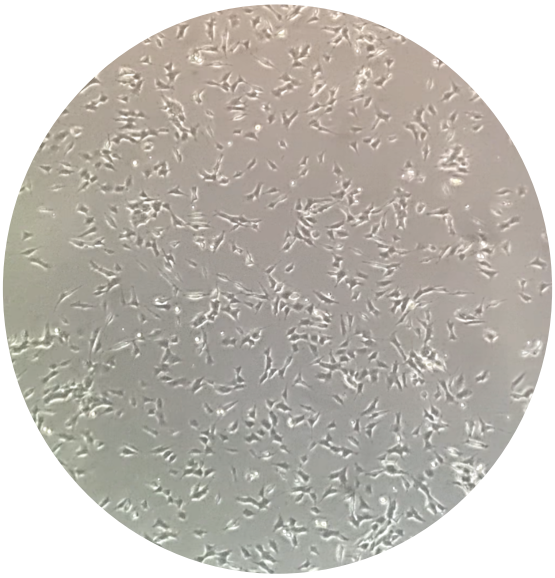

Fig. S1. Optical microscope images of confluent SH-SY5Y neuroblastoma cell line at 40X magnification power.

Table S1. Cell viability of SH-SY5Y neuroblastoma cell line to MeHg and different Se species co-exposure by MTT assay. Data are expressed as mean ± standard deviation (n = 5). * denotes p-value < 0.05, significantly difference from only MeHg-treated cells at the same concentration.

Species (Concentration)	**Cell viability (%)**	**Species (Concentration)**	**Cell viability (%)**
MeHg (1.5 μM)	86 ± 4	MeHg (1.5 μM) + SeMet (25 μM)	76 ± 4 *
		MeHg (1.5 μM) + SeMet (50 μM)	87 ± 5
MeHg (3 μM)	55 ± 4	MeHg (3 μM) + SeMet (25 μM)	69 ± 5 *
		MeHg (3 μM) + SeMet (50 μM)	73 ± 2 *
MeHg (1.5 μM)	86 ± 3	MeHg (1.5 μM) + SeMeSeCys (25 μM)	83 ± 8
		MeHg (1.5 μM) + SeMeSeCys (50 μM)	90 ± 5
MeHg (3 μM)	56 ± 3	MeHg (3 μM) + SeMeSeCys (25 μM)	97 ± 1 *
		MeHg (3 μM) + SeMeSeCys (50 μM)	99 ± 4 *
Citrate-SeNPs (25 μM)	89 ± 3	MeHg (1.5 μM) + citrate-SeNPs (25 μM)	91 ± 4 *
Citrate-SeNPs (50 μM)	78 ± 3	MeHg (1.5 μM) + citrate-SeNPs (50 μM)	65 ± 5 *
MeHg (1.5 μM)	82 ± 3	MeHg (3 μM) + citrate-SeNPs (25 μM)	66 ± 2 *
MeHg (3 μM)	54 ± 3	MeHg (3 μM) + citrate-SeNPs (50 μM)	59 ± 4
Ch-SeNPs (25 μM)	71 ± 4	MeHg (1.5 μM) + Ch-SeNPs (25 μM)	96 ± 3 *
Ch-SeNPs (50 μM)	62 ± 3	MeHg (1.5 μM) + Ch-SeNPs (50 μM)	88 ± 8
MeHg (1.5 μM)	81 ± 1	MeHg (3 μM) + Ch-SeNPs (25 μM)	78 ± 3 *
MeHg (3 μM)	47 ± 1	MeHg (3 μM) + Ch-SeNPs (50 μM)	87 ± 2 *

ARTÍCULO 6:

Técnicas (bio)analíticas para investigar el papel del selenio en la prevención de enfermedades neurodegenerativas y la neurotoxicidad inducida por neurotóxicos ambientales: As, Cd y Hg

(Bio)analytical approaches for investigating the role of selenium in preventing neurological disorders and neurotoxicity induced by environmental neurotoxicants: As, Cd and Hg

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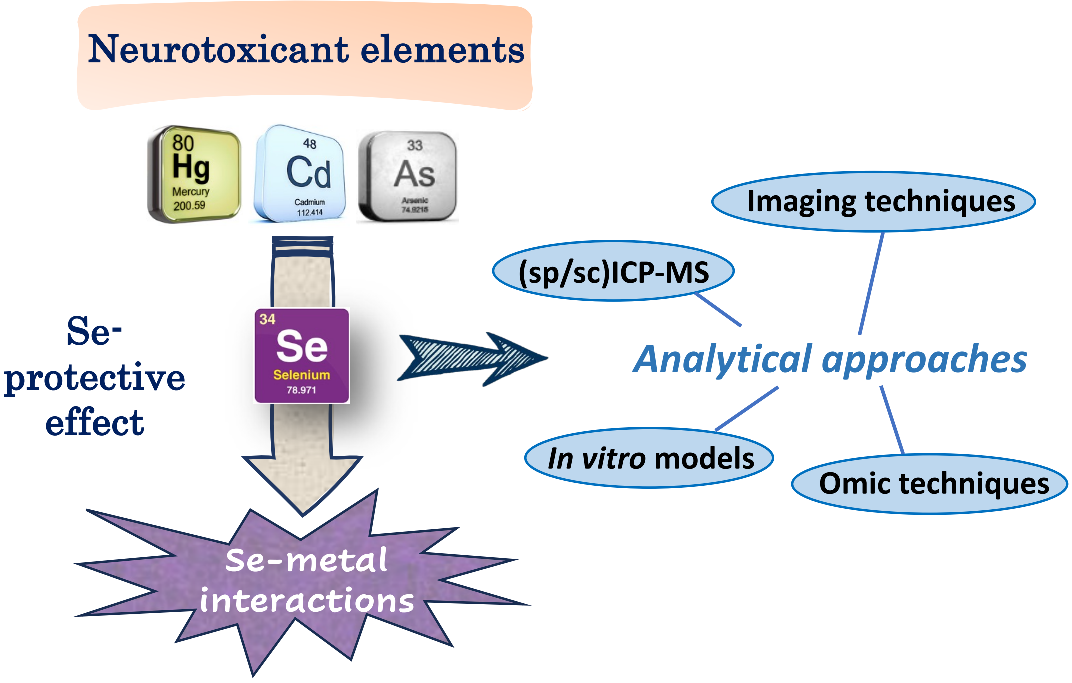

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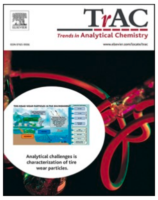

(Bio)analytical approaches for investigating the role of selenium in preventing neurological disorders and neurotoxicity induced by environmental neurotoxicants: As, Cd, and Hg

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ABSTRACT

There is increasing evidence that dysfunction of essential metal (Cu, Zn, Fe, Mn) homeostasis can lead to the appearance of several NDs. Other neurotoxic environmental metal/metalloids such as As, Cd and Hg contribute to neurological diseases by interacting with neurotransmitter receptors and protein binding sites of essential elements. Analytical chemists do not remain indifferent to the impact of neurological diseases. The development of analytical techniques to evaluate the role of endogenous and exogenous metals metals/metalloids in the development of neurogenerative diseases has increased in the last few years. This review summarizes the state of the art of the (bio)analytical techniques most applied together with *in vitro* models to evaluate the role of elements associated with neurological disorders with special emphasis on the role of selenium in neurodegeneration and in preventing the neurotoxic effects produced by environmental neurotoxins, in particular Cd, Hg and As.

1. Introduction

Neurogenerative diseases (NDs) are chronic diseases characterized by a damage or loss of the structure of neurons and a progressive deterioration of brain functions. This degradation can lead to physical disability and cognitive alterations (memory, language, calculation, behaviour, learning and emotion capacities) and even death. The most common NDs are Parkinson's (PD), Alzheimer's (AD), Huntington's (HD), prion's (transmissible spongiform encephalopathies) diseases, Amyotrophic Lateral Sclerosis (ALS) and Multiple Sclerosis (MS) among others, being Alzheimer's and Parkinson's diseases those that occur most often [1].

Although aging is considered the most prevalent factor for the appearance of NDs, other factors can contribute to the occurrence of these diseases such as: genetic, environment (neurotoxins such as metals and metalloids), biological (aggregation, abnormal folding, and accumulation of proteins), metabolic (oxidative stress, lipid peroxidation protein oxidation, DNA and RNA oxidation) and autoimmunity. Among the causes of NDs, protein aggregation, mitochondrial dysfunction, and oxidative-stress induced damage is recurrent phenomenon in many of the neurogenerative alterations [2,3].

Metals ions are broadly distributed in brain and play an important role in maintaining brain biological functions and central nervous system (CNS). Metals like copper (Cu), iron (Fe), zinc (Zn), manganese (Mn), and magnesium (Mg) are called "neurometals" and have significant roles in the appearance of neurological disorders as they are involved in the development, metabolisms, redox and synaptic transmission of CNS. Dysregulation of metal homeostasis (metal deficiency or excess) can cause different degrees of damage to the CNS [4,5]. Environmental pollution has also an impact in the development of neurological disorders. Arsenic (As), cadmium (Cd) and mercury (Hg) are considered as environmental neurotoxicants as they can contribute to the occurrence of NDs such as AD and PD. These environmental neurotoxic elements can interact with neurotransmitter receptors and with the protein binding sites of essential elements (Fe, Zn, Cu) [6,7].

Another trace element with antioxidant properties and with a relevant role in brain function is selenium (Se). The health-promoting benefits of Se are due to the relevant functions of selenoproteins in which Se is present as selenocysteine. Se levels in brain are around 90-110 ng mg^{-1} wet. Interestingly, when Se is depleted, brain Se levels remain constant suggesting the important role of Se and selenoproteins in maintaining brain metabolisms and preventing neurodegeneration [8].

The incidence of NDs has growth over the years and it is expected to reach the 152 million of people affected by 2050 with the subsequent

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Abbreviations

Ach	acetylcholine.
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AsB	arsenobetaine
BBB	blood-brain barrier
Ch-SeNPs	SeNPs functionalized with chitosan.
CNS	central nervous system
Cys	cysteine
DA	dopamine
DBcAMP	dibutyryl cyclic AMP
DHE	dihydroethidium or hydroethidine
DIO	iodothyronine deodinase
DMA	dimethylarsenic acid
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
ESC	Embryonic stem cells
ESI	electrospray ionization
EtHg	ethylmercury
FLE	*Ficus lyrate* (fiddle-leaf fig)
FTICR	Fourier-transform ion cyclotron resonance
GABA	γ-aminobutyric acid
GC	gas chromatography
Gpx	glutathione peroxidase
$[(GS)_2AsSe]^-$	seleno-bis-(S-glutathionyl) arsinium anion
GSH	glutathione
HD	Huntington's disease
HgSeNPs	mercury selenide nanoparticles
HPLC	high-performance liquid chromatography
HPLC-ESI-QTOF-MS	high-performance liquid chromatography coupled to electrospray ionization quadrupole time of flight mass spectrometry.
ICP-MS	inductively coupled plasma mass spectrometry.
ICP-MS/MS	inductively coupled plasma tandem mass spectrometry.
IP	propidium iodide
iPSCs	induced pluripotent stem cells.
LA	laser ablation
LC	liquid chromatography
LDH	lactate dehydrogenase
LIT/LTQ	linear ion trap.
MALDI	matrix-assisted laser desorption/ionization
MDA	malondialdehyde
MeHg	methylmercury
MMA	monomethylarsenic acid
MS	Multiple Sclerosis
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NDs	neurogenerative diseases
NMDA	N-methyl-D-aspartate
Nrf2	nuclear factor erythroid 2-related factor
PBCECs	porcine brain capillary endothelial cells
PCR	polymerase chain reaction
PD	Parkinson's disease
Q	quadrupole
QIT	quadrupole ion trap
RA	retinoic acid (RA)
RNA	Ribonucleic Acid
ROS	reactive oxygen species
RPLC	reversed phase chromatography
(RT)-PCR	real-time quantitative reverse transcription
SC-ICP-MS	single cell-inductively coupled plasma mass spectrometry.
SEC	size-exclusion chromatography
SeIP	selenoprotein P
SeIW	selenoprotein W
SeMeSeCys	selenomethyl selenocysteine
SeMet	selenomethionine
SeNPs	selenium nanoparticles
SOD	superoxide dismutase
spICP-MS	single particle-inductively coupled plasma mass spectrometry
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TEER	trans-endothelial electrical resistance
TEM	Transmission Electron Microscopy
TOF	time of flight
TPA	12-o-tetradecanoyl-phorbol-13-acetate
TrxR	3-thioredoxin reductase
UV-Vis	ultraviolet-visible spectrophotometry
WHO	World Health Organization
XAS	X-ray absorption spectroscopy
XRF	X-ray fluorescence spectrometry

economic, social and health impacts [1]. Analytical chemists have not remained indifferent to the impact of neurological diseases. In this line the development of analytical techniques to evaluate the role on endogenous and exogenous elements in the development of NDs has increased in the last years. Different analytical techniques are applied to investigate and understand the role of elements on neurological diseases [4]. Inductively coupled plasma-mass spectrometry is the technique of choice for determining elements at low levels in biological matrices (at $\mu g\ L^{-1}$ or $pg\ L^{-1}$) [9]. In the last years single-particle-ICPMS (spICP-MS) and single-cell-ICP-MS (scICP-MS) have emerged as powerful techniques for assessing quantitatively the impact of metal/metalloid-based nanoparticles and metal/metalloid in living systems [9]. On the other hand, LC-ICP-MS coupling has been extensively employed for assessing trace element speciation in biological matrices. Of special relevance has been the application of molecular mass spectrometry coupled to LC such as HPLC-ESI-MS/MS and high-resolution mass spectrometry (HPLC-ESI-QTOF-MS and HPLC-ESI-Orbitrap-MS instruments) for quantification and structural assignment of metallobiomolecules, metabolites and proteins [4]. Finally imaging techniques such electron microscopy or laser ablation ICP-MS (LA-ICP-MS), among others have allowed researchers to map metal distribution in tissues and cells [10].

Apart from the analytical approach, essential biological properties such as antioxidant activity (radical scavenging activity), cell viability (when using *in vitro* models) and neuroprotection capacity need to be addressed when evaluating the effect of different elements in brain function.

Consequently, this critical review recapitulates the state of the art of the (bio)analytical technique and *in vitro* methods most applied to evaluate the role of elements associated to neurological disorders with special emphasis on the role of Se in NDs and in preventing the neurotoxic effects produced by environmental neurotoxins such as metals, in particular Cd, Hg and As. To the best of our knowledge, there are no review paper directly addressing the use of bioanalytical techniques for investigating the role of selenium in preventing toxicity induced by environmental neurotoxicants Therefore, there is a need to present existing and future perspectives of this topic which we do believe of interest for both general and specialized chemical researchers. Understanding how As, Cd, Hg affects the brain, and the protective effect of Se is key to carry out accurate environmental and health risk. Given the persistent nature of these elements, their threat to human health and their role on the development of neurological disorders cannot be ignored.

2. Role of Se in the appearance of neurodegenerative diseases. Protective effect against the toxicity of environmental neurotoxic metals/metalloid such as As, Hg, Cd

Se is an essential element for human beings. The health promoting benefits of Se are related to the relevant role of Se proteins in which Se is present as selenocysteine, the 21st amino acid. To date dozens of selenoproteins families have been described although some of their functions have not been elucidated yet. The human selenoproteome contains 25 known selenoproteins with 5-SeCys containing Gpxs, 3-thioredoxin reductases (TrxRs), iodothyronine deiodinases (DIOs), selenoprotein P (SeIW), selenoprotein W (SeIW), as the most evaluated. Their functions are related to oxidative stress, thyroid hormone productions and metal detoxification, among others. Consequently, selenoproteins are implicated in a variety of diseases: cardiovascular diseases, thyroid disfunction and neurogenerative diseases [11]. SelP, Gpx and SelW are the three most highly expressed proteins in brain, suggesting that they may play an important role in brain functions [9]. In this line there is a growing interest in investigating the effects of Se on central nervous system [12, 13].

Se is essential for maintaining brain metabolisms. Se levels in brain is about 90–110 ng mg^{-1} wet. In cases of Se-deficiency, the human organisms maintain the Se level in brain by mobilizing Se from other organs. Interestingly, when Se is supplemented in combination with other essential elements, most of the other elements enter preferentially in the brain [14]. All these facts evidence the key role of Se in maintaining the correct metabolisms of brain. Se deficiency could lead to neurological disorders. For instance, plasma Se levels and Gpxs activity are significantly reduced in patients with AD disease. Parkinson disease has been also correlated with selenoproteins levels [8]. In a recent study, the highest concentration of SelP in serum was associated with a decrease of Aβ-amyloid content in serum [15] and some authors have suggested SelP in cerebrospinal fluid and blood as a biomarker related to dementia and cognitive aspects [16]. The dopamine pathway seems to be also dependent on Se status [17].

Se supplementation has been demonstrated to restore levels of Gpxs and SelP in brain. Selenite, SeMet and SeMeSeCys are the most common forms of Se used in Se supplements and their effect may be different. All these selenocompounds have shown to increase Se level in brain and antioxidant capacity in AD mice. However, SeMeSeCys notably enhances the activity of TrxR and reduces phosphorylation of the protein tau while SeMet greatly affects Gpx which is involved in the decrease of the Aβ-amyloid production [18]. Se behaves different in PD disease. Selenite provides a better neurological protective effect in PD mice than SeMet. Selenite seems to increase the activity of Gpx but not SeMet suggesting the protective effect of inorganic Se in preventing dopaminergic neuronal loss [19].

The conventional Se supplementation with organic and inorganic Se presents the drawbacks of low degree of absorption and a narrow margin of safety. In the last years Se nanoparticles (SeNPs) have attracted the interest as therapeutic agent due to its low toxicity, wide safety margin and increased permeability through gastrointestinal tract and blood-brain barrier (BBB) [20]. Moreover, the possibility of modifying the surface of SeNPs with functional groups with different properties (polyphenols, polyshacarides, proteins, peptides, and drugs) have allowed to produce SeNPs with enhanced bioactivity [21]. In this line, Se functionalized nanoparticles (SeNPs) have been synthesized for the therapy of neurodegenerative diseases [22] such as spinal cord injury [23], Huntington's disease [24] or Alzheimer's disease [25]. The application of curcumin-loaded SeNPs [26] and resveratrol-loaded SeNPs [27] has reduced the accumulation of Aβ-protein in the brain of AD mice and thus inhibiting Aβ-plaque aggregation and inflammation. SeNPs modified with glycine have improved the oxidation stress and the behaviour disorder of PD rats and decreased the loss of dopaminergic neurons [28]. *Ficus lyrata* (fiddle-leaf fig) extract (FLE) conjugated with Se nanoparticles (FLE-SeNPs) have shown an important neuroprotective action against $AlCl_3$-induced neurotoxicity by reversing oxidative damage and neuronal inflammation in rats exposed to $AlCl_3$ [29] while SeNPs alleviate Cd-induced cerebral damage trough regulating the expression of metal transporters and selenoproteins [30]. Biogenic SeNPs produced in presence of *Lactobacillus casei* ATCC 393 (*L. casei ATCC* 393) have effectively attenuated the Aβ25–35-induced toxicity in PC12 cells along with an increase of the oxidant capacity and an inhibition of ROS production [31]. The combination of SeNPs enriched with *L-Casei* also presents a regulatory effect on microbiota-gut-brain axis and the combination has been postulate as a food additive to prevent cognitive dysfunction due to AD [31].

Se also presents an important role against the toxicity of environmental neurotoxic elements such as As, Hg, Cd. Se acts as an antioxidant, helping protect against the harmful effects of As and Cd by counteracting the generation of ROS and reducing oxidative stress provoked by their presence [32,33]. In case of Hg, Se mitigates its adverse effects by participating in the formation of selenoproteins, including the enzyme Gpx, which has antioxidant properties and protects against mercury-induced oxidative stress [34]. However, it is important to note that the magnitude of these effects may depend on various factors, such as the relative concentrations of the elements, the chemical forms involved and long-term exposure [35,36].

In case of As, it has been demonstrated that Se reduces its toxicity in living organisms by interfering with its absorption, distribution, and excretion [37]. This is because Se competes with arsenic for the same binding sites in proteins and enzymes, thereby reducing its toxicity. They can also compete for the same transport and metabolism mechanisms in organisms, which means that high levels of one of these elements can decrease the absorption or biological availability of the other [38].

The formation of As-Se complexes, such as the seleno-bis-(S-glutathionyl) arsinium anion, $[(GS)_2AsSe]^-$, which is an inert compound formed in the bloodstream that is rapidly excreted from the liver into bile [37], plays a crucial role in the As detoxification process mediated by Se. $[(GS)_2AsSe]^-$ has been identified in the bile of rabbits [39,40] and rats [41].

Although the mechanisms of the interaction between Se and Cd through the formation of the Se-Cd insoluble complexes have not yet been elucidated, it is known that the appearance of Se-Cd complexes reduces the toxicity of Cd since its interaction with neuronal tissue is limited [42]. However, not only Cd-induced toxicity is decreased by Se-Cd interactions, but it has been also proved that toxic doses of Se (in form of selenide) can be alleviated through these biologically inert Se-Cd complexes, showing therefore synergistic effects [43].

Related to Hg, the key factor in the interaction between Se and Hg resides in an adequate Se:Hg molar ratio ≥ 1. Once the interaction occurs and a part of the Se is being sequestered by Hg, there must be enough remaining Se bioavailable for developing the corresponding biological functions of Se in an organism [44]. Hg has high affinity for Se ($ka = 10^{45}$, being approximatively 1 million-fold higher than for sulfur) and can specifically sequester it, especially in brain, decreasing therefore the selenoprotein synthesis. Nevertheless, the remaining sources of Se will maintain the Se supplies in brain up to 60 % [44,45]. Due to the complementary perspective of the Se-Hg interaction, it can be affirmed that Se protects against Hg toxicity, while, although seemingly opposite, Hg affects Se bioavailability reducing the activity of numerous Se-dependent enzymes.

As a result of this interaction, insoluble mercury selenide compounds are formed, including bis-[methylmercuric]selenide, selenocysteine-bound MeHg, selenoprotein P-bound HgSe clusters, and $HgSe_xS_{1-x}$ biomineral [46]. HgSe nanoparticles (HgSeNPs) are thought to be an end product of Hg detoxification formed by inorganic Hg binding Se after demethylation of MeHg [47]. HgSeNPs have been identified in different tissues of a wide range of animals: marine mammals (liver and pituitary tissues of beluga whale [48], liver, kidney, lung, spleen, pancreas, muscle and brain of striped dolphin [49], liver and brain of long-finned pilot whales [50] and liver of northern fur seal [51], seabirds (liver, kidney, brain and muscle of giant petrels [47,52], terrestrial birds of prey (liver of golden eagles [53], seafood (muscle tissues of fish species and shellfish [54] or even in human brain [55] and bladder stones [56].

Table 1 summarizes the mechanisms of neurotoxicity induced by Cd, Hg and As and their interaction with Se.

Due to all the beneficial Se properties and protective effects against toxic metals, potential applications of Se as protective element have been widely developed [22,66–68].

Understanding how As, Cd, Hg affects the brain, and the protective effect of Se and their role on the development of neurological disorders cannot be ignored and it requires the development and application of a plethora of (bio)analytical techniques to elucidate the mechanisms involved and to stablish a proper health and environmental risk assessment.

3. Analytical and bioanalytical approaches and *in vitro* assays to evaluate trace elements with neurotoxic effects

As it was previously indicated the elucidation of the mechanisms ruling the incidence of endogenous and exogenous elements on the development of NDs requires the application of a plethora of analytical techniques that provide different by complementary information. In the current section, an overview of the (bio)analytical techniques and *in vitro* methods most applied to evaluate the role of elements associated to neurological disorders, with special emphasis on the role of Se in NDs and in preventing the neurotoxic effects produced by As, Cd and Hg are presented and critically discussed. Table 2 compiles the (bio)analytical techniques discussed throughout this section.

Table 1
Mechanisms of neurotoxicity induced by Cd, As and Hg and the role of Se in preventing their toxic effects.

Neurotoxicant element	Mechanisms of neurotoxicity	Interaction with Se	References
Cd	- Increase of oxidative stress - Excessive generation of ROS - Essential metal disruption - Dysregulated Ca^{2+} homeostasis - Interaction with sulfhydryl groups	- Formation of Se-Cd insoluble complexes	[33,38,43,57–59]
As	- Increase of oxidative stress - Excessive generation of ROS - Inhibition of Nrf2 pathway → decrease of antioxidant defense. - Dysregulated Ca^{2+} homeostasis - Abnormal mitochondrial dynamics - DNA damage	- Competition for the same binding sites in proteins and enzymes - Selenium stimulates Nrf2 pathway → increase in Gpx, TrxR and SOD levels. - Formation of Se-As complexes: $[(GS)_2AsSe]^-$	[38–41,60–62]
Hg	- Increase of oxidative stress - Excessive generation of ROS - Dysregulated Ca^{2+} homeostasis - DNA damage - Disruption of selenoprotein synthesis - Interaction with sulfhydryl groups	- Increase of GSH synthesis - Selenium protects against Hg-induced oxidative stress. - Se-Hg interaction (Se:Hg molar ratio > 1) - Formation of insoluble Se-Hg compounds (HgSeNPs)	[44–47,63–65]

3.1. Cell models in neurotoxicity studies

In vitro neurotoxicity studies almost entirely rely on cell models. They constitute a first approach to study potential neurotoxicants and raise evidence of possibly mechanism of action and toxicity, which is quite difficult when using whole animals. *In vitro* assays offer the possibility to control the time of exposure and study the mechanistic processes under controlled and isolated conditions, giving information about cellular and molecular disruptions [92]. Current animals models lack of many essential human characteristics (human brain has a physiology very complex and different from other animal brains and with a unique pathology conditions of human neural diseases) in addition they are expensive, and their use raise ethical concerns.

In vitro neuronal systems present strong advantages, such as lack of ethical issues (except for human embryonic cells), easy manipulation and protocols, reduced costs because of the infinity lifespan of cell lines,

Table 2
Main (bio)analytical techniques for investigating the role of Se in preventing neurotoxicity induced by As, Cd and Hg.

(Bio)analytical technique	Measured parameter	Purpose of the analysis	References
Flow cytometry MTT assay / LDH assay	Cell death/ toxicity	Evaluation of the effect of elements in brain function	[69–72]
TBARS assay DPPH assay	Antioxidant capacity		[73]
Spectrophotometry methods using fluorescence and chemiluminescence probes	Reactive oxygen species (ROS)		[74]
ICP-MS	Total element quantification	Evaluation of elements contents at low levels in biological matrices	[4]
HPLC-ICP-MS	Chemical form of the target element		[10,75]
LA-ICP-MS	Elemental distribution from bioimaging		[4,9,75]
spICP-MS	NPs identification through quantitative parameters	Quantitatively assessment of the impact of elements-based NPs and elements in living systems	[76–80]
SC-ICP-MS	Accumulation and element uptake at single cell level		[81–83]
TEM	Map metal distribution in tissues and cells	Explore the internal structure of tissues, cells and organelles	[69,83]
Molecular mass spectrometry	Biomolecules quantification / Protein or peptide sequencing	Structural assignment and quantification of metallobiomolecules metabolites and proteins	[84–86]
Western blotting	Separation and identification of proteins	Information about molecular mechanisms through protein expression in biological matrices	[87,88]
Agarose gel electrophoresis / Comet assay	Genome DNA fragmentation	Evaluation of metal-induced DNA damage	[89,90]
PCR			[91]

short-life experiments, direct interactions neurotoxic-cell, exact measurements of cellular responses to neurotoxicants and the possibility to manipulate cellular environment [92–94]. *In vitro* neural systems suffer from several drawbacks that are lack of metabolizing activity (impossibility to identify metabolites), difficulties in mimicking microenvironment disturbances (studies are mostly based on non-real conditions of isolated unaltered neurotoxic compound without interactions with other elements), lack of heterogeneous cell to cell interactions or limited representation of the complexity of the nervous system [92,93,95].

The most common *in vitro* models are primary neuronal cultures, immortalized cell lines derived from neuronal or glial cells, co-culture, subcellular system such as isolated mitochondria or three-dimensional systems and BBB models, to name but a few [92,96–98]. Human neurons are differentiated into several subtypes such as cholinergic, adrenergic, dopaminergic, and GABAergic as most neurological diseases target one of these specific subtypes [99]. Consequently, a correct selection of the cell line for performing *in vitro* models is of paramount importance to get reliable results.

SH-SY5Y, a catecholaminergic human neuroblastoma cell line, is one of the most used cell lines for neurotoxicity studies. This *in vitro* model can be used either as immortalized human cell line or differentiated into a more relevant disease-specific phenotype by using differentiation-agent such as retinoic acid (RA), 12-o-tetradecanoyl-phorbol-13-acetate (TPA) or dibutyryl cyclic AMP (dBcAMP) [91,100–106]. These cells have been widely applied to study mechanisms of neurodegeneration. Branca et al. [104] evidenced differences in Se neuroprotection against Cd toxicity when using undifferentiated or differentiated human neuroblastoma SH-SY5Y cell line. Se treatment in undifferentiated cells co-exposed with Cd had beneficial effects increasing cell viability, whereas same conditions did not evidenced protection against Cd-induced neurotoxicity in differentiated cells, concluding that Se protection is dependent on the neuronal phenotype [104]. SH-SY5Y cell model has also been used for unravelling the mechanisms through which MeHg disrupts the expression of selenoenzymes, especially the selenoprotein W (SeW) [91]. SK-M-MC is another human neuroblastoma cell line used in neurodegenerative disease studies, which has predominately cholinergic phenotype. Vicente-Zurdo et al. [101] employed apart from SH-SY5Y cell line, mouse neuroblastoma cell line Neuro-2a to evaluate the neuroprotective activity of Se nanoparticles and selenoaminoacids (SeMet, SeMeSeCys and $SeCys_2$). MES23.5 and N27 cells represent other animal-derived neuronal cells used as *in vitro* models in neurotoxicity research. MES23.5 is a product of somatic fusion of rat embryonic mesencephalon cells and a neuroblastoma-glioma cell line whereas N27 cell line is an immortalized cell line derived from TH-positive fetal rat mesencephalic neuros [100].

Non-neuronal cell lines such as human immortalized ovarian carcinoma cells, HeLa, and hepatocyte lines, HepG2 and HepaRG cells, Human embryonic kidney 293 (HEK293), and rat pheochromocytoma (PC12) are also used as models for neurotoxicology assessment when they are exposed to specific neurotoxins [99]. Particularly, PC12 is widely used for neurotoxicity studies since, although PC12 is not isolated from a neuronal tumor, exhibits several characteristics of mature dopaminergic neurons [100,107]. Indeed, PC12 cells have been used for investigating the protective effects of Se on Hg-induced cytotoxicity [108], As-induced cytotoxicity [109] and Cd-induced cytotoxicity [32].

In vitro studies based on neuronal lineages derived from human stem cells or human neuronal precursors is a step forward in more complex and functionally active neuronal models. Stem cells can undergo differentiation and maturation in a similar way to how animal models behave. In this sense, human stem cells including embryonic (ESC) collected from blastocysts and induced pluripotent stem cells (iPSCs) derived from fibroblasts of human patient are widely used as *in vitro* system for assessing neurotoxicity [110]. Between them, iPSCs is reported to be one of the preferred cell models to simulate the complexity and functionality of *in vivo* neurological structures since they are relatively easy to collect and account for genetic variations among human patients [111]. Furthermore, ethical, and regulatory aspects implicit in the use of human ESC can be avoided [112]. Moreover, iPSCs can be differentiated into neural stem cells and later in glutaminergic, dopaminergic and GABAergic neurons [113–115]. For example, Prince et al. [112] considered iPSCs as a promising model to evaluate the selective neurotoxicity of methylmercury. However, the use of stem cell is still hampered by the need of applying very labour-intensive and costly procedures [116].

Lately, the use of 3D cells models has grown in interest and adds another level of complexity to neurotoxicity studies. These models include multiple cell types in a single cellular model, for example coculturing neurons with glial cells [99]. This approach favours a better replication of some aspects of the brain environment such as neuronal and glial cell interactions and incorporating the effects of blood flows [110], however.

3.2. *Cell-based in vitro blood-brain barrier models*

The human BBB controls the interplay, communication and molecular trafficking between the central nervous system and the periphery. Furthermore, astrocytes, vascular endothelial cells and pericytes are the cells that constitute the BBB and they are all grouped in what is known as neurovascular unit which is responsible of the regulation of cerebral blood and, by consequence, neuronal activity [117]. It has been reported that the transport of many substances proposed as therapeutic agents for brain-related diseases across the BBB is severely hindered [118]. Therefore, understanding the effect of chemical substances on BBB permeability, integrity and accumulation is a key aspect for developing novel therapies to fight against brain diseases and to better understand neurological disorders.

Cell-based *in vitro* models have been developed to elucidate the ability of certain substances to cross de BBB and how they affect to its integrity. In this sense, the Transwell-based models have been widely employed over the last decade as cell-based *in vitro* BBB models to study the ability of certain substances to cross the BBB [118]. Transwell device consists of two compartments: the apical and the basolateral zones. The apical (upper compartment) reproduces the lumen of the vessel and therefore mimics the blood side. The basolateral compartment mimics the parenchymal side of the BBB representing the nervous tissue (brain) (Figure 1) [119,120]. Both areas are separated by a membrane, frequently composed of a polycarbonate material, to recreate the architecture of the BBB and compartmentalize the cell culture [119,120]. Cells built up a tight monolayer on the membrane surface comparable to the epithelium of a brain micro vessel. It is important to highlight that, in this model, the co-culture of the main cell types of the BBB,

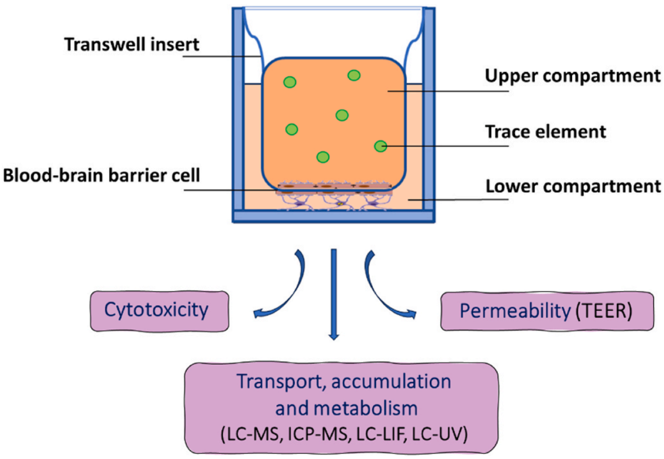

Fig. 1. Representation of a Transwell-based model employed as a cell-based *in vitro* BBB model and the analytical information that it provides.

endothelial cells, pericytes, and astrocytes, as well as the neurons of the brain tissues are integrated.

The substance under study is usually placed in the apical side of the cell barrier (cells grown on the Transwell insert). Once the incubated period has completed, some parameters such as membrane integrity, permeability, and/or the amount of the substance that has passed to the basal side or remained in the apical zone are monitored.

Methods for assessing the permeability are based on measurement of trans-endothelial electrical resistance (TEER) or monitoring the transport and accumulation of certain compounds between apical and basolateral compartments. Electrical resistance can be also used as a way of measuring the integrity of the barrier since gives information about the resistance of ions to be transported through a cell. TEER measurement is based on ohmic resistance or determining impedance across a wide spectrum of frequencies employing commercially available measurement systems or with custom built microfluidic implementations [121]. Highly sensitive analytical techniques are also required to follow the transport, accumulation, and metabolism of compounds of interest across the cell monolayers [122]. In this sense, separation techniques (chromatography and capillary electrophoresis) coupled with different detectors such as UV-Vis, laser-induced fluorescence, mass spectrometry or electrochemical detection are employed [122].

Moreover, in case of trace elements ICP-MS is the most widely employed technique. For example, Drobyshev et al. [123] investigated the capability of different Se species (selenite, selenoneine, and SeMeSeCys) to cross the BBB using primary porcine brain capillary endothelial cells (PBCECs) in a Transwell-based model and using ICP-MS as analytical technique for quantification purposes. Transfer studies indicated transport of selenite, selenoneine and SeMeSeCys from the blood-facing to the brain facing side being SeMeSeCys which exhibited the fastest and the most efficient transport. Additionally, the application of HPLC-ICP-MS evidenced no transformation of selenoneine in its methylated form during the transferred period by the endothelial cells [123]. Similarly, Lohren et al. [124] applied *an in vitro* blood-brain barrier based on Transwell using PBCECs primary culture for studying the transfer properties across the barrier model of MeHg, thiomersal and $HgCl_2$. ICP-MS was used for quantifying total Hg contents on the apical and basolateral side of the Transwell-based model. Results evidenced that $HgCl_2$-induced effects on the barrier integrity were comparable to that of organic mercury species. Moreover, authors concluded that MeHg and thiomersal crossed the barrier in both directions with a slight accumulation in the basolateral side and they provided the first evidence that BBB might transfer $HgCl_2$ out of the brain. On the other hand, analytical methods for determining gene and protein expression have been also used for elucidating the effect of arsenic exposure through drinking water to the BBB, such as Evans blue permeability assay, real-time PCR or western blot analysis [125].

3.3. Cell death assays/cell-based toxicity assays

It has been reported that one of the causes of the neurological disease is the death of nerve cells within the brain. Such cases have appeared in Alzheimer's disease [1] or in specific areas of the brain, for example in dopaminergic neurons located on subcortical structures of the brain in Parkinson's disease [99]. Thus, investigating neuronal cell lines viability can be used to assess neurotoxicity and to investigate the potential of certain compounds to be used as neuroprotective agents.

Cell health and growth can be determined by quantifying cell viability, proliferation, or apoptosis. Cell viability assays are frequently based on cell viability dyes which allows the quantification of dead cell by flow cytometry or spectrophotometry [69,126].

Flow cytometry is a powerful technique that provides information about different parameters of single cells from cultures in solution based on their fluorescent or light scattering characteristics. Flow cytometers employ lasers as light sources to produce both scattered and fluorescent light signal that are read by detectors such as photodiodes or photomultiplier tubes and converted into electronic and readable signals [127]. Moreover, it is uniquely suited to track the progression of apoptosis [128,129].

Viability dies employed for flow cytometry analysis are unable to penetrate intact cell membranes and, thus, only label dead cell by binding to DNA such as propidium iodide (PI); or by the binding of a dye to amines within a cell to determine if the cell membrane is intact [127]. Moreover, the detection of apoptosis by flow cytometry resides in multiple targets related to events associated with this programed cell death. For instance, the translocation of the plasma membrane during apoptosis can be monitored by means of using the Annexin V staining or the activation of Caspases can be targeted by using antibodies and dies [127].

Another widely used cell toxicity assay is based on the reduction of tetrazolium salts by metabolically active cell which is followed by a colorimetric-based measurement. In this assay, MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), a water-soluble tetrazolium salt, is reduced to a violet water-insoluble molecule called formazan by mitochondrial enzymes, being therefore a reliable indicator of the cellular metabolic activity. The violet water-insoluble formazan produced is then solubilized by a solvent such as dimethyl sulfoxide (DMSO) and its absorbance is measured at a wavelength which MTT-derived formazan absorbs the most (around 570) using a UV-Vis spectrophotometer [70]. The absorbance values of formazan are directly associated with intracellular reduction of MTT. This assay has been used during decades as a rapid and easy tool to study cell proliferation/viability, drug cytotoxicity, and mitochondrial/metabolic activity of cell [130]. However, MTT assay presents some strong drawbacks, such as chemical interferences (thus leading to inaccurate results) and limitations in detecting low cell numbers. Moreover, the conversion to formazan crystals depends on metabolic rate and number of mitochondria [131,132].

Cell death can be also studied by detecting certain metabolic enzyme such LDH. LDH is a cytoplasmic enzyme that is involve in the interconversion of pyruvate to L-lactate and NADH to NAD^+ during glycolysis, and the reverse reactions during the Cori cycle. This enzyme is released from the cytoplasm into the extracellular environment in response to cellular damage and toxicity [71,72]. The detection of LDH in cell culture is carried out through enzymatic reactions where LDH oxidizes lactate to pyruvate producing NADH which subsequently reacts with iodonitrotetrazolium chloride to form red water-soluble formazan. This compound can be detected using an UV-Vis spectrophotometer by measuring the absorbance at 490 nm [33,108,133]. The intensity of the absorbance measured correlates directly with the amount of LDH in the cell culture supernatant, which is, in turn, proportional to the number of dead or damage cells. Moreover, commercial kits are also available for LDH assay [134]. This is one of the most employed assays for studying the cytotoxicity induced by metals and metalloids.

The methods described above for assessing cell viability have been widely used to study the neuroprotective effect of Se against As, Cd and iHg. Hossain et al. [33], and Rahman et al. [109] reported the decreased in LDH levels found in cell culture supernatant after the co-exposure of Se with Cd and As, respectively. Similarly, Hossain et al. [108], by using flow cytometry analysis and LDH activity assay, found that Se had a defensive effect on iHg-influenced toxicity by increasing cell viability and avoiding cell membrane damage in PC12.

3.4. Antioxidant and oxidative stress reducing capacity

Oxidative stress and ROS production are involved in neurological diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis. It has long been suggested that the accumulation of ROS results in cell components damage (nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins) and that the imbalance between the generation of free radicals and ROS may be involved in the pathogenesis of the neurodegenerative disorders

[135–137].

Free radicals are generated from both endogenous and exogenous sources. Among the main endogenous sources are inflammation, infection, cancer, mental stress, aging or immune cell activation, while exogenous sources involve exposure to toxic elements (Cd, Hg, Pb, Fe, and As) and other environmental pollutants that enters the organisms and produce free radical as by-products when they are metabolized [138,139].

There are several methods to determine the antioxidant capacity of the molecules, such as thiobarbituric acid reactive substances (TBARS) assay and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.

DPPH$^{\cdot}$ scavenging assay is based on the reduction of DPPH$^{\cdot}$ to DPPH-H by an antioxidant. The DPPH$^{\cdot}$ radical is stable in the dark and presents a violet color, showing an absorption maximum at 515 nm. In the presence of increasing concentrations of antioxidant, the DPPH$^{\cdot}$ is reduced by protons given up by the antioxidant, turning from a less intense violet to yellow, indicating the presence of the DPPH-H species [73].

TBARS is a method to detect lipid peroxidation that consists of the measurement of a cleavage product of an endoperoxide of unsaturated fatty acids resulting from the oxidation of lipid substrates, the malondialdehyde (MDA). When MDA reacts with thiobarbituric acid (TBA) in the presence of heat and acid medium forms MDA-TBA$_2$ (with a stoichiometry 1:2), a pink chromogen that is measured spectrophotometrically at 532 nm. In the presence of antioxidant, the formation of the pink complex is reduced, thus decreasing the intensity of its absorption band [73].

Vettori et al. evidenced a significant increase in the levels of lipid peroxidation in PC12 cells exposed to MeHg by TBARS assay [140]. Moreover, Bovio et al. [141] treated SH-SY5Y cells with CdCl$_2$ for studying oxidative stress as one of the main mechanisms of Cd toxicity. By using TBARS assay, the occurrence of lipid peroxidation in cells treated with Cd was detected and they stated that this increase in oxidative stress level in Cd treated cells could possibly affect energy metabolism.

Brandao et al. [142] studied the effect of Se(IV) on Hg(II)-induced lipid peroxidation when co-exposure of both compounds in blood, showing that did not alter TBARS levels Glaser et al. [143] show similar results for Se(IV) and MeHg-induced neurotoxicity in mouse cerebral cortex, affirming that the co-treatment with both elements did not modify the increased levels of lipid peroxidation observed when single treatment with compounds.

With respect to ROS measurement, different techniques can be used. Among others, spectrophotometry methods or the use of fluorescence and chemiluminescence probes are the commonly ones. Fluorescent probes are often oxidant sensitive as they do not present fluorescent until being oxidized by oxygen species [74]. For instance, dihydroethidium (hydroethidine or DHE) is a well-recognized redox-sensitive fluorescent probe, which is oxidized by ROS and can be detected at 488 nm. Hossain et al. [108] measured ROS levels in PC12 cells exposed to inorganic Hg by using DHE staining, showing that iHg-induced cytotoxicity was due to ROS generation and oxidative damage. Moreover, ROS amount was lower when co-exposure with Se compared to the only Hg exposure.

3.5. Inductively coupled plasma mass spectrometry-based techniques

3.5.1. Total element concentrations in biological samples determined by inductively coupled plasma mass spectrometry

Dysregulation of metal homeostasis is reported to be associated with the progression of neurological diseases such as Alzheimer's, Parkinson's and Huntington's diseases, as well as amyotrophic lateral sclerosis [75]. Therefore, the study of metal distribution within different areas of the brain along with determining their content in biological fluids is crucial for understanding their role in neurological diseases but also for seeking unknown neuroprotective agents within trace elements. For this purpose, extremely sensitive techniques capable of identifying and quantifying metal and metalloids at low levels (μg L^{-1} or pg L^{-1}) as well as differentiating among their chemical forms are highly desirable. In this sense, ICP-MS is considered one of the most suitable techniques for detecting and quantifying elemental concentrations in biological samples due to its high sensitivity, precision, low limits of quantitation (at μg L^{-1} or pg L^{-1}), high linear dynamic range, capability of multi-elemental analysis, relatively short time of analysis and small amount of sample required [4]. When ICP-MS is hyphenated to a separation technique e.g. HPLC or electrophoresis, it enables determination of chemical species of elements. Chemical speciation analysis is indispensable in evaluating and understanding the role of elements in neurological diseases, and the impact of Se in preventing the neurotoxic effects of Cd, Hg and As [10]. Furthermore, in elements that poses at least two stable isotopes, ICP-MS allows the use of isotopic tracers to follow the metabolisms pathways [10]. Lately, ICP-MS/MS was used for the efficient removal of polyatomic interferences in the determination of P, S and Se, which significantly improved selectivity and limits of detection and importantly contributed to the determination of these biologically important elements in a variety of complex sample matrices [4]. Therefore, several authors have chosen ICP-MS for studying the presence of Ag, As, Ba, Ca, Ce, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Se, Pb, Rb, Sn, Sr, Ti, V, Y and Zn in brain samples and how their interactions affect the development of neurological disorders [144–149]. More recently, Oliveira et al. [150], compared the concentration of twelve metallic elements (Ba, Be, Ca, Co, Cr, Cu, Fe, Mg, Mo, Ni, Pb and Zn) in blood samples from patients with multiple sclerosis and healthy individuals through ICP-MS. In patients with multiple sclerosis, a reduction in Be, Cu, Cr, Co, Ni, Mg and Fe levels was found with respect to the control group, whereas concentration of lead in blood was significantly elevated in the patient group. Moreover, the use of ICP-MS is also indispensable in BBB studies to evaluate the ability of certain metal and metalloids compounds to cross the BBB which is crucial not only for investigating trace elements as neurotoxicants but also for studying their potential as neuroprotective agents [123–125,151].

3.5.2. Speciation analysis based on separation techniques coupled to inductively coupled plasma mass spectrometry (ICP-MS)

On the other hand, speciation analysis using chromatographic methods coupled to ICP-MS have a great potential for neurological studies since it allows to investigate the chemical form in which trace elements might accumulate in the brain and the potential association between metal/metalloid and biomolecules and its implication in the development of neurological diseases [10]. Among chromatographic methods, high-performance liquid chromatography (HPLC) with different separation mechanisms, namely, ion exchange (anion exchange and reversed-phase chromatography (RPLC)) and size-exclusion chromatography (SEC) are described to be the method of choice for brain studies [75]. In addition, capillary electrophoresis (CE) is another suitable separation technique for studying metallobiomolecules. For example, the combination of capillary electrophoresis with ICP-MS is also reported to study the concentration of elemental species bound to metallothioneins in the human brain [75]. Metallothioneins (MT) are cysteine-rich proteins which complex mainly with the metals Cu, Zn, and Cd and play a key role in the transport and storage of essential or toxic metals, being also involved in the detoxification of the latter. Particularly, Prange et al. [152] used CE-ICP-MS in a comparative study of the distributions of metallothioneins isoforms MT-1, MT-2 and MT-3 in brain samples taken from patients with Alzheimer's disease and from control groups in which Cu, Zn, Cd and S were monitored. Results evidenced differences in levels of MT-3 and MT-1 from patients with Alzheimer's disease compared to the control group.

Furthermore, Ajsuvakova et al. [153] performed the speciation of Fe, Cu, Zn and Mn in the serum of patients with Parkinson's disease and the results were compared with those obtained from control individuals.

The obtained data showed that despite the lack of significant group difference in total levels, metal speciation was significantly affected in Parkinson's disease patients. In particular, high concentration of low low-molecular weight species (amino acids)-bound copper were observed in Parkinson's disease patients which were associated with a significant decrease in cu/ceruloplamin copper producing free copper ions available to be coordinated with low-molecular weight species [153]. Likewise, levels of Mn-albumin complex in Parkinson's disease patients were 4-fold higher as compared to the respective value in the control group. In light of these results, the authors concluded that these differences could be considered as a sign of redistribution between high and low molecular weight ligands in patients with Parkinson's diseases [153].

On the other hand, Wang et al. [154] employed SEC, coupled to post-column isotope dilution ICP-MS to quantify Hg-containing protein fractions from the brain cytosol of female rats exposed to MeHg. Additionally, Cardoso et al. [155] studied the profiling of Se in the serum of Alzheimer's diseases patient using SEC-ICP-MS. Besides, speciation analysis using ICP-MS is also important to investigate transformation among different chemical species during the transport of metal and metalloids through the BBB since their potential neurotoxic or protective effects are going to depend on their chemical forms [123,156]. For example, arsenic neurotoxicity seems to be related with its inorganic forms overall with As (III) [123]; and the role of Se as neuroprotective agent appears to be associated with its organic forms mainly with the selenoamino acids and selenoproteins [11].

3.5.3. Bioimaging of tissues using laser ablation inductively coupled plasma mass spectrometry

However, the employment of ICP-MS, frequently after acid digestion of samples, has the drawback of losing spatial information of metal compound distribution, for example, in different brain areas [157]. In this sense, the use of laser ablation (LA) coupled to ICP-MS (LA-ICP-MS) enables direct analysis of solid samples. It is a powerful bioimaging technique that allows quantitative tracking of the spatial distribution of elements accumulated in the target tissues, for example in different brain regions. This makes it possible to study adverse effects caused by neurological disease processes. LA-ICP-MS is based on focusing a laser beam with short pulses (ns or fs) of high energy on the sample surface of a solid to generate small particles in an aerosol which is subsequently transported by a carrier gas (usually Ar, He or a mixture of both) to the plasma, where it is vaporized, atomized and ionized following mass spectrometric detection [4,9,75]. In order to acquire information on the distribution of elements from images obtained after LA-ICP-MS analysis, the use of appropriate software is required [158].

Nevertheless, quantitative imaging by LA-ICP-MS remains a challenge owing to the lack of matrix-matched certified reference materials for calibration [9]. In this sense, Liu et al. [157] proposed the use of isotope dilution (ID) technique for quantitative imaging of Cu and Zn in mouse brain sections by LA-ICP-MS. In turn, this technique could be also considered as a promising strategy to elucidate trace elements interactions which is crucial for neurotoxicity assessment. By the use of LA-ICP-MS, Liu et al. [157] investigated local distribution patterns of iHg and MeHg in brain tissue and other organs of mice after Hg and Se exposure, which enabled investigation of the Hg and Se interactions. The results seem to indicate that iHg mainly distributed in kidney, but also low levels were found in liver, spleen and brain, whereas in case of MeHg, low levels of Hg were found in kidney, liver and spleen but not in the brain. Moreover, an increase in Se level in all organs was observed in Hg and Se co-administration group which might indicate that Se promoted the absorption of Hg by organs [157]. Furthermore, LA-ICP-MS has been proposed for quantitative imaging of Fe and ferroportin (a transmembrane protein involved in iron transport) in Alzheimer's disease patients and healthy controls human brain sections, showing the potential of this technique to investigate neurological disorders [159].

3.5.4. Single particle inductively coupled plasma mass spectrometry (spICP-MS), a powerful tool for the determination of number concentration, size, and the number size distribution of inorganic nanoparticles

To date, inorganic nanoparticles have been used in the many industrial applications and personal care products. They are also increasingly used as carriers of drugs to target tissues in different medical applications for the diagnosis and therapy of various brain diseases and neurological disorders [160–162]. Nevertheless, studies in this regard are still in their early stage probably due to the existing analytical limitations for characterization and quantification of nanoparticles in the complex brain environment and the complexity of understanding nanoparticles biotransformation in living organisms [76]. This fact also hampers the assessment of (neuro)toxicity of nanomaterials, an indispensable issue that must be addressed since although they represent an important advance in several fields, they have also been reported to induce harmful effects in both living organisms and the environment [163]. In this sense, ICP-MS in single particle mode (spICP-MS) is a powerful analytical technique for both studying the potential of inorganic nanoparticles as neuroprotective agents and investigating their possible neurotoxic effects when they reach the organism unintentionally.

Briefly, spICP-MS is an analytical methodology which allows the identification of micro/nanoparticles in complex matrix and the determination of quantitative parameters such as mass and number concentration, at mass concentration levels down to the ng L^{-1}, as well as mean size and size distribution of nanoparticle dispersions. For spICP-Ms analysis highly diluted nanoparticle suspensions and very high data acquisition frequency are required to favour that just one particle reaches the plasma. Under such condition, nanoparticles are detected as individual pulses or events over a continuous baseline due to the background or the dissolved element. In this sense, the frequency of the pulses is associated with the number concentration of nanoparticles and the intensity of the events with the elemental mass content per nanoparticle, which, in turn, can provide information about the size of nanoparticle when additional information about its shape, composition and density is known (Figure 2) [77].

The fact that in spICP-MS analysis the nanoparticulate fraction (pulse or events) can be distinguished from the dissolved fraction of the element (continuous baseline signal), makes this technique remarkably suitable for the assessment of nanoparticles neurotoxicological effects. Namely, the toxicity of nanoparticles depends greatly on the ration between the nanosized and the dissolved elemental forms. Indeed, during last years some scientific articles which deal with nanoparticles neurotoxicity have been published [76,78–80]. However, most of them do not consider the contribution of ions from the dissolution process of nanoparticles to the neurotoxic effects.

Besides, spICP-MS was also applied in the investigations of the abilities of nanoparticles to cross the BBB and their transformations (the rate of dissolution, aggregation, etc.) during this process frequently studied by *in vitro* Transwell-based models. Changes in the properties of nanoparticles during BBB crossing could influence the way they exert neurotoxicity. Guo et al. [77] used an *in vitro* BBB model to explore the permeability of Ag, ZnO, CeO_2 and Fe_3O_4. Nanosized and the dissolved elemental fraction was determined by spICP-MS analysis. The results evidenced that the transport efficiency and number of particles passing through the BBB change significantly when the nanoparticles are prone to solubilize as it was the case of Ag, ZnO and CeO_2 nanoparticles. The study concluded that Ag and ZnO nanoparticles seemed to cross the BBB and enter the brain in the form of both particles and dissolved ions.

spICP-MS also constitutes a powerful strategy to detect the formation of micro/nano particles as consequence of interaction between neurotoxicants (Hg, Cd, As) and protective agents (Se) in brain tissues which may contribute to a better understanding on the role of trace element interactions in the development of neurotoxic effects. For instance, spICP-MS in combination with the complementary technique TEM allowed detecting HgSe micro/nano particles in brain tissues of giant

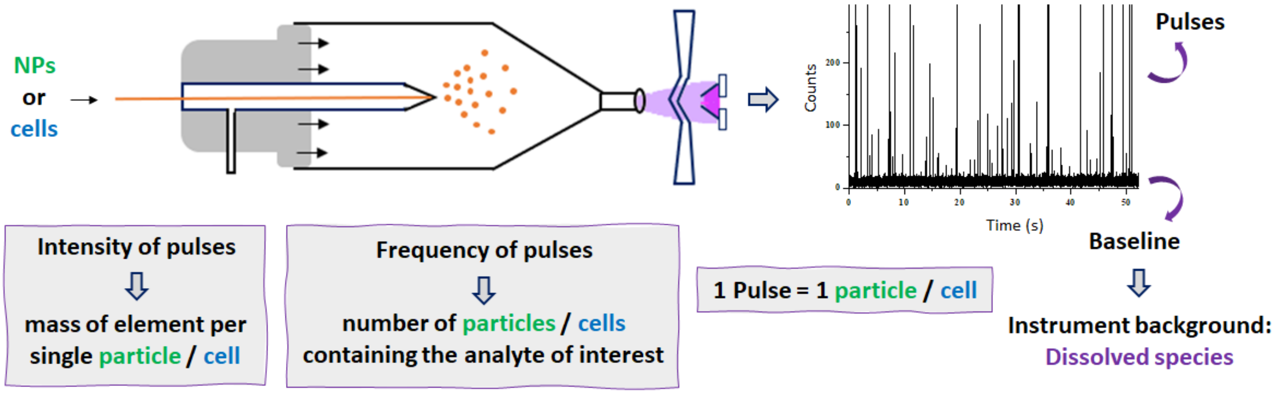

Fig. 2. Schematic representation of sp/scICP-MS fundamentals.

petrel (seabird) [53]. In addition, Cd particles associated with HgSe micro/nano particles were also observed [52].

3.5.5. *Single cell inductively coupled plasma mass spectrometry (scICP-MS) for analysis of elements at single cell levels*

Similarly, the use of ICP-MS in single cell mode (scICP-MS) could be of paramount importance for neurotoxicity studies as it enables to accurately determine the accumulation and uptake of neurotoxicant and neuroprotective agents at single cell levels in cell models considering the variability within cellular populations. The evaluation of cell-to-cell variations on stress resistance and/or element uptake can be a key aspect when evaluating the neurotoxicity or the outcome of a specific therapy [81].

scICP-MS is based on the same principle as the analysis of spICP-MS but in this case each registered event is the result of a single cell. In the appropriately diluted sample, the frequency of the events is directly related to the cell number concentration in the suspension and the intensity of the events is proportional to the mass of the monitored element accumulated by individual cells (Figure 2). Similarly, the intensity of the continuous baseline signal depends on the concentration of the element monitored in its dissolved form [81,82]. On the other hand, a key step for single-cell experiments is the selection of the sample introduction system since cells suspensions should be efficiently transported into the plasma maintaining the cell integrity and leading to high transport efficiencies. To achieve such conditions, two different approaches have been commonly used: the use of pneumatic nebulization-based sample introduction systems or laser ablation (LA-ICP-MS) to generate an aerosol of the cells previously fixed in solid surfaces [81].

For instance, the uptake and accumulation of different Se species, including SeNPs, in human (SH-SY5Y) and mouse (N2a) neuroblastoma cell lines related to Alzheimer's disease have been quantified at single cell level by using scICP-MS [101]. Authors found that high amounts of Se were accumulated by neuroblastoma cell lines when they were exposed to SeNPs in comparison with the Se accumulated when they were cultivated with selenoamino acids such as SeMet and SeMeSeCys. Moreover, the authors confirmed the high variability in terms of Se accumulation by neuroblastoma cell lines using the ICP-MS in single-cell mode. From our literature search, it appears that this is the first work in which scICP-MS has been considered for neurotoxicity studies. However, ICP-MS in single particle and single cell mode offers a great potential for determining the neurotoxic effects induced by metal and metalloids elements as ions and particulate forms, but also for elucidating their potential as neuroprotective agents.

3.6. *Transmission electron microscopy in neurotoxicity assessment*

As it has been described before, a common feature in many neurodegenerative diseases is the presence in the brain of abnormal aggregates of proteins, for example assembly of tau and Aβ amyloid fibrils in Alzheimer's disease and Lewy bodies containing filaments made of α-synuclein in Parkinson's disease. In this regard, Transmission Electron Microscopy (TEM) is a useful imaging technique with single cell resolution to investigate mechanisms of seeding and assembly of protein filaments associated to neurological disorders in presence of neurotoxicant or neuroprotective agents [69,83]. For example, Vicente-Zurdo et al. [164] employed TEM for studying the aggregation of $A\beta_{42}$ fibrils under the presence of Fe (II), Cu (II) and Zn (II) and after the co-exposure with different Se species including Ch-SeNPs (SeNPs functionalized with chitosan). The authors investigated the potential of different Se species for inhibiting the aggregation of $A\beta_{42}$ induced by transition biometals such as Fe (II), Cu (II) and Zn (II). Results evidenced that Ch-SeNPs were the only Se species able to inhibit the Fe(II) and Zn(II) metal-induced Aβ aggregation reducing both the number and fibril length, whereas Se aminoacids such as SeMet, SeCys and SeMeSeCys induced and increase in $A\beta_{42}$ aggregation. Moreover, Ch-SeNPs were able also to reduce considerably amyloid aggregation and to shorten fibril length when they added after exposure of $A\beta_{42}$ fibrils with Fe (II) (Figure 3) [164]. Additionally, amyloid protein aggregation induced by mercury has been also studied using other advanced imaging techniques such as Confocal Immunofluorescence Microscopy or Atomic Force Microscopy [165,166].

TEM also offers the possibility to explore the internal structure of tissues, cells and organelles supporting (neuro)toxicity studies in the biological system. For instance, Long et al. [167] observed that TiO_2 NPs were internalized by BV2, an immortalized brain microglia cell line model, in which swollen mitochondria were detected after exposure to TiO_2 NPs leading to mitochondrial dysfunction, one of the most recognized pathological processes in the ethology of neurological disorders.

In addition, other imaging techniques such as laser ablation LA-ICP-MS, X-ray absorption spectroscopy (XAS) and X-ray fluorescence spectrometry (XRF) and electron microscopy, among others have allowed researchers to map metal distribution in tissues and cells [4].

Due to its high-resolution, TEM has been claimed as a suitable imaging technique for elucidating the uptake and biodistribution within cell and tissue components since it offers great possibilities to study the localization and detailed track of nanoparticles within cell lines [168]. Obtaining information of nanoparticle localization inside cells of line models not only might provide insights into mechanisms of nanoparticles neurotoxicity, but also yield to valuable knowledge about the possibility that certain nanoparticles may act as neuroprotective agents. In this regard, Vicente-Zurdo et al. [101] employed TEM for tracking the

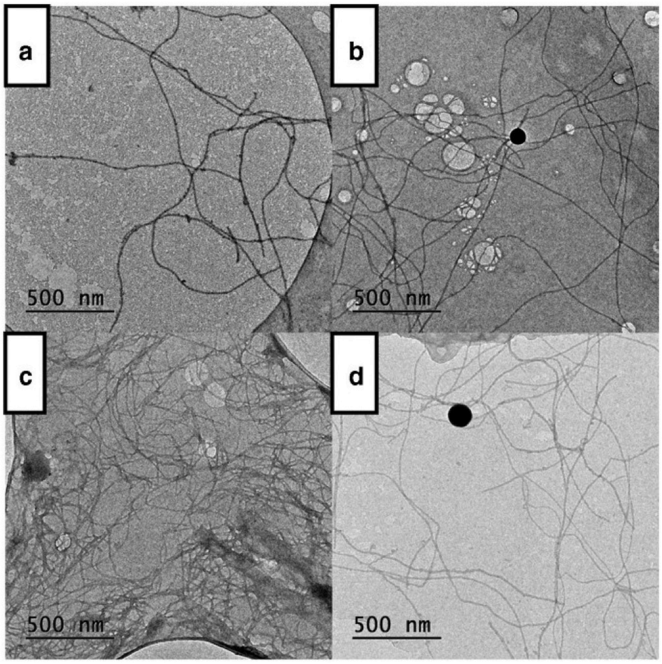

Fig. 3. TEM images of the disaggregation assays performed in 10 mM HCl medium ([Aβ_{1-42}] = 50 μM, [Fe(II)] = 50 μM, [Ch-SeNPs] = 50 μM). **a** Aβ_{1-42}, 24 h, 37 °C. **b** Aβ_{1-42} incubated at 37 °C during 48 h + Ch-SeNPs added the last 24 h. **c** Aβ_{1-42} + Fe(II) 24 h, 37 °C. **d** Aβ_{1-42} + Fe(II) incubated at 37 °C during 48 h + Ch-SeNPs added the last 24 h. Reproduced from reference [164] with permission from Springer Nature.

different stages of Ch-SeNPs uptake pathway in SH-SY5Y neuroblastoma cell line. TEM images showed that Ch-SeNPs were in the cytosol, mitochondria and attached to the external surface of the membrane in the early and intermediate stages as well as in the nuclei and nucleoli in an advanced stage (Figure 4). The authors suggest that these findings might indicate the possible implication of Ch-SeNPs in the inhibition of Aβ_{42} aggregates, the prevention of oxidative stress in mitochondria and the possible interaction with p-tau which is promising for AD treatment [101].

3.7. Omic techniques in the investigations of the molecular interactions

In terms of metallomics, it is also crucial to understand the localisation, speciation, and metabolism of metals and to study their connections and interactions with genes, proteins, or metabolites in the spatial dimension, referred to as spatial metallomics. Omics techniques such as genomic, metabolomic and proteomic have been widely used for understanding toxicological pathways or mechanisms using cell models. These different omics techniques are unique but complementary, being the multi-omics methods a more suitable choice since they allow a more comprehensive understanding of molecular changes contributing to cellular responses or molecular complexity of the brain, for instance.

Western blotting is one of the most commonly used analytical technique in molecular biology and proteomics to separate and identify proteins as well as post translational modifications on proteins providing semi-quantitative or quantitative data about the target protein in biological samples [87]. In this technique, a mixture of proteins is separated based on molecular weight through gel electrophoresis. Then, the gel is placed in contact with a membrane in which proteins migrate from the gel using an electrical current. After that, the membrane is further processed with antibodies specific for the target protein and visualized using enzyme-linked or fluorophore-conjugated secondary antibodies with colorimetric, fluorometric or chemiluminescent detection [88]. In this sense, western blot analysis has been widely used to investigate the molecular mechanisms responsible for Se-protection against metal-induced toxicity and apoptosis [104,108,109,169]. For instance, Hossain et al. [108] studied the protein expression of mTOR and Akt, involved in an important intracellular signaling pathway in regulating cell cycle. They evidenced that the protective effect of Se against inorganic Hg toxicity is strongly influenced by the activation of both proteins, being significantly increased when co-exposure of both elements in PC12 cells with respect to exposure of inorganic Hg alone. As also negatively modulates the mTOR/Akt signaling pathway in PC12 cells which contributes to the induction of apoptotic cell death, however, Se can reactivate the mTOR/Akt signaling pathway and thus ameliorate metal-induced toxicity [109].

Despite of its high implementation, western blotting presents some important drawbacks that limit its used, namely, the scarce availability of specific antibodies which means that only few proteins can be detected in a single experiment. In turn, mass spectrometry has been the analytical technique of choice in proteomic studies for protein characterization since it is a highly reliable and robust technique offering different analysis procedures and methods depending on the purpose of the investigation, from bottom-up (peptide-based approach) or top-down (protein-based approach) strategies to global (attempts to search differences in the proteome between samples by identifying and quantifying all the proteins) or target proteomics (allows higher sensitivity by reducing the number of detected proteins and optimizing the method) [84,85]. Mass spectrometric instrumentation for proteomics resides in using soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), capable of ionizing peptides or proteins, along with mass analyzers such as quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (TOF) or Fourier-transform ion cyclotron resonance (FTICR). Moreover, hybrid instruments (Q–q–Q, Q–q–LIT, Q–TOF, TOF–TOF and LTQ–FTICR) have been developed to combine the best features of each mass analyzer, providing additional precision, resolution, and robustness. Liquid chromatography is usually applied to achieve separation of proteins or peptides. Coupled to mass spectrometry techniques (LC-MS) it enables protein or peptide sequencing. Different modes of mass spectrometric detectors are used, e.g. tandem mass spectrometry MS/MS, MS using a triple quadrupole (Q-q-Q) detector, high resolution mass spectrometry (HR-MS) and time-of-flight (TOF) system [86].

On the other hand, to investigate metal-induced DNA damage, genome DNA fragmentation is usually evaluated by using agarose gel electrophoresis or the Comet assay, which is a rapid, sensitive, and simple method for detecting DNA damage at the level of individual cells [89]. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA attached to the nuclear matrix. Electrophoresis at high pH results in comet-like structures observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks, probably because of the loose of the supercoiling of the loops containing a break, being free to extend toward the anode. This is followed by visual analysis with staining of DNA with ethidium bromide, propidium iodide, Hoechst or DAPI [90]. For example, Hossain et al. [108] indicated that co-exposure of iHg and Se in PC12 cells resulted in higher amount of intact DNA extracted than in case of cells exposed to iHg alone, evidencing that that Se can inhibit DNA damage induced by iHg. Similar results were observed in case of Cd and Se co-exposure compared to Cd incubation in cells alone [33].

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments *in vitro*. It constitutes one the most widely used techniques in genomics. Real-time quantitative reverse transcription (RT)-PCR is usually selected for examining the influence of neurotoxic elements on a protein expression and is considered the best standard for the validation of high-throughput gene expression data. Kim et al. [91] investigated the mechanism of down-regulation of SeIW by MeHg through the alteration

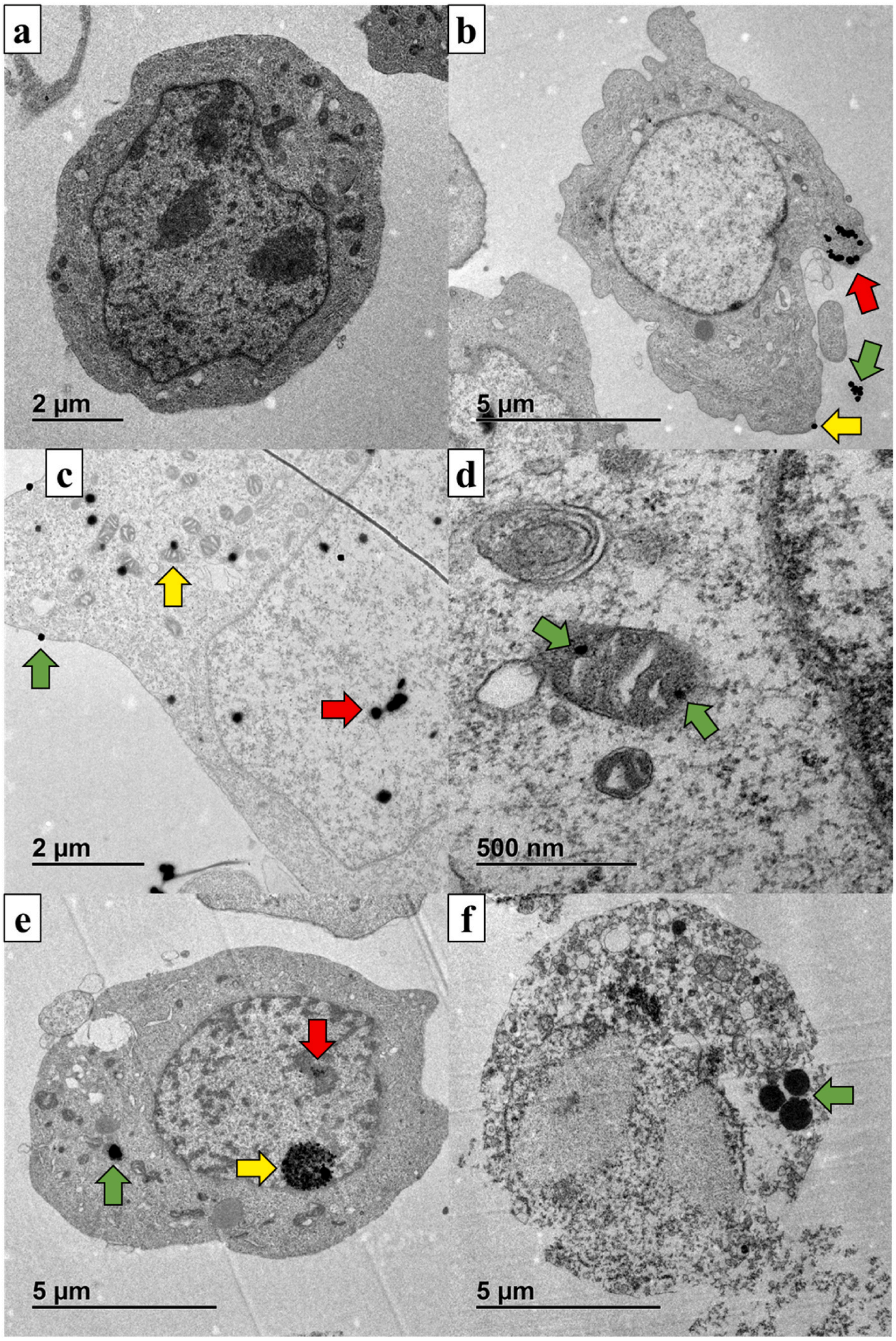

Fig. 4. Micrographs of Ch-SeNPs (pointed with green, yellow and red arrows) internalisation in SH-SY5Y cells observed by TEM. a) Control SH-SY5Y cell; b) SH-SY5Y cell at early stage of Ch-SeNPs internalisation; c) SH-SY5Y cell at intermediate stage of Ch-SeNPs internalisation; d) Ch-SeNPs location inside mitochondria; e) SH-SY5Y cell at advanced stage of Ch-SeNPs internalisation; f) Dead SH-SY5Y cell due to Ch-SeNPs effect. Reproduced from reference [101] with permission from Elsevier.

of SeIW mRNA expression of SH-SY5Y cells. Moreover, they showed that this down-regulation of SeIW by MeHg was independent of the generation of ROS but dependent on the depletion of intracellular GSH.

4. Conclusions and future perspectives

There is no doubt that further investigations are needed to elucidate the role that Se plays in preventing neurological disorders and neurotoxic effects from hazardous trace elements. In this sense, the combination of analytical techniques together with *in vitro models* and assays enables the evaluation of key aspects for (neuro)toxicological studies such as exposure concentration, absorption, accumulation in tissues, biotransformation, bioavailability, or excretion of metal/ metalloids of neurological relevance. In this context, ICP-MS in single-cell mode should be important in neurotoxicity studies, as it enables analysis at the single-cell level, providing new insights in order to better understand cellular heterogeneity, which is a key and a challenging factor to be investigated in some neurological diseases. Moreover, the lack of reliability of *in vivo* models for neurotoxicological studies the development of new or improved *in vitro* models are required.

CRediT authorship contribution statement

Beatriz Gómez-Gómez: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology. **Tamara Fernández-Bautista:** Writing – review & editing, Writing – original draft, Methodology. **Yolanda Madrid:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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References

[1] S. Alkahtani, N.S. AL-Johani, S. Alarifi, Mechanistic insights, treatment Paradigms, and Clinical progress in neurological disorders: current and future Prospects, Int. J. Mol. Sci. 24 (2023), https://doi.org/10.3390/ijms24021340.

[2] C.A. Ross, M.A. Poirier, Protein aggregation and neurodegenerative disease, Nat. Med. 10 (2004) S10, https://doi.org/10.1038/nm1066.

[3] K.J. Barnham, C.L. Masters, A.I. Bush, Neurodegenerative diseases and oxidatives stress, Nat. Rev. Drug Discov. 3 (2004) 205–214, https://doi.org/10.1038/nrd1330.

[4] J.R. De Jesus, M.A.Z. Arruda, Unravelling neurological disorders through metallomics-based approaches, Metallomics 12 (2020) 1878–1896, https://doi.org/10.1039/d0mt00234h.

[5] Y.Y. Zhang, X.S. Li, K. Di Ren, J. Peng, X.J. Luo, Restoration of metal homeostasis: a potential strategy against neurodegenerative diseases, Ageing Res. Rev. 87 (2023) 101931, https://doi.org/10.1016/j.arr.2023.101931.

[6] P. Nicotera, A. Rossi, Molecular mechanisms of metal neurotoxicity, J. Trace Elem, Electrolytes Health Dis. 7 (1993) 254–256.

[7] M. Nabi, N. Tabassum, Role of environmental Toxicants on neurodegenerative disorders, Front. Toxicol. 4 (2022) 1–20, https://doi.org/10.3389/ftox.2022.837579.

[8] B.R. Cardoso, B.R. Roberts, A.I. Bush, D.J. Hare, Selenium, selenoproteins and neurodegenerative diseases, Metallomics 7 (2015) 1213–1228, https://doi.org/10.1039/c5mt00075k.

[9] R.S. Amais, G.L. Donati, M.A. Zezzi Arruda, ICP-MS and trace element analysis as tools for better understanding medical conditions, TrAC - Trends Anal. Chem. 133 (2020), https://doi.org/10.1016/j.trac.2020.116094.

[10] B. Michalke, D. Willkommen, E. Drobyshev, N. Solovyev, The importance of speciation analysis in neurodegeneration research, TrAC - Trends Anal. Chem. 104 (2018) 160–170, https://doi.org/10.1016/j.trac.2017.08.008.

[11] F. Zhang, X. Li, Y. Wei, Selenium and selenoproteins in health, Biomolecules 13 (2023) 1–25, https://doi.org/10.3390/biom13050799.

[12] J. Chen, M.J. Berry, Selenium and selenoproteins in the brain and brain diseases, J. Neurochem. 86 (2003) 1–12, https://doi.org/10.1046/j.1471-4159.2003.01854.x.

[13] N.D. Solovyev, Importance of selenium and selenoprotein for brain function: from antioxidant protection to neuronal signalling, J. Inorg. Biochem. 153 (2015) 1–12, https://doi.org/10.1016/j.jinorgbio.2015.09.003.

[14] W. Ding, S. Wang, J. Gu, L. Yu, Selenium and human nervous system, Chinese Chem. Lett. 34 (2023), https://doi.org/10.1016/j.cclet.2022.108043.

[15] J. Luo, L. Su, X. He, Y. Du, N. Xu, R. Wu, Y. Zhu, T. Wang, R. Shao, F. W. Unverzagt, A.M. Hake, Y. Jin, S. Gao, Blood selenium and serum glutathione peroxidase levels were associated with serum β-amyloid in older adults, Biol. Trace Elem. Res. 201 (2023) 3679–3687, https://doi.org/10.1007/s12011-022-03480-4.

[16] M. Vinceti, T. Urbano, A. Chiari, T. Filippini, L.A. Wise, M. Tondelli, B. Michalke, M. Shimizu, Y. Saito, Selenoprotein P concentrations and risk of progression from mild cognitive impairment to dementia, Sci. Rep. 13 (2023) 1–10, https://doi.org/10.1038/s41598-023-36084-6.

[17] H.R. Rasekh, M.D. Davis, L.W. Cooke, E.A. Mazzio, R.R. Reams, K.F.A. Soliman, The effect of selenium on the central dopaminergic system: a microdialysis study, Life Sci 61 (1997) 1029–1035, https://doi.org/10.1016/S0024-3205(97)00610-3.

[18] Z.H. Zhang, J.Y. Peng, Y. Bin Chen, C. Wang, C. Chen, G.L. Song, Different effects and mechanisms of selenium compounds in improving pathology in alzheimer's disease, Antioxidants 12 (2023) 1–16, https://doi.org/10.3390/antiox12030702.

[19] C. Sun, Z. Du, X. Liu, Y. Yang, S. Zhou, C. Li, X. Cao, Q. Zhao, K. Wong, W. Chen, X. Dong, Selenium forms and dosages determined their biological actions in mouse models of Parkinson's disease, Nutrients 15 (2023), https://doi.org/10.3390/nu15010011.

[20] M.C. Zambonino, E.M. Quizhpe, L. Mouheb, A. Rahman, S.N. Agathos, S. A. Dahoumane, Biogenic selenium nanoparticles in biomedical sciences: properties, current trends, novel opportunities and emerging challenges in theranostic nanomedicine, Nanomaterials 13 (2023), https://doi.org/10.3390/nano13030424.

[21] R. Hu, X. Wang, L. Han, X. Lu, The developments of surface-functionalized selenium nanoparticles and their applications in brain diseases therapy, Biomimetics 8 (2023) 259, https://doi.org/10.3390/biomimetics8020259.

[22] X. Xiao, H. Deng, X. Lin, A.S.M. Ali, A. Viscardi, Z. Guo, L. Qiao, Y. He, J. Han, Selenium nanoparticles: properties, preparation methods, and therapeutic applications, Chem. Biol. Interact. 378 (2023) 110483, https://doi.org/10.1016/j.cbi.2023.110483.

[23] S. Rao, Y. Lin, Y. Du, L. He, G. Huang, B. Chen, T. Chen, Designing multifunctionalized selenium nanoparticles to reverse oxidative stress-induced spinal cord injury by attenuating ROS overproduction and mitochondria dysfunction, J. Mater. Chem. B. 7 (2019) 2648–2656, https://doi.org/10.1039/c8tb02520g.

[24] W. Cong, R. Bai, Y.F. Li, L. Wang, C. Chen, Selenium nanoparticles as an efficient nanomedicine for the therapy of Huntington's disease, ACS Appl. Mater. Interfaces. 11 (2019) 34725–34735, https://doi.org/10.1021/acsami.9b12319.

[25] D. Ji, X. Wu, D. Li, P. Liu, S. Zhang, D. Gao, F. Gao, M. Zhang, Y. Xiao, Protective effects of chondroitin sulphate nano-selenium on a mouse model of Alzheimer's disease, Int. J. Biol. Macromol. 154 (2020) 233–245, https://doi.org/10.1016/j.ijbiomac.2020.03.079.

[26] X. Huo, Y. Zhang, X. Jin, Y. Li, L. Zhang, A novel synthesis of selenium nanoparticles encapsulated PLGA nanospheres with curcumin molecules for the inhibition of amyloid β aggregation in Alzheimer's disease, J. Photochem. Photobiol. B Biol. 190 (2019) 98–102, https://doi.org/10.1016/j.jphotobiol.2018.11.008.

[27] O.A.R. Abozaid, M.W. Sallam, S. El-Sonbaty, S. Aziza, B. Emad, E.S.A. Ahmed, Resveratrol-selenium nanoparticles alleviate neuroinflammation and neurotoxicity in a rat model of alzheimer's disease by regulating sirt1/miRNA-134/gsk3β expression, Biol. Trace Elem. Res. 200 (2022) 5104–5114, https://doi.org/10.1007/s12011-021-03073-7.

[28] D. Yue, C. Zeng, S.K. Okyere, Z. Chen, Y. Hu, Glycine nano-selenium prevents brain oxidative stress and neurobehavioral abnormalities caused by MPTP in rats, J. Trace Elem. Med. Biol. 64 (2021) 126680, https://doi.org/10.1016/j.jtemb.2020.126680.

[29] S.S. Elganzoury, M.S. Abdelfattah, O.A. Habotta, M. El-khadragy, A.E. Abdel Moneim, M.S. Abdalla, Neuro-amelioration of Ficus lyrata (fiddle-leaf fig) extract conjugated with selenium nanoparticles against aluminium toxicity in rat brain: relevance to neurotransmitters, oxidative, inflammatory, and apoptotic events, Environ. Sci. Pollut. Res. 30 (2023) 65822–65834, https://doi.org/10.1007/s11356-023-26935-0.

[30] Y.-R. Xu, M. Talukder, C.-X. Li, C. Zhang, J. Ge, J.-L. Li, Nanoselenium alleviates cadmium-induced cerebral injury via regulating cerebral metal transporters and metal-regulatory transcription factor 1, J. Agric. Food Chem. 71 (2023) 9896–9907, https://doi.org/10.1021/acs.jafc.3c02121.

[31] L. Qiao, Y. Chen, X. Song, X. Dou, C. Xu, Selenium nanoparticles-enriched Lactobacillus casei ATCC 393 prevents cognitive dysfunction in mice through modulating microbiota-gut-brain Axis, Int. J. Nanomedicine. 17 (2022) 4807–4827, https://doi.org/10.2147/IJN.S374024.

[32] Z. Ren, H. Deng, Q. Wu, G. Jia, N. Wen, Y. Deng, L. Zhu, Z. Zuo, J. Deng, Effect of selenium on brain injury in chickens with subacute arsenic poisoning, Biol. Trace Elem. Res. 200 (2022) 330–338, https://doi.org/10.1007/s12011-021-02630-4.

[33] K.F. Binte Hossain, M.M. Rahman, M.T. Sikder, T. Saito, T. Hosokawa, M. Kurasaki, Inhibitory effects of selenium on cadmium-induced cytotoxicity in PC12 cells via regulating oxidative stress and apoptosis, Food Chem. Toxicol. 114 (2018) 180–189, https://doi.org/10.1016/j.fct.2018.02.034.

[34] Y.F. Li, Z. Dong, C. Chen, B. Li, Y. Gao, L. Qu, T. Wang, X. Fu, Y. Zhao, Z. Chai, Organic selenium supplementation increases mercury excretion and decreases oxidative damage in long-term mercury-exposed residents from Wanshan, China, Environ. Sci. Technol. 46 (2012) 11313–11318, https://doi.org/10.1021/es302241v.

[35] M. Ribeiro, N. Zephyr, J.A.L. Silva, M. Danion, T. Guérin, I. Castanheira, A. Leufroy, P. Jitaru, Assessment of the mercury-selenium antagonism in rainbow trout fish, Chemosphere 286 (2022), https://doi.org/10.1016/j.chemosphere.2021.131749.

[36] W. Ali, H. Zhang, M. Junaid, K. Mao, N. Xu, C. Chang, A. Rasool, M. Wajahat Aslam, J. Ali, Z. Yang, Insights into the mechanisms of arsenic-selenium interactions and the associated toxicity in plants, animals, and humans: a critical

review, Crit. Rev. Environ. Sci. Technol. 51 (2021) 704–750, https://doi.org/10.1080/10643389.2020.1740042.

[37] O. Ponomarenko, P.F. La Porte, S.P. Singh, G. Langan, D.E.B. Fleming, J. E. Spallholz, M. Alauddin, H. Ahsan, S. Ahmed, J. Gailer, G.N. George, I. J. Pickering, Selenium-mediated arsenic excretion in mammals: a synchrotron-based study of whole-body distribution and tissue-specific chemistry, Metallomics. 9 (2017) 1585–1595, https://doi.org/10.1039/c7mt00201g.

[38] I. Zwolak, The role of selenium in arsenic and cadmium toxicity: an updated review of scientific literature, Biol. Trace Elem. Res. 193 (2020) 44–63, https://doi.org/10.1007/s12011-019-01691-w.

[39] J. Gailer, G.N. George, I.J. Pickering, R.C. Prince, H.S. Younis, J.J. Winzerling, Biliary excretion of [(GS)2AsSe]- after intravenous injection of rabbits with arsenite and selenate, Chem. Res. Toxicol. 15 (2002) 1466–1471, https://doi.org/10.1021/tx025538s.

[40] S.A. Manley, G.N. George, I.J. Pickering, R.S. Glass, E.J. Prenner, R. Yamdagni, Q. Wu, J. Gailer, The seleno bis(S-glutathionyl) arsinium ion is assembled in erythrocyte lysate, Chem. Res. Toxicol. 19 (2006) 601–607, https://doi.org/10.1021/tx0503505.

[41] G.N. George, J. Gailer, O. Ponomarenko, P.F. La Porte, K. Strait, M. Alauddin, H. Ahsan, S. Ahmed, J. Spallholz, I.J. Pickering, Observation of the seleno bis-(S-glutathionyl) arsinium anion in rat bile, J. Inorg. Biochem. 158 (2016) 24–29, https://doi.org/10.1016/j.jinorgbio.2016.01.022.

[42] X. Hu, J.D. Chandler, J. Fernandes, M.L. Orr, L. Hao, K. Uppal, D.C. Neujahr, D. P. Jones, Y.M. Go, Selenium supplementation prevents metabolic and transcriptomic responses to cadmium in mouse lung, Biochim. Biophys. Acta - Gen. Subj. 1862 (2018) 2417–2426, https://doi.org/10.1016/j.bbagen.2018.04.009.

[43] M. Dauplais, M. Lazard, S. Blanquet, P. Plateau, Neutralization by metal ions of the toxicity of sodium selenide, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0054353.

[44] N.V.C. Ralston, C.R. Ralston, L.J. Raymond, Selenium health benefit values: updated criteria for mercury risk assessments, Biol. Trace Elem. Res. 171 (2016) 262–269, https://doi.org/10.1007/s12011-015-0516-z.

[45] L.J. Raymond, N.V.C. Ralston, Mercury: selenium interactions and health implications, Neurotoxicology 81 (2020) 294–299, https://doi.org/10.1016/j.neuro.2020.09.020.

[46] M.A.K. Khan, F. Wang, Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism, Environ. Toxicol. Chem. 28 (2009) 1567–1577, https://doi.org/10.1897/08-375.1.

[47] A. Manceau, A.C. Gaillot, P. Glatzel, Y. Cherel, P. Bustamante, In vivo formation of HgSe nanoparticles and Hg-tetraselenolate complex from methylmercury in seabirds-implications for the Hg-Se antagonism, Environ. Sci. Technol. 55 (2021) 1515–1526, https://doi.org/10.1021/acs.est.0c06269.

[48] F.E. Huggins, S.A. Raverty, O.S. Nielsen, N.E. Sharp, J.D. Robertson, N.V. C. Ralston, An XAFS investigation of mercury and selenium in Beluga whale tissues, Environ. Bioindic. 4 (2009) 291–302, https://doi.org/10.1080/15555270903404651.

[49] E. Nakazawa, T. Ikemoto, A. Hokura, Y. Terada, T. Kunito, S. Tanabe, I. Nakai, The presence of mercury selenide in various tissues of the striped dolphin: evidence from μ-XRF-XRD and XAFS analyses, Metallomics 3 (2011) 719–725, https://doi.org/10.1039/c0mt00106f.

[50] Z. Gajdosechova, A. Brownlow, N.T. Cottin, M. Fernandes, F.L. Read, D.S. Urgast, A. Raab, J. Feldmann, E.M. Krupp, Possible link between Hg and Cd accumulation in the brain of long-finned pilot whales (Globicephala melas), Sci. Total Environ. 545–546 (2016) 407–413, https://doi.org/10.1016/j.scitotenv.2015.12.082.

[51] T. Arai, T. Ikemoto, A. Hokura, Y. Terada, T. Kunito, S. Tanabe, I. Nakai, Chemical forms of mercury and cadmium accumulated in marine mammals and seabirds as determined by XAFS analysis, Environ. Sci. Technol. 38 (2004) 6468–6474, https://doi.org/10.1021/es040367u.

[52] K. El Hanafi, B. Gomez-Gomez, Z. Pedrero, P. Bustamante, Y. Cherel, D. Amouroux, Y. Madrid, Simple and rapid formic acid sample treatment for the isolation of HgSe nanoparticles from animal tissues, Anal. Chim. Acta. 1250 (2023) 340952, https://doi.org/10.1016/j.aca.2023.340952.

[53] S.T. Lancaster, G. Peniche, A. Alzahrani, M. Blanz, J. Newton, M.A. Taggart, W. T. Corns, E.M. Krupp, J. Feldmann, Mercury speciation in Scottish raptors reveals high proportions of inorganic mercury in Scottish golden eagles (Aquila chrysaetos): potential occurrence of mercury selenide nanoparticles, Sci. Total Environ. 829 (2022) 154557, https://doi.org/10.1016/j.scitotenv.2022.154557.

[54] Y. Suzuki, M. Kondo, H. Akiyama, Y. Ogra, Presence of nano-sized mercury-containing particles in seafoods, and an estimate of dietary exposure, Environ. Pollut. 307 (2022) 119555, https://doi.org/10.1016/j.envpol.2022.119555.

[55] M. Korbas, J.L. Odonoghue, G.E. Watson, I.J. Pickering, S.P. Singh, G.J. Myers, T. W. Clarkson, G.N. George, The chemical nature of mercury in human brain following poisoning or environmental exposure, ACS Chem. Neurosci. 1 (2010) 810–818, https://doi.org/10.1021/cn1000765.

[56] R. Moser, F. Zaccarini, T. Alber, R. Kerbl, First finding of tiemannite, HgSe, in human bladder stones: an electron microprobe study, Micron 138 (2020) 102928, https://doi.org/10.1016/j.micron.2020.102928.

[57] B. Wang, Y. Du, Cadmium and its neurotoxic effects, Oxid. Med. Cell. Longev. 2013 (2013), https://doi.org/10.1155/2013/898034.

[58] Y. Liu, S.P. Zhang, Y.Q. Cai, Cytoprotective effects of selenium on cadmium-induced LLC-PK1 cells apoptosis by activating JNK pathway, Toxicol. Vitr. 21 (2007) 677–684, https://doi.org/10.1016/j.tiv.2007.01.015.

[59] Y. jing Zhou, S. ping Zhang, C. wei Liu, Y. qing Cai, The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK1 cells, Toxicol. Vitr. 23 (2009) 288–294, https://doi.org/10.1016/j.tiv.2008.12.009.

[60] J.O. Fatoki, J.A. Badmus, Arsenic as an environmental and human health antagonist: a review of its toxicity and disease initiation, J. Hazard. Mater. Adv. 5 (2022) 100052, https://doi.org/10.1016/j.hazadv.2022.100052.

[61] M. Thakur, M. Rachamalla, S. Niyogi, A.K. Datusalia, S.J.S. Flora, Molecular mechanism of arsenic-induced neurotoxicity including neuronal dysfunctions, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms221810077.

[62] J.O. Fatoki, S. Kehinde, J. Badmus, A. Adekunle, Induction of oxidative stress: a possible mechanism for the arsenic induced catastrophes in male wistar rats, Adv. Life Sci. Technol. (2019) 22–32, https://doi.org/10.7176/alst/75-04.

[63] N.V.C. Ralston, L.J. Raymond, Mercury's neurotoxicity is characterized by its disruption of selenium biochemistry, Biochim. Biophys. Acta - Gen. Subj. 1862 (2018) 2405–2416, https://doi.org/10.1016/j.bbagen.2018.05.009.

[64] V.L. Cariccio, A. Samà, P. Bramanti, E. Mazzon, Mercury involvement in neuronal damage and in neurodegenerative diseases, Biol. Trace Elem. Res. 187 (2019) 341–356, https://doi.org/10.1007/s12011-018-1380-4.

[65] M. Farina, M. Aschner, J.B.T. Rocha, Oxidative stress in MeHg-induced neurotoxicity, Toxicol. Appl. Pharmacol. 256 (2011) 405–417, https://doi.org/10.1016/j.taap.2011.05.001.

[66] F. Azimi, M. Oraei, G. Gohari, S. Panahirad, A. Farmarzi, Chitosan-selenium nanoparticles (Cs–Se NPs) modulate the photosynthesis parameters, antioxidant enzymes activities and essential oils in Dracocephalum moldavica L. under cadmium toxicity stress, Plant Physiol. Biochem. 167 (2021) 257–268, https://doi.org/10.1016/j.plaphy.2021.08.013.

[67] B. Hawrylak-Nowak, S. Dresler, K. Rubinowska, R. Matraszek-Gawron, W. Woch, M. Hasanuzzaman, Selenium biofortification enhances the growth and alters the physiological response of lamb's lettuce grown under high temperature stress, Plant Physiol. Biochem. 127 (2018) 446–456, https://doi.org/10.1016/j.plaphy.2018.04.018.

[68] H. Huang, M. Li, M. Rizwan, Z. Dai, Y. Yuan, M.M. Hossain, M. Cao, S. Xiong, S. Tu, Synergistic effect of silicon and selenium on the alleviation of cadmium toxicity in rice plants, J. Hazard. Mater. 401 (2021), https://doi.org/10.1016/j.jhazmat.2020.123393.

[69] J.W. Linsley, T. Reisine, S. Finkbeiner, Cell death assays for neurodegenerative disease drug discovery, Expert Opin Drug Discov 14 (2019) 901–913, https://doi.org/10.1080/17460441.2019.1623784. Cell.

[70] M. Ghasemi, T. Turnbull, S. Sebastian, I. Kempson, The mtt assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms222312827.

[71] S. Kaja, A.J. Payne, Y. Naumchuk, P. Koulen, Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes Simon, Curr Protoc Toxicol 72 (2018), https://doi.org/10.1002/cptx.21.Quantification.

[72] N. Kumar, V. Kumari, C. Ram, B.S. Bharath Kumar, S. Verma, Impact of oral cadmium intoxication on levels of different essential trace elements and oxidative stress measures in mice: a response to dose, Environ. Sci. Pollut. Res. 25 (2018) 5401–5411, https://doi.org/10.1007/s11356-017-0868-3.

[73] İ. Gulcin, Antioxidants and antioxidant methods: an updated overview. https://doi.org/10.1007/s00204-020-02689-3, 2020.

[74] Y. Zhang, M. Dai, Z. Yuan, Methods for the detection of reactive oxygen species, Anal. Methods. 10 (2018) 4625–4638, https://doi.org/10.1039/c8ay01339j.

[75] C. Grochowski, E. Blicharska, P. Krukow, K. Jonak, M. Maciejewski, D. Szczepanek, K. Jonak, J. Flieger, R. Maciejewski, Analysis of trace elements in human brain: its aim, methods, and concentration levels, Front. Chem. 7 (2019), https://doi.org/10.3389/fchem.2019.00115.

[76] Z. Guo, P. Zhang, S. Chakraborty, A.J. Chetwynd, F.A. Monikh, C. Stark, H. Ali-Boucetta, S. Wilson, I. Lynch, E. Valsami-Jones, Biotransformation modulates the penetration of metallic nanomaterials across an artificial blood–brain barrier model, Proc. Natl. Acad. Sci. U. S. A. 118 (2021) 1–10, https://doi.org/10.1073/pnas.2105245118.

[77] F. Laborda, J. Jiménez-Lamana, E. Bolea, J.R. Castillo, Critical considerations for the determination of nanoparticle number concentrations, size and number size distributions by single particle ICP-MS, J. Anal. At. Spectrom. 28 (2013) 1220–1232, https://doi.org/10.1039/c3ja50100k.

[78] C. Aijie, L. Huimin, L. Jia, O. Lingling, W. Limin, W. Junrong, L. Xuan, H. Xue, S. Longquan, Central neurotoxicity induced by the instillation of ZnO and TiO nanoparticles through the taste nerve pathway, Nanomedicine 12 (2017) 2453–2470, https://doi.org/10.2217/nnm-2017-0171.

[79] R. You, Y.S. Ho, C.H.L. Hung, Y. Liu, C.X. Huang, H.N. Chan, S.L. Ho, S.Y. Lui, H. W. Li, R.C.C. Chang, Silica nanoparticles induce neurodegeneration-like changes in behavior, neuropathology, and affect synapse through MAPK activation, Part. Fibre Toxicol. 15 (2018) 1–18, https://doi.org/10.1186/s12989-018-0263-3.

[80] M. Zhou, L. Xie, C.J. Fang, H. Yang, Y.J. Wang, X.Y. Zhen, C.H. Yan, Y. Wang, M. Zhao, S. Peng, Implications for blood-brain-barrier permeability, in vitro oxidative stress and neurotoxicity potential induced by mesoporous silica nanoparticles: effects of surface modification, RSC Adv 6 (2016) 2800–2809, https://doi.org/10.1039/c5ra17517h.

[81] M. Montes-Bayón, M. Corte-Rodríguez, R. Álvarez-Fernández García, J. S. Fagundes, Biomedical Analysis by ICP-MS: A Focus on Single Cell Strategies, 1st ed., Elsevier B.V., 2022 https://doi.org/10.1016/bs.coac.2022.03.002.

[82] A.B.S. da Silva, M.A.Z. Arruda, Single-cell ICP-MS to address the role of trace elements at a cellular level, J. Trace Elem. Med. Biol. 75 (2023), https://doi.org/10.1016/j.jtemb.2022.127086.

[83] R.A. Crowther, Insights into neurodegeneration from electron microscopy studies, Biochem. Soc. Trans. 49 (2021) 2777–2786, https://doi.org/10.1042/BST20210719.

[84] N. Nguyen, D. Jennen, J. Kleinjans, Omics technologies to understand drug toxicity mechanisms, Drug Discov. Today. 27 (2022) 103348, https://doi.org/10.1016/j.drudis.2022.103348.

[85] L. Monaci, E. De Angelis, N. Montemurro, R. Pilolli, Comprehensive overview and recent advances in proteomics MS based methods for food allergens analysis, TrAC - Trends Anal. Chem. 106 (2018) 21–36, https://doi.org/10.1016/j.trac.2018.06.016.

[86] X. Han, A. Aslanian, J.R. Yates, Mass spectrometry for proteomics, Curr. Opin. Chem. Biol. 12 (2008) 483–490, https://doi.org/10.1016/j.cbpa.2008.07.024.

[87] M. Mishra, Shuchita Tiwari, A.V. Gomes, Protein purification and analysis: next generation Western blotting techniques, Expert Rev Proteomics 14 (2017) 1037–1053, https://doi.org/10.1080/14789450.2017.1388167. Protein.

[88] T. Mahmood, P.C. Yang, Western blot: technique, theory, and trouble shooting, N. Am. J. Med. Sci. 4 (2012) 429–434, https://doi.org/10.4103/1947-2714.100998.

[89] J.L. Luque-Garcia, P. Cabezas-Sanchez, D.S. Anunciação, C. Camara, Analytical and bioanalytical approaches to unravel the selenium-mercury antagonism: a review, Anal. Chim. Acta. 801 (2013) 1–13, https://doi.org/10.1016/j.aca.2013.08.043.

[90] J.L. Luque-Garcia, R. Sanchez-Díaz, I. Lopez-Heras, C. Camara, P. Martin, Bioanalytical strategies for in-vitro and in-vivo evaluation of the toxicity induced by metallic nanoparticles, TrAC - Trends Anal. Chem. 43 (2013) 254–268, https://doi.org/10.1016/j.trac.2012.11.004.

[91] Y.J. Kim, Y.G. Chai, J.C. Ryu, Selenoprotein W as molecular target of methylmercury in human neuronal cells is down-regulated by GSH depletion, Biochem. Biophys. Res. Commun. 330 (2005) 1095–1102, https://doi.org/10.1016/j.bbrc.2005.03.080.

[92] D.J. Barbosa, J.P. Capela, M. De Lourdes Bastos, F. Carvalho, In vitro models for neurotoxicology research, Toxicol. Res. (Camb). 4 (2015) 801–842, https://doi.org/10.1039/c4tx00043a.

[93] A. Kumaria, C.M. Tolias, In vitro models of neurotrauma, Br. J. Neurosurg. 22 (2008) 200–206, https://doi.org/10.1080/02688690701772413.

[94] G.J. Harry, M. Billingsley, A. Bruinink, I.L. Campbell, W. Classen, D.C. Dorman, C. Galli, D. Ray, R.A. Smith, H.A. Tilson, In vitro techniques for the assessment of neurotoxicity, Environ. Health Perspect. 106 (1998) 131–158, https://doi.org/10.1289/ehp.98106s1131.

[95] L. Lopez-Suarez, S. Al Awabdh, X. Coumoul, C. Chauvet, The SH-SY5Y human neuroblastoma cell line, a relevant in vitro cell model for investigating neurotoxicology in human: focus on organic pollutants, Neurotoxicology 92 (2022) 131–155, https://doi.org/10.1016/j.neuro.2022.07.008.

[96] Y. Cao, The uses of 3D human brain organoids for neurotoxicity evaluations: a review, Neurotoxicology 91 (2022) 84–93, https://doi.org/10.1016/j.neuro.2022.05.004.

[97] T. Chhibber, S. Bagchi, B. Lahooti, A. Verma, A. Al-Ahmad, M.K. Paul, G. Pendyala, R.D. Jayant, CNS organoids: an innovative tool for neurological disease modeling and drug neurotoxicity screening, Drug Discov. Today. 25 (2020) 456–465, https://doi.org/10.1016/j.drudis.2019.11.010.

[98] P. Balbuena, W. Li, G. Magnin-Bissel, J.B. Meldrum, M. Ehrich, Comparison of two blood-brain barrier in vitro systems: cytotoxicity and transfer assessments of malathion/oxon and lead acetate, Toxicol. Sci. 114 (2010) 260–271, https://doi.org/10.1093/toxsci/kfq001.

[99] S. Cetin, D. Knez, S. Gobec, J. Kos, A. Pišlar, Cell models for Alzheimer's and Parkinson's disease: at the interface of biology and drug discovery, Biomed. Pharmacother. 149 (2022), https://doi.org/10.1016/j.biopha.2022.112924.

[100] H.J. Heusinkveld, R.H.S. Westerink, Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity, Toxicol. Vitr. 45 (2017) 81–88, https://doi.org/10.1016/j.tiv.2017.07.030.

[101] D. Vicente-Zurdo, B. Gómez-Gómez, I. Romero-Sánchez, N. Rosales-Conrado, M. E. León-González, Y. Madrid, Cytotoxicity, uptake and accumulation of selenium nanoparticles and other selenium species in neuroblastoma cell lines related to Alzheimer's disease by using cytotoxicity assays, TEM and single cell-ICP-MS, Anal. Chim. Acta. 1249 (2023), https://doi.org/10.1016/j.aca.2023.340949.

[102] G. Sala, D. Marinig, C. Riva, A. Arosio, G. Stefanoni, L. Brighina, M. Formenti, L. Alberghina, A.M. Colangelo, C. Ferrarese, Rotenone down-regulates HSPA8/hsc70 chaperone protein in vitro: a new possible toxic mechanism contributing to Parkinson's disease, Neurotoxicology 54 (2016) 161–169, https://doi.org/10.1016/j.neuro.2016.04.018.

[103] J. Faria, J. Barbosa, O. Queirós, R. Moreira, F. Carvalho, R.J. Dinis-Oliveira, Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells, Toxicology 359–360 (2016) 1–10, https://doi.org/10.1016/j.tox.2016.06.010.

[104] J.J.V. Branca, G. Morucci, M. Maresca, B. Tenci, R. Cascella, F. Paternostro, C. Ghelardini, M. Gulisano, L. Di Cesare Mannelli, A. Pacini, Selenium and zinc: two key players against cadmium-induced neuronal toxicity, Toxicol. Vitr. 48 (2018) 159–169, https://doi.org/10.1016/j.tiv.2018.01.007.

[105] J.R. Sarkanen, J. Nykky, J. Siikanen, J. Selinummi, T. Ylikomi, T.O. Jalonen, Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells, J. Neurochem. 102 (2007) 1941–1952, https://doi.org/10.1111/j.1471-4159.2007.04676.x.

[106] H. Xicoy, B. Wieringa, G.J.M. Martens, The SH-SY5Y cell line in Parkinson's disease research: a systematic review, Mol. Neurodegener. 12 (2017) 1–11, https://doi.org/10.1186/s13024-017-0149-0.

[107] B.Z. Schmidt, M. Lehmann, S. Gutbier, E. Nembo, S. Noel, L. Smirnova, A. Forsby, J. Hescheler, H.X. Avci, T. Hartung, M. Leist, J. Kobolák, A. Dinnyés, In vitro acute and developmental neurotoxicity screening: an overview of cellular platforms and high-throughput technical possibilities, Arch. Toxicol. 91 (2017) 1–33, https://doi.org/10.1007/s00204-016-1805-9.

[108] K.F.B. Hossain, M.M. Rahman, M.T. Sikder, T. Hosokawa, T. Saito, M. Kurasaki, Selenium modulates inorganic mercury induced cytotoxicity and intrinsic apoptosis in PC12 cells, Ecotoxicol. Environ. Saf. 207 (2021) 111262, https://doi.org/10.1016/j.ecoenv.2020.111262.

[109] M.M. Rahman, R.A. Uson-Lopez, M.T. Sikder, G. Tan, T. Hosokawa, T. Saito, M. Kurasaki, Ameliorative effects of selenium on arsenic-induced cytotoxicity in PC12 cells via modulating autophagy/apoptosis, Chemosphere 196 (2018) 453–466, https://doi.org/10.1016/j.chemosphere.2017.12.149.

[110] A. Slanzi, G. Iannoto, B. Rossi, E. Zenaro, G. Constantin, In vitro models of neurodegenerative diseases, Front. Cell Dev. Biol. 8 (2020), https://doi.org/10.3389/fcell.2020.00328.

[111] J. Xie, K. Wettschurack, C. Yuan, Review: in vitro cell platform for understanding developmental toxicity, Front. Genet. 11 (2020) 1–8, https://doi.org/10.3389/fgene.2020.623117.

[112] L.M. Prince, M. Aschner, A.B. Bowman, Human-induced pluripotent stems cells as a model to dissect the selective neurotoxicity of methylmercury, Biochim. Biophys. Acta - Gen. Subj. 1863 (2019), https://doi.org/10.1016/j.bbagen.2019.02.002.

[113] L. D'Aiuto, Y. Zhi, D. Kumar Das, M.R. Wilcox, J.W. Johnson, L. Mc Clain, M. L. Macdonald, R. Di Maio, M.E. Schurdak, P. Piazza, L. Viggiano, R. Sweet, P. R. Kinchington, A.G. Bhattacharjee, R. Yolken, V.L. Nimgaonka, Large-scale generation of human ipsc-derived neural stem cells/early neural progenitor cells and their neuronal differentiation, Organogenesis 10 (2014) 365–377, https://doi.org/10.1080/15476278.2015.1011921.

[114] G.W. Anderson, P.J.M. Deans, R.D.T. Taylor, P. Raval, D. Chen, H. Lowder, S. Murkerji, L.C. Andreae, B.P. Williams, D.P. Srivastava, Characterisation of neurons derived from a cortical human neural stem cell line CTX0E16, Stem Cell Res. Ther. 6 (2015) 1–20, https://doi.org/10.1186/s13287-015-0136-8.

[115] S. Suzuki, W. Akamatsu, F. Kisa, T. Sone, K. ichi Ishikawa, N. Kuzumaki, H. Katayama, A. Miyawaki, N. Hattori, H. Okano, Efficient induction of dopaminergic neuron differentiation from induced pluripotent stem cells reveals impaired mitophagy in PARK2 neurons, Biochem. Biophys. Res. Commun. 483 (2017) 88–93, https://doi.org/10.1016/j.bbrc.2016.12.188.

[116] J.C.M. Schlachetzki, S.W. Saliba, A.C.P. de Oliveira, Studying neurodegenerative diseases in culture models, Rev. Bras. Psiquiatr. 35 (2013) 92–100, https://doi.org/10.1590/1516-4446-2013-1159.

[117] A. Williams-Medina, M. Deblock, D. Janigro, In vitro models of the blood–brain barrier: tools in translational medicine, Front. Med. Technol. 2 (2020) 1–20, https://doi.org/10.3389/fmedt.2020.623950.

[118] A. Pérez-López, A.I. Torres-Suárez, C. Martín-Sabroso, J. Aparicio-Blanco, An overview of in vitro 3D models of the blood-brain barrier as a tool to predict the in vivo permeability of nanomedicines, Adv. Drug Deliv. Rev. 196 (2023), https://doi.org/10.1016/j.addr.2023.114816.

[119] H. Franke, H.J. Galla, C.T. Beuckmann, Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood-brain barrier in vitro, Brain Res. Protoc. 5 (2000) 248–256, https://doi.org/10.1016/S1385-299X(00)00020-9.

[120] I. Mármol, S. Abizanda-Campo, J.M. Ayuso, I. Ochoa, S. Oliván, Towards novel biomimetic in vitro models of the blood–brain barrier for drug permeability evaluation, Bioengineering 10 (2023), https://doi.org/10.3390/bioengineering10050572.

[121] B. Srinivasan, L. Shuler, J.J. Hickman, TEER measuments in cells. https://doi.org/10.1177/2211068214561025.TEER, 2015.

[122] C.D.K. Sloan, P. Nandi, T.H. Linz, J.V. Aldrich, K.L. Audus, S.M. Lunte, Analytical and biological methods for probing the blood-brain barrier, Annu Rev Anal Chem 5 (2012) 505–531, https://doi.org/10.1146/annurev-anchem-062011-143002.Analytical.

[123] E. Drobyshev, S. Raschke, R.A. Glabonjat, J. Bornhorst, F. Ebert, D. Kuehnelt, T. Schwerdtle, Capabilities of selenoneine to cross the in vitro blood–brain barrier model, Metallomics 13 (2021) 1–9, https://doi.org/10.1093/mtomcs/mfaa007.

[124] H. Lohren, J. Bornhorst, R. Fitkau, G. Pohl, H.J. Galla, T. Schwerdtle, Effects on and transfer across the blood-brain barrier in vitro-Comparison of organic and inorganic mercury species, BMC Pharmacol. Toxicol. 17 (2016) 1–12, https://doi.org/10.1186/s40360-016-0106-5.

[125] N. Yan, H. Jing, J. Wang, Z. Li, K. Xu, Q. Wang, J. Zheng, L. Shi, X. Cao, X. Duan, Arsenic induces blood−brain barrier disruption and regulates T lymphocyte subpopulation differentiation in the cerebral cortex and Hippocampus associated with the Nrf2 pathway in vivo, Biol. Trace Elem. Res. 201 (2023) 3981–3993, https://doi.org/10.1007/s12011-022-03500-3.

[126] B.S. Cummings, R.G. Schnellmann, Measurement of cell death in mammalian cells, Curr Protoc Pharmacol 1 (2004) 1–30, https://doi.org/10.1002/cpz1.210.

[127] K.M. McKinnon, Flow cytometry: an overview, Curr Protoc Immunol 120 (2019), https://doi.org/10.1007/978-94-017-0623-0_1.

[128] C. Cottet-Rousselle, X. Ronot, X. Leverve, J.F. Mayol, Cytometric assessment of mitochondria using fluorescent probes, Cytom. Part A 79 A (2011) 405–425, https://doi.org/10.1002/cyto.a.21061.

[129] M. Van Engeland, F.C.S. Ramaekers, B. Schutte, C.P.M. Reutelingsperger, A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture, Cytometry 24 (1996) 131–139, https://doi.org/10.1002/(SICI)1097-0320(19960601)24:2<131::AID-CYTO5>3.0.CO;2-M.

[130] J.C. Stockert, R.W. Horobin, L.L. Colombo, A. Blázquez-Castro, Tetrazolium salts and formazan products in Cell Biology: viability assessment, fluorescence imaging, and labeling perspectives, Acta Histochem 120 (2018) 159–167, https://doi.org/10.1016/j.acthis.2018.02.005.
[131] A. Van Tonder, A.M. Joubert, A.D. Cromarty, Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays, BMC Res. Notes. 8 (2015), https://doi.org/10.1186/s13104-015-1000-8.
[132] S.E. Winikoff, H.J. Zeh, R. DeMarco, M.T. Lotze, Cytolytic assays. https://doi.org/10.1016/B978-0-12-455900-4.50291-9, 2005.
[133] M.M. Rahman, J. Ukiana, R. Uson-Lopez, M.T. Sikder, T. Saito, M. Kurasaki, Cytotoxic effects of cadmium and zinc co-exposure in PC12 cells and the underlying mechanism, Chem. Biol. Interact. 269 (2017) 41–49, https://doi.org/10.1016/j.cbi.2017.04.003.
[134] G. Fotakis, J.A. Timbrell, In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride, Toxicol. Lett. 160 (2006) 171–177, https://doi.org/10.1016/j.toxlet.2005.07.001.
[135] Y. Christen, Oxidative stress and Alzheimer disease, Am. J. Clin. Nutr. 71 (2000), https://doi.org/10.1093/ajcn/71.2.621s.
[136] R.P. Singh, S. Sharad, S. Kapur, Free radicals and oxidative stress in neurodegenerative diseases : relevance of dietary antioxidants, Jiacm. 5 (2004) 218–225.
[137] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla, A. Bitto, Oxidative stress: harms and benefits for human health, Oxid. Med. Cell. Longev. 2017 (2017), https://doi.org/10.1155/2017/8416763.
[138] M. Valko, H. Morris, M. Cronin, Metals, toxicity and oxidative stress, curr, Med. Chem. 12 (2005) 1161–1208, https://doi.org/10.2174/0929867053764635.
[139] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (2007) 44–84, https://doi.org/10.1016/j.biocel.2006.07.001.
[140] M.V. Vettori, M. Goldoni, A. Caglieri, D. Poli, G. Folesani, S. Ceccatelli, A. Mutti, Antagonistic effects of methyl-mercury and PCB153 on PC12 cells after a combined and simultaneous exposure, Food Chem. Toxicol. 44 (2006) 1505–1512, https://doi.org/10.1016/j.fct.2006.04.009.
[141] F. Bovio, P. Melchioretto, M. Forcella, P. Fusi, C. Urani, Cadmium promotes glycolysis upregulation and glutamine dependency in human neuronal cells, Neurochem. Int. 149 (2021) 105144, https://doi.org/10.1016/j.neuint.2021.105144.
[142] R. Brandão, F.S. Lara, L.B. Pagliosa, F.A. Soares, J.B.T. Rocha, C.W. Nogueira, M. Farina, Hemolytic effects of sodium selenite and mercuric chloride in human blood, Drug Chem. Toxicol. 28 (2005) 397–407, https://doi.org/10.1080/01480540500262763.
[143] V. Glaser, E.M. Nazari, Y.M.R. Müller, L. Feksa, C.M.D. Wannmacher, J.B. T. Rocha, A.F. de Bem, M. Farina, A. Latini, Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mouse cerebral cortex, Int. J. Dev. Neurosci. 28 (2010) 631–637, https://doi.org/10.1016/j.ijdevneu.2010.07.225.
[144] A. Rembach, D.J. Hare, M. Lind, C.J. Fowler, R.A. Cherny, C. McLean, A.I. Bush, C.L. Masters, B.R. Roberts, Decreased copper in alzheimer's disease brain is predominantly in the soluble extractable fraction, Int. J. Alzheimers. Dis. 2013 (2013), https://doi.org/10.1155/2013/623241.
[145] N. Krebs, C. Langkammer, W. Goessler, S. Ropele, F. Fazekas, K. Yen, E. Scheurer, Assessment of trace elements in human brain using inductively coupled plasma mass spectrometry, J. Trace Elem. Med. Biol. 28 (2014) 1–7, https://doi.org/10.1016/j.jtemb.2013.09.006.
[146] D. Dahlberg, J. Ivanovic, E. Mariussen, B. Hassel, High extracellular levels of potassium and trace metals in human brain abscess, Neurochem. Int. 82 (2015) 28–32, https://doi.org/10.1016/j.neuint.2015.02.003.
[147] P. Ramos, A. Santos, N.R. Pinto, R. Mendes, T. Magalhães, A. Almeida, Anatomical regional differences in selenium levels in the human brain, Biol. Trace Elem. Res. 163 (2015) 89–96, https://doi.org/10.1007/s12011-014-0160-z.
[148] P. Ramos, A. Santos, E. Pinto, N.R. Pinto, R. Mendes, T. Magalhães, A. Almeida, Alkali metals levels in the human brain tissue: anatomical region differences and age-related changes, J. Trace Elem. Med. Biol. 38 (2016) 174–182, https://doi.org/10.1016/j.jtemb.2016.03.018.
[149] M. Korvela, A.L. Lind, M. Wetterhall, T. Gordh, M. Andersson, J. Pettersson, Quantification of 10 elements in human cerebrospinal fluid from chronic pain patients with and without spinal cord stimulation, J. Trace Elem. Med. Biol. 37 (2016) 1–7, https://doi.org/10.1016/j.jtemb.2016.06.003.
[150] M. de Oliveira, T.M.R. Gianeti, F.C.G. da Rocha, P.N. Lisboa-Filho, M. Piacenti-Silva, A preliminary study of the concentration of metallic elements in the blood of patients with multiple sclerosis as measured by ICP-MS, Sci. Rep. 10 (2020) 1–8, https://doi.org/10.1038/s41598-020-69979-9.
[151] J. Baj, G. Teresiński, B. Kowalska, T. Krajka, G. Buszewicz, A. Forma, W. Flieger, K.H. Karakuła, P. Kędzierawski, T. Cywka, J. Flieger, ICP-MS multi-elemental analysis of the human meninges collected from sudden death victims in south-eastern Poland, Molecules 27 (2022) 1–17, https://doi.org/10.3390/molecules27061911.
[152] A. Prange, D. Schaumlöffel, P. Brätter, A.N. Richarz, C. Wolf, Species analysis of metallothionein isoforms in human brain cytosols by use of capillary electrophoresis hyphenated to inductively coupled plasma-sector field mass spectrometry, Analytical and Bioanalytical Chemistry 371 (2001) 764–774, https://doi.org/10.1007/s002160101019.
[153] O.P. Ajsuvakova, A.A. Tinkov, D. Willkommen, A.A. Skalnaya, A.B. Danilov, A. A. Pilipovich, M. Aschner, A.V. Skalny, B. Michalke, M.G. Skalnaya, Assessment of copper, iron, zinc and manganese status and speciation in patients with Parkinson's disease: a pilot study, J. Trace Elem. Med. Biol. 59 (2020) 126423, https://doi.org/10.1016/j.jtemb.2019.126423.
[154] M. Wang, W.Y. Feng, H.J. Wang, Y. Zhang, J. Li, B. Li, Y.L. Zhao, Z.F. Chai, Analysis of mercury-containing protein fractions in brain cytosol of the maternal and infant rats after exposure to a low-dose of methylmercury by SEC coupled to isotope dilution ICP-MS, J. Anal. At. Spectrom. 23 (2008) 1112–1116, https://doi.org/10.1039/b802124d.
[155] B.R. Cardoso, D.J. Hare, A.I. Bush, Q.X. Li, C.J. Fowler, C.L. Masters, R.N. Martins, K. Ganio, A. Lothian, S. Mukherjee, E.A. Kapp, B.R. Roberts, Selenium levels in serum, red blood cells, and cerebrospinal fluid of alzheimer's disease patients: a report from the Australian imaging, biomarker & lifestyle flagship study of ageing (aibl), J. Alzheimer's Dis. 57 (2017) 183–193, https://doi.org/10.3233/JAD-160622.
[156] T. Kiguchi, Y. Yoshino, B. Yuan, S. Yoshizawa, T. Kitahara, D. Akahane, M. Gotoh, T. Kaise, H. Toyoda, K. Ohyashiki, Speciation of arsenic trioxide penetrates into cerebrospinal fluid in patients with acute promyelocytic leukemia, Leuk. Res. 34 (2010) 403–405, https://doi.org/10.1016/j.leukres.2009.08.001.
[157] J. Liu, L. Zheng, Q. Li, L. Feng, B. Wang, M. Chen, M. Wang, J. Wang, W. Feng, Isotope dilution LA-ICP-MS for quantitative imaging of trace elements in mouse brain sections, Anal. Chim. Acta. 1273 (2023) 341524, https://doi.org/10.1016/j.aca.2023.341524.
[158] R. Weiskirchen, S. Weiskirchen, P. Kim, R. Winkler, Software solutions for evaluation and visualization of laser ablation inductively coupled plasma mass spectrometry imaging (LA-ICP-MSI) data: a short overview, J. Cheminform. 11 (2019) 1–21, https://doi.org/10.1186/s13321-019-0338-7.
[159] M. Cruz-Alonso, B. Fernandez, A. Navarro, S. Junceda, A. Astudillo, R. Pereiro, Laser ablation ICP-MS for simultaneous quantitative imaging of iron and ferroportin in hippocampus of human brain tissues with Alzheimer's disease, Talanta 197 (2019) 413–421, https://doi.org/10.1016/j.talanta.2019.01.056.
[160] S. Zia, A. Islam Aqib, A. Muneer, M. Fatima, K. Atta, T. Kausar, C.N.F. Zaheer, I. Ahmad, M. Saeed, A. Shafique, Insights into nanoparticles-induced neurotoxicity and cope up strategies, Front. Neurosci. 17 (2023) 1–13, https://doi.org/10.3389/fnins.2023.1127460.
[161] N. Zhao, Z. Quicksall, Y.W. Asmann, Y. Ren, Network approaches for omics studies of neurodegenerative diseases, Front. Genet. 13 (2022) 1–6, https://doi.org/10.3389/fgene.2022.984338.
[162] D.M. Teleanu, C. Chircov, A.M. Grumezescu, R.I. Teleanu, Neurotoxicity of nanomaterials: an up-to-date overview, Nanomaterials 9 (2019) 1–14, https://doi.org/10.3390/nano9010096.
[163] R. Akçan, H.C. Aydogan, M.Ş. Yildirim, B. Taştekin, N. Sağlam, Nanotoxicity: a challenge for future medicine, Turkish J. Med. Sci. 50 (2020) 1180–1196, https://doi.org/10.3906/sag-1912-209.
[164] D. Vicente-Zurdo, I. Romero-Sánchez, N. Rosales-Conrado, M.E. León-González, Y. Madrid, Ability of selenium species to inhibit metal-induced Aβ aggregation involved in the development of Alzheimer's disease, Anal. Bioanal. Chem. 412 (2020) 6485–6497, https://doi.org/10.1007/s00216-020-02644-2.
[165] F. Arnhold, K.H. Gührs, A. von Mikecz, Amyloid domains in the cell nucleus controlled by nucleoskeletal protein lamin B1 reveal a new pathway of mercury neurotoxicity, PeerJ 2015 (2015), https://doi.org/10.7717/peerj.754.
[166] C. Wallin, M. Friedemann, S.B. Sholts, A. Noormägi, T. Svantesson, J. Jarvet, P. M. Roos, P. Palumaa, A. Gräslund, S.K.T.S. Wärmländer, Mercury and alzheimer's disease: Hg(II) ions display specific binding to the amyloid-β peptide and hinder its fibrillization, Biomolecules 10 (2020), https://doi.org/10.3390/biom10010044.
[167] T.C. Long, J. Tajuba, P. Sama, N. Saleh, C. Swartz, J. Parker, S. Hester, G. V. Lowry, B. Veronesi, Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro, Environ. Health Perspect. 115 (2007) 1631–1637, https://doi.org/10.1289/ehp.10216.
[168] M. Malatesta, Transmission electron microscopy as a powerful tool to investigate the interaction of nanoparticles with subcellular structures, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms222312789.
[169] L.X. Li, J.H. Chu, X.W. Chen, P.C. Gao, Z.Y. Wang, C. Liu, R.F. Fan, Selenium ameliorates mercuric chloride-induced brain damage through activating BDNF/TrKB/PI3K/AKT and inhibiting NF-κB signaling pathways, J. Inorg. Biochem. 229 (2022) 111716, https://doi.org/10.1016/j.jinorgbio.2022.111716.

VII. DISCUSIÓN INTEGRADORA

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En este apartado se discuten de forma global los resultados derivados de los trabajos de investigación presentados en esta Tesis Doctoral, donde se ha abordado la identificación de biomoléculas y especies de selenio y mercurio presentes en pescados, productos derivados y procesados del pescado. Asimismo, también se ha investigado el efecto protector de distintas especies de selenio frente a la neurotoxicidad inducida por el mercurio empleando la línea celular de neuroblastoma humano (SH-SY5Y).

El **selenio** constituye un micronutriente esencial con importantes propiedades antioxidantes. Sin embargo, dicha esencialidad es dependiente de la dosis ingerida, su forma química y su biodisponibilidad, siendo las especies inorgánicas (Se(IV), Se(VI)) más tóxicas que las especies orgánicas (SeMet, SeCys, SeMeSeCys, γ–Glu-SeMeSeCys, entre otras). De entre las especies orgánicas, la SeCys es la que se incorpora a las proteínas y confiere el carácter esencial a este elemento. Por otro lado, como antagonista de dicha esencialidad, nos encontramos al **mercurio**, cuyo significado biológico se limita a su toxicidad, si bien es cierto que esta es dependiente de la dosis ingerida, su forma química y su biodisponibilidad, siendo el $MeHg^+$ la especie más tóxica, por su tendencia a bioacumularse y biomagnificarse a lo largo de la cadena trófica.

Los **pescados** constituyen una importante fuente de selenio en nuestra dieta, además de contribuir a la ingesta de otros nutrientes esenciales, como proteínas de alto valor nutricional y ácidos grasos. Sin embargo, también pueden acumular cantidades relativamente elevadas de elementos tóxicos, como es el mercurio, sobre todo en aquellos pescados que se sitúan en los niveles más altos de la cadena trófica marina. Bajo este contexto, llevar a cabo estudios, no solamente acerca de los contenidos totales de selenio y mercurio, sino de sus especies, es de especial relevancia para una correcta evaluación de la calidad y seguridad de estos alimentos.

En este contexto, es importante mencionar el incremento del consumo de **alimentos derivados del pescado** como sustitutos del pescado fresco o congelado. Debido a que estos alimentos procesados son cada vez más consumidos, también pueden suponer una fuente importante de selenio y de mercurio en nuestra dieta al estar ambos elementos presentes en los mismos y en las materias primas a partir de las cuales se producen. Hasta la fecha, no existían datos en la literatura científica sobre los niveles de selenio y mercurio en este tipo de productos.

Los pescados, productos derivados y procesados considerados en la investigación recogida en la presente Tesis Doctoral son alimentos de consumo frecuente y han sido adquiridos en supermercados locales. Entre los pescados seleccionados se encuentran el **atún** (*Thunnus*) y el **pez espada** (*Xiphias gladius*), por estar en la parte superior de la cadena trófica marina y, por consiguiente, presentar niveles relativamente elevados de selenio y mercurio. De igual modo, para investigar si hay diferencias, bajo este contexto, entre pescados con diferente alimentación, es decir, un pescado salvaje y otro proveniente de acuicultura, se seleccionaron también el **salmón rosado salvaje** (*Oncorhynchus gorbuscha*) y el **salmón de piscifactoría** (*Salmo salar*). En todos los casos, la parte del pescado evaluada fue el músculo, ya que es la parte comestible del mismo.

Por otro lado, en lo que respecta a los productos derivados comercializados, las huevas son un ejemplo claro de dichos productos. Se han considerado tres tipos: **huevas de lumpo** (*Cyclopterus lumpus*), **huevas de trucha** (*Oncorhynchus mykiss*) y **huevas de salmón rosado salvaje** (*Oncorhynchus gorbuscha*). Finalmente, también se han incluido productos procesados derivados del pescado elaborados a base de surimi, como son los comúnmente denominados **palitos de cangrejo**, las **gulas** y los **tallarines elaborados a partir de bacalao o salmón**.

Los **artículos 1 y 2** se centraron en la evaluación del selenio y del mercurio presentes en los pescados, productos derivados y procesados. Para ello, se estudiaron los contenidos totales y las especies de ambos elementos, así como la distribución del selenio y mercurio asociado a biomoléculas (proteínas). Asimismo, a partir de los contenidos totales de estos elementos en los distintos productos considerados, se calcularon las **relaciones molares Se:Hg** y el índice ***Selenium Health Benefit Value*** o **$\mathbf{HBV_{Se}}$,** indispensable para evaluar los riesgos-beneficios asociados a la exposición al mercurio por el consumo de este tipo de alimentos. Fruto de la interacción existente entre el selenio y el mercurio, aunque no del todo elucidada, la hipótesis más aceptada sugiere que la toxicidad del mercurio se ve reducida cuando hay un exceso molar de selenio en los tejidos, es decir, cuando la relación molar Se:Hg es mayor que 1. De este modo, da lugar a la formación de los compuestos inertes e insolubles de HgSe, favoreciendo así la disminución de la toxicidad del mercurio. De la misma manera, el parámetro HBV_{Se} es crucial para evaluar cuánto selenio queda disponible para el correcto desarrollo de sus funciones biológicas, una vez producida su interacción con el mercurio. Así, valores positivos indican que, aparentemente, no hay un riesgo para el consumidor por la toxicidad asociada al mercurio (Ralston *et al.*, 2019).

La determinación de los contenidos totales de selenio y mercurio en las muestras de interés se llevó a cabo mediante un tratamiento de muestra basado en una digestión ácida asistida en un horno microondas y posterior detección por ICP-MS, para el caso del selenio, y mediante análisis directo, sin previo tratamiento de muestra, por DMA, para el caso del mercurio.

En cuanto a los resultados obtenidos en el análisis del **músculo de los pescados**, las concentraciones de selenio y mercurio total varían entre (0.68 ± 0.05) y (2.2 ± 0.1) mg kg^{-1} y entre (0.011 ± 0.001) y (1.45 ± 0.04) mg kg^{-1}, respectivamente. Como ha sido comentado anteriormente, el atún y el pez espada, que se encuentran en los niveles superiores de la cadena trófica, siendo por tanto peces grandes y depredadores, presentan los niveles más altos de ambos analitos, debido al proceso de biomagnificación que tiene lugar. Sin embargo, al igual que los demás pescados, el atún y el pez espada, aun con valores relativamente elevados de mercurio (> 0.7 mg kg^{-1}), presentan valores de relaciones molares de Se:Hg mayores que 1, y valores del parámetro HBV_{Se} positivos. Cabe destacar las diferencias estadísticamente significativas (p-valor < 0.05) evidenciadas entre los valores de mercurio de los dos tipos de salmones analizados (piscifactoría y salvaje), presentando mayores contenidos de mercurio el salmón rosado salvaje, lo que se puede atribuir a las diferencias en las condiciones de vida que llevan, tanto del lugar donde habitan como en su tipo de alimentación.

Debido a que tanto el selenio como el mercurio se encuentran en el pescado unidos principalmente a proteínas, inicialmente, se procedió a la extracción de los tres tipos de proteínas existentes en el tejido muscular (parte comestible del pescado): **proteínas sarcoplasmáticas, miofibrilares y álcali-solubles**. Este enfoque lo diferencia de la gran mayoría de trabajos existentes que únicamente abordan estos estudios en las proteínas solubles del músculo del pescado, sin hacer mayor diferenciación. Para ello, y debido a la diferencia de solubilidad que presentan, se llevó a cabo una extracción de proteínas secuencial en el músculo de los pescados considerados. Primeramente, las proteínas sarcoplasmáticas se extrajeron en una disolución salina de baja fuerza iónica (tampón fosfato 0.05 mol L^{-1}, pH 7.5), seguidamente, las proteínas miofibrilares en una disolución salina de alta fuerza iónica (tampón fosfato 0.05 mol L^{-1} y KCl 0.45 mol L^{-1}, pH 7.5) y, finalmente, para la extracción de las proteínas álcali-solubles se utilizó una disolución de NaOH 0.1 mol L^{-1}, pH 13.2. Además, se añadió en todas las extracciones el cóctel de inhibidor de proteasas para impedir la degradación proteolítica durante el procedimiento de extracción.

Una vez realizadas las extracciones, se analizaron mediante HPLC-UV-Vis y HPLC-ICP-MS, empleando la columna cromatográfica de exclusión por tamaños (SEC) Superdex 200, que proporciona un rango de separación de tamaños moleculares entre 10 y 600 kDa. Los perfiles cromatográficos obtenidos mediante HPLC-UV-VIS evidenciaron que la diferencia más notable que se aprecia entre los perfiles cromatográficos del mismo tipo de proteínas extraídas de los diferentes pescados analizados reside en las proteínas sarcoplasmáticas extraídas del atún y del pez espada. Mientras que la mayoría de las proteínas sarcoplasmáticas extraídas para el atún tienen una masa molecular de en torno a 4 kDa (pico mayoritario), las extraídas para el pez espada presentan, además, masas moleculares alrededor de 7 y 13 kDa, en similar proporción, y de unos 48 kDa, siendo estas últimas el pico mayoritario obtenido en el cromatograma. Estas diferencias pueden atribuirse, entre otras cosas, al distinto contenido en grasa que presentan estos pescados, ya que el atún es comúnmente considerado un pescado azul o graso, con un contenido en grasa que supera el 5 %, mientras que el pez espada es un pescado semigraso, con un contenido en grasa cercano al 2 %. Otra posible explicación radica en la mioglobina, proteína incluida en el grupo de las sarcoplasmáticas y responsable del transporte de oxígeno en el músculo. A diferencia de lo que sucede en el pez espada, la mioglobina constituye alrededor del 80 % de todas las hemoproteínas presentes en el atún, siendo la principal responsable de su color rojo oscuro (Hashimoto *et al.*, 1979).

En cuanto a la distribución de selenio y mercurio en las proteínas, los análisis por HPLC-ICP-MS de los extractos proteicos evidenciaron que el selenio se encuentra asociado de forma mayoritaria a biomoléculas de baja masa molecular, de unos 8 kDa, para todos los tipos de proteínas extraídas de todos los pescados analizados (atún, pez

espada, salmón de piscifactoría y salmón rosado salvaje). Sin embargo, en el caso del mercurio, los perfiles obtenidos son más variables entre las muestras, constatando además picos correspondientes a mercurio unido a biomoléculas de mayores masas moleculares, sobre todo en el caso de las proteínas miofibrilares y álcali-solubles, a excepción de las proteínas miofibrilares extraídas del músculo de los pescados salmón de piscifactoría y salmón rosado salvaje, donde apenas se observaron picos correspondientes a mercurio. En relación con estos dos últimos pescados, cabe destacar que los perfiles de selenio y mercurio unidos a biomoléculas para los tres tipos de proteínas extraídas de ambos son muy similares, no constatando diferencias significativas en los perfiles de distribución de ambos analitos. Esto sugiere que el selenio y el mercurio se unen de forma preferente a compuestos de una determinada masa molecular, independiente de la alimentación y el desarrollo del salmón. Finalmente, es importante mencionar que **se obtuvieron picos cromatográficos donde el selenio y el mercurio aparecían asociados a la misma masa molecular: en el caso de las proteínas sarcoplasmáticas de unos 64 y 12 kDa, y de las proteínas álcali-solubles de unos 574 kDa, extraídas el músculo del atún, lo que podría evidenciar algún tipo de interacción entre estos elementos.**

Posteriormente se determinó la concentración de selenio asociada a cada una de las fracciones proteicas. Los resultados varían según el tipo de pescado, aunque el resultado de la suma del contenido de selenio extraído de todas las fracciones fue superior al 84 % del contenido de selenio total. **Para el atún, la fracción proteica con mayor cantidad de selenio, en relación con su concentración de selenio total, es la sarcoplasmática, a diferencia de lo que ocurre en el resto de los pescados**. **Para el pez espada y el salmón de piscifactoría, la fracción miofibrilar es la que evidencia mayor contenido en selenio, y para el salmón rosado salvaje es la álcali-soluble**.

A continuación, y para completar el estudio, también se realizó el análisis de especies de selenio en las tres fracciones proteicas aisladas del músculo del pescado. El proceso de hidrólisis enzimática para obtener los selenoaminoácidos es un paso crítico en el tratamiento de la muestra para su posterior análisis en los estudios de especiación. En este caso, los extractos proteicos obtenidos se sometieron a una hidrolisis enzimática con proteasa XIV (aislada de *Streptomyces griseus*). En el caso de las proteínas álcali-solubles, y previamente a la adición de la enzima, fue necesario un ajuste del pH a 7-8 para que la proteasa alcanzase su actividad óptima, ya que los extractos proteicos se encontraban inicialmente a un pH excesivamente básico (13.2). Posteriormente, las muestras conjuntamente con la enzima se incubaron en un baño termostático durante toda la noche (aproximadamente 16 h) a una temperatura controlada de 37 °C.

En cuanto al análisis de especiación de selenio, la separación y posterior detección de las especies de interés se llevó a cabo mediante el acoplamiento HPLC-ICP-MS, empleando la columna de fase inversa Phenomenex Kinetex EVO C18 (150x3 mm, 5 µm). Por otro lado, para una correcta identificación de las especies de selenio, que es uno de los grandes problemas de la especiación, es necesario utilizar otra técnica complementaria, ya que la identificación no debe estar únicamente basada en los tiempos de retención de los compuestos y a experimentos de enriquecimiento, pues puede darse la coelución de especies. Es por ello por lo que se empleó como técnica confirmatoria la cromatografía de líquidos acoplada a espectrometría de masas en tándem (HPLC-ESI-MS/MS), utilizando la misma columna cromatográfica debido a la compatibilidad que presenta con el sistema de detección. Estos análisis llevados a cabo permitieron la **identificación y confirmación de la presencia de SeMeSeCys en los hidrolizados enzimáticos de las proteicas sarcoplasmáticas, miofibrilares y álcali-solubles de todos los pescados analizados (atún, pez espada, salmón de piscifactoría y salmón rosado salvaje)**.

Adicionalmente, también se realizó la especiación de selenio en las proteínas solubles en Tris-HCl, ya que son las más comúnmente estudiadas en la literatura científica. Para ello, las muestras de músculo de pescado fueron tratadas con la proteasa inespecífica aislada de *Streptomyces griseus* (proteasa XIV) en medio extractante Tris-HCl (pH 7.5), con incubación en un baño termostático durante toda la noche (aproximadamente 16 h) y a una temperatura controlada de 37 °C. Los análisis por HPLC-ICP-MS, y confirmados por HPLC-ESI-MS/MS, volvieron a constatar la **presencia de SeMeSeCys en todos los pescados, y en el caso del atún también se identificó SeMet**. Las biotransformaciones que sufre el selenio en los procesos de incorporación desde el medio marino (agua, plantas, etc.) a la cadena trófica y su posterior biomagnificación son los responsables de que especies como SeMet y SeMeSeCys estén presentes en los tejidos de los pescados.

En el caso del mercurio, los estudios de especiación también se realizaron mediante el acoplamiento HPLC-ICP-MS, utilizando una columna de fase inversa, Phenomenex Kinetex EVO C18 (150x3 mm, 5 µm), previa extracción de las especies, ahora empleando un compuesto con propiedades complejantes hacia el mercurio, como es la cisteína, y concretamente se empleó una disolución de L-cisteína·HCl·H_2O al 1 % (w/v), aplicando calor en una estufa, a 60 °C, durante 2 h. Cabe destacar que el análisis por ICP-MS presenta problemas de efecto memoria. Sin embargo, este problema puede ser resuelto utilizando una disolución de oro de 500 µg L^{-1} preparada al 2 % (v/v) en ácido nítrico para arrastrar los restos de mercurio que se quedan en el sistema.

Las especies metilmercurio ($MeHg^+$) y mercurio inorgánico (Hg^{2+}) fueron identificadas en todas las muestras de pescado, siendo el $MeHg^+$ la especie mayoritaria en todos los casos. Sin embargo, se evidenciaron diferencias significativas entre las proporciones de $MeHg^+$ y Hg^{2+} de las muestras analizadas, constatando, y en consonancia con otros autores (Forsyth *et al.*, 2004), que **no existe un factor de conversión fijo que correlacione la cantidad de $MeHg^+$ existente con respecto al contenido total de mercurio presente en un pescado**, ya que depende de parámetros biológicos como el nivel trófico en que se sitúen, la edad, el tamaño, el sexo, el metabolismo o los hábitos de alimentación. Los resultados obtenidos muestran que, **en los pescados depredadores, como el atún y el pez espada, la concentración de $MeHg^+$ supone más del 90 % del contenido total de mercurio ((110 ± 6) y (96 ± 3) %, respectivamente), a diferencia de lo que ocurre en los pescados situados en eslabones inferiores de la cadena trófica**. Esto es, entre otros motivos, debido a que la estabilidad de las especies de mercurio es distinta, es decir, el $MeHg^+$ permanece más tiempo en un organismo que el Hg^{2+}, por tanto, las especies depredadoras y los peces de edad más avanzada tendrán mayores concentraciones de la especie metilada (Povari, 2003; Forsyth *et al.*, 2004; Park & Zheng, 2012). En este sentido, **el salmón de piscifactoría y el salmón rosado salvaje analizados, que se sitúan por debajo del atún y del pez espada en la cadena trófica, presentan las mayores concentraciones relativas de Hg^{2+} obtenidas de todas las muestras consideradas en este estudio, (35 ± 1) y (27 ± 4) %, respectivamente.**

Además de los pescados, también se han considerado en estos estudios las **huevas de pescado**, ya que son un producto comercial derivado del pescado, que podemos encontrar fácilmente en el supermercado y que se consume con cierta frecuencia. Además, destacan por sus beneficios nutricionales, ya que constituyen una fuente de proteínas de alta calidad, ácidos grasos omega-3, vitaminas y minerales, como selenio, hierro, calcio o zinc. De igual manera, en esta Tesis Doctoral se han considerado también **productos procesados derivados del pescado**, elaborados a base de surimi, como son los palitos de cangrejo, las gulas y dos tipos de tallarines elaborados a base de bacalao y de salmón, respectivamente. Estos alimentos están suponiendo nuevas maneras de introducir la ingesta de pescado en nuestra dieta, debido a la versatilidad que ofrecen en las comidas o a la mínima preparación culinaria que requieren, entre otros aspectos. Sin embargo, es relevante mencionar que, hoy en día, no existen estudios en la literatura científica que evalúen la exposición del consumidor con respecto al selenio, al mercurio y a sus respectivas especies, por el consumo de estos productos.

Estos productos derivados presentan valores relativamente elevados de selenio total, especialmente en el caso de las huevas de los pescados analizados, comprendidos entre (1.3 ± 0.2) y (4.4 ± 0.4) mg kg^{-1}, perteneciendo este último valor, especialmente elevado, a las huevas de salmón rosado salvaje. Estas concentraciones encontradas en las huevas son comparables, e incluso superiores, a lo encontrado en pescados y, especialmente, a las altas concentraciones que poseen los pescados depredadores (atún y pez espada), en torno a 2 mg kg^{-1}. Por su parte, los productos procesados derivados del pescado evidenciaron menores concentraciones de selenio total que para el caso de las huevas, con valores de (0.78 ± 0.08), (0.75 ± 0.05), (0.74 ± 0.03) y (0.79 ± 0.02) mg kg^{-1} para los palitos de cangrejo, las gulas, los tallarines de bacalao y los tallarines de salmón, respectivamente. Con relación al mercurio, tanto las huevas como los productos procesados arrojaron valores de mercurio total relativamente bajos en comparación con el músculo de los pescados analizados, estando todos por debajo de 0.06 mg kg^{-1}. Por tanto, las relaciones molares Se:Hg fueron mayores que 1 y los valores del parámetro HBV_{Se} positivos, tanto para las huevas como para los productos derivados analizados.

Estos resultados son concordantes con lo reportado por otros autores (Zhang *et al.*, 2020), pudiendo concluir que **el músculo de pescado parece ser un órgano de almacenamiento de mercurio, mientras que las huevas constituyen una importante fuente de selenio.**

A tenor de los resultados obtenidos, podemos constatar que, de manera general, **todas las opciones, tanto de pescados como de sus productos derivados y procesados, son seguras, *a priori*, en base a las relaciones molares Se:Hg y al parámetro HBV_{Se} obtenidos y que, por tanto, un consumo moderado de estos pescados/productos no tendría consecuencias negativas asociadas con el mercurio en la salud humana.**

En lo que respecta a la especiación del mercurio, en el caso de los tres tipos de huevas consideradas (de lumpo, de trucha y de salmón rosado salvaje) no se consiguió identificar ninguna especie, debido al bajo contenido de mercurio total que presentan. En cuanto a los productos derivados elaborados a partir de pescado (palitos de cangrejo, gulas, tallarines de bacalao y tallarines de salmón), como se ha comentado anteriormente, **es esperable que contengan una cantidad relativamente menor de mercurio en forma de $MeHg^+$, ya que estos productos se elaboran principalmente a partir de especies de peces no depredadores**, como el abadejo, la merluza o el salmón (en este caso, de piscifactoría). Los resultados obtenidos para estas muestras están en consonancia con esta afirmación, obteniéndose concentraciones de Hg^{2+} comprendidas entre (0.004 ± 0.001) y (0.006 ± 0.001) mg kg^{-1}, lo que representa entre un 10 y un 20 % del contenido total. En lo que respecta al $MeHg^+$, se encontraron concentraciones comprendidas entre (0.018 ± 0.003) y (0.051 ± 0.002) mg kg^{-1}, lo que corresponde a porcentajes de $MeHg^+$, en general, menores que en el caso de los pescados, llegando a representar, por ejemplo, para el caso de los tallarines de salmón un (62 ± 5) % del contenido de mercurio total.

Por otro lado, en relación con los estudios de especiación de selenio en estos productos, previamente se sometieron a un proceso de extracción de proteínas solubles, utilizando Tris-HCl (pH 7.5) como extractante, y posterior hidrólisis enzimática por acción de la proteasa inespecífica aislada de *Streptomyces griseus* (proteasa XIV). Las muestras se incubaron en un baño termostático durante toda la noche (aproximadamente 16 h) a una temperatura controlada de 37 °C. Algunos trabajos de especiación recogidos en la literatura científica evidencian menores tiempos de extracción requeridos al emplear la sonda de ultrasonidos. Sin embargo, otros han demostrado que su uso puede no ser adecuado dependiendo de la matriz objeto de análisis, así como de las especies a determinar en la misma, proporcionando mejores resultados un tratamiento de muestra más suave mediante una incubación a temperatura controlada, y evitando así la posible degradación de especies. Esto lleva a la obtención de mejores perfiles cromatográficos como consecuencia, probablemente, de una mayor eficiencia en la actuación de las enzimas al emplear tiempos de extracción más prolongados. Mediante este procedimiento se obtuvieron eficiencias de extracción para los productos derivados analizados superiores al (77 ± 3) %.

Una vez extraídas las especies de selenio, la separación y posterior detección de estas se llevó a cabo mediante análisis por HPLC-ICP-MS, utilizando la columna de intercambio aniónico, Hamilton PRP-X100 (250x4.1 mm, 10 µm), y posterior confirmación por HPLC-ESI-MS/MS, empleando una columna de fase inversa, Kinetex EVO C18 (150x3 mm, 5 µm). En este último caso se empleó un mecanismo cromatográfico distinto con una doble finalidad: como técnica cromatográfica confirmatoria alternativa y debido a la compatibilidad que presenta con el sistema HPLC-ESI-MS/MS.

Análogamente a lo que ocurría en el músculo de los pescados, **se evidenció la presencia de SeMet y SeMeSeCys, tanto en las huevas como en los productos procesados derivados del pescado, siendo la SeMet la especie mayoritaria en todos los casos**. Las concentraciones de SeMet y SeMeSeCys obtenidas se situaron entre (0.31 ± 0.04) y (2.40 ± 0.07) mg kg^{-1}, y entre (0.13 ± 0.01) y (0.69 ± 0.03) mg kg^{-1}, respectivamente, presentando los tres tipos de huevas analizadas (de lumpo, de trucha y de salmón rosado salvaje) las concentraciones más elevadas de ambas especies, al igual que ocurría con los contenidos totales en selenio.

Ambas especies mayoritarias de selenio, SeMet y SeMeSeCys, fueron identificadas tanto en el músculo de pescados frecuentemente consumidos, como en alimentos comerciales derivados del pescado. Como es bien sabido, la SeMet sirve como almacén de selenio en las proteínas, constituyendo las proteínas que contienen selenio, ya que es capaz de sustituir a la Met. Además, es la forma de selenio más fácilmente absorbida por el organismo, siendo una manera estable de almacenarlo. Por su parte, la SeMeSeCys, un aminoácido no proteico, es considerada la forma de selenio más eficiente en su efecto antioxidante y anticarcinogénico, ofreciendo protección frente a algunos tipos de cáncer.

Los procesos de elaboración de los productos procesados del pescado pueden afectar a la distribución de los nutrientes y componentes tóxicos. Por ello, resulta interesante hacer un seguimiento de estos dos elementos, selenio y mercurio, en cada una de las etapas del proceso de producción, determinando tanto los contenidos totales como las especies, desde las materias primas hasta los productos finales, pasando por los productos intermedios. En este contexto, en el **artículo 3** se incluyeron las materias primas, los productos intermedios y los productos finales procedentes de las cadenas de producción de los productos procesados derivados del pescado estudiados en los trabajos anteriores: gulas, palitos de cangrejo y tallarines de salmón. Todas las muestras estudiadas en cada una de las etapas de una misma cadena de producción pertenecían a un mismo lote, es decir, las materias primas analizadas eran las utilizadas posteriormente para fabricar los productos intermedios y finales considerados. Todas estas muestras fueron suministradas directamente por las empresas colaboradoras en esta investigación: Angulas Aguinaga y Grupo Nueva Pescanova.

Las líneas de producción correspondientes a los palitos de cangrejo y gulas hacen referencia a las designadas como **líneas de producción 1 y 2**, respectivamente. Las materias primas empleadas en ambas son las mismas: abadejo (*Gadus chalcogrammus*) y merluza del Pacífico Norte (*Merluccius productus*), dando lugar a los productos intermedios palitos de cangrejo semiterminados y gulas semiterminadas, que, una vez llevado a cabo el proceso de pasteurización, proporcionan los productos finales. Por otro lado, para la elaboración de los tallarines de salmón, correspondientes a la **línea de producción 3**, se utilizaron dos salmones de piscifactoría criados con distinta alimentación (*Salmo salar*) y merluza del Pacífico (*Merluccius productus*). El proceso general de elaboración de estos productos con base en surimi se esquematiza en la **Figura 7** (apartado 2.1 de la Introducción).

Con respecto a las líneas de producción 1 y 2 y a los contenidos en selenio total, no se evidenciaron diferencias estadísticamente significativas (p-valor < 0.05) entre las materias primas, es decir, entre el abadejo y la merluza del Pacífico Norte, con concentraciones de (0.68 ± 0.03) y (0.69 ± 0.05) mg kg^{-1}, respectivamente. Tampoco se constataron diferencias estadísticamente significativas (p-valor < 0.05) entre las muestras analizadas del producto semiterminado y producto final para un mismo alimento procesado. **Mientras que los palitos de cangrejo no presentaron diferencias estadísticamente significativas (p-valor < 0.05) en su contenido en selenio total con respecto al de sus materias primas, las gulas sí las mostraron con respecto al abadejo**. Atendiendo al contenido en mercurio total, destaca la merluza, con la concentración más alta obtenida para las muestras de estas cadenas de producción 1 y 2, (0.039 ± 0.001) mg kg^{-1}, presentando diferencias estadísticamente significativas (p-valor < 0.05) con el resto de muestras de estas cadenas de producción. De igual modo, es relevante mencionar que **los productos finales tienen menor contenido en mercurio que las materias primas empleadas en su elaboración**. Es importante tener en cuenta que la cantidad de pescado en estos productos finales representa menos de un 50 % del total de los ingredientes empleados.

En relación con la tercera línea de producción, empleada en la elaboración de los tallarines de salmón, las tres materias primas (salmón de piscifactoría 1, salmón de piscifactoría 2 y merluza) evidencian diferencias estadísticamente significativas (p-valor < 0.05), no solo entre sus concentraciones en selenio total ((0.41 ± 0.06), (0.61 ± 0.01) y (0.89 ± 0.03) mg kg^{-1}, respectivamente), sino también entre las de mercurio total ((0.031 ± 0.001), (0.0239 ± 0.0006) y (0.0471 ± 0.0009) mg kg^{-1}, respectivamente). Finalmente, aunque con ligeras diferencias, **los niveles de selenio y mercurio total encontrados en el producto final, (0.72 ± 0.03) y (0.0345 ± 0.001) mg kg^{-1}, respectivamente, son del orden de los encontrados en las materias primas**.

Además**, tanto las materias primas como los productos de las tres líneas de producción presentan valores de relaciones molares de Se:Hg mayores que 1 y valores del parámetro HBV_{Se} positivos**, por lo que el consumo tanto de los pescados como los productos procesados elaborados a partir de los mismos no tendría, *a priori*, efectos negativos en la salud del consumidor asociados con la toxicidad del mercurio.

La legislación vigente sobre los límites máximos permitidos de mercurio para los productos pesqueros y el músculo de los pescados está regulada por la EFSA (EFSA, 2012), estando fijado un límite máximo de 0.5 mg kg^{-1} para el contenido total de mercurio en estos alimentos, a excepción del músculo de algunos pescados específicos, para los que aplica un límite de 1.0 mg kg^{-1}, como son el atún (*Thunnus species,* *Euthynnus species, Katsuwonus pelamis*), el pez espada (*Xiphias gladius*), el tiburón (todas las especies), el rape (*Lophius species*), la anguila (*Anguilla species*), el gallo (*Lepidorhombus*) o el esturión (*Acipenser species*), entre otros. Sin embargo, algunos peces depredadores como el atún, por ejemplo, pueden llegar a contener hasta 1.5 mg kg^{-1} de mercurio, ya que puede haber variaciones en la concentración dependiendo de las características específicas del ecosistema.

En lo que respecta a los pescados, productos derivados y procesados considerados en este trabajo, todos ellos cumplen con la legislación establecida por la EFSA, si estos últimos son considerados productos pesqueros, ya que no existe legislación específica para ellos.

En relación con las especies de selenio identificadas mediante HPLC-ICP-MS y confirmadas por HPLC-ESI-MS/MS, **se constató la presencia de SeMeSeCys, SeMet y SeMetO en todas las muestras analizadas**. Esta última especie, la selenometionina oxidada, es un compuesto que aparece fruto de la oxidación de la SeMet durante el almacenamiento o durante el proceso de extracción de las especies.

La SeMet fue la especie de selenio mayoritaria en todas las muestras analizadas, cuadruplicando en algunos casos la concentración de la SeMeSeCys. En este sentido, la SeMet representa entre un 44 y un 65 % del selenio total en las materias primas correspondientes a las líneas de producción 1 y 2 (abadejo y merluza), mientras que la SeMeSeCys representa en torno a un 16-18 % para estas muestras. Con respecto a la tercera línea de producción podemos sacar las mismas conclusiones, obteniendo porcentajes de 39-56 % de SeMet para las materias primas (salmón de piscifactoría 1, salmón de piscifactoría 2 y merluza), a diferencia de lo obtenido para la SeMeSeCys (18-30 %).

Comparando las concentraciones de las especies obtenidas de los productos finales con las materias primas empleadas en su fabricación, se puede afirmar que **el proceso de elaboración no afecta a la integridad de estas, pero sí a su concentración**, ya que tanto para las líneas de producción 1 y 2, como para la línea de producción 3, en general, casi en todos los casos, se han obtenido porcentajes de SeMet y SeMeSeCys menores en los productos finales que en el caso de las materias primas. Esto puede ser principalmente debido, y como ya se comentó anteriormente, a que la cantidad de pescado en estos productos representa menos de un 50 % del total de los ingredientes empleados.

Para realizar una estimación más precisa de la exposición a estos elementos es necesario considerar su bioaccesibilidad, es decir, la fracción del elemento teóricamente liberada de la matriz del alimento en el tracto gastrointestinal y, por tanto, disponible para su posterior absorción en la mucosa intestinal. Para ello, la aplicación de un proceso de digestión gastrointestinal *in vitro* permitió determinar la bioaccesibilidad del selenio y del mercurio a lo largo de las líneas de producción de los productos procesados de interés. **Los porcentajes de bioaccesibilidad de selenio y mercurio obtenidos en las materias primas (abadejo, merluza y salmón de piscifactoría) y los productos finales (palitos de cangrejo, gulas y tallarines de salmón) variaron entre 20-39 % para el selenio y 9-37 % para el mercurio.**

Atendiendo a los resultados obtenidos para el selenio, no se aprecia de forma clara diferencias entre los valores de bioaccesibilidad entre las materias primas y productos finales, en ninguno de los productos analizados. Sin embargo, cabe destacar que las gulas proporcionaron los mayores porcentajes de bioaccesibilidad, lo que puede ser atribuido a las etapas implicadas en el proceso de elaboración, que incluye un desgrasado a 90 °C. La aplicación de calor durante la fase de desgrasado induce la pérdida de agua y la degradación de las proteínas, favoreciendo la liberación del selenio unido a biomoléculas, lo que podría aumentar su bioaccesibilidad. Se podría concluir, por tanto, que **los procesos de elaboración empleados para obtener los productos finales desde las materias primas, aparentemente, no afectan negativamente a la bioaccesibilidad del selenio**. Sin embargo, también es importante conocer cuáles son las especies químicas en las que se encuentra el selenio bioaccesible en la fracción gastrointestinal, para lo que se recurrió al análisis de especies en dichas fracciones mediante HPLC-ICP-MS, y posterior confirmación por HPLC-ESI-MS/MS. Los resultados indicaron **la presencia de las especies SeMeSeCys y SeMet en las fracciones gastrointestinales, siendo, de nuevo, la SeMet la forma de selenio más abundante para todas las muestras analizadas**. Si bien es cierto que la integridad de las especies se mantiene a lo largo de las cadenas de producción, la concentración de SeMet en la fracción bioaccesible es mayor en el caso de las materias primas (33-42 %) que en los productos finales (16-19 %). Esto también sería extrapolable a la SeMeSeCys, constatándose porcentajes ligeramente superiores de bioaccesibilidad para las materias primas (12-19 %) respecto a los productos finales (8-10 %).

Con respecto a la bioaccesibilidad del mercurio, se obtuvieron porcentajes ligeramente inferiores (8-31 %) respecto al selenio en las muestras analizadas, menos en el caso de los tallarines de salmón, en los que alcanzó un (37 ± 2) %. A este respecto, y en base a los resultados de relaciones molares Se:Hg bioaccesibles, así como del parámetro HBV_{Se} en la fracción bioaccesible obtenidos, **ninguna de las muestras analizadas debería considerarse un riesgo para la salud en relación a la toxicidad del mercurio**.

La literatura científica acerca de estudios de bioaccesibilidad de elementos esenciales, como el selenio, o elementos tóxicos, como el mercurio, no solo es muy escasa, sino que existe una gran variabilidad en los resultados reportados. En este sentido, se han obtenidos valores de bioaccesibilidad de selenio de 70-96 % para el pez espada, de 50-100 % para el salmón o de 17-91 % para la merluza. De igual modo, en el caso del mercurio, de 17-87 % para el pez espada o de 9-19 % para el atún (Cabañero *et al.*, 2004; 2007; Torres-Escribano *et al.*, 2010; Torres-Escribano *et al.*, 2011; Calatayud *et al.*, 2012). Dicha variabilidad puede ser atribuida a las diferencias en la procedencia, la composición y la matriz del pescado analizado, así como las distintas condiciones experimentales empleadas en el proceso de digestión gastrointestinal *in vitro*, tales como el pH, las enzimas y sales utilizadas o el modo de agitación.

Con el fin de completar los estudios de bioaccesibilidad, se empleó **la línea celular de adenocarcinoma colorrectal humano (Caco-2),** frecuentemente utilizada como modelo *in vitro* para simular las células epiteliales intestinales humanas. Esta línea celular fue expuesta a las **fracciones bioaccesibles de las muestras procedentes de las digestiones gastrointestinales (abadejo, merluza, salmón de piscifactoría, palitos de cangrejo, gulas y tallarines de salmón) y se estudió la citotoxicidad causada por dichos extractos mediante el ensayo MTT**.

Para ello, en primer lugar, se optimizó el número de células empleadas en los ensayos ($24x10^3$ células por pocillo) y se evaluó el efecto del blanco de la digestión gastrointestinal, que contiene la mezcla de enzimas y sales (pancreatina 3 mg mL^{-1}, sales biliares 0.3 mg mL^{-1} y NaCl 0.15 M), en la viabilidad celular. Estos experimentos previos son de gran importancia ya que pueden ser claves a la hora de obtener resultados correctos y representativos. Se probaron distintas diluciones del blanco de digestión (1/2, 1/3, 1/4, 1/5, 1/7 y 1/10) y se observó que todas provocaban una reducción de en torno al 30 % en la viabilidad celular. En base a esto, se seleccionó la mínima dilución posible, es decir 1/2, para simular la situación más realista y se aplicó tanto a los blancos de digestión como a las fracciones bioaccesibles. Bajo estas condiciones de ensayo, se observó que, tras 2 h de exposición, **los extractos gastrointestinales tanto de las materias primas como de los productos finales no afectaron significativamente a la viabilidad celular**, obteniendo resultados similares a los del blanco intestinal (en torno al 70 %), lo que sugiere la ausencia de toxicidad de los componentes de los extractos gastrointestinales (incluidos el selenio y el mercurio) en las muestras analizadas.

Con el propósito de profundizar en la identificación de las especies de selenio presentes en pescados y productos derivados, el **artículo 4** se centró en estudiar **la presencia de selenoneína y su análogo sulfurado, ergotioneína,** en este tipo de alimentos. La especie selenoneína fue identificada por primera vez en 2010 en la sangre del atún**.** Más de una década después, se ha identificado también en otras matrices biológicas de origen animal marino, como la ballena beluga, el delfín, la tortuga marina, las aves marinas y en varios pescados (pez espada, caballa, sardina o tilapia), evidenciando, por tanto, su transferencia a lo largo de la cadena trófica marina (Anan *et al.*, 2011; Yamashita *et al.*, 2011; Achouba *et al.*, 2019; El Hanafi *et al.*, 2022).

Aunque se le asigna una menor toxicidad que a otras especies de selenio, la selenoneína no contribuye en la síntesis de selenoproteínas, y, por tanto, tiene poca incidencia en el metabolismo del selenio. Sin embargo, el interés por la selenoneína radica fundamentalmente en su elevado poder antioxidante y en el importante papel que juega en la detoxificación frente al $MeHg^+$, promoviendo su desmetilación previa formación de los compuestos insolubles estables de HgSe (Little *et al.*, 2024).

Por otro lado, debido a que los animales, entre ellos los seres humanos, no son capaces de sintetizar la selenoneína, **es importante establecer las fuentes de obtención de este compuesto de selenio,** siendo la principal a través de la dieta**.**

La selenoneína posee una estrecha y directa relación con su compuesto análogo que contiene azufre, la ergotioneína. Una vez consumida, la selenoneína se transporta a través de las membranas celulares por el transportador de ergotioneína, OCTN1. Además, se ha demostrado que es más resistente a la degradación oxidativa irreversible en comparación con la ergotioneína (Little *et al.*, 2024). La ergotioneína, por su parte, se ha estudiado de forma mayoritaria en alimentos de origen vegetal y carnes, pero no en alimentos de origen marino. También se trata de un potente agente antioxidante y, por tanto, una evaluación de la presencia conjunta con la selenoneína en matrices biológicas podría ayudar a conocer la relación entre ambos compuestos.

Bajo este contexto, la siguiente investigación llevada a cabo (**artículo 4**) se centró en la **identificación simultánea de selenoneína y ergotioneína en pescados y productos derivados frecuentemente consumidos mediante la optimización de un método de análisis por HPLC-ESI-MS/MS**. Las muestras seleccionadas fueron atún, pez espada, salmón de piscifactoría, salmón rosado salvaje, huevas de lumpo, huevas de trucha, huevas de salmón rosado salvaje, palitos de cangrejo, gulas, tallarines de bacalao y tallarines de salmón.

Primeramente, se llevó a cabo la **optimización de las condiciones de extracción evaluando la eficiencia de extracción en base al contenido total de selenio**, para lo que se seleccionó el atún, como muestra representativa, ya que es uno de los peces con mayor contenido en selenio. Los parámetros considerados fueron el tipo de extractante y su concentración, la masa de muestra, y la metodología de extracción. Dentro de la metodología se evaluaron dos procedimientos 1) extracción mecánica con bolas de vidrio de 2 mm de diámetro combinada con agitación en vórtex y posterior baño de ultrasonidos; 2) sonda de ultrasonidos. En la literatura científica se ha descrito que la selenoneína se extrae principalmente en disoluciones acuosas (Anan *et al.*; 2011; Kroepfl *et al.*, 2015; El Hanafi *et al.*, 2022), por ello en este estudio se evaluaron como extractantes seis disoluciones acuosas distintas compuestas de: acetato de amonio, formiato de amonio, fosfato sódico, citrato potásico o Tris-HCl, todas ellas en concentraciones de 100 y 200 mmol L^{-1} a pH 7.4.

Fijando una masa de muestra inicial de 1.5 g y una extracción con bolas de vidrio combinada con vórtex (3000 rpm) y baño de ultrasonidos (720 W, 3.1 % de amplitud) durante 60 s, se obtuvieron porcentajes de extracción superiores al 40 % en todos los casos, consiguiendo las mayores eficiencias de extracción, (64 ± 2) y (47 ± 2) %, cuando se emplearon como extractantes el acetato de amonio y el formiato de amonio, respectivamente, ambos en concentración 100 mmol L^{-1}. Para mejorar dichos resultados, se varió la cantidad de muestra empleada (0.5-1.5 g), manteniendo las condiciones de extractante y concentración anteriormente optimizadas. Los mayores porcentajes de selenio extraído se obtuvieron cuando se empleó 1 g de muestra, resultando en (82 ± 1) y (60 ± 7) %, utilizando 100 mmol L^{-1} de acetato de amonio y formiato de amonio, respectivamente. Finalmente, y con el objetivo de mejorar las eficiencias de extracción también se evaluó el empleo de la sonda de ultrasonidos (20 % amplitud). Asimismo, se ensayaron tiempos de extracción de 60 y 120 s para ambas condiciones. En el caso del empleo de acetato de amonio 100 mmol L^{-1}, los mejores resultados se obtuvieron con bolas de vidrio en combinación con vórtex y baño de ultrasonidos durante 60 s, con una eficiencia de extracción del (106 ± 3) %. En relación al formiato de amonio 100 mmol L^{-1}, los mejores resultados, se obtuvieron con el empleo de la sonda de ultrasonidos durante 120 s, siendo la eficiencia de extracción de un (104 ± 5) %.

Por lo tanto, las dos condiciones óptimas de extracción, en base al selenio total presente en el atún fueron las siguientes:

- 1 g de muestra con 2.0 mL de acetato de amonio 100 mmol L^{-1} a pH 7.4, adición de 0.9 g de bolas de vidrio y posterior tratamiento durante 60 s con la combinación de vórtex (3000 rpm) y baño de ultrasonidos (720 W, 3.1 % de amplitud).
- 1 g de muestra con 2.0 mL de formiato de amonio 100 mmol L^{-1} a pH 7.4 y posterior sonicación durante 120 s con la sonda de ultrasonidos (20 % de amplitud).

A continuación, una vez optimizado el procedimiento de extracción, **se empleó la técnica SEC-ICP-MS como método de *screening* para estudiar la presencia de compuestos de selenio unidos a biomoléculas de bajo peso molecular en dichos extractos acuosos, concretamente en los obtenidos mediante la extracción con acetato de amonio (primera condición óptima descrita anteriormente), para todas las muestras**. Mediante el empleo de dos columnas de exclusión molecular con distintos rangos de separación, Superdex 200 (10-600 kDa) y Superdex Peptide (0.1-7 kDa), se pudo estimar que **el selenio en esta fracción soluble está principalmente unido a biomoléculas de bajo peso molecular (< 10 kDa) para todos los pescados, productos**

derivados y productos procesados analizados. En los perfiles cromatográficos obtenidos de selenio mediante la columna Superdex 200, las fracciones mayoritarias que contiene selenio eluyeron a tiempos de retención de entre 30 y 40 min, lo que se correspondería con la zona de separación de los compuestos con menores pesos moleculares que esta columna es capaz de retener (10 y < 10 kDa). Es por ello por lo que se procedió a analizar también estas fracciones empleando la columna Superdex Peptide, donde se pudo observar un mayor número de picos cromatográficos para los pescados y los productos derivados (huevas), correspondientes a biomoléculas con selenio de distintos pesos moleculares, aproximadamente de 0.1, 0.5, 1, 5 y 7 kDa. Sin embargo, para los productos procesados, la mayor parte del selenio eluyó fundamentalmente en una sola fracción a un tiempo de retención de 42 min, correspondiendo con pesos moleculares de alrededor de 0.1 kDa.

Si bien es cierto que una de las principales desventajas de la técnica SEC-ICP-MS es su baja resolución, lo cual dificulta obtener una separación adecuada de los selenocompuestos, proporciona información valiosa acerca del tamaño aproximado de biomoléculas con selenio presentes en el extracto soluble. En este sentido, se ha comprobado que **la mayor parte del selenio existente en la fracción soluble del músculo de los pescados, los productos derivados y procesados analizados se encuentra unido a biomoléculas de bajo peso molecular, formando selenocompuestos, entre los cuales se encuentran las selenoproteínas y otros compuestos como la selenoneína, cuya masa molecular (275 g/mol o 0.275 kDa) se sitúa en la zona de bajos pesos moleculares.**

Seguidamente, para la separación e identificación inequívoca de selenoneína y ergotioneína en los extractos, estos se analizaron mediante HPLC-ESI-MS/MS utilizando una columna de fase inversa, Phenomenex Kinetex EVO C18 (150x3 mm, 5 µm). La fase móvil seleccionada fue 95 % agua (0.1 % ácido fórmico) y 5 % acetonitrilo (0.1 % ácido fórmico), y se trabajó en modo de elución isocrático, con un flujo de 0.5 mL min^{-1} y un volumen de inyección de 5 µL. Debido a la similitud estructural de ambas moléculas, se observó un comportamiento cromatográfico similar entre los patrones de selenoneína y ergotioneína, estando sus tiempos de retención muy próximos entre sí: 1.4 y 1.3 min, respectivamente. Su determinación simultánea se llevó a cabo mediante la monitorización de dos transiciones MRM (monitorización de reacciones múltiples) características de cada analito (cuantificación y confirmación).

Una vez establecidas las condiciones de operación del equipo, se llevó a cabo la validación del método en términos de linealidad, LOD y LOQ. En ambos casos se obtuvieron buenas correlaciones lineales, con coeficientes de determinación superiores a 0.9991 en el intervalo de concentración evaluado (1-500 µg L^{-1}). Además, los límites de detección y cuantificación del método desarrollado fueron de 0.0011 y 0.0035 µg g^{-1} para la selenoneína y de 0.0009 y 0.0030 µg g^{-1} para la ergotioneína, respectivamente.

Asimismo, los estudios de recuperación llevados a cabo a una concentración de 30 µg L^{-1} para cada uno de los analitos empleando las condiciones de extracción y de medida optimizadas proporcionaron recuperaciones comprendidas entre 76-93 %. Esto podría descartar el posible efecto matriz derivado de una supresión de ionización inducida por la misma.

Seguidamente, se llevó a cabo el análisis de las muestras de pescados y productos derivados y procesados de pescado tras aplicar las dos condiciones de extracción óptimas, y la cuantificación se llevó a cabo con un calibrado en solvente. De esta forma, se consiguió identificar y cuantificar la ergotioneína en las muestras de atún, pez espada, salmón de piscifactoría y en las huevas de lumpo, de trucha y de salmón rosado salvaje. Sin embargo, solo se identificó la selenoneína en las muestras de atún y pez espada. Asimismo, es importante resaltar que no existieron diferencias significativas entre las concentraciones obtenidas por ambos procedimientos de extracción.

En este sentido, **el atún y las huevas de salmón rosado salvaje son los que poseen la mayor cantidad de ergotioneína** de entre todas las muestras analizadas, con concentraciones de (3.5 ± 0.2) y (3.3 ± 0.3) µg g^{-1}, respectivamente, seguidos de las huevas de trucha, cuya concentración resultó ser de (2.7 ± 0.5) µg g^{-1}. En las huevas de lumpo también se evidenció la presencia de este compuesto a una concentración de (0.082 ± 0.004) µg g^{-1}. En cuanto al salmón, **la ergotioneína parece estar presente solo en el músculo del salmón de piscifactoría, con una concentración de (0.03 ± 0.01) µg g^{-1}, ya que no se detectó en el salmón rosado salvaje**. La ergotioneína tampoco está presente en los productos derivados como los palitos de cangrejo, las gulas y los tallarines de bacalao y de salmón, lo que sugiere que, o no está presente desde un inicio en las materias primas utilizadas para la elaboración de estos productos o que el proceso de elaboración induce pérdidas de este compuesto. Otra posible explicación radica en que la concentración que presenten puede estar por debajo del LOD del método empleado.

Por otro lado, **en lo que respecta a la selenoneína, los resultados obtenidos evidenciaron que estaba presente en concentraciones de (0.306 ± 0.006) y (0.14 ± 0.02) µg g^{-1} en el músculo del atún y del pez espada, respectivamente**. Sin embargo, este compuesto de selenio no se detectó ni en salmón (de piscifactoría y rosado salvaje), ni en huevas de pescado, ni en productos procesados derivados del pescado. La no detectabilidad de la selenoneína también ha sido descrita por otros autores (Yamashita *et al.*, 2011), sin embargo, esta es la primera vez que se investiga la presencia de selenoneína en huevas de pescado y productos procesados derivados del pescado. Aunque se encontraron contenidos de selenio total elevados en algunas de las huevas de pescado, como por ejemplo en las huevas de salmón rosado salvaje, con cantidades de (4.4 ± 0.4) µg g^{-1}, no se detectó selenoneína. Por lo tanto, **si bien es cierto que las huevas de pescado pueden suponer una notable fuente de selenio, este parece acumularse en forma de selenoaminoácidos y no como selenoneína**.

Cabe destacar que en la literatura científica apenas se describen métodos de extracción para la detección simultánea de la selenoneína y la ergotioneína, identificándose generalmente de manera individual, en ocasiones con elevados tiempos de extracción (hasta 40 min) o con varias etapas de purificación mediante el empleo de diferentes columnas cromatográficas (Seko *et al.*, 2021; Kitsanayanyong *et al.*, 2022). Sin embargo, en este trabajo se ha descrito la extracción e identificación de ambos compuestos en pescados, productos derivados y procesados, detectándose **la coexistencia de selenoneína y ergotioneína en el músculo del atún y del pez espada**, que son peces depredadores. Si bien es cierto que son necesarios más estudios para dilucidar las diferencias o similitudes entre estos dos compuestos en lo que respecta a su absorción a partir de los alimentos. Los peces asimilan la selenoneína y la ergotioneína a través de la dieta, ya que estos compuestos solo son sintetizados por bacterias y hongos. Sin embargo, algunos estudios también sugieren que la selenoneína puede sintetizarse biológicamente a partir de la ergotioneína en los eritrocitos y otras células animales ricas en glutatión y ergotioneína (Yamashita & Yamashita, 2010).

Siguiendo en este contexto, la presencia de las especies metiladas de selenoneína y ergotioneína, la Se-metilselenoneína y S-metilergotioneína, respectivamente, fue también estudiada en los pescados, productos derivados y procesados por HPLC-ESI-MS/MS, monitorizando dos transiciones MRM características de cada compuesto (cuantificación y confirmación). Los resultados obtenidos evidenciaron la **posible presencia de Se-metilselenoneína en el músculo del atún y de S-metilergotioneína en todas las muestras donde la ergotioneína fue detectada**, es decir, en el músculo del atún, del pez espada y del salmón de piscifactoría, así como en las huevas de lumpo, de trucha y de salmón rosado salvaje. Al no disponer de patrones de estos compuestos, ya que no son comerciales y su síntesis es compleja, se llevó a cabo un análisis de identificación inequívoca mediante cromatografía líquida acoplada a un analizador cuadrupolo-tiempo de vuelo (HPLC-QTOF-MS) que permite obtener medidas de masa exacta de los iones. Sin embargo, no se pudo confirmar la presencia de ninguno de los dos compuestos en ninguna muestra, lo que podría ser debido a la relativa baja sensibilidad del QTOF en modo *full-scan* comparado con la excelente sensibilidad proporcionada por el triple cuadrupolo en modo MRM. Por tanto, serían necesarios estudios adicionales para identificar de forma inequívoca la presencia de los compuestos metilados de selenoneína y ergotioneína en estas matrices alimentarias. Cabe destacar que ciertos estudios sugieren que la Se-metilselenoneína se produce como metabolito y forma de excreción de la selenoneína, metilándose en el hígado y en los riñones, mediante una vía metabólica única, distinta a la de otros compuestos de selenio, no afectando por tanto a la síntesis de selenoproteínas (Seko *et al.*, 2023). De igual modo, se trata de una de las principales formas de selenio excretadas en la orina, además de los selenoazúcares. Sin embargo, aparte de ser detectada en la sangre, el hígado y los riñones, también se ha identificado en el músculo de pescados como el atún, la caballa o la sardina y es por ello que se cree que también su presencia puede derivar directamente del consumo (Ohta & Suzuki, 2008; Klein *et al.*, 2011; Achouba *et al.*, 2023).

La especie metilada de la ergotioneína, S-metilergotioneína, por su parte, ya ha sido identificada en sangre humana (Achouba *et al.*, 2023). Aunque tampoco se han dilucidado completamente los mecanismos de metilación y el metabolismo asociado, debe tener similitud al de la Se-metilselenoneína. Por tanto, es evidente que son necesarios más estudios e investigaciones acerca de estos compuestos metilados para dilucidar los metabolismos asociados.

Para concluir, en la presente Tesis Doctoral se ha investigado el **efecto protector del selenio frente a la neurotoxicidad inducida por el mercurio** empleando distintas técnicas, entre ellas la técnica ***single-cell* ICP-MS (SC-ICP-MS)** y modelos *in vitro*, utilizando la **línea celular de neuroblastoma humano (SH-SY5Y)**. La técnica SC-ICPMS permite evaluar la acumulación de elementos esenciales/tóxicos en poblaciones celulares, teniendo en cuenta la heterogeneidad que estas presentan en relación a su comportamiento frente a un factor externo. Por lo tanto, esta técnica es de una gran utilidad para llevar a cabo estudios neurotoxicológicos en los que están involucradas líneas celulares.

Bajo este contexto, en el **artículo 5** se recogen los estudios relacionados con el efecto protector de las distintas especies de selenio identificadas en los pescados, frente a la citoxicidad que ejerce el $MeHg^+$. Además de las especies orgánicas de selenio, también se evaluó el potencial efecto protector de las nanopartículas de selenio, disponibles comerciales y estabilizadas con citrato, citrato-SeNPs (de 80 nm de tamaño), y químicamente sintetizadas en el laboratorio modificadas con quitosano, Ch-SeNPs, y, por tanto, de tamaño también conocido: (60 ± 15) nm.

La línea celular elegida para llevar a cabo los ensayos fue la de neuroblastoma humano (SH-SY5Y) debido a que es una de las más empleadas en estudios de neurotoxicidad, ya que, entre otras cosas, tiene un origen neuronal.

En primer lugar, se determinó **la concentración letal del $MeHg^+$ para el 50 % de la población (LC_{50}) mediante el ensayo MTT, concluyéndose una concentración 3 µM del tóxico**. De igual modo, es importante conocer el efecto citotóxico de los compuestos de selenio de interés. En un estudio previo, se observó que las especies SeMet y SeMeSeCys presentaban una baja citotoxicidad en la línea celular SH-SY5Y (LC_{50} > 1000 µM), lo cual era de esperar debido a que forman parte de los aminoácidos que contienen selenio y que desempeñan una función biológica en el organismo (Vicente-Zurdo *et al.*, 2023). En relación con las SeNPs, los estudios de citotoxicidad realizados evidenciaron una disminución en la viabilidad celular desde el (100 ± 4) % hasta un (89 ± 3) y un (78 ± 3) % cuando las células fueron expuestas a concentraciones de 25 y 50 µM de SeNPs recubiertas con citrato (citrato-SeNPs), respectivamente, y a un (71 ± 4) y un (62 ± 3) % cuando las células fueron expuestas a concentraciones de 25 y 50 µM de SeNPs recubiertas de quitosano (quitosano-SeNPs), respectivamente.

Seguidamente, se evaluó la influencia de las distintas especies orgánicas de selenio y nanopartículas de selenio en la citotoxicidad inducida por el $MeHg^+$ empleando ensayos de coexposición. Estos ensayos de viabilidad celular se realizaron a concentraciones de 3 y 1.5 µM del tóxico, correspondientes con su LC_{50} y LC_{20}, respectivamente, siendo las concentraciones seleccionadas de SeMet, SeMeSeCys y SeNPs de 25 y 50 µM.

Los resultados obtenidos mostraron que, a elevadas dosis de $MeHg^+$ (LC_{50}), la coexposición con SeMet o SeMeSeCys (25 o 50 µM) produce un aumento significativo en la viabilidad celular, disminuyendo la muerte celular inducida por la toxicidad del $MeHg^+$. En concreto, la viabilidad celular aumentó significativamente desde un (55 ± 4) % hasta un (69 ± 5) y un (73 ± 2) % cuando se añadió SeMet en concentración 25 o 50 µM, respectivamente, y desde un (56 ± 3) % hasta un (97 ± 1) y un (99 ± 4) % cuando se añadió SeMeSeCys en concentración 25 o 50 µM, respectivamente. Por tanto, los mejores resultados fueron obtenidos con la especie SeMeSeCys, siendo independiente de la concentración empleada. Sin embargo, cuando se adicionaron dosis más bajas de $MeHg^+$ (LC_{20}) a los cultivos celulares, la coexposición con las especies SeMet o SeMeSeCys a concentraciones de 25 o 50 µM no produjo un aumento en la viabilidad celular. En lo que respecta a las nanopartículas de selenio, **aunque se ha demostrado que las quitosano-SeNPs son más tóxicas que las citrato-SeNPs para las células SH-SY5Y, las primeras mostraron un mayor efecto protector frente a la toxicidad inducida por el $MeHg^+$ para todas las concentraciones probadas**. En este sentido, a altas dosis de $MeHg^+$ (LC_{50}), la coexposición con quitosano-SeNPs (25 o 50 µM) aumentó significativamente la viabilidad celular, obteniendo los mejores resultados, (87 ± 3) %, con una concentración de 50 µM. Con dosis inferiores de $MeHg^+$ (LC_{20}) adicionadas a los cultivos celulares, la coexposición con quitosano-SeNPs a una concentración de 25 µM resultó en un aumento de la viabilidad celular de hasta el (96 ± 3) %, no presentando diferencias estadísticamente significativas (p-valor < 0.05) con respecto al grupo control.

Este aumento en la viabilidad celular otorgado a las quitosano-SeNPs, mayor que en el caso de emplear citrato-SeNPs, puede ser atribuido al agente de recubrimiento, ya que el tamaño medio de ambos tipos de nanopartículas es similar ((60 ± 15) y 80 nm, respectivamente). Además, en un estudio previo, se observó que las células SH-SY5Y eran capaces de internalizar las quitosano-SeNPs, siendo localizadas en el citosol, en las mitocondrias, adheridas al exterior de la membrana celular y en el interior del núcleo y de los nucleolos (Vicente-Zurdo *et al.*, 2023), lo que podría facilitar el efecto protector de las mismas frente a la toxicidad inducida por el $MeHg^+$. De igual modo, las quitosano-SeNPs podrían interaccionar con el $MeHg^+$ añadido en el medio de cultivo, dificultando así su absorción por parte de las células.

En definitiva, los resultados obtenidos en estos ensayos evidencian que tanto las especies orgánicas de selenio, en especial la SeMeSeCys, como las formas particuladas, exhiben efectos de protección en la línea celular SH-SY5Y frente a la toxicidad inducida por el $MeHg^+$. Sin embargo y debido a la mayor citotoxidad exhibida por las formas particuladas, las especies SeMet y SeMeSeCys fueron seleccionadas para llevar a cabo los siguientes estudios de acumulación celular y SC-ICP-MS.

Primeramente, se determinó el porcentaje total de mercurio acumulado por las células SH-SY5Y expuestas durante 48 h a diferentes condiciones (1.5 µM $MeHg^+$ o 1.5 µM $MeHg^+$ + 50 µM SeMet/SeMeSeCys) mediante ICP-MS, previa mineralización de la muestra mediante digestión ácida. Además, se determinó el porcentaje total de mercurio en el medio de cultivo, y en el PBS, con el fin de completar el balance de masas. **Los resultados mostraron que, para todas las condiciones, solo el 3 % del $MeHg^+$ añadido al medio de cultivo fue acumulado por las células, no observando diferencias estadísticamente significativas (p-valor < 0.05) entre las tres condiciones de exposición**. Además, el balance de masas fue cuantitativo en todos los casos, corroborando que el mercurio restante no absorbido por las células permaneció en el medio de cultivo.

Sin embargo, en estos estudios de acumulación no se ha tenido en cuenta la heterogeneidad celular a la hora de asimilar los elementos añadidos a los medios de cultivo. Para este fin, se empleó la técnica ICP-MS en modo de detección de células individuales (SC-ICP-MS) para evaluar la acumulación de mercurio a nivel de células individuales expuestas durante 48 h a las condiciones anteriormente mencionadas (1.5 µM $MeHg^+$ o 1.5 µM $MeHg^+$ + 50 µM SeMet/SeMeSeCys). En la configuración del equipo se empleó un nebulizador concéntrico de alto rendimiento en combinación con una cámara de nebulización de consumo total. Primeramente, se optimizaron las condiciones de fijación celular con PFA para asegurar que el máximo número de células llegasen al plasma. Para ello, se añadieron diferentes concentraciones de PFA (0, 1 y 4 %) al medio de cultivo y se incubaron durante toda la noche. Mediante la monitorización del isótopo ^{55}Mn, elemento celular constitutivo, por SC-ICP-MS se evaluó la eficiencia de transporte de las células dentro del equipo, calculada dividiendo el número de eventos detectados por SC-ICP-MS entre el número de células introducidas en el sistema, contadas previamente al microscopio con la cámara de Neubauer. **La mejor eficiencia de nebulización, (93 ± 6) %, se obtuvo cuando las células fueron fijadas con PFA al 4 %**. Cuando se fijaron con PFA al 1 %, la eficiencia de nebulización fue del (55 ± 2) % y, al no utilizar fijación, solo el (10 ± 3) % de las células introducidas en el sistema consiguieron alcanzar el plasma. **Estos resultados muestran la necesidad de llevar a cabo una etapa de fijación celular al realizar este tipo de análisis, debido a la gran fragilidad que presentan.**

Seguidamente, tras la exposición de las células durante 48 h a las condiciones mencionadas (1.5 µM $MeHg^+$ o 1.5 µM $MeHg^+$ + 50 µM SeMet/SeMeSeCys) y posteriormente fijadas con PFA al 4 %, se aplicó la dilución adecuada en cada caso para que, monitorizando el isótopo ^{202}Hg, cada evento registrado correspondiese a una sola célula. En función de la frecuencia de los eventos y de la intensidad de los mismos, se obtuvo información acerca de la masa de dicho elemento que contenía cada una de ellas. **Los histogramas elaborados a partir de los datos obtenidos por SC-ICP-MS, en los que se muestra el número de células que acumulan una determinada masa de mercurio, evidencian la variabilidad con la que las células individuales acumulan $MeHg^+$, para todos los casos de exposición evaluados**.

En general, cuando el selenio es añadido al medio de cultivo en coexposición con el $MeHg^+$, un mayor número de células acumulan menos cantidad de mercurio (5 fg Hg celula^{-1}) comparado con las células expuestas solo a $MeHg^+$. Por ejemplo, alrededor del 76 y del 64 % de células acumulan hasta una cantidad de 5 fg Hg célula^{-1} cuando se añaden 50 µM SeMeSeCys y 50 µM SeMet, en combinación con 1.5 µM $MeHg^+$, respectivamente. Sin embargo, cuando no hay coexposición con selenio, este porcentaje de células que acumula la menor cantidad de mercurio disminuye hasta un 54 %, siendo mayor el porcentaje de células que acumulan una mayor cantidad de mercurio. A tenor de estos resultados, parece que se ha podido determinar mediante SC-ICP-MS el efecto protector de las especies de selenio SeMeSeCys y SeMet evaluadas frente a la acumulación de $MeHg^+$ por parte de las células SH-SY5Y, ya que estas no son capaces de asimilar tanta cantidad de mercurio como lo hacen cuando son solamente expuestas a $MeHg^+$.

Este hecho también puede corroborarse atendiendo a las cantidades máximas de acumulación de mercurio por célula individual. En los casos de exposición solo a 1.5 µM $MeHg^+$ y de coexposición de 1.5 µM $MeHg^+$ con 50 µM SeMeSeCys, algunas células son capaces de acumular hasta 60 fg Hg célula^{-1}, a diferencia de la condición de coexposición de 1.5 µM $MeHg^+$ con 50 µM SeMet, donde esta concentración se ve disminuida hasta la mitad, 30 fg Hg célula^{-1}. Por lo tanto, **no solamente el efecto protector del selenio frente a la toxicidad del $MeHg^+$ se hace evidente en base a una disminución del porcentaje de células que acumulan mayores cantidades de mercurio, sino también en base a una reducción de los niveles máximos del tóxico que pueden ser acumulados por las células**. **Este efecto protector se podría explicar en base a la interacción entre el selenio y el mercurio en el medio de cultivo, que dificultaría, por tanto, una internalización de mercurio por parte de las células, disminuyendo así su absorción**.

Finalmente, los datos obtenidos por SC-ICP-MS se compararon con los resultados obtenidos del valor medio de cantidad de mercurio acumulado, en fg por célula individual, con el calculado mediante el análisis por ICP-MS convencional. Para ello, se dividió el contenido total de mercurio obtenido tras el análisis por ICP-MS, previa digestión ácida de los cultivos celulares para cada condición, entre el número de células presentes en los cultivos contadas al microscopio con la cámara de Neubauer. En todos los casos, **los valores reportados por ICP-MS convencional fueron más elevados que los obtenidos mediante SC-ICP-MS, concretamente entre 12.9 y 16 veces mayores, aunque todos ellos se encontraban en el mismo orden de magnitud**. La información descrita en la literatura científica en relación a la comparación entre los valores de acumulación proporcionados por el ICP-MS en modo convencional y en modo de detección de células individuales también evidencia la obtención de mayores valores de acumulación para el caso del ICP-MS en modo convencional, con relaciones entre las concentraciones que van desde 0.8 hasta 37 para elementos como titanio, plata, arsénico, cobre, hierro o azufre, en distintas líneas celulares derivadas de especies de acuicultura, humanas, levaduras o de ratas (López-Serrano *et al.*, 2018; Meyer *et al.*, 2018; Cao *et al.*, 2020; Tanaka *et al.*, 2020; Suárez-Oubiña *et al.*, 2023).

Las conclusiones derivadas de este estudio no hubieran sido posibles de obtener utilizando únicamente el ICP-MS en modo convencional, ya que este no tiene en cuenta la enorme variabilidad celular, obteniendo los mismos resultados de valor medio de mercurio acumulado por célula para las tres condiciones de exposición, sin poder concluir diferencia alguna. Esto pone de manifiesto el gran potencial que tiene la técnica SC-ICP-MS en este tipo de estudios contribuyendo a una mejor comprensión del potencial papel neuroprotector de las especies de selenio consideradas frente a la toxicidad inducida por el $MeHg^+$ a nivel celular.

Finalmente, es necesario resaltar la importancia de la combinación de la técnica SC-ICP-MS y otras técnicas (bio)analíticas, junto con modelos *in vitro* empleando líneas celulares/bacterias, para una correcta evaluación de la exposición, acumulación y biotransformación de contaminantes ambientales con efecto neurotóxico para elucidar el mecanismo de elementos neuroprotectores, como el selenio. Según previsiones, las enfermedades neurodegenerativas asociadas a estos elementos afectarán a 152 millones de personas en 2050.

En este contexto, en la última parte de esta Tesis Doctoral se presenta el **artículo 6**, donde se ha llevado a cabo una **revisión bibliográfica con el objetivo de exponer el estado del arte de las técnicas (bio)analíticas y los métodos *in vitro* más empleados para evaluar el papel de ciertos elementos en las afecciones neurológicas haciendo**

especial hincapié en el potencial del selenio para prevenir los efectos neurotóxicos de contaminantes ambientales como pueden ser el cadmio, mercurio o arsénico.

VIII. CONCLUSIONES

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De los resultados obtenidos en los diferentes trabajos expuestos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

1. Para todas las muestras de pescados, productos derivados y procesados analizados, se obtuvieron relaciones molares Se:Hg mayores que uno y valores del parámetro HBV_{Se} positivos, por lo que se puede decir que, de manera general, el consumo tanto de los pescados como de los productos derivados y procesados no tendría, *a priori*, efectos negativos en la salud del consumidor asociados con la toxicidad del mercurio.

2. Los contenidos totales de selenio y mercurio encontrados en las muestras objeto de estudio evidenciaron que el músculo del pescado es el órgano preferente de almacenamiento de mercurio, mientras que las huevas constituyen una importante fuente de selenio.

3. Los estudios de especiación de selenio por HPLC-ICP-MS y confirmados por HPLC-ESI-MS/MS pusieron de manifiesto la presencia de la especie SeMeSeCys en las fracciones sarcoplasmáticas, miofibrilares y álcali-solubles de los pescados analizados, así como en las proteínas solubles en Tris-HCl extraídas de los mismos. De igual modo, se constató la presencia de SeMet en las proteínas solubles en Tris-HCl extraídas del músculo del atún. Para los productos derivados y procesados del pescado también se identificaron las mismas especies, SeMet y SeMeSeCys, siendo la SeMet la mayoritaria en todas las muestras analizadas.

4. En cuanto a la especiación de mercurio, las especies $MeHg^+$ y Hg^{2+} fueron identificadas en las muestras de pescado y los productos derivados, siendo el $MeHg^+$ la especie mayoritaria en todos los casos. Sin embargo, existen diferencias significativas entre las proporciones de $MeHg^+$ y Hg^{2+} de las muestras analizadas. En los pescados depredadores, como el atún y el pez espada, la concentración de $MeHg^+$ supone más del 90 % del contenido total de mercurio, ((110 ± 6) y (96 ± 3) %, respectivamente). Sin embargo, el salmón de piscifactoría y el salmón rosado salvaje presentan las mayores concentraciones relativas de Hg^{2+} obtenidas, (35 ± 1) y (27 ± 4) %, respectivamente. Por su parte, en los productos procesados, en general, se observaron menores porcentajes de $MeHg^+$ que en el caso de los pescados, llegando a representar, por ejemplo, para el caso de los tallarines de salmón un (62 ± 5) % del contenido de mercurio total. No se consiguió identificar ninguna especie de mercurio, debido al bajo contenido de mercurio total que presentan, en los tres tipos de huevas consideradas (de lumpo, de trucha y de salmón salvaje).

5. En relación con los productos procesados, las etapas implicadas en su fabricación pueden afectar a los niveles y especies de selenio y mercurio. En este sentido, el análisis de las muestras obtenidas en cada una de las etapas del proceso de producción, y dentro de un mismo lote, proporcionadas por las empresas Angulas Aguinaga y Nueva Pescanova España, evidenció la presencia de las especies SeMet y SeMeSeCys, las cuales fueron identificadas tanto en las materias primas como en los productos intermedios y finales.

6. Los porcentajes de bioaccesibilidad de selenio y mercurio obtenidos en las materias primas (abadejo, merluza y salmón de piscifactoría) y los productos finales considerados (palitos de cangrejo, gulas y tallarines de salmón) variaron entre 20-39 % para el selenio, y 9-37 % para el mercurio, no observándose diferencias significativas entre los dos grandes grupos. Por lo tanto, los procesos de elaboración empleados, aparentemente, tampoco afectan negativamente a la bioaccesibilidad del selenio. Además, las especies de selenio identificadas en las muestras también se observaron en las fracciones gastrointestinales, siendo, de nuevo, la SeMet la forma de selenio más bioaccesible para todas las muestras analizadas. Asimismo, aunque se ha comprobado que se mantiene la integridad de las especies a lo largo de las cadenas de producción, las concentraciones de SeMet y SeMeSeCys en la fracción bioaccesible son mayores en el caso de las materias primas (33-42 y 12-19 %, respectivamente) que en los productos finales (16-19 y 8-10 %, respectivamente). Por otro lado, se puede concluir que los extractos gastrointestinales tanto de las materias primas como de los productos finales no afectaron significativamente a la viabilidad de las células Caco-2 tras 2 h de exposición, obteniéndose resultados comparables a los de los blancos (en torno al

70 %), lo que sugiere la ausencia de toxicidad de los componentes de los extractos gastrointestinales (incluidos el selenio y el mercurio) en las muestras analizadas.

7. El desarrollo de un método de análisis por HPLC-ESI-MS/MS, previa optimización de las condiciones de extracción, permitió la determinación simultánea de selenoneína y ergotioneína en pescados, productos derivados y procesados. Los resultados obtenidos evidenciaron la presencia de ergotioneína en las muestras de pescado y en las distintas variedades de huevas consideradas, y la presencia de selenoneína en el músculo del atún y del pez espada. Por lo tanto, parece que ambos compuestos antioxidantes pueden coexistir, al menos, en los peces depredadores. Sin embargo, no se detectaron ninguna de ellas en productos de pescado procesados, lo que revela que el proceso de elaboración podría afectar a la presencia de estos compuestos. Además, como resultados preliminares, se ha sugerido la posible presencia de los compuestos metilados S-metilergotioneína y Se-metilselenoneína en el músculo de pescado y las huevas, aunque se requieren más estudios para confirmarlo.

8. Los estudios de citotoxicidad realizados empleando la línea celular SH-SY5Y mostraron que las especies de selenio identificadas en los pescados y productos derivados y procesados, SeMet y SeMeSeCys, ejercen un mayor efecto protector que las SeNPs (citrato-SeNPs y Ch-SeNPs) frente a la muerte celular inducida por el $MeHg^+$ a altas concentraciones del tóxico (LC_{50}, 3 µM). Los mejores resultados fueron obtenidos con la especie SeMeSeCys, siendo independiente de la concentración empleada (25 o 50 µM), mostrando las mayores viabilidades celulares, de hasta (99 ± 4) %, comparables con las del grupo control.

9. Mediante la aplicación de la técnica SC-ICP-MS se pudo deducir que, para la línea celular SH-SY5Y, cuando el selenio (SeMet o SeMeSeCys) es añadido al medio de cultivo en coexposición con el $MeHg^+$, un mayor número de células acumulan menos cantidad de mercurio, comparado con las células expuestas solo a $MeHg^+$. En concreto, la coexposición de SeMet con $MeHg^+$ condujo a una reducción en la cantidad máxima de acumulación de mercurio por célula individual, llegándose a reducir hasta la mitad (de 60 fg Hg $célula^{-1}$ a 30 fg Hg $célula^{-1}$). Sin embargo, mediante análisis por ICP-MS convencional no se obtuvieron diferencias de

acumulación de mercurio entre las condiciones de exposición mencionadas. De este modo, la aplicación de la técnica SC-ICP-MS permitió obtener información más precisa, al tener en cuenta la heterogeneidad celular, poniendo de manifiesto su elevado potencial.

IX. CONCLUSIONS

XIX. CONCLUSIONS

The following conclusions can be drawn from the results obtained in the different works presented in this Doctoral Thesis:

1. For all fish samples and fish-derived and processed products, Se:Hg molar ratios greater than one and positive HBV_{Se} parameter values were obtained, suggesting that, in general, the consumption of these fish and fish-based products would not have, apparently, negative effects on consumers' health related to mercury toxicity.
2. Based on the total selenium and mercury contents found in samples, it seems to be that fish muscle appears to be a storage organ for mercury, while fish roe constitutes an important source of selenium.
3. Selenium speciation studies performed by HPLC-ICP-MS and confirmed by HPLC-ESI-MS/MS revealed that SeMeSeCys is present in the sarcoplasmic, myofibrillar and alkali-soluble fractions extracted from fish, as well as in their Tris-HCl soluble proteins. Similarly, the presence of SeMet was found in Tris-HCl soluble proteins extracted from tuna muscle. The same species, SeMet and SeMeSeCys, were also identified in fish-derived and processed products, being SeMet the major selenium species in all the samples analyzed.
4. Regarding mercury speciation, $MeHg^+$ and Hg^{2+} were identified in fish samples and fish-derived products, being $MeHg^+$ the major mercury species found in all cases. However, statistically significant differences were observed between the relative $MeHg^+$ and Hg^{2+} contents in the analyzed samples. In predatory fish, such as tuna and swordfish, their $MeHg^+$ contents accounts for more than 90 % of total mercury contents ((110 ± 6) and (96 ± 3) %, respectively). However, farmed salmon and wild salmon showed the highest relative Hg^{2+} concentrations, (35 ± 1) and (27 ± 4) % of total mercury content, respectively. On the other hand, processed fish-derived products, in general, presented lower percentages of $MeHg^+$, compared to fish, with values of (62 ± 5) % of total mercury content, in the case of salmon noodles. However, in the case of fish roe

(lumpfish, trout and wild salmon), no mercury species could be identified, due to their low mercury levels.

5. With respect to fish-processed products, the manufacturing processes involved could alter the concentration of selenium and mercury and species distribution. In this sense, the analysis of samples of all the stages of the production lines of the same batch of these products, provided by companies Angulas Aguinaga and Nueva Pescanova España, evidenced the unaltered presence of SeMet and SeMeSeCys from the raw materials to the finished products.

6. The percentages of selenium and mercury bioaccessibility obtained in the raw materials (pollock, hake and farmed salmon) and the final products considered (crab sticks, surimi-derived elvers and salmon noodles) ranged between 20-39 % for selenium and 9-37 % for mercury, with no significant differences observed between the two large groups. Therefore, the processing processes employed, apparently, do not affect the selenium bioaccessibility. Furthermore, the selenium species identified in the samples were also observed in the gastrointestinal fractions, being SeMet the most bioaccessible form of selenium for all samples analyzed. Additionally, although it was found that the integrity of the species was maintained throughout the production lines, the concentrations of SeMet and SeMeSeCys in the bioaccessible fractions were higher in the case of raw materials (33-42 and 12-19 %, respectively) than in the final products (16-19 and 8-10 %, respectively). On the other hand, it can be concluded that the gastrointestinal extracts of both raw materials and final products did not significantly affect the viability of Caco-2 cells after 2 h of exposure, obtaining similar results as for the blank solutions (around 70 %), suggesting the absence of toxicity of the components of the gastrointestinal extracts (including selenium and mercury) in the samples analyzed.

7. The development of a method of analysis by HPLC-ESI-MS/MS, after optimization of the extraction conditions, allowed us the simultaneous analysis of selenoneine and ergothioneine in fish, fish-derived and processed products. The results obtained showed the presence of ergothioneine in the muscle of all fish samples and in all the different varieties of fish roe considered, and the presence of selenoneine in the muscle of tuna and swordfish. Therefore, it seems that both antioxidant compounds may coexist, at least, in predatory fish. However, neither selenoneine nor ergothioneine were detected in fish-processed products, revealing that the processing might affect the presence of these compounds. In addition, as preliminary results, the possible presence of the methylated compounds S-methylergothioneine and Se-methylselenoneine in fish muscle and fish roe has been suggested, although further confirmatory analyses are required.

8. Cytotoxicity studies carried out using SH-SY5Y cell line showed that the selenium species identified in fish and fish-derived and processed products, SeMet and SeMeSeCys, exhibited a greater protective effect than SeNPs (citrate-SeNPs and Ch-SeNPs) against cell death induced by $MeHg^+$ at high levels of exposure (LC_{50}, 3 µM). The best results were obtained with SeMeSeCys, regardless the concentration employed (25 or 50 µM), showing the highest cell viabilities, up to (99 ± 4) %, comparable with those of the control group.

9. By employing SC-ICP-MS technique, it was possible to determine that, for the SH-SY5Y cell line, when selenium (SeMet or SeMeSeCys) is added to the culture medium in co-exposure with $MeHg^+$, a greater number of cells accumulate less quantity of mercury, compared to those cells exposed only to $MeHg^+$. In particular, co-exposure of SeMet with $MeHg^+$ led to a reduction in the maximum amount of mercury accumulation per individual cell, up to the half (from 60 fg Hg $cell^{-1}$ to 30 fg Hg $cell^{-1}$). However, by conventional ICP-MS analysis, no differences in mercury accumulation were obtained between the aforementioned exposure conditions. Thus, the application of the SC-ICP-MS technique allowed us to obtain more precise information by considering cellular heterogeneity, highlighting its high potential.

X. REFERENCIAS BIBLIOGRÁFICAS

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Aaseth, J., Alexander, J., Alehagen, U., Tinkov, A., Skalny, A., Larsson, A., Crisponi, G., & Nurchi, V. M. (2021). The Aging Kidney—As Influenced by Heavy Metal Exposure and Selenium Supplementation. *Biomolecules, 11(8)*, 1–11. https://doi.org/10.3390/biom11081078

Abad-Álvaro, I., Peña-Vázquez, E., Bolea, E., Bermejo-Barrera, P., Castillo, J. R., & Laborda, F. (2016). Evaluation of number concentration quantification by single-particle inductively coupled plasma mass spectrometry: microsecond vs. millisecond dwell times. *Analytical and Bioanalytical Chemistry, 408(19)*, 5089–5097. https://doi.org/10.1007/s00216-016-9515-y

Achouba, A., Dumas, P., Ouellet, N., Little, M., Lemire, M., & Ayotte, P. (2019). Selenoneine is a major selenium species in beluga skin and red blood cells of Inuit from Nunavik. *Chemosphere, 229*, 549–558. https://doi.org/10.1016/j.chemosphere.2019.04.191

Achouba, A., Dumas, P., & Ayotte, P. (2023). Simultaneous determination of ergothioneine, selenoneine, and their methylated metabolites in human blood using ID-LC–MS/MS. *Analytical and Bioanalytical Chemistry, 415(29)*, 7259–7267. https://doi.org/10.1007/s00216-023-04994-z

Ajsuvakova, O. P., Tinkov, A. A., Willkommen, D., Skalnaya, A. A., Danilov, A. B., Pilipovich, A. A., Aschner, M., Skalny, A. V., Michalke, B., & Skalnaya, M. G. (2020). Assessment of copper, iron, zinc and manganese status and speciation in patients with Parkinson's disease: A pilot study. *Journal of Trace Elements in Medicine and Biology, 59(October 2019)*, 126423. https://doi.org/10.1016/j.jtemb.2019.126423

Alcântara, D. B., Dionísio, A. P., Artur, A. G., Silveira, B. K. S., Lopes, A. F., Guedes, J. A. C., Luz, L. R., Nascimento, R. F., Lopes, G. S., Hermsdorff, H. H. M., & Zocolo, G. J. (2022). Selenium in Brazil nuts: An overview of agronomical aspects, recent trends in analytical chemistry, and health outcomes. *Food Chemistry, 372(March 2021)*. https://doi.org/10.1016/j.foodchem.2021.131207

Allan, C. B., Lacourciere, G. M., & Stadtman, T. C. (1999). Responsiveness of selenoproteins to dietary selenium. *Annual Review of Nutrition, 19*, 1–16. https://doi.org/10.1146/annurev.nutr.19.1.1

Álvarez-Fernández García, R., Corte-Rodríguez, M., Macke, M., Leblanc, K. L., Mester, Z., Montes-Bayón, M., & Bettmer, J. (2020). Addressing the presence of biogenic selenium nanoparticles in yeast cells: Analytical strategies based on ICP-TQ-MS. *Analyst, 145(4)*, 1457–1465. https://doi.org/10.1039/c9an01565e

Alzate, A., Cañas, B., Pérez-Munguí, S., Hernández-Mendoza, H., Pérez-Conde, C., Gutiérrez, A. M., & Cámara, C. (2007). Evaluation of the inorganic selenium biotransformation in selenium-enriched yogurt by HPLC-ICP-MS. *Journal of Agricultural and Food Chemistry, 55(24)*, 9776–9783. https://doi.org/10.1021/jf071596d

Amais, R. S., Donati, G. L., & Zezzi Arruda, M. A. (2020). ICP-MS and trace element analysis as tools for better understanding medical conditions. *TrAC - Trends in Analytical Chemistry, 133*. https://doi.org/10.1016/j.trac.2020.116094

Anan, Y., Ishiwata, K., Suzuki, N., Tanabe, S., & Ogra, Y. (2011). Speciation and identification of low molecular weight selenium compounds in the liver of sea turtles. *Journal of Analytical Atomic Spectrometry, 26(1),* 80–85. https://doi.org/10.1039/c0ja00031k

Andersen, O., & Nielsen, J. B. (1994). Effects of simultaneous low-level dietary supplementation with inorganic and organic selenium on whole-body, blood, and organ levels of toxic metals in mice. *Environmental Health Perspectives, 102(SUPPL. 3)*, 321–324. https://doi.org/10.1289/ehp.94102s3321

Anouar, Y., Lihrmann, I., Falluel-Morel, A., & Boukhzar, L. (2018). Selenoprotein T is a key player in ER proteostasis, endocrine homeostasis and neuroprotection. *Free Radical Biology and Medicine, 127(May)*, 145–152. https://doi.org/10.1016/j.freeradbiomed.2018.05.076

Antony Jesu Prabhu, P., Holen, E., Espe, M., Silva, M. S., Holme, M. H., Hamre, K., Lock, E. J., & Waagbø, R. (2020). Dietary selenium required to achieve body homeostasis and attenuate pro-inflammatory responses in Atlantic salmon post-smolt exceeds the present EU legal limit. *Aquaculture, 526(April)*, 735413. https://doi.org/10.1016/j.aquaculture.2020.735413

Arai, T., Ikemoto, T., Hokura, A., Terada, Y., Kunito, T., Tanabe, S., & Nakai, I. (2004). Chemical forms of mercury and cadmium accumulated in marine mammals and seabirds as determined by XAFS analysis. *Environmental Science and Technology, 38(24)*, 6468–6474. https://doi.org/10.1021/es040367u

Arcagni, M., Juncos, R., Rizzo, A., Pavlin, M., Fajon, V., Arribére, M. A., Horvat, M., & Ribeiro Guevara, S. (2018). Species- and habitat-specific bioaccumulation of total mercury and methylmercury in the food web of a deep oligotrophic lake. *Science of the Total Environment, 612,* 1311–1319. https://doi.org/10.1016/j.scitotenv.2017.08.260

Arevalo, R. Jr. (2014). Laser ablation ICP–MS and laser fluorination GS–MS. *Treatise on Geochemistry (Second Edition)*. Oxford: Elsevier, p. 425-441. https://doi.org/10.1016/B978-0-08-095975-7.01432-7

Azad, A. M., Frantzen, S., Bank, M. S., Nilsen, B. M., Duinker, A., Madsen, L., & Maage, A. (2019). Effects of geography and species variation on selenium and mercury molar ratios in Northeast Atlantic marine fish communities. *Science of the Total Environment, 652*, 1482–1496. https://doi.org/10.1016/j.scitotenv.2018.10.405

B'Hymer, C., & Caruso, J. A. (2006). Selenium speciation analysis using inductively coupled plasma-mass spectrometry. *Journal of Chromatography A, 1114(1*), 1–20. https://doi.org/10.1016/j.chroma.2006.02.063

Balbuena, P., Li, W., Magnin-Bissel, G., Meldrum, J. B., & Ehrich, M. (2010). Comparison of two blood-brain barrier in vitro systems: Cytotoxicity and transfer assessments of malathion/oxon and lead acetate. *Toxicological Sciences, 114(2)*, 260–271. https://doi.org/10.1093/toxsci/kfq001

Barbosa, D. J., Capela, J. P., De Lourdes Bastos, M., & Carvalho, F. (2015). In vitro models for neurotoxicology research. *Toxicology Research, 4(4)*, 801–842. https://doi.org/10.1039/c4tx00043a

Beck, M. A., Esworthy, R. S., Ho, Y. S., & Chu, F. F. (1998). Glutathione peroxidase protects mice from viral-induced myocarditis. *The FASEB Journal, 12(12)*, 1143–1149. https://doi.org/10.1096/fasebj.12.12.1143

Beckett, G. J., & Arthur, J. R. (2005). Selenium and endocrine systems. *Journal of Endocrinology, 184(3)*, 455–465. https://doi.org/10.1677/joe.1.05971

Behne, D., Hammel, C., Pfeifer, H., Rötlilein, D., Gessner, H., & Kyriakopoulos, A. (1998). Speciation of selenium in the mammalian organism. *Analyst, 123(5)*, 871–873. https://doi.org/10.1039/a708699g

Bellinger, F. P., Bellinger, M. T., Seale, L. A., Takemoto, A. S., Raman, A. V., Miki, T., Manning-Boğ, A. B., Berry, M. J., White, L. R., & Ross, G. W. (2011). Glutathione peroxidase 4 is associated with neuromelanin in substantia nigra and dystrophic axons in putamen of Parkinson's brain. *Molecular Neurodegeneration, 6(1)*. https://doi.org/10.1186/1750-1326-6-8

Berntssen, M. H. G., Betancor, M., Caballero, M. J., Hillestad, M., Rasinger, J., Hamre, K., Sele, V., Amlund, H., & Ørnsrud, R. (2018). Safe limits of selenomethionine and selenite supplementation to plant-based Atlantic salmon feeds. *Aquaculture*, 495(May), 617–630. https://doi.org/10.1016/j.aquaculture.2018.06.041

Bertrand G. (1912). On the role of trace substances in agriculture. *Eighth International Congress of Applied Chemistry*, *28*, 30-40

Bin, H., Gui-bin, J., & Zhe-ming, N. (1998). Determination of methylmercury in biological samples and sediments by capillary gas chromatography coupled with atomic absorption spectrometry after hydride derivatization and solid phase microextraction. *Journal of Analytical Atomic Spectrometry, 13(10)*, 1141–1144.

Bisi, T. L., Lepoint, G., Azevedo, A. D. F., Dorneles, P. R., Flach, L., Das, K., Malm, O., & Lailson-Brito, J. (2012). Trophic relationships and mercury biomagnification in Brazilian tropical coastal food webs. *Ecological Indicators, 18,* 291–302. https://doi.org/10.1016/j.ecolind.2011.11.015

Bloom, N. (1989). Determination of Picogram Levels of Methylmercury by Aqueous Phase Ethylation, Followed by Cryogenic Gas Chromatography with Cold Vapour Atomic Fluorescence Detection. *Fisheries and Aquatic Sciences, 46*. www.nrcresearchpress.com

Branca, J. J. V., Morucci, G., Maresca, M., Tenci, B., Cascella, R., Paternostro, F., Ghelardini, C., Gulisano, M., Di Cesare Mannelli, L., & Pacini, A. (2018). Selenium and zinc: Two key players against cadmium-induced neuronal toxicity. *Toxicology in Vitro, 48*, 159–169. https://doi.org/10.1016/j.tiv.2018.01.007

Branco, V., Canário, J., Lu, J., Holmgren, A., & Carvalho, C. (2012). Mercury and selenium interaction in vivo: Effects on thioredoxin reductase and glutathione peroxidase. *Free Radical Biology and Medicine, 52(4)*, 781–793. https://doi.org/10.1016/j.freeradbiomed.2011.12.002

Branco, V., Godinho-Santos, A., Gonçalves, J., Lu, J., Holmgren, A., & Carvalho, C. (2014). Mitochondrial thioredoxin system as a primary target for mercury compounds. *Toxicology Letters, 229(2014)*, S57–S58. https://doi.org/10.1016/j.toxlet.2014.06.234

Branco, V., Lu, J., Holmgren, A., & Carvalho, C. (2015). Effect of mercury compounds over the redox state of thioredoxin 1 and 2 and its relation with glutathione levels and glutaredoxin activity in human neuroblastoma cells. *Toxicology Letters, 238(2)*, S314. https://doi.org/10.1016/j.toxlet.2015.08.898

Brigelius-Flohé, R. (2015). The evolving versatility of selenium in biology. *Antioxidants and Redox Signaling, 23(10)*, 757–760. https://doi.org/10.1089/ars.2015.6469

Burbano, X., Miguez-Burbano, M. J., McCollister, K., Zhang, G., Rodriguez, A., Ruiz, P., Lecusay, R., & Shor-Posner, G. (2002). Impact of a selenium chemoprevention clinical trial on hospital admissions of HIV-infected participants. *HIV Clinical Trials, 3(6)*, 483–491. https://doi.org/10.1310/A7LC-7C9V-EWKF-2Y0H

Burger, J., Gaines, K. F., Boring, C. S., Stephens, W. L., Snodgrass, J., & Gochfeld, M. (2001). Mercury and selenium in fish from the Savannah River: Species, trophic level, and locational differences. *Environmental Research, 87(2)*, 108–118. https://doi.org/10.1006/enrs.2001.4294

Burger, J. & Gochfeld, M. (2011). Mercury and Selenium Levels in 19 Species of Saltwater Fish from New Jersey as a Function of Species, Size, and Season. *Science of the Total Environment, 409 (8)*, 1418–1429. https://doi.org/10.1016/j.scitotenv.2010.12.034.

Burgess, N. M., & Meyer, M. W. (2008). Methylmercury exposure associated with reduced productivity in common loons. *Ecotoxicology, 17(2)*, 83–91. https://doi.org/10.1007/s10646-007-0167-8

Burk, R. F., Jordan, H. E., & Kiker, K. W. (1977). Some effects of selenium status on inorganic mercury metabolism in the rat. *Toxicology and Applied Pharmacology, 40(1)*, 71–82. https://doi.org/10.1016/0041-008X(77)90118-1

Cabañero, A. I., Madrid, Y., & Cámara, C. (2004). Selenium and mercury bioaccessibility in fish samples: An in vitro digestion method. *Analytica Chimica Acta, 526(1)*, 51–61. https://doi.org/10.1016/j.aca.2004.09.039

Cabañero, A. I., Madrid, Y., & Cámara, C. (2005a). Enzymatic probe sonication extraction of Se in animal-based food samples: A new perspective on sample preparation for total and Se speciation analysis. *Analytical and Bioanalytical Chemistry, 381(2)*, 373–379. https://doi.org/10.1007/s00216-004-2798-4

Cabañero, A. I., Madrid, Y., & Cámara, C. (2005b). Study of mercury-selenium interaction in chicken liver by size exclusion chromatography inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry, 20(9)*, 847–855. https://doi.org/10.1039/b503216d

Cabañero, A. I., Madrid, Y., & Cámara, C. (2007). Mercury-selenium species ratio in representative fish samples and their bioaccessibility by an in vitro digestion method. *Biological Trace Element Research, 119(3)*, 195–211. https://doi.org/10.1007/s12011-007-8007-5

Caito, S. W., Milatovic, D., Hill, K. E., Aschner, M., Burk, R. F., & Valentine, W. M. (2011). Progression of neurodegeneration and morphologic changes in the brains of juvenile mice with selenoprotein P deleted. *Brain Research, 1398*, 1–12. https://doi.org/10.1016/j.brainres.2011.04.046.Progression

Calatayud, M., Devesa, V., Virseda, J. R., Barberá, R., Montoro, R., & Vélez, D. (2012). Mercury and selenium in fish and shellfish: Occurrence, bioaccessibility and uptake by Caco-2 cells. *Food and Chemical Toxicology, 50(8)*, 2696–2702. https://doi.org/10.1016/j.fct.2012.05.028

Cámara C., & Pérez-Conde C. (2011). Análisis de trazas. En: Análisis químico de trazas. Cámara, C. & Pérez-Conde, C. (Eds.), pp. 20-69. Editorial *Síntesis S.A.*, España

Cao, Y., Feng, J., Tang, L., Yu, C., Mo, G., & Deng, B. (2020). A Highly Efficient Introduction System for Single Cell- ICP-MS and Its Application to Detection of Copper in Single Human Red Blood Cells. *Talanta, 206 (April),* 120174. https://doi.org/10.1016/j.talanta.2019.120174.

Cao, Y. (2022). The uses of 3D human brain organoids for neurotoxicity evaluations: A review. *NeuroToxicology, 91(May)*, 84–93. https://doi.org/10.1016/j.neuro.2022.05.004

Capelo, J. L., Ximénez-Embún, P., Madrid-Albarrán, Y., & Cámara, C. (2004). Enzymatic Probe Sonication: Enhancement of Protease-Catalyzed Hydrolysis of Selenium Bound to Proteins in Yeast. *Analytical Chemistry, 76(1)*, 233–237. https://doi.org/10.1021/ac034871d

Capelo, J. L., Fernandez, C., Pedras, B., Santos, P., Gonzalez, P., & Vaz, C. (2006). Trends in selenium determination/speciation by hyphenated techniques based on AAS or AFS. *Talanta, 68(5)*, 1442–1447. https://doi.org/10.1016/j.talanta.2005.08.027

Carvalho, C. M. L., Lu, J., Zhang, X., Arnér, E. S. J., & Holmgren, A. (2011). Effects of selenite and chelating agents on mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning. *The FASEB Journal, 25(1)*, 370–381. https://doi.org/10.1096/fj.10-157594

Cetin, S., Knez, D., Gobec, S., Kos, J., & Pišlar, A. (2022). Cell models for Alzheimer's and Parkinson's disease: At the interface of biology and drug discovery. *Biomedicine and Pharmacotherapy, 149(April)*. https://doi.org/10.1016/j.biopha.2022.112924

Chan, Q., & Caruso, J. A. (2012). A metallomics approach discovers selenium-containing proteins in selenium-enriched soybean. *Analytical and Bioanalytical Chemistry, 403(5)*, 1311–1321. https://doi.org/10.1007/s00216-012-5948-0

Chassaigne, H., Chéry, C. C., Bordin, G., & Rodriguez, A. R. (2002). Development of new analytical methods for selenium speciation in selenium-enriched yeast material. *Journal of Chromatography A, 976(1–2*), 409–422. https://doi.org/10.1016/S0021-9673(02)00945-7

Chelegão, R., Carioni, V. M. O., Naozuka, J., & Nomura, C. S. (2016). Feasibility of using AAS for the characterization of a tuna fish candidate reference material for total hg and methyl-Hg measurement. *Journal of the Brazilian Chemical Society, 27(4)*, 712–718. https://doi.org/10.5935/0103-5053.20150320

Chen, W., Wee, P., & Brindle, I. D. (2000). Elimination of the memory effects of gold, mercury and silver in inductively coupled plasma atomic emission spectroscopy. *Journal of Analytical Atomic Spectrometry, 15(4)*, 409–413. https://doi.org/10.1039/a908658g

Chen, J., & Berry, M. J. (2003). Selenium and selenoproteins in the brain and brain diseases. *Journal of Neurochemistry, 86(1)*, 1–12. https://doi.org/10.1046/j.1471-4159.2003.01854.x

Chen, S., Chen, Y., Zhang, Y., Kuang, X., Liu, Y., Guo, M., Ma, L., Zhang, D., & Li, Q. (2020). Iron Metabolism and Ferroptosis in Epilepsy. *Frontiers in Neuroscience, 14(December)*. https://doi.org/10.3389/fnins.2020.601193

Chhibber, T., Bagchi, S., Lahooti, B., Verma, A., Al-Ahmad, A., Paul, M. K., Pendyala, G., & Jayant, R. D. (2020). CNS organoids: an innovative tool for neurological disease modeling and drug neurotoxicity screening. *Drug Discovery Today, 25(2)*, 456–465. https://doi.org/10.1016/j.drudis.2019.11.010

Chung, Y. W., Jeong, D., Noh, O. J., Park, Y. H., Kang, S. I., Lee, M. G., Lee, T. H., Yim, M. Bin, & Kim, I. Y. (2009). Antioxidative role of selenoprotein W in oxidant-induced mouse embryonic neuronal cell death. *Molecules and Cells, 27(5)*, 609–613. https://doi.org/10.1007/s10059-009-0074-3

Clark, L. C., Combs, G. F., Turnbull, B. W., Slate, E. H., Chalker, D. K., Chow, J., Davis, L. S., Glover, R. A., Graham, G. F., Gross, E. G., Krongrad, A., Lesher, J. L., Park, H. K., Sanders, B. B., Smith, C. L., & Taylor, R. (1996). Effects of Selenium Supplementation With Carcinoma of the Skin. *Jama, 276,* 1957–1963. https://doi.org/10.1001/jama.1996.03540240035027

Clarkson, T. W., & Magos, L. (2006). The toxicology of mercury and its chemical compounds. *Critical Reviews in Toxicology, 36(8)*, 609–662. https://doi.org/10.1080/10408440600845619

Combs, G. F. (2001). Selenium in global food systems. *British Journal of Nutrition, 85(5)*, 517–547. https://doi.org/10.1079/bjn2000280

Córdoba-Tovar, L., Marrugo-Negrete, J., Barón, P. R., & Díez, S. (2022). Drivers of biomagnification of Hg, As and Se in aquatic food webs: A review. *Environmental Research, 204(October 2021)*. https://doi.org/10.1016/j.envres.2021.112226

Corte-Rodríguez, M., Álvarez-Fernández García, R., Blanco, E., Bettmer, J., & Montes-Bayón, M. (2017). Quantitative Evaluation of Cisplatin Uptake in Sensitive and Resistant Individual Cells by Single-Cell ICP-MS (SC-ICP-MS). *Analytical Chemistry, 89(21)*, 11491–11497. https://doi.org/10.1021/acs.analchem.7b02746

Corte-Rodríguez, M., Blanco-González, E., Bettmer, J., & Montes-Bayón, M. (2019). Quantitative Analysis of Transferrin Receptor 1 (TfR1) in Individual Breast Cancer Cells by Means of Labeled Antibodies and Elemental (ICP-MS) Detection. *Analytical Chemistry, 91(24)*, 15532–15538. https://doi.org/10.1021/acs.analchem.9b03438

Corte-Rodríguez, M., Álvarez-Fernández, R., García-Cancela, P., Montes-Bayón, M., & Bettmer, J. (2020). Single cell ICP-MS using on line sample introduction systems: Current developments and remaining challenges. *TrAC - Trends in Analytical Chemistry, 132*. https://doi.org/10.1016/j.trac.2020.116042

Crews, H. M., Clarke, P. A., Lewis, D. J., Owen, L. M., Strutt, P. R., & Izquierdo, A. (1996). Investigation of Selenium Speciation in In Vitro Gastrointestinal Extracts of Cooked Cod by High-performance Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry and Electrospray Mass Spectrometry. *Journal of Analytical Atomic Spectrometry, 11(December)*, 1177–1182. https://doi.org/10.1039/JA9961101177

Da Rocha, M. S., Soldado, A.B., Blanco-González, E. & Sanz-Medel, A. (2000). Speciation of mercury compounds by capillary electrophoresis coupled on-line with quadrupole and double-focusing inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry 15*, 513–518. https://doi.org/10.1039/a909880a.

Dahlberg, D., Ivanovic, J., Mariussen, E., & Hassel, B. (2015). High extracellular levels of potassium and trace metals in human brain abscess. *Neurochemistry International, 82*, 28–32. https://doi.org/10.1016/j.neuint.2015.02.003

Daniels, L. A. (1996). Selenium metabolism and function. *Biological Trace Element Research, 54(1)*, 185–199.

Day, J. A., Kannamkumarath, S. S., Yanes, E. G., Montes-Bayón, M., & Caruso, J. A. (2002). Chiral speciation of Marfey's derivatized DL-selenomethionine using capillary electrophoresis with UV and ICP-MS detection. *Journal of Analytical Atomic Spectrometry, 17(1)*, 27–31. https://doi.org/10.1039/b104679a

de Freitas, A. S., Funck, V. R., Rotta, M. dos S., Bohrer, D., Mörschbächer, V., Puntel, R. L., Nogueira, C. W., Farina, M., Aschner, M., & Rocha, J. B. T. (2009). Diphenyl diselenide, a simple organoselenium compound, decreases methylmercury-induced cerebral, hepatic and renal oxidative stress and mercury deposition in adult mice. *Brain Research Bulletin, 79(1)*, 77–84. https://doi.org/10.1016/j.brainresbull.2008.11.001

Degueldre, C., & Favarger, P. Y. (2003). Colloid analysis by single particle inductively coupled plasma-mass spectroscopy: A feasibility study. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 217(1–3)*, 137–142. https://doi.org/10.1016/S0927-7757(02)00568-X

Degueldre, C., & Favarger, P. Y. (2004). Thorium colloid analysis by single particle inductively coupled plasma-mass spectrometry. *Talanta, 62(5)*, 1051–1054. https://doi.org/10.1016/j.talanta.2003.10.016

Degueldre, C., Favarger, P. Y., Rossé, R., & Wold, S. (2006a). Uranium colloid analysis by single particle inductively coupled plasma-mass spectrometry. *Talanta, 68(3)*, 623–628. https://doi.org/10.1016/j.talanta.2005.05.006

Degueldre, C., Favarger, P. Y., & Wold, S. (2006b). Gold colloid analysis by inductively coupled plasma-mass spectrometry in a single particle mode. *Analytica Chimica Acta, 555(2)*, 263–268. https://doi.org/10.1016/j.aca.2005.09.021

De Jesus, J. R., & Arruda, M. A. Z. (2020). Unravelling neurological disorders through metallomics-based approaches. *Metallomics, 12(12)*, 1878–1896. https://doi.org/10.1039/d0mt00234h

De Souza, S. S., Rodrigues, J. L., De Oliveira Souza, V. C., & Barbosa, F. (2010). A fast sample preparation procedure for mercury speciation in hair samples by high-performance liquid chromatography coupled to ICP-MS. *Journal of Analytical Atomic Spectrometry, 25(1)*, 79–83. https://doi.org/10.1039/b911696f

Dietz, C., Madrid, Y., & Cámara, C. (2001). Mercury speciation using the capillary cold trap coupled with microwave-induced plasma atomic emission spectroscopy. *Journal of Analytical Atomic Spectrometry, 16(12)*, 1397–1402. https://doi.org/10.1039/b107110f

Díez, S., & Bayona, J. M. (2002). Determination of methylmercury in human hair by ethylation followed by headspace solid-phase microextraction-gas chromatography-cold-vapour atomic fluorescence spectrometry. *Journal of Chromatography A, 963(1–2)*, 345–351. https://doi.org/10.1016/S0021-9673(02)00140-1

Dong, Z., Liu, Y., Dong, G., & Wu, H. (2021). Effect of boiling and frying on the selenium content, speciation, and in vitro bioaccessibility of selenium-biofortified potato (Solanum tuberosum L.). *Food Chemistry, 348(October 2020)*, 129150. https://doi.org/10.1016/j.foodchem.2021.129150

Duffield, A. J., Thomson, C. D., Hill, K. E. & Williams, S. (1999). An Estimation of Selenium Requirements for New Zealanders. *The American Journal of Clinical Nutrition*, *70 (5)*, 896–903. https://doi.org/10.1093/ajcn/70.5.896.

Dumont, E. (2006). Hyphenated techniques for speciation of Se in biological matrices. Thesis in Sciences: Chemistry. Ghent University. Faculty of Sciences, Ghent, Belgium

Dumont, E., Ogra, Y., Vanhaecke, F., Suzuki, K. T., & Cornelis, R. (2006). Liquid chromatography-mass spectrometry (LC-MS): A powerful combination for selenium speciation in garlic (Allium sativum). *Analytical and Bioanalytical Chemistry, 384(5)*, 1196–1206. https://doi.org/10.1007/s00216-005-0272-6

Ebdon, L., Foulkes, M. E., Le Roux, S., & Muñoz-Olivas, R. (2002). Cold vapour atomic fluorescence spectrometry and gas chromatography-pyrolysis-atomic fluorescence spectrometry for routine determination of total and organometallic mercury in food samples. *Analyst, 127(8)*, 1108–1114. https://doi.org/10.1039/b202927h

EFSA. (2012). Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal*, *10(12)*. https://doi.org/10.2903/j.efsa.2012.2985

EFSA. (2014). Scientific Opinion on Dietary Reference Values for selenium. *EFSA Journal*, *12(10)*, 1–67. https://doi.org/10.2903/j.efsa.2014.3846

El Hanafi, K., Pedrero, Z., Ouerdane, L., Marchán Moreno, C., Queipo-Abad, S., Bueno, M., Pannier, F., Corns, W. T., Cherel, Y., Bustamante, P., & Amouroux, D. (2022). First Time Identification of Selenoneine in Seabirds and Its Potential Role in Mercury Detoxification. *Environmental Science & Technology, 56 (5)*, 3288-3298. https://doi.org/10.1021/acs.est.1c04966

El Hanafi, K., Gómez-Gómez, B., Pedrero, Z., Bustamante, P., Cherel, Y., Amouroux, D., & Madrid, Y. (2023). Simple and rapid formic acid sample treatment for the isolation of HgSe nanoparticles from animal tissues. *Analytica Chimica Acta, 1250(February)*, 340952. https://doi.org/10.1016/j.aca.2023.340952

Enamorado-Báez, S. M., Abril, J. M., & Gómez-Guzmán, J. M. (2013). Determination of 25 Trace Element Concentrations in Biological Reference Materials by ICP-MS following Different Microwave-Assisted Acid Digestion Methods Based on Scaling Masses of Digested Samples. *ISRN Analytical Chemistry, 2013*, 1–14. https://doi.org/10.1155/2013/851713

Ey, J., Schömig, E., & Taubert, D. (2007). Dietary sources and antioxidant effects of ergothioneine. *Journal of Agricultural and Food Chemistry, 55(16)*, 6466–6474. https://doi.org/10.1021/jf071328f

Faria, J., Barbosa, J., Queirós, O., Moreira, R., Carvalho, F., & Dinis-Oliveira, R. J. (2016). Comparative study of the neurotoxicological effects of tramadol and tapentadol in SH-SY5Y cells. *Toxicology, 359–360*, 1–10. https://doi.org/10.1016/j.tox.2016.06.010

Farina, M., Aschner, M., & Rocha, J. B. T. (2011a). Oxidative stress in MeHg-induced neurotoxicity. *Toxicology and Applied Pharmacology, 256(3)*, 405–417. https://doi.org/10.1016/j.taap.2011.05.001

Farina, M., Rocha, J. B. T., & Michael, A. (2011b). Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies. *Life Sci., 90(2)*, 133–154. https://doi.org/10.1016/j.lfs.2011.05.019

FDA/EPA. (2004). What you Need to Know about Mercury in Fish and Shellfish, *Food and Drugs Administration/Environmental Protection Agency*. https://www.fda.gov/food/environmental-contaminants-food/fdaepa-2004-advice-what-you-need-know-about-mercury-fish-and-shellfish

Fernández-Bañares, F., Cabre, E., Esteve, M., Mingorance, M. D., Abad-Lacruz, A., Lachica, M., Gil, A., & Gassull, M. A. (2002). Serum selenium and risk of large size colorectal adenomas in a geographical area with a low selenium status.

The American Journal of Gastroenterology, 97(8), 2103–2108. https://doi.org/10.1111/j.1572-0241.2002.05930.x

Fernández-Bautista, T., Gómez-Gómez, B., Palacín-García, R., Gracia-Lor, E., Pérez-Corona, T., & Madrid, Y. (2022). Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS. *Talanta, 237(October 2021)*. https://doi.org/10.1016/j.talanta.2021.122922

Fernández-Bautista, T., Gómez-Gómez, B., Vicente-Zurdo, D., & Madrid, Y. (2023). Single-cell ICP-MS for evaluating the Se-protective effect against $MeHg^+$-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y). *Analytical and Bioanalytical Chemistry, 0123456789*. https://doi.org/10.1007/s00216-023-05021-x

Forsyth, D. S., Casey, V., Dabeka, R. W., & McKenzie, A. (2004). Methylmercury Levels in Predatory Fish Species Marketed in Canada. *Food Additives & Contaminants, 21 (9)*, 849–856. https://doi.org/10.1080/02652030400004259.

Fox, T. E., Van den Heuvel, E. G. H. M., Atherton, C. A., Dainty, J. R., Lewis, D. J., Langford, N. J., Crews, H. M., Luten, J. B., Lorentzen, M., Sieling, F. W., van Aken-Schneyder, P., Hoek, M., Kotterman, M. J. J., van Dael, P., & Firweather-Tail, S. J. (2004). Biovailability of selenium from fish, yeast and selenate: A comparative study in humans using stable isotopes. *European Journal of Clinical Nutrition, 58(2)*, 343–349. https://doi.org/10.1038/sj.ejcn.1601787

Flowers, B., Poles, A., & Kastrati, I. (2022). Selenium and breast cancer – An update of clinical and epidemiological data. *Archives of Biochemistry and Biophysics, 732(August)*, 109465. https://doi.org/10.1016/j.abb.2022.109465

Francesconi, K. A., & Pannier, F. (2004). Selenium metabolites in urine: A critical overview of past work and current status. *Clinical Chemistry, 50(12)*, 2240–2253. https://doi.org/10.1373/clinchem.2004.039875

Franco, J. L., Posser, T., Dunkley, P. R., Dickson, P. W., Mattos, J. J., Martins, R., Bainy, A. C. D., Marques, M. R., Dafre, A. L., & Farina, M. (2009). Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. *Free Radical Biology and Medicine, 47(4)*, 449–457. https://doi.org/10.1016/j.freeradbiomed.2009.05.013

Gajdosechova, Z., Brownlow, A., Cottin, N. T., Fernandes, M., Read, F. L., Urgast, D. S., Raab, A., Feldmann, J., & Krupp, E. M. (2016). Possible link between Hg and Cd accumulation in the brain of long-finned pilot whales (Globicephala melas). *Science of the Total Environment, 545–546*, 407–413. https://doi.org/10.1016/j.scitotenv.2015.12.082

Gammelgaard, B., Stürup, S., & Christensen, M. V. (2012). Human urinary excretion and metabolism of 82Se-enriched selenite and selenate determined by LC-ICP-MS. *Metallomics, 4(2)*, 149–155. https://doi.org/10.1039/c2mt00163b

Ganther, H. E. (1978). Modification of methylmercury toxicity and metabolism by selenium and vitamin E: Possible mechanisms. *Environmental Health Perspectives, VOL. 25*, 71–76. https://doi.org/10.1289/ehp.782571

Ghasemi, M., Turnbull, T., Sebastian, S., & Kempson, I. (2021). The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International Journal of Molecular Sciences, 22(23)*. https://doi.org/10.3390/ijms222312827

Gladyshev, V. N., & Hatfield, D. L. (1999). Selenocysteine-containing proteins in mammals. *Journal of Biomedical Science, 6(3)*, 151–160. https://doi.org/10.1007/BF02255899

Glaser, V., Moritz, B., Schmitz, A., Dafré, A. L., Nazari, E. M., Rauh Müller, Y. M., Feksa, L., Straliottoa, M. R., De Bem, A. F., Farina, M., Da Rocha, J. B. T., & Latini, A. (2013). Protective effects of diphenyl diselenide in a mouse model of brain toxicity. *Chemico-Biological Interactions, 206(1)*, 18–26. https://doi.org/10.1016/j.cbi.2013.08.002

Glynn, A. W., Ilbäck, N. G., Brabencova, D., Carlsson, L., Enqvist, E. C., Netzel, E., & Oskarsson, A. (1993). Influence of sodium selenite on ^{203}Hg absorption, distribution, and elimination in male mice exposed to methyl^{203}Hg. *Biological Trace Element Research, 39(1*), 91–107. https://doi.org/10.1007/BF02783813

Glynn, A. W., & Lmd, Y. (1995). Effect of Long-Term Sodium Selenite Supplementation on Levels and Distribution of Mercury in Blood, Brain and Kidneys of Methyl Mercury-Exposed Female Mice. *Pharmacology & Toxicology, 77*, 41–47. https://doi.org/10.1111/j.1600-0773.1995.tb01912.x

Gómez-Ariza, J. L., Caro De La Torre, M. A., Giráldez, I., & Morales, E. (2004). Speciation analysis of selenium compounds in yeasts using pressurised liquid extraction and liquid chromatography-microwave-assisted digestion-hydride generation-atomic fluorescence spectrometry. *Analytica Chimica Acta, 524(1-2 SPEC. ISS.)*, 305–314. https://doi.org/10.1016/j.aca.2004.02.044

Gómez-Gómez, B., Corte-Rodríguez, M., Pérez-Corona, M. T., Bettmer, J., Montes-Bayón, M., & Madrid, Y. (2020). Combined single cell and single particle ICP-TQ-MS analysis to quantitatively evaluate the uptake and biotransformation of tellurium nanoparticles in bacteria. *Analytica Chimica Acta, 1128*, 116–128. https://doi.org/10.1016/j.aca.2020.06.058

Grøn, C., & Andersen, L. (2003). Human Bioaccessibility of Heavy Metals and PAH from Soil. Environmental Project No. 840 2003, Technology Programme for Soil and Groundwater Contamination. *Danish Environmental Protection Agency, 840*, 11.

Groombridge, A. S., Miyashita, S. I., Fujii, S. I., Nagasawa, K., Okahashi, T., Ohata, M., Umemura, T., Takatsu, A., Inagaki, K., & Chiba, K. (2013). High sensitive elemental analysis of single yeast cells (saccharomyces cerevisiae) by time-resolved inductively-coupled plasma mass spectrometry using a high efficiency cell introduction system. *Analytical Sciences, 29(6)*, 597–603. https://doi.org/10.2116/analsci.29.597

Gründemann, D., Harlfinger, S., Golz, S., Geerts, A., Lazar, A., Berkels, R., Jung, N., Rubbert, A., & Schömig, E. (2005). Discovery of the ergothioneine transporter. *Proceedings of the National Academy of Sciences of the United States of America, 102(14),* 5256–5261. https://doi.org/10.1073/pnas.0408624102

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology, 30(11)*, 591–600. https://doi.org/10.1016/j.tibtech.2012.08.001

Guzmán, J. L., Reyes, L. H., Rahman, G. M. M., & Kingston, H. M. S. (2009). Simultaneous extraction of arsenic and selenium species from rice products by microwave-assisted enzymatic extraction and analysis by ion chromatography-inductively coupled plasma-mass spectrometry. *Journal of Agricultural and Food Chemistry, 57(8)*, 3005–3013. https://doi.org/10.1021/jf803598k

Hambright, W. S., Fonseca, R. S., Chen, L., Na, R., & Ran, Q. (2017). Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration. *Redox Biology, 12(January)*, 8–17. https://doi.org/10.1016/j.redox.2017.01.021

Hammerschmidt, C. R., & Fitzgerald, W. F. (2001). Formation of artifact methylmercury during extraction from a sediment reference material. *Analytical Chemistry, 73(24)*, 5930–5936. https://doi.org/10.1021/ac010721w

Hammerschmidt, C. R., & Fitzgerald, W. F. (2006). Methylmercury in freshwater fish linked to atmospheric mercury deposition. *Environmental Science and Technology, 40(24),* 7764–7770. https://doi.org/10.1021/es061480i

Hansen, J. C., Kristensen, P., & Westergaard, I. (1981). The influence of selenium on mercury distribution in mice after exposure to low dose Hg^0 vapours. *Journal of Applied Toxicology, 1(3),* 149–153. https://doi.org/10.1002/jat.2550010304

Hardy, R. W., Oram, L. L., & Möller, G. (2010). Effects of dietary selenomethionine on cutthroat trout (Oncorhynchus clarki bouvieri) growth and reproductive performance over a life cycle. *Archives of Environmental Contamination and Toxicology, 58(1)*, 237–245. https://doi.org/10.1007/s00244-009-9392-x

Harry, G. J., Billingsley, M., Bruinink, A., Campbell, I. L., Classen, W., Dorman, D. C., Galli, C., Ray, D., Smith, R. A., & Tilson, H. A. (1998). In vitro techniques for the assessment of neurotoxicity. *Environmental Health Perspectives, 106(SUPPL. 1)*, 131–158. https://doi.org/10.1289/ehp.98106s1131

Hashimoto, K., Watabe, S., Kono, M., & Shiro, K. (1979). Muscle Protein Composition of Sardine and Mackerel. *Bulletin of the Japanese Society for the Science of Fish, 45 (11)*, 1435–1441. https://doi.org/10.2331/suisan.45.1435.

Hawkes, W. C., & Hornbostel, L. (1996). Effects of dietary selenium on mood in healthy men living in a metabolic research unit. *Biological Psychiatry, 39(2)*, 121–128. https://doi.org/10.1016/0006-3223(95)00085-2

Hempel, M., Hintelmann, H., & Wilken, R.-D. (1992). Determination of organic mercury species in soils by high-performance liquid chromatography with ultraviolet detection. *Analyst, 117(March)*, 669–672.

Heusinkveld, H. J., & Westerink, R. H. S. (2017). Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity. *Toxicology in Vitro, 45(February)*, 81–88. https://doi.org/10.1016/j.tiv.2017.07.030

Hineman, A., & Stephan, C. (2014). Effect of dwell time on single particle inductively coupled plasma mass spectrometry data acquisition quality. *Journal of Analytical Atomic Spectrometry, 29(7)*, 1252–1257. https://doi.org/10.1039/c4ja00097h

Hinojosa Reyes, L., Guzmán Mar, J. L., Rahman, G. M. M., Seybert, B., Fahrenholz, T., & Kingston, H. M. S. (2009). Simultaneous determination of arsenic and selenium species in fish tissues using microwave-assisted enzymatic extraction and ion chromatography-inductively coupled plasma mass spectrometry. *Talanta, 78(3)*, 983–990. https://doi.org/10.1016/j.talanta.2009.01.003

Hintelmann, H., Falter, R., Ilgen, G., & Evans, R. D. (1997). Determination of artifactual formation of monomethylmercury (CH_3Hg^+) in environmental samples using stable Hg^{2+} isotopes with ICP-MS detection: Calculation of contents applying Species Specific Isotope Addition. *Fresenius' Journal of Analytical Chemistry, 358(3)*, 363–370. https://doi.org/10.1007/s002160050431

Holben, D. H., & Smith, A. M. (1999). The diverse role of selenium within selenoproteins: A review. *Journal of the American Dietetic Association, 99(7)*, 836–843. https://doi.org/10.1016/S0002-8223(99)00198-4

Horvat, M., Liang, L., & Bloom, N. S. (1993). Comparison of distillation with other current isolation methods for the determination of methyl mercury compounds in low level environmental samples Part II. Water. *Analytica Chimica Acta, 282*, 153–168.

Hossain, K. F. B., Rahman, M. M., Sikder, M. T., Hosokawa, T., Saito, T., & Kurasaki, M. (2021). Selenium modulates inorganic mercury induced cytotoxicity and intrinsic apoptosis in PC12 cells. *Ecotoxicology and Environmental Safety, 207(September 2020)*, 111262. https://doi.org/10.1016/j.ecoenv.2020.111262

Hu, T., Liang, Y., Zhao, G., Wu, W., Li, H., & Guo, Y. (2019). Selenium Biofortification and Antioxidant Activity in *Cordyceps militaris* Supplied with Selenate, Selenite, or Selenomethionine. *Biological Trace Element Research, 187(2)*, 553–561. https://doi.org/10.1007/s12011-018-1386-y

Huang, J. H. (2005). Artifact formation of methyl- and ethyl-mercury compounds from inorganic mercury during derivatization using sodium tetra(n-propyl)borate. *Analytica Chimica Acta, 532(2)*, 113–120. https://doi.org/10.1016/j.aca.2004.10.057

Huang, S. S. Y., Strathe, A. B., Wang, W. F., Deng, D. F., Fadel, J. G., & Hung, S. S. O. (2012). Selenocompounds in juvenile white sturgeon: Evaluating blood, tissue, and urine selenium concentrations after a single oral dose. *Aquatic Toxicology, 109*, 158–165. https://doi.org/10.1016/j.aquatox.2011.12.009

Huerta, V. D., Reyes, L. H., Marchante-Gayón, J. M., Sánchez, M. L. F., & Sanz-Medel, A. (2003). Total determination and quantitative speciation analysis of selenium in yeast and wheat flour by isotope dilution analysis ICP-MS. *Journal of Analytical Atomic Spectrometry, 18(10)*, 1243–1247. https://doi.org/10.1039/b305827a

Huggins, F. E., Raverty, S. A., Nielsen, O. S., Sharp, N. E., Robertson, J. D., & Ralston, N. V. C. (2009). An XAFS investigation of mercury and selenium in Beluga whale tissues. *Environmental Bioindicators, 4(4)*, 291–302. https://doi.org/10.1080/15555270903404651

Hung, R. J., Spaeth, C. S., Yesilyurt, H. G., & Terman, J. R. (2013). Selr Reverses Mical-Mediated Oxidation of Actin to Regulate F-Actin Dynamics. *Nature Cell Biology, 15(12)*, 1445–1454. https://doi.org/10.1038/ncb2871

Hurd D. L. & Kipling J.J. (1964). Origin and growth of physical science, Part 2. *Penguin Books*, Reino Unido

Ip, C., Thompson, H. J., Zhu, Z., & Ganther, H. E. (2000). In vitro and in vivo studies of methylseleninic acid: Evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. *Cancer Research, 60(11)*, 2882–2886

Ip C, & Dong Y. (2001). Methylselenocysteine modulates proliferation and apoptosis biomarkers in premalignant lesions of the rat mammary gland. *Anticancer Research*, *21(2A)*, 863-867. PMID: 11396176

Itano, K., Kawai, S., Miyazaki, N., Tatsukawa, R. & Fujiyama, T. (1984). Body Burdens and Distribution of Mercury and Selenium in Striped Dolphins. *Agriculture and Biological Chemistry*, *48*, 1117-1121

Jaramillo, F., Peng, L., & Gatlin, D. M. (2009). Selenium nutrition of hybrid striped bass (Morone chrysops × M. saxatilis) bioavailability, toxicity and interaction with vitamin e. *Aquaculture Nutrition, 15(2),* 160–165. https://doi.org/10.1111/j.1365-2095.2008.00579.x

Jenkins, D. J. A., Spence, J. D., Giovannucci, E. L., Kim, Y. in, Josse, R., Vieth, R., Blanco Mejia, S., Viguiliouk, E., Nishi, S., Sahye-Pudaruth, S., Paquette, M., Patel, D., Mitchell, S., Kavanagh, M., Tsirakis, T., Bachiri, L., Maran, A., Umatheva, N., McKay, T., ... & Sievenpiper, J. L. (2018). Supplemental Vitamins and Minerals for CVD Prevention and Treatment. *Journal of the American College of Cardiology, 71(22*), 2570–2584. https://doi.org/10.1016/j.jacc.2018.04.020

Jonsson, S., Skyllberg, U., Nilsson, M. B., Lundberg, E., Andersson, A., & Björn, E. (2014). Differentiated availability of geochemical mercury pools controls methylmercury levels in estuarine sediment and biota. *Nature Communications, 5*. https://doi.org/10.1038/ncomms5624

Joshi, D., Mittal, D. K., Shukla, S., Srivastav, A. K., & Srivastav, S. K. (2014). Methylmercury toxicity: Amelioration by selenium and water-soluble chelators as N-acetyl cysteine and dithiothreitol. *Cell Biochemistry and Function, 32(4)*, 351–360. https://doi.org/10.1002/cbf.3023

Kahn-Kirby, A. H., Amagata, A., Maeder, C. I., Mei, J. J., Sideris, S., Kosaka, Y., Hinman, A., Malone, S. A., Bruegger, J. J., Wang, L., Kim, V., Shrader, W. D., Hoff, K. G., Latham, J. C., Ashley, E. A., Wheeler, M. T., Bertini, E., Carrozzo, R., Martinelli, D., ... & Holst, C. R. (2019). Targeting ferroptosis: A novel therapeutic strategy for the treatment of mitochondrial disease-related epilepsy. *PLoS ONE, 14(3),* 1–24. https://doi.org/10.1371/journal.pone.0214250

Kannamkumarath, S. S., Wrobel, K., & Wuilloud, R. G. (2005). Studying the distribution pattern of selenium in nut proteins with information obtained from SEC-UV-ICP-MS and CE-ICP-MS. *Talanta, 66(1),* 153–159. https://doi.org/10.1016/j.talanta.2004.10.010

Kasper, D., Forsberg, B. R., De Almeida, R., Bastos, W. R., & Malm, O. (2015). Metodologias de coleta, preservação e armazenamento de amostras de água para análise de mercúrio - Uma revisão. *Quimica Nova, 38(3)*, 410–418. https://doi.org/10.5935/0100-4042.20150020

Khan, M. A. K., & Wang, F. (2009). Mercury-selenium compounds and their toxicological significance: Toward a molecular understanding of the mercury-selenium antagonism. *Environmental Toxicology and Chemistry, 28(8)*, 1567–1577. https://doi.org/10.1897/08-375.1

Khan, M. A. K., & Wang, F. (2010). Chemical demethylation of methylmercury by selenoamino acids. *Chemical Research in Toxicology, 23(7)*, 1202–1206. https://doi.org/10.1021/tx100080s

Kim, Y. J., Chai, Y. G., & Ryu, J. C. (2005). Selenoprotein W as molecular target of methylmercury in human neuronal cells is down-regulated by GSH depletion. *Biochemical and Biophysical Research Communications, 330(4)*, 1095–1102. https://doi.org/10.1016/j.bbrc.2005.03.080

Kim, B. M., Choi, A. L., Ha, E. H., Pedersen, L., Nielsen, F., Weihe, P., Hong, Y. C., Budtz-Jørgensen, E., & Grandjean, P. (2014). Effect of hemoglobin adjustment on the precision of mercury concentrations in maternal and cord blood. *Environmental Research, 132*, 407–412. https://doi.org/10.1016/j.envres.2014.04.030

Kitsanayanyong, L., Ishikawa, Y., & Ohshima, T. (2022). Putative Ergothioneine Transporter of Salmonids as a Functional Tool for Ergothioneine Supplementation. *Aquaculture, 547*, 737496. https://doi.org/10.1016/j.aquaculture.2021.737496.

Klein, M., Ouerdane, L., Bueno, M., & Pannier, F. (2011). Identification in human urine and blood of a novel selenium metabolite, Se-methylselenoneine, a potential biomarker of metabolization in mammals of the naturally occurring selenoneine, by HPLC coupled to electrospray hybrid linear ion trap-orbital ion trap MS. *Metallomics, 3(5)*, 513–520. https://doi.org/10.1039/c0mt00060d

Korbas, M., Odonoghue, J. L., Watson, G. E., Pickering, I. J., Singh, S. P., Myers, G. J., Clarkson, T. W., & George, G. N. (2010). The chemical nature of mercury in human brain following poisoning or environmental exposure. *ACS Chemical Neuroscience, 1*, 810–818. https://doi.org/10.1021/cn1000765

Korvela, M., Lind, A. L., Wetterhall, M., Gordh, T., Andersson, M., & Pettersson, J. (2016). Quantification of 10 elements in human cerebrospinal fluid from chronic pain patients with and without spinal cord stimulation. *Journal of Trace Elements in Medicine and Biology, 37*, 1–7. https://doi.org/10.1016/j.jtemb.2016.06.003

Kozaki, D., Mori, M., Hamasaki, S., Doi, T., Tanihata, S., Yamamoto, A., Takahashi, T., Sakamoto, K., & Funado, S. (2021). Simple mercury determination using an enclosed quartz cell with cold vapour-atomic absorption spectrometry. *Analytical Methods, 13(9)*, 1106–1109. https://doi.org/10.1039/d0ay02232b

Krebs, N., Langkammer, C., Goessler, W., Ropele, S., Fazekas, F., Yen, K., & Scheurer, E. (2014). Assessment of trace elements in human brain using inductively coupled plasma mass spectrometry. *Journal of Trace Elements in Medicine and Biology, 28(1)*, 1–7. https://doi.org/10.1016/j.jtemb.2013.09.006

N. Kroepfl, N., K.B. Jensen, K.B., K.A. Francesconi, K.A., & Kuehnelt, D. (2015). Human excretory products of selenium are natural constituents of marine fish muscle. *Analytical and Bioanalytical Chemistry, 407*, 7713–7719. https://doi.org/10.1007/s00216-015-8936-3.

Kroepfl, N., Francesconi, K. A., Schwerdtle, T., & Kuehnelt, D. (2019). Selenoneine and ergothioneine in human blood cells determined simultaneously by HPLC/ICP-QQQ-MS. *Journal of Analytical Atomic Spectrometry, 34(1)*, 127–134. https://doi.org/10.1039/c8ja00276b

Kubáň, P., Houserová, P., Kubáň, P., Hauser, P. C., & Vlastimil, K. (2007). Mercury speciation by CE: A review. *Electrophoresis, 28(1–2)*, 58–68. https://doi.org/10.1002/elps.200600457

Kubáň, P., Pelcová, P., Margetínová, J., & Kubáň, V. (2009). Mercury speciation by CE: An update. *Electrophoresis, 30(1)*, 92–99. https://doi.org/10.1002/elps.200800382

Kumaria, A., & Tolias, C. M. (2008). In vitro models of neurotrauma. *British Journal of Neurosurgery, 22(2)*, 200–206. https://doi.org/10.1080/02688690701772413

Laborda, F., Jiménez-Lamana, J., Bolea, E., & Castillo, J. R. (2013). Critical considerations for the determination of nanoparticle number concentrations, size and number size distributions by single particle ICP-MS. *Journal of Analytical Atomic Spectrometry, 28(8)*, 1220–1232. https://doi.org/10.1039/c3ja50100k

Laborda, F., Bolea, E., & Jimenez-Lamana, J. (2014). Single Particle Inductively Coupled Plasma Mass Spectrometry: A Powerful Tool for Nanoanalysis. *Analytical Chemistry, 86*, 2270–2278. https://doi.org/10.1021/ac402980q

Lancaster, S. T., Peniche, G., Alzahrani, A., Blanz, M., Newton, J., Taggart, M. A., Corns, W. T., Krupp, E. M., & Feldmann, J. (2022). Mercury speciation in Scottish raptors reveals high proportions of inorganic mercury in Scottish golden eagles (Aquila chrysaetos): Potential occurrence of mercury selenide nanoparticles. *Science of The Total Environment, 829*, 154557. https://doi.org/10.1016/j.scitotenv.2022.154557

Lavoie, R. A., Jardine, T. D., Chumchal, M. M., Kidd, K. A., & Campbell, L. M. (2013). Biomagnification of mercury in aquatic food webs: A worldwide meta-analysis. *Environmental Science and Technology, 47(23)*, 13385–13394. https://doi.org/10.1021/es403103t

Le, K. T., & Fotedar, R. (2014). Toxic effects of excessive levels of dietary selenium in juvenile yellowtail kingfish (*Seriola lalandi*). *Aquaculture, 433*, 229–234. https://doi.org/10.1016/j.aquaculture.2014.06.021

Lee, T. H. & Jiang, S. J. (2000). Determination of mercury compounds by capillary electrophoresis inductively coupled plasma mass spectrometry with microconcentric nebulization. *Analytica Chimica Acta, 413*, 197-205. doi:10.1016/S0003-2670(00)00807-2

Leiter, O., Zhuo, Z., Rust, R., Wasielewska, J. M., Grönnert, L., Kowal, S., Overall, R. W., Adusumilli, V. S., Blackmore, D. G., Southon, A., Ganio, K., McDevitt, C. A., Rund, N., Brici, D., Mudiyan, I. A., Sykes, A. M., Rünker, A. E., Zocher, S., Ayton, S., … & Walker, T. L. (2022). Selenium mediates exercise-induced adult neurogenesis and reverses learning deficits induced by hippocampal injury and aging. *Cell Metabolism, 34(3)*, 408-423.e8. https://doi.org/10.1016/j.cmet.2022.01.005

Lemes, M., & Wang, F. (2009). Methylmercury speciation in fish muscle by HPLC-ICP-MS following enzymatic hydrolysis. *Journal of Analytical Atomic Spectrometry, 24(5)*, 663–668. https://doi.org/10.1039/b819957b

Letavayová, L., Vlčková, V., & Brozmanová, J. (2006). Selenium: From cancer prevention to DNA damage. *Toxicology, 227(1–2)*, 1–14. https://doi.org/10.1016/j.tox.2006.07.017

Li, X., Yin, D., Yin, J., Chen, Q., & Wang, R. (2014). Dietary selenium protect against redox-mediated immune suppression induced by methylmercury exposure. *Food and Chemical Toxicology, 72,* 169–177. https://doi.org/10.1016/j.fct.2014.07.023

Li, J., He, Q., Wu, L., Sun, J., Zheng, F., Li, L., Liu, W., & Liu, J. (2020). Ultrasensitive speciation analysis of mercury in waters by headspace solid-phase microextraction coupled with gas chromatography-triple quadrupole mass spectrometry. *Microchemical Journal, 153(August 2019)*, 1–6. https://doi.org/10.1016/j.microc.2019.104459

Li, L. X., Chu, J. H., Chen, X. W., Gao, P. C., Wang, Z. Y., Liu, C., & Fan, R. F. (2022). Selenium ameliorates mercuric chloride-induced brain damage through activating BDNF/TrKB/PI3K/AKT and inhibiting NF-κB signaling pathways. *Journal of Inorganic Biochemistry, 229(December 2021)*, 111716. https://doi.org/10.1016/j.jinorgbio.2022.111716

Liang, L., Evens, C., Lazoff, S., Woods, J. S., Cernichiari, E., Horvat, M., Martin, M. D., & DeRouen, T. (2000). Determination of methyl mercury in whole blood by ethylation-GC-CVAFS after alkaline digestion-solvent extraction. *Journal of Analytical Toxicology, 24(5)*, 328–332. https://doi.org/10.1093/jat/24.5.328

Lim, D., Gründemann, D., & Seebeck, F. P. (2019). Total Synthesis and Functional Characterization of Selenoneine. *Angewandte Chemie - International Edition, 58(42)*, 15026–15030. https://doi.org/10.1002/anie.201908967

Lin, Y. H. (2014). Effects of dietary organic and inorganic selenium on the growth, selenium concentration and meat quality of juvenile grouper *Epinephelus* *malabaricus*. *Aquaculture, 430,* 114–119. https://doi.org/10.1016/j.aquaculture.2014.03.048

Lindemann, T., Prange, A., Dannecker, W., & Neidhart, B. (2000). Stability studies of arsenic, selenium, antimony and tellurium species in water, urine, fish and soil extracts using HPLC/ICP-MS. *Fresenius' Journal of Analytical Chemistry, 368(2–3)*, 214–220. https://doi.org/10.1007/s002160000475

Little, M., Achouba, A., Dumas, P., Ouellet, N., Ayotte, P., & Lemire, M. (2019). Determinants of selenoneine concentration in red blood cells of Inuit from Nunavik (Northern Québec, Canada). *Environmental International, 127(March)*, 243–252. https://doi.org/10.1016/j.envint.2018.11.077

Little, M., Achouba, A., Ayotte, P., & Lemire, M. (2024). Emerging evidence on selenoneine and its public health relevance in coastal populations: a review and case study of dietary Se among Inuit populations in the Canadian Arctic. *Nutrition Research Review, 8,* 1-10. https://doi.org/10.1017/S0954422424000039.

Liu, W., & Lee, H. K. (1999). Chemical modification of analytes in speciation analysis by capillary electrophoresis, liquid chromatography and gas chromatography. *Journal of Chromatography A, 834(1–2)*, 45–63. https://doi.org/10.1016/S0021-9673(98)00962-5

Liu, Z., Xue, A., Chen, H., & Li, S. (2019). Quantitative determination of trace metals in single yeast cells by time-resolved ICP-MS using dissolved standards for calibration. *Applied Microbiology and Biotechnology, 103(3)*, 1475–1483. https://doi.org/10.1007/s00253-018-09587-w

Liu, T., Bolea-Fernandez, E., Mangodt, C., De Wever, O., & Vanhaecke, F. (2021). Single-event tandem ICP-mass spectrometry for the quantification of chemotherapeutic drug-derived Pt and endogenous elements in individual human cells. *Analytica Chimica Acta, 1177*. https://doi.org/10.1016/j.aca.2021.338797

López-Bellido Garrido, F.J., & López Bellido, L. (2013). Selenio y salud; valores de referencia y situación actual de la población española. Nutrición Hospitalaria, *28 (5)*, 1396-1406. https://doi.org/10.3305/nh.2013.28.5.6634

López-Serrano Oliver, A., Baumgart, S., Bremser, W., Flemig, S., Wittke, D., Grützkau, A., Luch, A., Haase, A., & Jakubowski, N. (2018). Quantification of silver nanoparticles taken up by single cells using inductively coupled plasma mass spectrometry in the single cell measurement mode. *Journal of Analytical Atomic Spectrometry, 33 (7)*, 1256–1263. https://doi.org/10.1039/c7ja00395a.

Lopez-Suarez, L., Awabdh, S. Al, Coumoul, X., & Chauvet, C. (2022). The SH-SY5Y human neuroblastoma cell line, a relevant in vitro cell model for investigating neurotoxicology in human: Focus on organic pollutants. *NeuroToxicology, 92(February)*, 131–155. https://doi.org/10.1016/j.neuro.2022.07.008

Lorentzen, M., Maage, A., & Julshamn, K. (1994). Effects of dietary selenite or selenomethionine on tissue selenium levels of Atlantic salmon (*Salmo salar*). *Aquaculture, 121(4)*, 359–367. https://doi.org/10.1016/0044-8486(94)90270-4

Loscalzo, M. D. J. (2014). Keshan Disease, Selenium Deficiency, and the Selenoproteome. *The New England Journal of Medicine Clinical, 370(18)*, 1756–1760. https://doi.org/10.1177/2514183x18789327

Lu, J., & Holmgren, A. (2014). The thioredoxin antioxidant system. *Free Radical Biology and Medicine, 66*, 75–87. https://doi.org/10.1016/j.freeradbiomed.2013.07.036

Ma, S., He, M., Chen, B., Deng, W., Zheng, Q., & Hu, B. (2016). Magnetic solid phase extraction coupled with inductively coupled plasma mass spectrometry for the speciation of mercury in environmental water and human hair samples. *Talanta, 146*, 93–99. https://doi.org/10.1016/j.talanta.2015.08.036

Manceau, A., Bourdineaud, J. P., Oliveira, R. B., Sarrazin, S. L. F., Krabbenhoft, D. P., Eagles-Smith, C. A., Ackerman, J. T., Stewart, A. R., Ward-Deitrich, C., Del Castillo Busto, M. E., Goenaga-Infante, H., Wack, A., Retegan, M., Detlefs, B., Glatzel, P., Bustamante, P., Nagy, K. L., & Poulin, B. A. (2021a). Demethylation of Methylmercury in Bird, Fish, and Earthworm. *Environmental Science and Technology, 55(3)*, 1527–1534. https://doi.org/10.1021/acs.est.0c04948

Manceau, A., Gaillot, A. C., Glatzel, P., Cherel, Y., & Bustamante, P. (2021b). In Vivo Formation of HgSe Nanoparticles and Hg-Tetraselenolate Complex from Methylmercury in Seabirds-Implications for the Hg-Se Antagonism. *Environmental Science and Technology, 55(3)*, 1515–1526. https://doi.org/10.1021/acs.est.0c06269

Maraldi, T., Riccio, M., Zambonin, L., Vinceti, M., De Pol, A., & Hakim, G. (2011). Low levels of selenium compounds are selectively toxic for a human neuron cell line through ROS/RNS increase and apoptotic process activation. *NeuroToxicology, 32(2)*, 180–187. https://doi.org/10.1016/j.neuro.2010.10.008

Marcocci, C., Kahaly, G. J., Krassas, G. E., Bartalena, L., Prummel, M., Stahl, M., Altea, M. A., Nardi, M., Pitz, S., Boboridis, K., Sivelli, P., von Arx, G., Mourits, M. P., Baldeschi, L., Bencivelli, W., & Wiersinga, W. (2011). Selenium and the Course of Mild Graves' Orbitopathy. *New England Journal of Medicine, 364(20)*, 1920–1931. https://doi.org/10.1056/nejmoa1012985

Marcocci, C., Leo, M., & Altea, M. A. (2012). Oxidative Stress in Graves' Disease. *European Thyroid Journal, 1(2)*, 80–87. https://doi.org/10.1159/000337976

Mavrakis, E., Mavroudakis, L., Lydakis-Simantiris, N., & Pergantis, S. A. (2019). Investigating the uptake of arsenate by chlamydomonas reinhardtii cells and its effect on their lipid profile using single cell ICP-MS and Easy Ambient Sonic-Spray Ionization-MS. *Analytical Chemistry, 91(15),* 9590–9598. https://doi.org/10.1021/acs.analchem.9b00917

Mazan, S., Gilon, N., Crétier, G., Rocca, J. L., & Mermet, J. M. (2002). Inorganic selenium speciation using HPLC-ICP-hexapole collision/reaction cell-MS. *Journal of Analytical Atomic Spectrometry, 17(4),* 366–370. https://doi.org/10.1039/b108267c

McCurdy, E., & Potter, D. (2001). Optimising ICP-MS for the determination of trace metals in high matrix samples. *Atomic Spectrometry, 3,* 1–4.

McKenzie, R. C., Beckett, G. J., & Arthur, J. R. (2006). Effects of selenium on immunity and aging. *Selenium: Its Molecular Biology and Role in Human Health, Second Edition, 311–322*. https://doi.org/10.1007/0-387-33827-6_27

McKinnon, K. M. (2019). Flow cytometry: an overview. *Current Protocols in Immunology, 120*. https://doi.org/10.1007/978-94-017-0623-0_1

Meija, J., Montes-Bayón, M., Le Duc, D. L., Terry, N., & Caruso, J. A. (2002). Simultaneous monitoring of volatile selenium and sulfur species from se accumulating plants (wild type and genetically modified) by GC/MS and GC/ICPMS using solid-phase microextraction for sample introduction. *Analytical Chemistry, 74(22)*, 5837–5844. https://doi.org/10.1021/ac020285t

Mengel, H., & Karlog, O. (1980). Studies on the Interaction and Distribution of Selenite, Mercuric, Methoxyethyl Mercuric and Methyl Mercuric Chloride in Rats. 11. Analysis of the Soluble Proteins and the Precipitates of Liver and Kidney Homogenates. *Acta Pharmacologica et Toxicologica, 46,* 25–31.

Meyer, S., López-Serrano, A., Mitze, H., Jakubowski, N., & Schwerdtle, T. (2018). Single-cell analysis by ICP-MS/MS as a fast tool for cellular bioavailability studies of arsenite. *Metallomics, 10(1)*, 73–76. https://doi.org/10.1039/c7mt00285h

Montaño, M. D., Badiei, H. R., Bazargan, S., & Ranville, J. F. (2014). Improvements in the detection and characterization of engineered nanoparticles using spICP-MS with microsecond dwell times. *Environmental Science: Nano, 1(4)*, 338–346. https://doi.org/10.1039/c4en00058g

Montes-Bayón, M., Molet, M. J. D., González, E. B., & Sanz-Medel, A. (2006). Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (*Allium sativum* and *Brassica juncea*) and Se speciation by HPLC-ICP-MS. *Talanta, 68(4)*, 1287–1293. https://doi.org/10.1016/j.talanta.2005.07.040

Montes-Bayón, M., Corte-Rodríguez, M., Álvarez-Fernández García, M., Fagundes & J.S. (2022). Biomedical analysis by ICP-MS: A focus on single cell strategies, *1st ed., Elsevier B.V.* https://doi.org/10.1016/bs.coac.2022.03.002

Moreda-Piñeiro, J., Moreda-Piñeiro, A., Romarís-Hortas, V., Domínguez-González, R., Alonso-Rodríguez, E., López-Mahía, P., Muniategui-Lorenzo, S., Prada-Rodríguez, D., & Bermejo-Barrera, P. (2013). In vitro bioavailability of total selenium and selenium species from seafood. *Food Chemistry, 139(1–4)*, 872–877. https://doi.org/10.1016/j.foodchem.2013.01.116

Moreda-Piñeiro, J., Moreda-Piñeiro, A., & Bermejo-Barrera, P. (2017). In vivo and in vitro testing for selenium and selenium compounds bioavailability assessment in foodstuff. *Critical Reviews in Food Science and Nutrition, 57(4)*, 805–833. https://doi.org/10.1080/10408398.2014.934437

Moreno, F., García-Barrera, T., & Gómez-Ariza, J. L. (2013). Simultaneous speciation and preconcentration of ultra trace concentrations of mercury and selenium species in environmental and biological samples by hollow fiber liquid phase microextraction prior to high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry. *Journal of Chromatography A, 1300*, 43–50. https://doi.org/10.1016/j.chroma.2013.02.083

Moreno-Martín, G., Sanz-Landaluze, J., León-González, M. E., & Madrid, Y. (2020). Insights into the accumulation and transformation of Ch-SeNPs by *Raphanus sativus* and *Brassica juncea*: Effect on essential elements uptake. *Science of the Total Environment, 725*. https://doi.org/10.1016/j.scitotenv.2020.138453

Moreno-Martín, G., Sanz-Landaluze, J., León-González, M. E., & Madrid, Y. (2021). In vivo quantification of volatile organoselenium compounds released by bacteria exposed to selenium with HS-SPME-GC-MS. Effect of selenite and selenium nanoparticles. *Talanta, 224*. https://doi.org/10.1016/j.talanta.2020.121907.

Moscoso-Pérez, C., Moreda-Piñeiro, J., López-Mahía, P., Muniategui-Lorenzo, S., Fernández-Fernández, E., & Prada-Rodríguez, D. (2008). Pressurized liquid extraction followed by high performance liquid chromatography coupled to hydride generation atomic fluorescence spectrometry for arsenic and selenium speciation in atmospheric particulate matter. *Journal of*

Chromatography A, 1215(1–2), 15–20. https://doi.org/10.1016/j.chroma.2008.10.100

Moser, R., Zaccarini, F., Alber, T., & Kerbl, R. (2020). First finding of tiemannite, HgSe, in human bladder stones: An electron microprobe study. *Micron, 138(June)*, 102928. https://doi.org/10.1016/j.micron.2020.102928

Mounicou, S., McSheehy, S., Szpunar, J., Potin-Gautier, M., & Lobinski, R. (2002). Analysis of selenized yeast for selenium speciation by size-exclusion chromatography and capillary zone electrophoresis with inductively coupled plasma mass spectrometric detection (SEC-CZE-ICP-MS). *Journal of Analytical Atomic Spectrometry, 17(1)*, 15–20. https://doi.org/10.1039/b107943n

Mykkanen, H. M., & Metsaniitty, L. (1987). Selenium-mercury interaction during intestinal absorption of 75Se compounds in chicks. *Journal of Nutrition, 117(8)*, 1453–1458. https://doi.org/10.1093/jn/117.8.1453

Nakazawa, E., Ikemoto, T., Hokura, A., Terada, Y., Kunito, T., Tanabe, S., & Nakai, I. (2011). The presence of mercury selenide in various tissues of the striped dolphin: Evidence from μ-XRF-XRD and XAFS analyses. *Metallomics, 3(7)*, 719–725. https://doi.org/10.1039/c0mt00106f

Navarro-Alarcon, M., & Cabrera-Vique, C. (2008). Selenium in food and the human body: A review. *Science of the Total Environment, 400(1–3)*, 115–141. https://doi.org/10.1016/j.scitotenv.2008.06.024

Neve, J. (1996). Selenium as a risk factor for cardiovascular diseases. *Journal of Cardiovascular Risk, 3*, 42–47. https://doi.org/10.3389/fpsyt.2016.00025

Newland, M. C., Reed, M. N., LeBlanc, A., & Donlin, W. D. (2006). Brain and blood mercury and selenium after chronic and developmental exposure to methylmercury. *NeuroToxicology, 27(5)*, 710–720. https://doi.org/10.1016/j.neuro.2006.05.007

Nogara, P. A., Oliveira, C. S., Schmitz, G. L., Piquini, P. C., Farina, M., Aschner, M., & Rocha, J. B. T. (2019). Methylmercury's chemistry: From the environment to the mammalian brain. *Biochimica et Biophysica Acta - General Subjects, 1863(12)*, 129284. https://doi.org/10.1016/j.bbagen.2019.01.006

Ohi, G., Nishigaki, S., Seki, H., Tamura, Y., Maki, T., Konno, H., Ochiai, S., Yamada, H., Shimamura, Y., Mizoguchi, I., & Yagyu, H. (1976). Efficacy of selenium in tuna and selenite in modifying methylmercury intoxication. *Environmental Research, 12(1)*, 49–58. https://doi.org/10.1016/0013-9351(76)90008-6

Ohta, Y., & Suzuki, K. T. (2008). Methylation and demethylation of intermediates selenide and methylselenol in the metabolism of selenium. *Toxicology and Applied Pharmacology, 226(2)*, 169–177. https://doi.org/10.1016/j.taap.2007.09.011

Ohta, Y., Kobayashi, Y., Konishi, S., & Hirano, S. (2009). Speciation analysis of selenium metabolites in urine and breath by HPLC- and GC-inductively coupled plasma-MS after administration of selenomethionine and methylselenocysteine to rats. *Chemical Research in Toxicology, 22(11)*, 1795–1801. https://doi.org/10.1021/tx900202m

Olesik, J. W., & Gray, P. J. (2012). Considerations for measurement of individual nanoparticles or microparticles by ICP-MS: Determination of the number of particles and the analyte mass in each particle. *Journal of Analytical Atomic Spectrometry, 27(7)*, 1143–1155. https://doi.org/10.1039/c2ja30073g

Oliveira, C. S., Piccoli, B. C., Aschner, M., & Rocha, J. B. T. (2017). Chemical Speciation of Selenium and Mercury as Determinant of Their Neurotoxicity. In *Advances in Neurobiology (Vol. 18)*. https://doi.org/10.1007/978-3-319-60189-2_4

Olmedo, P., Hernández, A. F., Pla, A., Femia, P., Navas-Acien, A., & Gil, F. (2013). Determination of essential elements (copper, manganese, selenium and zinc) in fish and shellfish samples. Risk and nutritional assessment and mercury-selenium balance. *Food and Chemical Toxicology, 62*, 299–307. https://doi.org/10.1016/j.fct.2013.08.076

Olson, G. E., Winfrey, V. P., Hill, K. E., & Burk, R. F. (2008). Megalin mediates selenoprotein p uptake by kidney proximal tubule epithelial cells. *Journal of*

Biological Chemistry, 283(11), 6854–6860. https://doi.org/10.1074/jbc.M709945200

Oomen, A. G., Rompelberg, C. J. M., Van De Kamp, E., Pereboom, D. P. K. H., De Zwart, L. L., & Sips, A. J. A. M. (2004). Effect of Bile Type on the Bioaccessibility of Soil Contaminants in an In Vitro Digestion Model. *Archives of Environmental Contamination and Toxicology, 46(2)*, 183–188. https://doi.org/10.1007/s00244-003-2138-2

Pace, H. E., Rogers, N. J., Jarolimek, C., Coleman, V. A., Higgins, C. P., & Ranville, J. F. (2011). Erratum: Determining transport efficiency for the purpose of counting and sizing nanoparticles via single particle inductively coupled plasma mass spectrometry. *Analytical Chemistry, 84(10)*, 4633. https://doi.org/10.1021/ac300942m

Palacios, Ò., Ruiz Encinar, J., Schaumlöffel, D., & Lobinski, R. (2006). Fractionation of selenium-containing proteins in serum by multiaffinity liquid chromatography before size-exclusion chromatography-ICPMS. *Analytical and Bioanalytical Chemistry, 384(6),* 1276–1283. https://doi.org/10.1007/s00216-005-0286-0

Palmer, J. H., & Parkin, G. (2015). Protolytic Cleavage of Hg-C Bonds Induced by 1-Methyl-1,3-dihydro-2 H -benzimidazole-2-selone: Synthesis and Structural Characterization of Mercury Complexes. *Journal of the American Chemical Society, 137(13)*, 4503–4516. https://doi.org/10.1021/jacs.5b00840

Panel, E., & Chain, F. (2012). Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA Journal, 10(12)*. https://doi.org/10.2903/j.efsa.2012.2985

Park, J. D., & Zheng, W. (2012). human exposure and health effects of inorganic and elemental mercury. *Journal of Preventive Medicine and Public Health, 45 (6)*, 344–352. https://doi.org/10.3961/jpmph.2012.45.6.344.

Pedrero, Z., Ruiz Encinar, J., Madrid, Y., & Cámara, C. (2007). Application of species-specific isotope dilution analysis to the correction for selenomethionine oxidation in Se-enriched yeast sample extracts during storage. *Journal of*

Analytical Atomic Spectrometry, 22(9), 1061–1066. https://doi.org/10.1039/b704807f

Pedrero, Z., & Madrid, Y. (2009). Novel approaches for selenium speciation in foodstuffs and biological specimens: A review. *Analytica Chimica Acta, 634(2)*, 135–152. https://doi.org/10.1016/j.aca.2008.12.026

Pedrero, Z., Ouerdane, L., Mounicou, S., Lobinski, R., Monperrus, M., & Amouroux, D. (2014). Hemoglobin as a major binding protein for methylmercury in white-sided dolphin liver. *Analytical and Bioanalytical Chemistry, 406(4)*, 1121–1129. https://doi.org/10.1007/s00216-013-7274-6

Peeler, J. C., & Weerapana, E. (2019). Chemical Biology Approaches to Interrogate the Selenoproteome. *Accounts of Chemical Research, 52(10)*, 2832–2840. https://doi.org/10.1021/acs.accounts.9b00379

Peretz, A., Neve, J., Duchateau, J., & Famaey, J. P. (1992). Adjuvant Treatment of Recent Onset Rheumatoid Arthritis by Selenium Supplementation: Preliminary Observations. *British Journal of Rheumatology, 31*, 281–286.

Pérez, P. A., Bravo, M. A., & Quiroz, W. (2020). Total mercury bias in soil analysis by CV-AFS: Causes, consequences and a simple solution based on sulfhydryl cotton fiber as a clean-up step. *Analytical Methods, 12(29)*, 3756–3762. https://doi.org/10.1039/d0ay01035a

Pietilä, H., Perämäki, P., Piispanen, J., Majuri, L., Starr, M., Nieminen, T., Kantola, M., & Ukonmaanaho, L. (2014). Determination of methyl mercury in humic-rich natural water samples using N2-distillation with isotope dilution and on-line purge and trap GC-ICP-MS. *Microchemical Journal, 112*, 113–118. https://doi.org/10.1016/j.microc.2013.10.002

Pitts, M. W., Reeves, M. A., Hashimoto, A. C., Ogawa, A., Kremer, P., Seale, L. A., & Berry, M. J. (2013). Deletion of selenoprotein M leads to obesity without cognitive deficits. *Journal of Biological Chemistry, 288(36)*, 26121–26134. https://doi.org/10.1074/jbc.M113.471235

Porvari, P. (2003). Sources and fate of mercury in aquatic ecosystems. *Monographs of the Boreal Environment Research, 23,* 1–53.

https://helda.helsinki.fi/bitstream/handle/10138/39340/BERMon_23.pdf?sequence=1.

Prince, L. M., Aschner, M., & Bowman, A. B. (2019). Human-induced pluripotent stems cells as a model to dissect the selective neurotoxicity of methylmercury. *Biochimica et Biophysica Acta - General Subjects, 1863(12)*. https://doi.org/10.1016/j.bbagen.2019.02.002

Qvarnström, J., & Frech, W. (2002). Mercury species transformations during sample pre-treatment of biological tissues studied by HPLC-ICP-MS. *Journal of Analytical Atomic Spectrometry, 17(11)*, 1486–1491. https://doi.org/10.1039/b205246f

Qvarnström,Ralston, N. V. C., Kaneko, J. J., & Raymond, L. J. (2019). Selenium health benefit values provide a reliable index of seafood benefits vs. risks. *Journal of Trace Elements in Medicine and Biology, 55(May)*, 50–57. https://doi.org/10.1016/j.jtemb.2019.05.009

Rahman, M. M., Uson-Lopez, R. A., Sikder, M. T., Tan, G., Hosokawa, T., Saito, T., & Kurasaki, M. (2018). Ameliorative effects of selenium on arsenic-induced cytotoxicity in PC12 cells via modulating autophagy/apoptosis. *Chemosphere, 196*, 453–466. https://doi.org/10.1016/j.chemosphere.2017.12.149

Rahmanto, A. S., & Davies, M. J. (2012). Selenium-containing amino acids as direct and indirect antioxidants. *IUBMB Life, 64(11)*, 863–871. https://doi.org/10.1002/iub.1084

Ralston, N. V. C., Ralston, C. R., Blackwell, J. L., & Raymond, L. J. (2008). Dietary and Tissue Selenium in Relation to Methylmercury Toxicity. *Neurotoxicology, 29 (5)*, 802–811. https://doi.org/10.1016/j.neuro.2008.07.007.

Ralston, N. V. C., Ralston, C. R., & Raymond, L. J. (2016). Selenium Health Benefit Values: Updated Criteria for Mercury Risk Assessments. *Biological Trace Element Research, 171(2),* 262–269. https://doi.org/10.1007/s12011-015-0516-z

Ralston, N. V. C., Kaneko, J. J., & Raymond, L. J. (2019). Selenium Health Benefit Values Provide a Reliable Index of Seafood Benefits vs. Risks. *Journal of Trace Elements in Medicine and Biology, 55 (May),* 50–57. https://doi.org/10.1016/j.jtemb.2019.05.009.

Ramalhosa, E., Segade, S. R., Pereira, E., Vale, C., & Duarte, A. (2001). Microwave-assisted extraction for methylmercury determination in sediments by high performance liquid chromatography-cold vapour-atomic fluorescence spectrometry. *Journal of Analytical Atomic Spectrometry, 16(6)*, 643–647. https://doi.org/10.1039/b010229f

Ramos, P., Santos, A., Pinto, N. R., Mendes, R., Magalhães, T., & Almeida, A. (2015). Anatomical Regional Differences in Selenium Levels in the Human Brain. *Biological Trace Element Research, 163(1–2)*, 89–96. https://doi.org/10.1007/s12011-014-0160-z

Ramos, P., Santos, A., Pinto, E., Pinto, N. R., Mendes, R., Magalhães, T., & Almeida, A. (2016). Alkali metals levels in the human brain tissue: Anatomical region differences and age-related changes. *Journal of Trace Elements in Medicine and Biology, 38*, 174–182. https://doi.org/10.1016/j.jtemb.2016.03.018

Rayman, M. (2000). The importance of Selenium on human health. *Lancet, 356(7)*, 233–241.

Rayman, M. P. (2005). Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society, 64(4)*, 527–542. https://doi.org/10.1079/pns2005467

Rayman, M. P., Infante, H. G., & Sargent, M. (2008). Food-chain selenium and human health: Spotlight on speciation. *British Journal of Nutrition, 100(2)*, 238–253. https://doi.org/10.1017/S0007114508922522

Rayman, M. P. (2012). Selenium and human health. *The Lancet, 379(9822)*, 1256–1268. https://doi.org/10.1016/S0140-6736(11)61452-9

Rembach, A., Hare, D. J., Lind, M., Fowler, C. J., Cherny, R. A., McLean, C., Bush, A. I., Masters, C. L., & Roberts, B. R. (2013). Decreased copper in alzheimer's disease brain is predominantly in the soluble extractable fraction.

International Journal of Alzheimer's Disease, 2013. https://doi.org/10.1155/2013/623241

Rider, S. A., Davies, S. J., Jha, A. N., Clough, R., & Sweetman, J. W. (2010). Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (Oncorhynchus mykiss) diet. *Journal of Animal Physiology and Animal Nutrition, 94(1)*, 99–110. https://doi.org/10.1111/j.1439-0396.2008.00888.x

Rodrigues, J. L., de Souza, S. S., de Oliveira Souza, V. C., & Barbosa, F. (2010). Methylmercury and inorganic mercury determination in blood by using liquid chromatography with inductively coupled plasma mass spectrometry and a fast sample preparation procedure. *Talanta, 80(3)*, 1158–1163. https://doi.org/10.1016/j.talanta.2009.09.001

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., & Hoekstra, W. G. (1973). Selenium: Biochemical Role as a Component of Glutathione Peroxidase. *Science, 179*, 588–590.

Ruiz Chaves, I. (2016). Metodologías analíticas utilizadas actualmente para la determinación de mercurio en músculo de pescado. *Pensamiento Actual, 16(26)*, 113. https://doi.org/10.15517/pa.v16i26.25187

Ruiz-De-Cenzano, M., López-Salazar, O., Cervera, M. L., & De La Guardia, M. (2016). Non-chromatographic speciation of mercury in mushrooms. *Analytical Methods, 8(8)*, 1774–1779. https://doi.org/10.1039/c5ay03227j

Ruus, A., Øverjordet, I. B., Braaten, H. F. V., Evenset, A., Christensen, G., Heimstad, E. S., Gabrielsen, G. W., & Borgå, K. (2015). Methylmercury biomagnification in an Arctic pelagic food web. *Environmental Toxicology and Chemistry, 34(11),* 2636–2643. https://doi.org/10.1002/etc.3143

Sakamoto, M., Yasutake, A., Kakita, A., Ryufuku, M., Chan, H. M., Yamamoto, M., Oumi, S., Kobayashi, S., & Watanabe, C. (2013). Selenomethionine protects against neuronal degeneration by methylmercury in the developing rat cerebrum. *Environmental Science and Technology, 47(6)*, 2862–2868. https://doi.org/10.1021/es304226h

Sala, G., Marinig, D., Riva, C., Arosio, A., Stefanoni, G., Brighina, L., Formenti, M., Alberghina, L., Colangelo, A. M., & Ferrarese, C. (2016). Rotenone down-regulates HSPA8/hsc70 chaperone protein in vitro: A new possible toxic mechanism contributing to Parkinson's disease. *NeuroToxicology, 54*, 161–169. https://doi.org/10.1016/j.neuro.2016.04.018

Sappey, C., Legrand-Poels, S., Best-Belpomme, M., Favier, A., Rentier, B., & Piette, J. (1994). Stimulation of Glutathione Peroxidase Activity Decreases HIV Type 1 Activation after Oxidative Stress. *AIDS Research and Human Retroviruses, 10(11)*, 1451–1461. https://doi.org/10.1089/aid.1994.10.1451

Sarkanen, J. R., Nykky, J., Siikanen, J., Selinummi, J., Ylikomi, T., & Jalonen, T. O. (2007). Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. *Journal of Neurochemistry, 102(6)*, 1941–1952. https://doi.org/10.1111/j.1471-4159.2007.04676.x

Sathiya Kamatchi, T., Mohamed Subarkhan, M. K., Ramesh, R., Wang, H., & Małecki, J. G. (2020). Investigation into antiproliferative activity and apoptosis mechanism of new arene Ru(ii) carbazole-based hydrazone complexes. *Dalton Transactions, 49(32)*, 11385–11395. https://doi.org/10.1039/d0dt01476a

Scharpf, M., Schweizer, U., Arzberger, T., Roggendorf, W., Schomburg, L., & Köhrle, J. (2007). Neuronal and ependymal expression of selenoprotein P in the human brain. *Journal of Neural Transmission, 114(7)*, 877–884. https://doi.org/10.1007/s00702-006-0617-0

Schmidt, B. Z., Lehmann, M., Gutbier, S., Nembo, E., Noel, S., Smirnova, L., Forsby, A., Hescheler, J., Avci, H. X., Hartung, T., Leist, M., Kobolák, J., & Dinnyés, A. (2017). In vitro acute and developmental neurotoxicity screening: an overview of cellular platforms and high-throughput technical possibilities. *Archives of Toxicology, 91(1)*, 1–33. https://doi.org/10.1007/s00204-016-1805-9

Schomburg, L., & Köhrle, J. (2008). On the importance of selenium and iodine metabolism for thyroid hormone biosynthesis and human health. *Molecular Nutrition and Food Research, 52(11)*, 1235–1246. https://doi.org/10.1002/mnfr.200700465

Schram, E., Pedrero, Z., Cámara, C., Van Der Heul, J. W., & Luten, J. B. (2008). Enrichment of African catfish with functional selenium originating from garlic. *Aquaculture Research, 39(8)*, 850–860. https://doi.org/10.1111/j.1365-2109.2008.01938.x

Schwarz, K., & Foltz, C. M. (1957). Selenium as an Integral Part of Factor 3 Against Dietary Necrotic Liver Degeneration. *Journal of the American Chemical Society, 79(12)*, 3292–3293. https://doi.org/10.1021/ja01569a087

Scientific Committee on Food. (2000). Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Selenium. http://ec.europa.eu/food/fs/sc/scf/out80g_en.pdf

Seko, T., Uchida, H., Yamashita, Y., & Yamashita, M. (2021). Novel method for separating selenoneine reduced monomer and ergothioneine from fission yeast extracts. *Separation and Purification Technology, 254,* 117607. https://doi.org/10.1016/j.seppur.2020.117607.

Seko, T., Uchida, H., Sato, Y., Imamura, S., Ishihara, K., Yamashita, Y., & Yamashita, M. (2023). Selenoneine is methylated in the bodies of mice and then excreted in urine as Se-methylselenoneine. *Biological Trace Element Research*, No. 0123456789. https://doi.org/10.1007/s12011-023-03936-1.

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H. G., & Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *Journal of Trace Elements in Medicine and Biology, 47(May 2017)*, 124–133. https://doi.org/10.1016/j.jtemb.2018.02.005

Selin, N. E. (2009). Global biogeochemical cycling of mercury: A review. *Annual Review of Environment and Resources, 34*, 43–63. https://doi.org/10.1146/annurev.environ.051308.084314

Semple, K. T., Doick, K. J., Jones, K. C., Burauel, P., Craven, A., & Harms, H. (2004). Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environmental Science and Technology, 38(12)*. https://doi.org/10.1021/es040548w

Seppänen, K., Laatikainen, R., Salonen, J. T., Kantola, M., Lötjönen, S., Harri, M., Nurminen, L., Kaikkonen, J., & Nyyssönen, K. (1998). Mercury-binding capacity of organic and inorganic selenium in rat blood and liver. *Biological Trace Element Research, 65(3)*, 197–210. https://doi.org/10.1007/BF02789096

Sheikh, T. J., Patel, B. J., & Joshi, D. V. (2011). Effect of mercuric chloride on oxidative stress and target organ pathology in wistar rat. *Journal of Applied Pharmaceutical Science, 1(7)*, 59–61.

Shengyu, C., Yinhua, L., Yuanhong, L., Jinbo, Z., Can, F., Hao, X., & Changjiang, Z. (2022). Selenium alleviates heart remodeling through Sirt1/AKT/GSK-3β pathway. *International Immunopharmacology, 111(August)*, 109158. https://doi.org/10.1016/j.intimp.2022.109158

Singh, A. D., Khanna, K., Kour, J., Dhiman, S., Bhardwaj, T., Devi, K., Sharma, N., Kumar, P., Kapoor, N., Sharma, P., Arora, P., Sharma, A., & Bhardwaj, R. (2023). Critical review on biogeochemical dynamics of mercury (Hg) and its abatement strategies. *Chemosphere, 319(January)*. https://doi.org/10.1016/j.chemosphere.2023.137917

Siwek, M., Noubar, A. B., Bergmann, J., Niemeyer, B., & Galunsky, B. (2006). Enhancement of enzymatic digestion of Antarctic krill and successive extraction of selenium organic compounds by ultrasound treatment. *Analytical and Bioanalytical Chemistry, 384(1)*, 244–249. https://doi.org/10.1007/s00216-005-0163-x

Slanzi, A., Iannoto, G., Rossi, B., Zenaro, E., & Constantin, G. (2020). In vitro Models of Neurodegenerative Diseases. *Frontiers in Cell and Developmental Biology, 8(May)*. https://doi.org/10.3389/fcell.2020.00328

Sloth, J. J., & Larsen, E. H. (2000). Application of inductively coupled plasma dynamic reaction cell mass spectrometry for measurement of selenium isotopes, isotope ratios and chromatographic detection of selenoamino acids. *Journal of Analytical Atomic Spectrometry, 15(6)*, 669–672. https://doi.org/10.1039/b001798l

Soriano-Garcia, M. (2012). Organoselenium Compounds as Potential Therapeutic and Chemopreventive Agents: A Review. *Current Medicinal Chemistry, 11(12)*, 1657–1669. https://doi.org/10.2174/0929867043365053

Spallholz, J. E., Boylan, L. M., & Larsen, H. S. (1990). Advances in understanding selenium's role in the immune system. *Annals of the New York Academy of Sciences, 587*, 123–139. https://doi.org/10.1111/j.1749-6632.1990.tb00140.x

Spiller, H. A. (2018). Rethinking mercury: the role of selenium in the pathophysiology of mercury toxicity. *Clinical Toxicology, 56(5)*, 313–326. https://doi.org/10.1080/15563650.2017.1400555

Stockert, J. C., Horobin, R. W., Colombo, L. L., & Blázquez-Castro, A. (2018). Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta Histochemica, 120(3)*, 159–167. https://doi.org/10.1016/j.acthis.2018.02.005

Suárez-Oubiña, C., Herbello-Hermelo, P., Mallo, N., Vázquez, M., Cabaleiro, S., Pinheiro, I., Rodríguez-Lorenzo, L., Espiña, B., Bermejo-Barrera, P., & Moreda-Piñeiro, A. (2023). Single-cell ICP-MS for studying the association of inorganic nanoparticles with cell lines derived from aquaculture species. *Analytical and Bioanalytical Chemistry, 0123456789*. https://doi.org/10.1007/s00216-023-04723-6

Sundström, H., Korpela, H., Viinikka, L., & Kauppila, A. (1984). Serum selenium and glutathione peroxidase, and plasma lipid peroxides in uterine, ovarian or vulvar cancer, and their responses to antioxidants in patients with ovarian cancer. *Cancer Letters, 24(1)*, 1–10. https://doi.org/10.1016/0304-3835(84)90073-9

Suzuki, K. T. (2005). Metabolomics of selenium: Se metabolites based on speciation studies. *Journal of Health Science, 51(2)*, 107–114. https://doi.org/10.1248/jhs.51.107

Syversen, T., & Kaur, P. (2012). The toxicology of mercury and its compounds. *Journal of Trace Elements in Medicine and Biology, 26(4)*, 215–226. https://doi.org/10.1016/j.jtemb.2012.02.004

Szpunar, J. (2005). Advances in analytical methodology for bioinorganic speciation analysis: Metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics. *Analyst, 130(4)*, 442–465. https://doi.org/10.1039/b418265k

Tanaka, Y. ki, Iida, R., Takada, S., Kubota, T., Yamanaka, M., Sugiyama, N., Abdelnour, Y., & Ogra, Y. (2020). Quantitative elemental analysis of a single cell by using inductively coupled plasma-mass spectrometry in fast time-resolved analysis mode. *ChemBioChem, 21 (22)*, 3266–3272. https://doi.org/10.1002/cbic.202000358.

Tapiero, H., Townsend, D. M., & Tew, K. D. (2003). The antioxidant role of selenium and seleno-compounds. *Biomedicine and Pharmacotherapy, 57(3*), 134–144. https://doi.org/10.1016/S0753-3322(03)00035-0

Taylor, E. W., Nadimpalli, R. G., & Ramanathan, C. S. (1997). Genomic structures of viral agents in relation to the biosynthesis of selenoproteins. *Biological Trace Element Research, 56(1)*, 63–91. https://doi.org/10.1007/BF02778984

Thibaud, Y., & Cossa, D. (1989). An international intercalibration for methylmercury in biological tissue. *Applied Organometallic Chemistry, 3(3)*, 257–266. https://doi.org/10.1002/aoc.590030309

Tian, M., Hui, M., Thannhauser, T. W., Pan, S., & Li, L. (2017). Selenium-induced toxicity is counteracted by sulfur in broccoli (*Brassica oleracea L. var. italica*). *Frontiers in Plant Science, 8(August)*, 1–13. https://doi.org/10.3389/fpls.2017.01425

Tinggi, U. (2003). Essentiality and toxicity of selenium and its status in Australia: A review. *Toxicology Letters, 137(1–2),* 103–110. https://doi.org/10.1016/S0378-4274(02)00384-3

Tirtom, V. N., Goulding, Ş., & Henden, E. (2008). Application of a wool column for flow injection online preconcentration of inorganic mercury(II) and methyl mercury species prior to atomic fluorescence measurement. *Talanta, 76(5)*, 1212–1217. https://doi.org/10.1016/j.talanta.2008.05.038

Torres, D. P., Borges, D. L. G., Frescura, V. L. A., & Curtius, A. J. (2009). A simple and fast approach for the determination of inorganic and total mercury in aqueous slurries of biological samples using cold vapor atomic absorption spectrometry and in situ oxidation. *Journal of Analytical Atomic Spectrometry, 24(8)*, 1118–1122. https://doi.org/10.1039/b816622f

Torres-Escribano, S., Ruiz, A., Barrios, L., Vélez, D. & Montoro, R. (2011). Influence of mercury bioaccessibility on exposure assessment associated with consumption of cooked predatory fish in Spain. *Journal of the Science of Food and Agriculture, 91(6)*, 981–986. https://doi.org/10.1002/jsfa.4241.

Torres-Escribano, S., Vélez, D. & Montoro, R. (2010). Mercury and methylmercury bioaccessibility in swordfish. *Food Additives and Contaminants - Part A, 27 (3)*, 327–337. https://doi.org/10.1080/19440040903365272.

Toulis, K. A., Anastasilakis, A. D., Tzellos, T. G., Goulis, D. G., & Kouvelas, D. (2010). Selenium supplementation in the treatment of Hashimoto's thyroiditis: A systematic review and a meta-analysis. *Thyroid, 20(10)*, 1163–1173. https://doi.org/10.1089/thy.2009.0351

Townsend, D. M., Tew, K. D., & Tapiero, H. (2003). The importance of glutathione in human disease. *Biomedicine and Pharmacotherapy, 57(3)*, 145–155. https://doi.org/10.1016/S0753-3322(03)00043-X

Tu, Q., Qian, J., & Frech, W. (2000). Rapid determination of methylmercury in biological materials by GC-MIP-AES or GC-ICP-MS following simultaneous ultrasonicassisted in situ ethylation and solvent extraction. *Journal of*

Analytical Atomic Spectrometry, 15(12), 1583–1588. https://doi.org/10.1039/b006987f

Turiel-Fernández, D., Gutiérrez-Romero, L., Corte-Rodriguez, M., Bettmer, J., & Montes-Bayón, M. (2021). Ultrasmall iron oxide nanoparticles cisplatin (IV) prodrug nanoconjugate: ICP-MS based strategies to evaluate the formation and drug delivery capabilities in single cells. *Analytica Chimica Acta, 1159*. https://doi.org/10.1016/j.aca.2021.338356

Uden, P. C., Boakye, H. T., Kahakachchi, C., & Tyson, J. F. (2004). Selective detection and identification of Se containing compounds - Review and recent developments. *Journal of Chromatography A, 1050(1)*, 85–93. https://doi.org/10.1016/j.chroma.2004.05.077

Ulusoy, Ş., Mol, S., Karakulak, F. S., & Kahraman, A. E. (2019). Selenium-Mercury Balance in Commercial Fish Species from the Turkish Waters. *Biological Trace Element Research, 191(1)*, 207–213. https://doi.org/10.1007/s12011-018-1609-2

Van Tonder, A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Research Notes, 8(1)*. https://doi.org/10.1186/s13104-015-1000-8

Vanderpas, J. B., Contempre, B., Duale, N. L., Goossens, W., Bebe, N., Thorpe, R., Ntambue, K., Dumont, J., Thilly, C. H., & Diplock, A. T. (1990). Iodine and selenium deficiency associated with cretinism in northern Zaire1-3. *American Journal of Clinical Nutrition, 52(6)*, 1087–1093. https://doi.org/10.1093/ajcn/52.6.1087

Vicente-Zurdo, D., Gómez-Gómez, B., Pérez-Corona, M. T., & Madrid, Y. (2019). Impact of fish growing conditions and cooking methods on selenium species in swordfish and salmon fillets. *Journal of Food Composition and Analysis, 83(August)*, 103275. https://doi.org/10.1016/j.jfca.2019.103275

Vicente-Zurdo, D., Gómez-Gómez, B., Romero-Sánchez, I., Rosales-Conrado, N., León-González, M. E., & Madrid, Y. (2023). Cytotoxicity, uptake and accumulation of selenium nanoparticles and other selenium species in neuroblastoma cell lines related to Alzheimer's disease by using cytotoxicity assays, TEM and single cell-ICP-MS. *Analytica Chimica Acta, 1249(February)*. https://doi.org/10.1016/j.aca.2023.340949

Vinceti, M., Vicentini, M., Wise, L. A., Sacchettini, C., Malagoli, C., Ballotari, P., Filippini, T., Malavolti, M., & Rossi, P. G. (2018). Cancer incidence following long-term consumption of drinking water with high inorganic selenium content. *Science of the Total Environment, 635*, 390–396. https://doi.org/10.1016/j.scitotenv.2018.04.097

Virtamo, J., Valkeila, E., Alfthan, G., Punsar, S., Huttunen, J. K., & Karvonen, M. J. (1985). Serum selenium and the risk of coronary heart disease and stroke. *American Journal of Epidemiology, 122(2)*, 276–282. https://doi.org/10.1093/oxfordjournals.aje.a114099

Wallschläger, D., & London, J. (2004). Determination of inorganic selenium species in rain and sea waters by anion exchange chromatography-hydride generation-inductively-coupled plasma-dynamic reaction cell-mass spectrometry (AEC-HG-ICP-DRC-MS). *Journal of Analytical Atomic Spectrometry, 19(9)*, 1119–1127. https://doi.org/10.1039/b401616e

Wang, L., Sagada, G., Wang, R., Li, P., Xu, B., Zhang, C., Qiao, J., & Yan, Y. (2022). Different forms of selenium supplementation in fish feed: The bioavailability, nutritional functions, and potential toxicity. *Aquaculture, 549(December 2021)*, 737819. https://doi.org/10.1016/j.aquaculture.2021.737819

Westöö, G. (1967). Determination of methylmercury compounds in foodstuffs. *Acta Chemica Scandinavica, 21(1967)*, 1790–1800.

Wilber C.G. (1980). Toxicology of selenium: a review. *Clinical Toxicology, 17(2)*, 171-230

Wirth, E. K., Conrad, M., Winterer, J., Wozny, C., Carlson, B. A., Roth, S., Schmitz, D., Bornkamm, G. W., Coppola, V., Tessarollo, L., Schombure, L., Köhrle, J., Hateld, D. L., & Schweizer, U. (2010). Neuronal selenoprotein expression is required for interneuron development and prevents seizures and neurodegeneration. *The FASEB Journal, 24(3)*, 844–852. https://doi.org/10.1096/fj.09-143974

Wischhusen, P., Parailloux, M., Geraert, P. A., Briens, M., Bueno, M., Mounicou, S., Bouyssiere, B., Antony Jesu Prabhu, P., Kaushik, S. J., Fauconneau, B., & Fontagné-Dicharry, S. (2019). Effect of dietary Se in rainbow trout (Oncorhynchus mykiss) broodstock on antioxidant status, its parental transfer and oxidative status in the progeny. *Aquaculture, 507(December 2018)*, 126–138. https://doi.org/10.1016/j.aquaculture.2019.04.006

Wojcieszek, J., & Ruzik, L. (2016). Operationally defined species characterization and bioaccessibility evaluation of cobalt, copper and selenium in Cape gooseberry (Physalis Peruviana L.) by SEC-ICP MS. *Journal of Trace Elements in Medicine and Biology, 34,* 15–21. https://doi.org/10.1016/j.jtemb.2015.12.001

WHO (World Health Organization). (2003). Selenium in drinking-water: background document for development of WHO guidelines for drinking-water quality. WHO/SDE/WSH/03.04/13. https://cdn.who.int/media/docs/default-source/wash-documents/wash-chemicals/selenium.pdf?sfvrsn=d3b3dcc7_6

WHO (World Health Organization). (2005). Mercury in health care. http://www.who.int/water_sanitation_health/medicalwaste/mercurypolpap230506.pdf

WHO (World Health Organization). (2008). Guidance for identifying populations at risk from mercury exposure. https://www.who.int/publications/m/item/guidance-for-identifying-populations-at-risk-from-mercury-exposure

Xicoy, H., Wieringa, B., & Martens, G. J. M. (2017). The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration, 12(1)*, 1–11. https://doi.org/10.1186/s13024-017-0149-0

Xie, J., Wettschurack, K., & Yuan, C. (2020). Review: In vitro Cell Platform for Understanding Developmental Toxicity. *Frontiers in Genetics, 11(December)*, 1–8. https://doi.org/10.3389/fgene.2020.623117

Xie, M., Sun, X., Li, P., Shen, X., & Fang, Y. (2021). Selenium in cereals: Insight into species of the element from total amount. *Comprehensive Reviews in Food Science and Food Safety, 20(3)*, 2914–2940. https://doi.org/10.1111/1541-4337.12748

Xiong, Y., Huang, Y., Li, L., Liu, Y., Liu, L., Wang, L., Tong, L., Wang, F., & Fan, B. (2023). A Review of Plant Selenium-Enriched Proteins/Peptides: Extraction, Detection, Bioavailability, and Effects of Processing. *Molecules, 28(3)*. https://doi.org/10.3390/molecules28031223

Xu, Q., Zhao, L., Wang, Y., Xie, Q., Yin, D., Feng, X., & Wang, D. (2018). Bioaccumulation characteristics of mercury in fish in the Three Gorges Reservoir, China. *Environmental Pollution, 243*, 115–126. https://doi.org/10.1016/j.envpol.2018.08.048

Yamashita, Y., Yabu, T., & Yamashita, M. (2010). Discovery of the strong antioxidant selenoneine in tuna and selenium redox metabolism. *World Journal of Biological Chemistry, 1(5)*, 144. https://doi.org/10.4331/wjbc.v1.i5.144

Yamashita, Y., & Yamashita, M. (2010). Identification of a novel selenium-containing compound, selenoneine, as the predominant chemical form of organic selenium in the blood of bluefin tuna. *Journal of Biological Chemistry, 285(24)*, 18134–18138. https://doi.org/10.1074/jbc.C110.106377

Yamashita, Y., Amlund, H., Suzuki, T., Hara, T., Hossain, M. A., Yabu, T., Touhata, K., & Yamashita, M. (2011). Selenoneine, total selenium, and total mercury content in the muscle of fishes. *Fisheries Science, 77(4)*, 679–686. https://doi.org/10.1007/s12562-011-0360-9

Yamashita, M., Yamashita, Y., Suzuki, T., Kani, Y., Mizusawa, N., Imamura, S., Takemoto, K., Hara, T., Hossain, M. A., Yabu, T., & Touhata, K. (2013). Selenoneine, a Novel Selenium-Containing Compound, Mediates Detoxification Mechanisms against Methylmercury Accumulation and

Toxicity in Zebrafish Embryo. *Marine Biotechnology, 15(5)*, 559–570. https://doi.org/10.1007/s10126-013-9508-1

Yang, L., Sturgeon, R. E., Wolf, W. R., Goldschmidt, R. J., & Mester, Z. (2004). Determination of selenomethionine in yeast using CNBr derivatization and species specific isotope dilution GC ICP-MS and GC-MS. *Journal of Analytical Atomic Spectrometry, 19(11)*, 1448–1453. https://doi.org/10.1039/b410543e

Yoo, M. H., Gu, X., Xu, X. M., Kim, J. Y., Carlson, B. A., Patterson, A. D., Cai, H., Gladyshev, V. N., & Hatfield, D. L. (2010). Delineating the role of glutathione peroxidase 4 in protecting cells against lipid hydroperoxide damage and in alzheimer’s disease. *Antioxidants and Redox Signaling, 12(7)*, 819–827. https://doi.org/10.1089/ars.2009.2891

Yoo, S. E., Chen, L., Na, R., Liu, Y., Rios, C., Van Remmen, H., Richardson, A., & Ran, Q. (2012). Gpx4 ablation in adult mice results in a lethal phenotype accompanied by neuronal loss in brain. *Free Radical Biology and Medicine, 52(9)*, 1820–1827. https://doi.org/10.1016/j.freeradbiomed.2012.02.043

Yoshinaga, J., Suzuki, T., Hongo, T., Minagawa, M., Ohtsuka, R., Kawabe, T., Inaoka, T., & Akimichi, T. (1992). Mercury concentration correlates with the nitrogen stable isotope ratio in the animal food of papuans. *Ecotoxicology and Environmental Safety, 24(1)*, 37–45. https://doi.org/10.1016/0147-6513(92)90033-Y

Yu, S. Y., Zhu, Y. J., & Li, W. G. (1997). Protective role of Selenium against hepatitis B virus and primary liver cancer in Qidong. *Biological Trace Element Research, 56(1)*, 117–124. https://doi.org/10.1007/bf02778987

Yu, M. W., Horng, I. S., Hsu, K. H., Chiang, Y. C., Liaw, Y. F., & Chen, C. J. (1999). Plasma selenium levels and risk of hepatocellular carcinoma among men with chronic hepatitis virus infection. *American Journal of Epidemiology, 150(4)*, 367–374. https://doi.org/10.1093/oxfordjournals.aje.a010016

Yuan, W., Yang, N., & Li, X. (2016). Advances in Understanding How Heavy Metal Pollution Triggers Gastric Cancer., *BioMed Research International, 2016*. https://doi.org/10.1155/2016/7825432

Zhang, W. B., Xue, J. J., Yang, X. A., & Wang, S. B. (2011). Determination of inorganic and total mercury in seafood samples by a new ultrasound-assisted extraction system and cold vapor atomic fluorescence spectrometry. *Journal of Analytical Atomic Spectrometry, 26(10)*, 2023–2029. https://doi.org/10.1039/c1ja10145e

Zhang, H., Guo, C., Feng, H., Shen, Y., Wang, Y., Zeng, T., & Song, S. (2020). Total mercury, methylmercury, and selenium in aquatic products from coastal cities of China: Distribution characteristics and risk assessment. *Science of the Total Environment, 739*, 140034. https://doi.org/10.1016/j.scitotenv.2020.140034

Zhang, S., & Zhou, M. (2020). Comparison of DMA-80 and ICP-MS Combined with Closed-Vessel Microwave Digestion for the Determination of Mercury in Coal. *Journal of Analytical Methods in Chemistry, 2020*. https://doi.org/10.1155/2020/8867653

Zhang, X., Wang, Q., Zhang, J., Song, M., Shao, B., Han, Y., Yang, X., & Li, Y. (2022). The Protective Effect of Selenium on T-2-Induced Nephrotoxicity Is Related to the Inhibition of ROS-Mediated Apoptosis in Mice Kidney. *Biological Trace Element Research, 200(1)*, 206–216. https://doi.org/10.1007/s12011-021-02614-4

Zhang, F., Li, X., & Wei, Y. (2023). Selenium and Selenoproteins in Health. *Biomolecules, 13(5)*, 1–25. https://doi.org/10.3390/biom13050799

Zhu, S., Chen, B., He, M., Huang, T., & Hu, B. (2017). Speciation of mercury in water and fish samples by HPLC-ICP-MS after magnetic solid phase extraction. *Talanta, 171(April)*, 213–219. https://doi.org/10.1016/j.talanta.2017.04.068

Zwolak, I. (2020). The Role of Selenium in Arsenic and Cadmium Toxicity: an Updated Review of Scientific Literature. *Biological Trace Element Research, 193(1)*, 44–63. https://doi.org/10.1007/s12011-019-01691-w

XI. GLOSARIO DE TÉRMINOS

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AAS	Espectroscopia de absorción atómica (*Atomic Absorption Spectrometry*)
ADN	Ácido desoxirribonucleico
AFS	Espectroscopia de fluorescencia atómica (*Atomic Fluorescence Spectroscopy*)
Caco-2	Células de adenocarcinoma colorrectal humano
CE	Electroforesis capilar (*Capillary Electrophoresis*)
Ch-SeNPs	Nanopartículas de selenio estabilizadas con quitosano
Citrato-SeNPs	Nanopartículas de selenio recubiertas con quitosano
COD	Carbono orgánico disuelto
CV	Vapor frío (*Cold Vapour*)
Cys	Cisteína
Dio	Yodotironina deiodinasa
DMA	Analizador directo de mercurio (*Direct Mercury Analizer*)
DPPH	2,2-difenil-1-picrilhidracilo
ECD	Detector de captura electrónica (*Electron Capture Detector*)
EFSA	Agencia Europea de Seguridad Alimentaria (*European Food Safety Authority*)
EPA	Agencia de Protección Ambiental de Estados Unidos (*Environmental Protection Agency*)
ESC	Células madre embrionarias humanas (*Embryonic Stem Cells*)
ESI	Ionización por electrospray (*Electrospray Ionization*)
FDA	Administración de Alimentos y Medicamentos *(Food and Drug Administration)*
FID	Detector de ionización en llama (*Flame Ionization Detector*)
GC	Cromatografía de gases (*Gas Chromatography*)
GF	Cámara de grafito (*Graphite Furnace*)
GPx	Glutatión peroxidasa
HBV_{Se}	*Selenium Health Benefit Value*
HECIS	Sistema de Introducción Celular de Alta Eficacia (*High Efficiency Cell Introduction System*)

HEK293	Células de riñón embrionario humano inmortalizado
HeLa	Células de carcinoma de ovario humano inmortalizado
HepaRG	Células hepáticas humanas
HepG2	Células de cáncer hepático humano
HG	Generación de hidruros (*Hydride Generation*)
HgSe	Tiemannita
HPLC	Cromatografía de líquidos de alta eficacia (*High Performance Liquid Chromatography*)
HS	Espacio de cabeza (Head-Space)
ICP-MS	Espectrometría de masas con plasma de acoplamiento inductivo (*Inductively Coupled Plasma Mass Spectrometry*)
ICP-OES	Espectroscopia de emisión atómica con plasma de acoplamiento inductivo (*Inductively Coupled Plasma Optical Emission Spectroscopy*)
IE	Intercambio iónico (*Ion Exchange*)
iPSCs	Células madre pluripotentes inducidas (*induced Pluripotent Stem Cells)*
LA	Ablación láser (*Laser Ablation*)
LC_{50}	Concentración letal media (*Median lethal concentration*)
MAE	Extracción asistida por microondas (*Microwave-Assisted Extraction*)
MALDI	Desorción/ionización láser asistida por matriz (*Matrix-Assisted Laser Desorption/Ionization*)
Met	Metionina
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
MSPE	Extracción en fase sólida magnética (*Magnetic Solid-Phase Extraction*)
MsrB	Selenoproteína R
MT	Metalotioneína
MTT	Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol
N27	Línea celular inmortalizada derivada de neuronas mesencefálicas fetales de rata TH-positivas
Neuro-2a	Células de neuroblastoma de ratón
NOAEL	Nivel sin efecto adverso observado (*Non-Observed Adverse Effects Level*)

NP	Fase normal (*Normal Phase*)
OCTN1	Transportador de ergotioneína
PC12	Células de feocromocitoma de rata
PDMS	Polidimetilsiloxano
PLE	Extracción con líquidos presurizados (*Pressurised Liquid Extraction*)
ROS	Especies reactivas de oxígeno (*Reactive Oxygen Species*)
RP	Fase inversa (*Reverse Phase*)
SAM	S-adenosilmetionina
SC-ICP-MS	Espectrometría de masas con plasma de acoplamiento inductivo en modo de detección de células individuales (*Single-Cell Inductively Coupled Plasma Mass Spectrometry)*
SEC	Cromatografía de exclusión molecular (*Size-Exclusion Chromatography*)
SeI15	Selenoproteína de 15 kDa
SeI18	Selenoproteína de 18 kDa
SeIM	Selenoproteína M
SeIN	Selenoproteína N
SeIP	Selenoproteína P
SeIR	Selenoproteína R
SeIT	Selenoproteína T
SeIW	Selenoproteína W
SH-SY5Y	Células de neuroblastoma humano
SPE	Extracción en fase sólida (*Solid-Phase Extraction*)
SP-ICP-MS	Espectrometría de masas con plasma de acoplamiento inductivo en modo de detección de partículas individuales (*Single-Particle Inductively Coupled Plasma Mass Spectrometry*)
SPME	Microextracción en fase sólida (*Solid-Phase Microextraction*)
SPS2	Selenofosfato sintetasa 2
tARN	Ácido ribonucléico de transferencia
TBARS	Sustancias reactivas del ácido tiobarbitúrico
TEM	Microscopia electrónica de transmisión (*Transmission Electron Microscopy*)
TOF	Analizador de tiempo de vuelo (*Time-of-Flight*)

TRx	Tiorredoxina reductasa
UAE	Extracción asistida por ultrasonidos (*Ultrasound-Assisted Extraction*)
UGA	Codón Uracilo-Guanina-Adenina
UL	Nivel más alto de ingesta tolerable (*Tolerable Upper Intake Level*)
UV	Radiación ultravioleta (*Ultraviolet radiation*)
Vis	Radiación visible (*Visible radiation*)
VIH	Virus de Inmunodeficiencia Humana
WHO	Organización Mundial de la Salud (*World Health Organization*)

XII. ANEXOS

XII. ANEXO: PRODUCCIÓN CIENTÍFICA

Artículos científicos incluidos en la Tesis Doctoral

- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes by SEC-UV-ICP-MS and HPLC-ESI-MS/MS. *Talanta*. **2022**, 237:122922.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. Selenium Health Benefit Values and Hg and Se speciation studies for elucidating the quality and safety of non-traditional food products derived from fish. *Food Chem*. **2024**, 435:137544.
- Fernández-Bautista, T.; Gómez-Gómez B.; Vicente-Zurdo, D.; Madrid, Y. Single-cell ICP-MS for evaluating the Se-protective effect against MeHg+-induced neurotoxicity in human neuroblastoma cell line (SH-SY5Y). *Anal. Bioanal. Chem*. **2024**, 416:2749-2759.
- Gómez-Gómez B.; Fernández-Bautista, T.; Madrid, Y. (Bio)analytical approaches for investigating the role of selenium in preventing neurological disorders and neurotoxicity induced by environmental neurotoxicants: As, Cd and Hg. *Trends Anal. Chem*. **2024**, 117661.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. Investigating the presence of selenoneine, ergothioneine and selenium-containing biomolecules in fish and fish-derived commercial products. *J. Agric. Food Chem*. Enviado (jf-2024-02610a).
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. Se and Hg in processed fish-derived products and their fish raw materials: occurrence, Se:Hg molar ratio, HBV_{Se} index, bioaccessibility and Caco-2 cells toxicity. *Food Chem.* Enviado (FOODCHEM-D-24-05527).

Otros artículos científicos

- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. Determinación de especies de selenio y mercurio en pescados de elevado consumo mediante HPLC-ICP-MS y HPLC-ESI-MS/MS. *Actualidad Analítica*. **2023**, 81:47-51.
- El Hanafi, K.[1]; Fernández-Bautista, T.[1]; Ouerdane, L.; Corns, W. T.; Bueno, M.; Fontagné, S.; Amouroux, D.; Pedrero-Zayas, Z. Revealing dietary mercury and selenium dynamics: biomolecular speciation in aquaculture Rainbow Trout. *Environ. Sci. Technol*. Enviado (es-2024-05365m).

  [1] El Hanafi, K. y Fernández-Bautista, T. tuvieron la misma contribución en el artículo.

Comunicaciones a congresos

- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Determinación de selenio y mercurio en las proteínas solubles de pescados de consumo habitual*. **V Jornada de Promoción de la Investigación Básica para Estudiantes de Ciencias e Ingenierías**, Móstoles (España), septiembre de **2021**. Tipo de comunicación: oral.
- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Analysis of Se and Hg biomolecules distribution and Se speciation in poorly studied protein fractions of muscle tissues of highly consumed fishes*. **RSEQ Symposium 2021**, online, septiembre de **2021**. Tipo de comunicación: póster.
- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Distribution de biomolécules avec Se and Hg et spéciation du Se dans différentes fractions protéiques extraites des poissons par HPLC-UV-ICP-MS et HPLC-ESI-MS/MS*. **9ème édition de Spectr'atom**, Pau (Francia), mayo de **2022**. Tipo de comunicación: póster.
- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Estudio de biomoléculas y especies con Se en fracciones proteicas (sarcoplasmáticas, miofibrilares y álcali-solubles) de pescados de consumo frecuente mediante HPLC-UV-ICP-MS et HPLC-ESI-MS/MS*. **Jornada de Especiación**

de la XXIII Reunión de la Sociedad Española de Química Analítica, Oviedo (España), julio de **2022**. Tipo de comunicación: oral.

- Fernández-Bautista, T.; Gómez-Gómez B.; Palacín-García, R.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Análisis de Se y Hg en fracciones proteicas extraídas del músculo de pescados de consumo habitual. Evaluación de su unión a biomoléculas e interacción*. **XXIII Reunión de la Sociedad Española de Química Analítica**, Oviedo (España), julio de **2022**. Tipo de comunicación: póster.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Determinación de especies de Se y Hg e identificación de partículas de HgSe en pescados y productos derivados mediante HPLC-ICP-MS y spICP-MS*. **VII Simposio Anual en Química Avanzada**, Madrid (España), julio de **2023**. Tipo de comunicación: oral.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Determination of Se and Hg species and HgSe particles found in fish and derived products by HPLC-ICP-MS and spICP-MS*. **European Winter Conference on Plasma Spectrochemistry**. Ljubljana (Eslovenia), enero de **2023**. Tipo de comunicación: oral.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Identification of selenoneine and ergothioneine in highly consumed fish and derived products*. **European Winter Conference on Plasma Spectrochemistry**. Ljubljana (Eslovenia), enero de **2023**. Tipo de comunicación: póster.
- Gómez-Gómez B.; Fernández-Bautista, T.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Surveillance des changements dans les contenus totaux en Se et Hg et leurs espèces au cours de l'élaboration des produits ultra-transformés dérivés du poisson*. **10ème édition de Spectr'atom**, Pau (Francia), junio de **2024**. Tipo de comunicación: póster.
- Fernández-Bautista, T.; Gómez-Gómez B.; Gracia-Lor, E.; Pérez-Corona, T.; Madrid, Y. *Determinación de Hg, Se y especiación de Se en productos procesados derivados de pescados y sus materias primas. Estudios de bioaccesibilidad y toxicidad mediante ensayos in vitro*. **Jornada de Especiación de la XXIV Reunión de la Sociedad**

Española de Química Analítica, Zaragoza (España), julio de **2024**. Tipo de comunicación: oral.

- Gómez-Gómez B.; Fernández-Bautista, T.; Merino-Sánchez, J..; Madrid, Y. *Single-cell ICP-MS como herramienta analítica en estudios de neurotoxicidad. Evaluación del efecto protector de distintas especies de selenio frente a la toxicidad del mercurio en células de neuroblastoma humano (SH-SY5Y)*. **XXIV Reunión de la Sociedad Española de Química Analítica**, Zaragoza (España), julio de **2024**. Tipo de comunicación: oral.

Estancias de investigación

Estancia en el Grupo de investigación *Chimie et Microbiologie de l'Environnement*, dirigido por el Dr. David Amouroux, del *Institut des Sciences Analytiques et de Physico-Chimie pour l'Environnement et les Matériaux* (**IPREM**) en Pau (Francia), del **15 de febrero al 15 de mayo de 2022** para el desarrollo de metodologías analíticas para la determinación de compuestos de selenio (selenoneína) en matrices de pescados.