UNIVERSIDAD MIGUEL HERNÁNDEZ DE ELCHE

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PROGRAMA DE DOCTORADO EN RECURSOS Y TECNOLOGÍAS AGRARIAS, AGROAMBIENTALES Y ALIMENTARIAS

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JESÚS ENRIQUE CHAN HIGUERA

TESIS DOCTORAL

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Jumbo Squid (*Dosidicus gigas*) Pigments: Chemical structure, antioxidant and antimicrobial activity and their application as food additive

Jesús Enrique Chan Higuera
Master in Food Science and Technology

Director: Ángel Antonio Carbonell Barrachina

Codirector: Josafat Marina Ezquerra Brauer

Thesis for the degree of Doctor from the Miguel Hernández University of Elche

Orihuela, 2020

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CALIDAD DEL COMPENDIO DE CADA PUBLICACIÓN

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New icing media for quality enhancement of chilled hake (*Merluccius merluccius*) using a jumbo squid (*Dosidicus gigas*) skin extract

Autores: Josafat Marina Ezquerra-Brauer, José M. Miranda, **Jesús Enrique Chan-Higuera**, Jorge Barros-Velázquez, Santiago Pedro Aubourg-Martínez.

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Editor: Andrew Waterhouse, Mark Shepherd

ISSN: 0022-5142

Ámbito de la publicación: Agriculture and Multidisciplinary

Categoría JCR	Categoría del Cuartil	Rango	Factor de Impacto	Factor de impacto últimos 5 años
Agriculture and Multidisciplinary	Q1	9/57	2.422	2.733

Jumbo Squid (*Dosidicus Gigas*) Skin Pigments: Chemical Analysis and Evaluation of Antimicrobial and Antimutagenic Potential

Autores: Jesús Enrique Chan-Higuera, Ángel Antonio Carbonell-Barrachina, José Luis Cárdenas-López, Miroslava Kačániová, Armando Burgos-Hernández, Josafat Marina Ezquerra-Brauer

Revista: *Journal of Microbiology, Biotechnology and Food Sciences*

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Editor: Lukáš Hleba

Ámbito de la publicación: Agriculture and Biological Science

Categoría SCOPUS	Categoría del Cuartil	Rango	Factor de Impacto	Factor de Impacto de los Últimos Cinco Años
Agriculture and Biological Science	Q4	218/257	0.33	0.33

Xanthommatin is Behind the Antioxidant Activity of the Skin of *Dosidicus gigas*.

Autores: Jesús Enrique Chan-Higuera, Hisila del Carmen Santacruz-Ortega, Ángel Antonio Carbonell-Barrachina, Armando Burgos-Hernández, Rosario Maribel Robles-Sánchez, Susana Gabriela Cruz-Ramírez, Josafat Marina Ezquerra-Brauer

Revista: *Molecules*

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Editor: Derek J. McPhee

ISSN: 1420-3049

Ámbito de la publicación: Chemistry and Multidisciplinary

Categoría JCR	Categoría del Cuartil	Rango	Factor de Impacto	Factor de impacto últimos 5 años
Chemistry and Multidisciplinary	Q2	136/299	3.060	3.380

Evaluation of *Dosidicus gigas* skin extract as an antioxidant and preservative in tuna paté

Autores: Jesús Enrique Chan-Higuera, Josafat Marina Ezquerra-Brauer, Leontina Lipan, Marina Cano-Lamadrid, Roberta Rizzitano, Ángel Antonio Carbonell-Barrachina

Revista: *Foods*

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ISSN: 2304-8158

Ámbito de la publicación: Food Science and Technology

Categoría JCR	Categoría del Cuartil	Rango	Factor de Impacto	Factor de impacto últimos 5 años
Food Science & Technology	Q2	36/135	3.011	

Prof. Dr. D. Ángel Antonio Carbonell Barrachina, Catedrático de Universidad y Coordinador del Programa de Doctorado Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada **"Pigmentos de Calamar Gigante (*Dosidicus gigas*): Estructura Química, Actividad Antioxidante y Antimicrobiana y su Aplicación como Aditivo Alimentario)"** del que es autor el Químico en Alimentos **D. Jesús Enrique Chan Higuera** ha sido realizada bajo la dirección del **Dr. Ángel Antonio Carbonell Barrachina,** profesor de la UMH y de la codirección de la **Dra. Josafat Marina Ezquerra Brauer**, profesora de la Universidad de Sonora (UNISON, México), actuando como tutor la Dra. Francisca Hernández García (UMH). Considero que la tesis es conforme en cuanto a forma y contenido a los requerimientos del Programa de Doctorado ReTos-AAA, por tanto, es apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a 22 de enero de dos mil veinte.

UNIVERSITAS Miguel Hernández

ANGEL ANTONIO| CARBONELL| BARRACHINA

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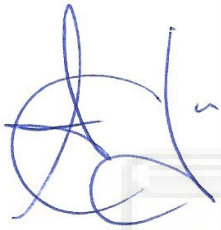
Programa Doctorado ReTos-AAA

Prof. Dr. Ángel A. Carbonell Barrachina

Esta memoria ha sido presentada por **D. Jesús Enrique Chan Higuera,** Químico en Alimentos y Máster en Ciencia y Tecnología de los Alimentos, para obtener el grado de doctor.

D. Jesús Enrique Chan Higuera

Esta Tesis Doctoral ha sido dirigida por el **Dr. Ángel Antonio Carbonell Barrachina,** Catedrático de Universidad de la Universidad Miguel Hernández, del Departamento Tecnología Agroalimentaria y codirigida por la **Dra. Josafat Marina Ezquerra Brauer,** catedrática de la Universidad de Sonora, del Departamento de Investigación y Posgrado en Alimentos.



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Dr. Ángel Antonio Carbonell Barrachina Dra. Josafat Marina Ezquerra Brauer

Orihuela, a 22 de enero de 2020

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A goro cachetes de goro. Sin tu apoyo, tu ayuda, tu paciencia y tu conocimiento de química, no hubiera podido interpretar los espectros de IR, ni de RMN. Woaini.

A mis padres; Óscar y María Teresa

A mis hermanos; Trina, Óscar y Ana

A mis sobrinos y sobrinas; Óscar, Alejandro, Ivana y Luisana

ÍNDICE

ABREVIACIONES Y SÍMBOLOS

Abreviaciones

ABTS	2,2'-azino-bis(3-etilbenzotiazolin-6-ácido sulfónico)
TLC	Cromatografía de capa fina
3-HXK	3-Hidroxiquinurenina
TBARS	Sustancias reactivas al ácido tiobarbitúrico
KYN	Quinurenina
Trp	Triptófano
HPLC-DAD diodos	Cromatografía líquida de alta eficiencia con detector de arreglo de
DHX	Dihidroxantomatina
XAc	Ácido xanturénico

PRODUCCIÓN CIENTÍFICA DURANTE EL PERIODO PREDOCTORAL

ARTÍCULOS PUBLICADOS EN REVISTAS CIENTÍFICAS

Ezquerra-Brauer, J. M., Miranda, J. M., **Chan-Higuera, J. E.**, Barros-Velázquez, J., Aubourg, S. P. 2017. New icing media for quality enhancement of chilled hake (*Merluccius merluccius*) using a jumbo squid (*Dosidicus gigas*) skin extract. *Journal of the Science of Food and Agriculture.* 97 (10): 3412-3419. https://doi.org/10.1002/jsfa.8192

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ESTRUCTURA DE LA TESIS

Para la elaboración de la presente Tesis Doctoral se ha seguido la metodología basada en la publicación de artículos de investigación. Con esta Tesis Doctoral se pretende obtener el título de Doctor, para ello en la redacción de la misma, se ha seguido la normativa vigente de la Universidad Miguel Hernández de Elche.

La Tesis Doctoral se estructura en las siguientes partes:

1. Introducción.
2. Objetivos.
3. Resumen de la Metodología.
4. Publicaciones Científicas.
5. Resumen de los Resultados, Discusión y Conclusiones.
6. Conclusiones Generales e Investigaciones Futuras.
7. Referencias Bibliográficas.

La **Introducció**n contiene una breve revisión bibliográfica sobre los omocromos, tanto su rol como pigmentos como su actividad biológica. Y por último esta primera parte también incluye una breve revisión de las aplicaciones de estos compuestos en la industria alimentaria. En la segunda parte se describen los **Objetivos** estimados en la presente tesis doctoral.

En la siguiente parte se detalla un **Resumen de la Metodología** utilizada, para la recopilación de los resultados y entender el diseño y la preparación de las muestras, además incluye los programas informáticos utilizados en los análisis estadísticos de los datos. A continuación, se recogen las publicaciones científicas publicadas que componen esta tesis doctoral.

- La **Primera Publicación** trata acerca de la aplicación de extractos de piel de calamar gigante en un sistema de enhielado de filetes de merluza, evaluando la calidad microbiológica, sensorial y de apreciación. Se utilizó un extracto obtenido con etanol y ácido acético. Este artículo se encuentra publicado en la revista *Journal of the Science of Food and Agriculture.*
- La **Segunda Publicación** contiene los resultados de la extracción de los pigmentos de la piel del calamar gigante. En ella se evaluaron las actividades

biológicas como agente antioxidante y antimicrobiano. Se utilizaron tres ensayos de actividad antioxidante y cepas de bacterias, levaduras y mohos. Este artículo está publicado en la revista *Journal of Microbiology, Biotechnology and Food Sciences*.

- La **Tercera Publicación** aborda parte de la caracterización química de los compuestos responsables de la actividad antioxidante observada en el extracto de piel de calamar gigante. Se utilizaron técnicas de caracterización estructural por métodos espectrofotométricos y espectrométricos. Este artículo se publicó en la revista *Molecules*.
- La **Cuarta Publicació**n aborda la aplicación de los extractos de piel de calamar gigante en la formulación de un producto alimentario de origen marino, para establecer su efecto sobre la calidad sensorial y microbiológica, así como la estabilidad oxidativa del alimento a lo largo de su vida de anaquel. Se emplearon ensayos físicos, químicos, microbiológicos y sensoriales para establecer el efecto de la adición del extracto. Este artículo se publicó en la revista *Foods.*

El **Resumen de los Resultados, Discusión y Conclusiones** muestra un resumen con los resultados más interesantes e importantes conseguidos en los estudios, una discusión general de los mismos y las conclusiones de cada publicación. A continuación, se recogen las **Conclusiones Generales** obtenidas de los estudios realizados en esta Tesis Doctoral. En la siguiente parte se presentan las Investigaciones Futuras. Y en la última parte se recogen las **Referencias Bibliográficas** consultadas para la elaboración de esta memoria, sin tener en cuenta el apartado de publicaciones científicas.

RESUMEN

Los pigmentos desempeñan un papel clave en el aposematismo, atracción, mimetismo, camuflaje y protección contra la radiación UV. Dentro de los pigmentos que desarrollan estas funciones están los omocromos. Los omocromos, al igual que otros pigmentos, producen color en el organismo que los sintetiza, así como ejercer diversas actividades biológicas. Los omocromos pueden actuar como antioxidantes y su mecanismo antioxidante generalmente sigue los siguientes mecanismos: pueden unir metales y ejercer actividad quelante, y pueden actuar como antioxidantes primarios eliminando radicales, como el oxígeno singlete y los aniones superóxido. Para explorar una nueva fuente de compuestos con múltiples potenciales, el objetivo de esta tesis doctoral fue documentar el potencial antioxidante y antimicrobiano de forma in vitro, así como de forma aplicada en la formulación de paté de atún.

ABSTRACT

Pigments play a key role in aposematism, attraction, mimicry, camouflage and protection against UV radiation. Among the pigments that develop these functions are the ommochromes. Ommochromes, like other pigments, produce color in the organism that synthesizes them, as well as exercising various biological activities. Ommochromes can act as antioxidants and their antioxidant mechanism generally follows the following mechanisms: they can bind metals and exert chelating activity, and they can act as primary antioxidants by eliminating radicals, such as singlet oxygen and superoxide anions. To explore a new source of compounds with multiple potentials, the objective of this doctoral thesis was to document the antioxidant and antimicrobial potential in vitro, as well as in the form of tuna pâté.

1. INTRODUCCIÓN.

1. INTRODUCCIÓN

La coloración en los animales cumple más que el propósito de ser un atractivo visual, Los pigmentos desempeñan un papel clave en las funciones biológicas, dentro de las cuales están el aposematismo, atracción, mimetismo, camuflaje y protección contra la radiación UV. Se agrupan comúnmente por sus similitudes físicas y químicas (Endler y Greenwood, 1988). A la fecha se han detectados diferentes tipos de pigmentos en los organismos marinos, entre los cuáles se encuentran los omocromos.

Los omocromos son compuestos coloreados que se encuentran en invertebrados, como cefalópodos, crustáceos e insectos, específicamente artrópodos (Shamim *et al*., 2014). Su estructura química es parte del grupo tricíclico de compuestos y tiende a solubilizarse en ácidos y álcalis (Van den Branden y Decleir, 1976). Todos los omocromos derivan del aminoácido triptófano, que se considera tóxico en altas concentraciones, y su síntesis está relacionada con el proceso de eliminación de tóxicos (Stratakis, 1979).

Según sus características físicas, los omocromos se clasifican en ominas y omatinas. Las ominas tienen un alto peso molecular, son de color oscuro y son estables a valores básicos de pH. Las omatinas, por otro lado, tienen un bajo peso molecular, son lábiles a los álcalis y están asociadas con tonalidades más claras (Brooks, 1985).

Los dos órganos principales en invertebrados donde se pueden encontrar omocromos son la piel y los ojos; esto se relaciona primordialmente con su función biológica como sustancias fotoprotectoras contra la luz en el océano (Ostrovsky *et al*., 1987). Los omocromos prácticamente nunca se encuentran como compuestos aislados. Se sintetizan como gránulos, y unidos a ellos se encuentran proteínas y iones de calcio. Debido a su papel como antioxidantes, han sido estudiados por su efecto sobre la actividad biológica (Sawada *et al*., 2002; Sawada *et al*., 2007).

Esta introducción abordará los omocromos y su método de extracción, aislamiento, identificación (incluidas las proteínas a las que tienden a unirse), prevalencia en la naturaleza, actividad biológica, tendencias futuras de investigación y aplicaciones prácticas.

1.1. Antecedentes

La investigación sobre omocromos se remonta a casi 130 años. En el período comprendido entre 1940 y 1970, se publicaron casi cincuenta artículos científicos sobre el metabolismo del triptófano en pigmentos, incluidos algunos sobre la identificación de omocromos. Los estudios innovadores fueron exploratorios y trataron las vías metabólicas y la expresión génica relacionadas con estos pigmentos en *Drosophila melanogaster*, incluida la síntesis de xantomatina y omatina D. La metodología descrita todavía se usa hoy en día para preparar un estándar de HPLC (Riou y Christides, 2010). Con respecto a los estudios de omocromos en cefalópodos marinos, las primeras publicaciones sobre el tema informaron el papel y la funcionalidad de los pigmentos en calamares y sepias. La vía metabólica que produce omocromos se trazó analizando principalmente los descubrimientos realizados en insectos. Una revisión a menudo citada es el trabajo de Linzen (1974) que discutió la síntesis de omocromos y promovió la clasificación de ominas y omatinas (se muestra más información sobre las características físicas y químicas de los omocromos en la **Tabla 1**). Aunque aceptado por biólogos y bioquímicos por igual, la primera investigación que propone la agrupación de omocromos no puede atribuirse a un solo trabajo de investigación o estudio. A partir de entonces, la información generada sobre los omocromos se centró en su importancia en la coloración dinámica y la visión de los invertebrados.

Tabla 1. Características Físicas y Químicas de los Omocromos.

Característica	Subdivisión de los Omocromos		
	Ominas	**Omatinas**	**Omidinas**
Coloración	Violeta a café	Amarillo a rojo	Amarillo a rojo
Peso molecular	>700 g/mol	400-700 g/mol	No reportado; similar a las omatinas
Solubilidad	Escasa en solventes polares	Buena en solventes polares	Mayor que las omatinas
Estabilidad según el pH	Estable bajo condiciones ácidas y alcalinas	Lábil a pH alcalino	Lábil a pH alcalino
Estabilidad oxidativa	Estable bajo condiciones reductoras y oxidantes	Tiende a autooxidarse	Estable en condiciones reductoras
¿Asociado a proteínas?	Sí	No reportado	No reportado
Ejemplo	Omina A	Xantomatina	Criptomidina

Fuente: Elaboración propia.

1.2. Métodos de Extracción

El estudio de los omocromos a menudo requiere de su extracción de un órgano específico del animal estudiado. Se han informado varios métodos de extracción desde que se descubrieron los omocromos. Las condiciones más comunes implican el uso de metanol acidificado como disolvente de extracción. Los informes explican que permite una selección efectiva de pigmentos y es lo suficientemente específico como para evitar la extracción de otros compuestos coloreados (Van den Branden y Decleir, 1976). Los omocromos son moléculas altamente no polares casi insolubles en agua pura, moderadamente solubles en tampones salinos y solubles en disolventes orgánicos próticos polares. El metanol puro puede y ha sido utilizado para este propósito, sin embargo, no es el solvente más eficiente porque la velocidad de extracción es lenta y requiere condiciones más severas. La acidificación del medio de extracción con un ácido inorgánico ayuda a superar estos inconvenientes. Como se ve en la **Figura 1**, que muestra una vía de síntesis de omocromos, los grupos amina están presentes en su estructura. En condiciones ácidas, los átomos de nitrógeno son protonados y forman un resto cargado positivamente en la molécula, lo que aumenta su solubilidad en metanol.

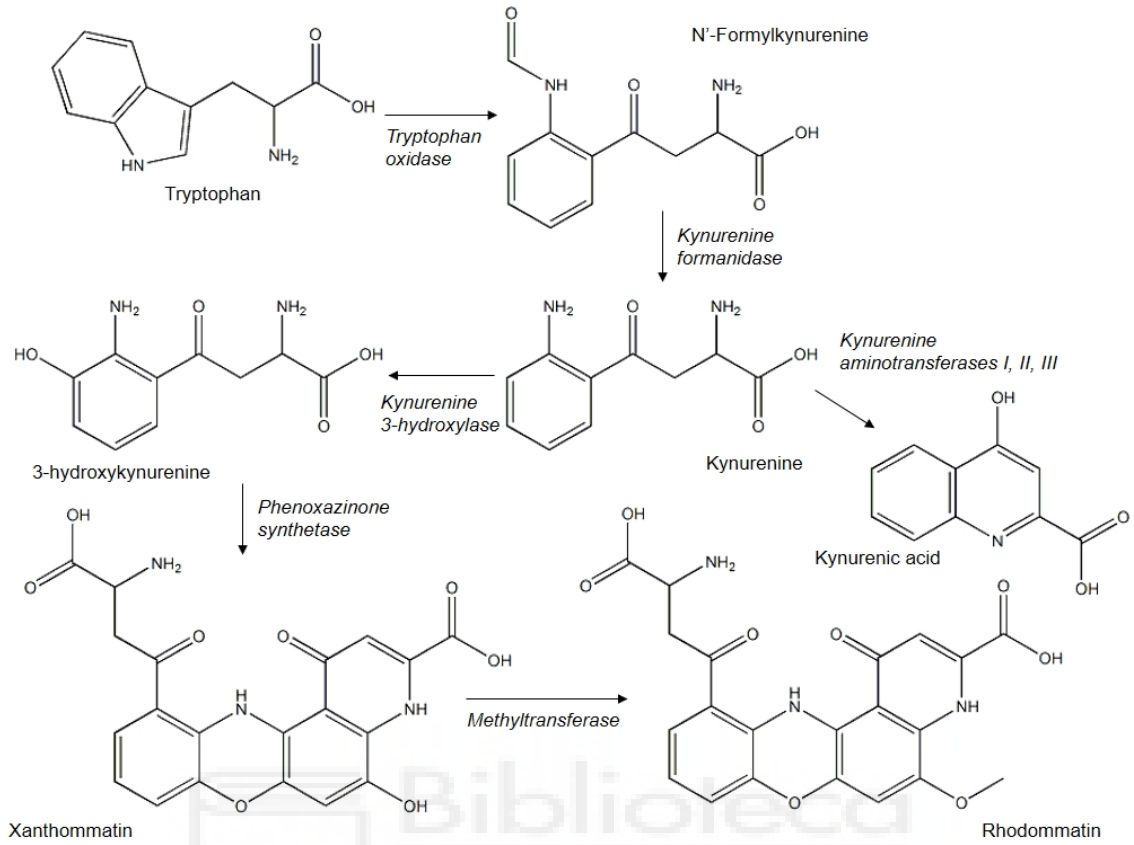

Figura 1. Vía metabólica de la síntesis de los omocromos. Fuente: Elaboración propia.

A hacer notar que las tendencias actuales se centran en hacer que los procesos químicos sean menos perjudiciales al ambiente mediante el uso de reactivos no tóxicos y ecológicos. Al ser el metanol una sustancia carcinógena y mutagénica conocida, se ha sugerido una combinación de etanol y ácido acético como sustituto. El extracto obtenido, usando estas condiciones, mostró actividad antioxidante y antimicrobiana, pero no hubo evidencia sólida suficiente de la presencia de omocromos, aparte de las pruebas de solubilidad y los espectros UV-visibles (Aubourg *et al*., 2016; Ezquerra-Brauer *et al*., 2016; Ezquerra-Brauer *et al*., 2017). También debe tenerse en cuenta que el etanol, aunque no es un solvente completamente inocuo, es significativamente menos tóxico que el metanol. Un método de extracción etanólica puede considerarse una metodología más segura que podría ampliar otras aplicaciones.

Como se señaló, el comportamiento de solubilidad ha sido una prueba presunta de la presencia de omocromos en extractos (Van den Branden y Decleir, 1976). Sin embargo, los resultados de las pruebas de solubilidad no siempre son concluyentes, especialmente

cuando se realizan con un extracto crudo. Para superar este obstáculo, los extractos de omocromos se han sometido a técnicas cromatográficas para determinar los componentes y su naturaleza química.

1.3. Técnicas de Separación e Identificación de Omocromos

Analizar un extracto crudo tiene ciertas implicaciones. Por ejemplo, no se puede atribuir resultados a un compuesto o compuestos específicos. Cuando se trata de la actividad o funcionalidad biológica, los efectos sinérgicos y/o antagonistas no se contemplan o se subestiman, lo que hace que la interpretación del resultado no sea lo suficientemente precisa y sea más difícil llegar a una conclusión.

Antes del desarrollo de métodos más sensibles, la cromatografía en capa fina (TLC) era la técnica más citada que separaba los componentes de un extracto crudo. Hoy en día, la TLC continúa siendo una herramienta preliminar útil para estimar las características y el número de compuestos en una muestra.

Los primeros artículos sobre extractos de omocromos hacían alusión al número de bandas, factores de retención y coloración. La TLC permitió la separación e identificación de omocromos y pteridinas (pigmentos con características similares). Contrariamente al disolvente de extracción, las condiciones de TLC variaron de una publicación a otra. La mayoría de los informes coincidieron en el uso de una mezcla fuertemente polar de solventes contra una placa de celulosa; otros sugirieron la combinación opuesta. Las condiciones utilizadas con frecuencia fueron metanol: ácido fórmico como fase móvil y placas de celulosa como fase estacionaria (Hori y Riddiford, 1981). Al tratar de identificar omocromos de las alas de *Precis coenia*, se descubrió que estas condiciones eran efectivas para la identificación, pero no para la recuperación de fracciones porque las bandas eran irregulares y estaban mal definidas (Nijhout, 1997). Los cambios sugeridos para superar esto fueron fenol: agua y gel de sílice como fases móviles y estacionarias, respectivamente. TLC encuentra su base en las diferencias de polaridad entre las fases y la muestra. En este caso, el resto polar parcial del fenol interactúa con el agua formando enlaces de hidrógeno, mientras que el anillo aromático es la contraparte no polar de la molécula que puede interactuar con los pigmentos no cargados. También se informó que, bajo estas condiciones, también se puede lograr la diferenciación de omocromos oxidados y reducidos.

La cromatografía líquida de alto rendimiento se ha utilizado para separar e identificar los omocromos presentes en un extracto. Un artículo de 2010 aborda en profundidad este tipo de análisis mediante el desarrollo de una metodología HPLC-DAD (Riou y Christides, 2010). Se identificaron cinco precursores y tres omocromos, junto con un metabolito no identificado. La técnica cuantificó omocromos en extractos metanólicos de *Misumenia vatia*. Se mencionó que la presencia de otros pigmentos estaba excluida debido al tipo de solvente y su acidez. Entre los pigmentos y otras moléculas que no se extrajeron, enumeraron pterinas, guanina, ácido úrico y carotenoides. Estas conclusiones se basan en la comparación con otra bibliografía; no se realizaron controles ni pruebas para descartar por completo la posibilidad de la eliminación de estos compuestos o cualquier otro, para el caso. Sin embargo, este método se considera innovador porque ofrece y demuestra los medios para estudiar omocromos a un nivel más preciso.

Dependiendo del objetivo del estudio, la identificación de omocromos, sus metabolitos y precursores puede realizarse mediante TLC, escaneos en UV-Vis, HPLC u otras técnicas cromatográficas o espectrométricas. Pero la información que se puede producir seguramente puede ayudar a construir una discusión sólida de datos y concluir con evidencia más sólida.

1.4. Proteínas Unidas a Omocromos

Los omocromos prácticamente nunca se encuentran como compuestos aislados. Se sintetizan como gránulos y se unen a proteínas y iones de calcio. Se ha comprobado que las extracciones metanólicas eliminan las proteínas que acompañan ominas y omatinas. Se teoriza que los pigmentos y las proteínas están fuertemente unidos y no se pueden separar fácilmente (Coates *et al*., 2005; Sawada *et al*., 2007).

Las proteínas de unión a omocromos no son de un solo tipo. Son diversos y su papel en el metabolismo cambia a medida que avanza la vida del insecto. Se aisló una proteína de 32 kDa de *Bombyx mori*. Se pensaba que era una proteína de la familia de las hexamerinas y se planteó la hipótesis de que funciona como un reservorio de aminoácidos aromáticos durante la etapa larval de *B. mori*. Curiosamente, la proteína purificada era roja: esto demuestra que también puede actuar como pigmentos (Sawada *et al*., 2007).

Las proteínas de otras fuentes de insectos se han relacionado con omocromos. Se han encontrado en la hemolinfa de *B. mori* y se unieron específicamente a la xantomatina. La función se asoció a una proteína de almacenamiento de tipo arilforina. Se obtuvo una proteína de 24 kDa de los ojos de *Hyalopora cecropia*. Estaba covalentemente unida a xantomatina y una omina no identificada. Se evaluó el comportamiento redox del extracto de proteína, así como los cambios de absorción de luz. La coloración dependiente del estado de oxidación es otra característica utilizada para identificar y clasificar los omocromos, por lo tanto, esto confirma su presencia (Ajami y Riddiford, 1971). Se extrajo otra proteína coloreada de la hemolinfa de *Manduca sexta*. Sus principales características físicas fueron una coloración amarilla, un peso molecular estimado de 31 kDa y dos picos de absorción máxima a 278 y 405 nm. Estructuralmente, consistía en casi 70% de láminas beta y el omocromo unido era la omatina D. Para interrumpir las interacciones con la proteína, se utilizaron metanol y cloroformo (Martel y Law, 1991). Esto puede considerarse como una de las pocas condiciones de desnaturalización que permiten la liberación del omocromo sin dañar la proteína ni la estructura del pigmento. En cuanto a la función de estas proteínas, la evidencia sugiere que las proteínas de unión a omocromo tienen un papel en el transporte de los metabolitos de triptófano y están involucradas en la síntesis de pigmentos.

Un análisis detallado de los cromatóforos cutáneos de *Sepia officinalis* reveló que los gránulos de pigmento no solo estaban formados por omocromos, sino también por proteínas. Una adición de NaOH al extracto de cromatóforo mostró que, a medida que aumentaba la concentración de álcali, el color pasaba del marrón oscuro al rojo. Debido al efecto de los agentes alcalinos en las proteínas, su disrupción se atribuyó como el factor principal en el cambio de coloración. Las dos proteínas estructurales principales que se encuentran en los gránulos de pigmento fueron la cristalina y la reflectina (Deravi *et al*., 2014). En este caso particular, los omocromos juegan un papel en la coloración dinámica de los calamares. Las dos proteínas estudiadas, como lo sugieren sus nombres, están relacionadas con la forma en que la luz interactúa con los cromatóforos y otras estructuras presentes en la piel del calamar.

La expresión de la proteína de unión a omocromo está regulada por varios genes: claro, granate, rosa, carmín, clavel y clarete, por nombrar algunos. Dependiendo de la especie, la actividad del gen será diferente (Coates *et al*., 2005; Sawada *et al*., 2002), y determinará la apariencia final del órgano del que se recuperan los pigmentos.

1.5. Distribución e Importancia Biológica de Omocromos en Insectos

Los omocromos se encuentran en invertebrados terrestres y marinos. El órgano en el que se encuentran varía de una especie a otra, pero la región de almacenamiento más frecuente son los ojos y la piel. En la **Tabla 2**, se resumen las características de los omocromos encontrados en cefalópodos e insectos.

La coloración de los insectos es el resultado de la interacción y expresión de al menos tres tipos de pigmentos: (i) melanina, (ii) ommocromos y (iii) pteridinas. Diferentes apariencias visuales y patrones de color emergen de la combinación de estos compuestos, y también de su interacción con el ácido úrico (Kato et al., 2006).

De las larvas de *Manduca sexta*, se han obtenido omocromos. El pigmento principal encontrado en los extractos fue dihidroxantomatina (DHX), que es de una tonalidad ladrillo, junto con 3-hidroxiquinurenina (3-HXK), recuperada específicamente de la piel de las larvas. Otro compuesto presente era una omatina no identificada. El espectro IR fue similar al 3-HXK. Al ser un precursor de los omocromos, el compuesto no identificado podría ser parte de la vía anabólica de los pigmentos en *M. sexta* (Hori y Riddiford, 1981). Un análisis estructural adicional podría confirmar esta teoría, específicamente utilizando otras herramientas de caracterización química. Si la estructura muestra algún parecido con los precursores del grupo tricíclico de compuestos, es casi seguro que está relacionado con el metabolismo de omocromos.

El proceso de pérdida de coloración de la araña cangrejo, *Misumena vatia*, implica la degradación de los gránulos omocrómicos; no se debe a una dilución o una translocación de los pigmentos. Los análisis realizados a los diferentes gránulos demostraron que el catabolismo y el anabolismo de estos pigmentos ocurren simultáneamente en las células de *M. vatia* (Insausti y Casas, 2009). El metabolismo ininterrumpido de los omocromos respalda la teoría de que se sintetizan como una forma de eliminación del triptófano, ya que permite la transformación de este aminoácido en compuestos menos tóxicos para los insectos. La quinurenina (KYN) y su forma oxidada también se identificaron en gránulos de pigmento. La coloración resultante de este artrópodo es una mezcla de quinureninas y omocromos (Insausti y Casas, 2008).

Tabla 2. Principales omocromos encontrados en invertebrados terrestres y marinos.

Organismo	Tipo de omocromo	Región anatómica	Coloración	Referencia
Aedes aegypti	XAN, 3-HK†	Ojos	Roja	Li *et al*. (1999)
Aldrichina grahami	3-HK			Wadano *et al*. (1993)
Anopheles stephensi	Ácido xanturénico	Glándulas salivales, intestinos		Okech *et al*. (2006)
Armadillidium vulgare	3-HK	Exoesqueleto	Café oscuro, violeta	Hasegawa *et al*. (1999)
Bombyx mori	Proteínas asociadas a omocromos		Rojo	Sawada *et al*. (2002); Sawada *et al*. (2007)
Carausius morosus	XAN, omina no identificada	Epidermis		Stratakis (1979)
Drosophila melanogaster	Quinurenina, 3-HK	Ojos	Roja	Yagi y Ogawa (1996)
Manduca sexta	DHX, omatina no identificada	Piel de la larva	Rojo	Hori y Riddiford (1981)
Misumena vatia	Quinurenina, 3-HK			Insausti y Casas (2008)
Mysis relicta		Ojos		Dontsov *et al*. (1999)
Papilio graphium weiskei	Ominas, derivado de pteridinas	Alas	Rosa a violeta	Barbier (1983)
Teuthowenia pellucida	Ominas	Ojos		Evans *et al*. (2015)
Triatoma infestans	XAN	Ojos	Rojo, violeta oscuro	Moraes *et al*. (2005)
Vanessa cardui	XAN, DHX, compuesto no identificado	Alas	Rojo	Reed y Nagy (2005)

†*3-HK: 3-hidroxiquinurenina, DHX: dihidroxantomatina, XAN, xantomatina*

Durante la investigación acerca de la coloración de una mariposa, se obtuvo un omocromo del grupo de las ominas de las alas de *Papilio graphium weiskei*. El aspecto general de *P. graphium weiskei* se determinó principalmente por la combinación de dos tipos de pigmentos: un derivado de pterina y una omina (Barbier, 1983). Mientras que otros compuestos coloreados se recuperaron mediante la extracción con solvente de las partes verde y azul del ala, los pigmentos de los fragmentos rosa y violeta se eliminaron después de un tratamiento con ácido. Esta evidencia corrobora las propiedades únicas y distintivas de los omocromos en comparación con otros pigmentos encontrados en la misma región anatómica.

Un estudio que comparó los compuestos de *Armadillidium vulgare* mostró la presencia de omocromos en el exoesqueleto de muestras pigmentadas y albinas. Estos

pigmentos fueron los principales contribuyentes de su coloración marrón-violeta oscura. En los organismos albinos, los órganos externos y algunos internos eran de color amarillo o marrón claro; mientras que las mismas áreas en los insectos pigmentados eran de color marrón intenso. Se creía que la xantomatina (XAN) era responsable de la coloración, pero no se encontró en ninguno de los grupos. Por el contrario, se encontró 3-HXK en concentraciones más bajas en organismos albinos (Hasegawa *et al.,* 1999). Esta evidencia sugiere una posible explicación de la falta de coloración: que la vía de síntesis de omocromos en *A. vulgare* depende directamente de la cantidad de 3-HXK.

Se encontró que la coloración roja del ojo de *Aedes aegypti* se sintetizó desde las primeras etapas de desarrollo larvario. Esta evidencia sugiere que la síntesis de omocromos está activa durante toda la vida del insecto. Los niveles de XAN aumentaron a medida que avanzó el ciclo de vida de la pupa. Los niveles de 3-HXK fueron los más altos en las primeras etapas de *A. aegypti*. Debido al papel de esta molécula en la ruta de síntesis omocrómica, su presencia en las pupas sugiere que su papel principal es como precursor de pigmento (Li *et al*., 1999).

En el caso de los pigmentos aislados de *Triatoma infestans*, se descubrió que XAN era el componente principal, y era responsable de la coloración de los ojos rojos y negros. Dado que las ominas presentan un pico de absorción típico a 520 nm, la ausencia de esta señal en los espectros recolectados de los extractos sugirió que las ominas no fueron los principales contribuyentes en la coloración de *T. infestans* (Moraes *et al*., 2005). Como las ominas no eran responsables de la coloración, la identificación de los responsables de la coloración de los ojos de este insecto habría confirmado o refutado la presencia de compuestos de la familia de los omocromos.

La dieta tiene un efecto directo sobre la síntesis de omocromo, como se demostró en la alimentación de un mutante *Drosophila melanogaster* con KYN y 3-HXK. Se observó un efecto sobre la formación de un pigmento ocular que normalmente no está presente en el tipo salvaje, pero el tamaño de los gránulos de pigmento aumentó después de la administración de 3-HXK. El número de gránulos aumentó cuando se alimentó con KYN, permaneciendo igual que el grupo de control. La transformación de 3-HXK a XAN tiene lugar dentro de los gránulos de pigmento. En cuanto a la síntesis omocrómica, se propuso que se llevase a cabo como una descarga vesicular del aparato de Golgi (Yagi y Ogawa, 1996). Evidencia como esta demuestra el efecto de factores exógenos, lo que sugiere que la ingesta de los propios omocromos desencadena o aumenta la síntesis. En

un estudio realizado en *Vanessa cardui* ayudó con el destino final de almacenamiento de triptófano (Trp). La inyección abdominal de Trp marcado en la hemolinfa de pupas de *V. cardui* permitió un análisis detallado de este aminoácido en mariposas. Se encontraron metabolitos Trp coloreados en todas las manchas rojas en sus alas (Reed y Nagy, 2005). Se determinó que la aparición de escamas rojas en las alas era el resultado de la interacción de melanina y omocromos.

Como uno de los metabolitos Trp en los insectos, la concentración de ácido xanturénico (XAc) aumentó después del tratamiento con este aminoácido en las larvas de *Anopheles stephensi*, en comparación con las alimentadas con agua o una solución de azúcar. Sin embargo, se encontró una diferencia significativa entre la concentración de XAc entre machos y hembras. Los mosquitos hembras produjeron más XAc cuando se les dio Trp, en comparación con los mosquitos machos. La acumulación de pigmentos fue más evidente en las glándulas salivales y el intestino medio. Durante el proceso de alimentación de sangre, la concentración de XAc alcanzó su punto máximo en las mujeres, ya que desencadena la gametogénesis. Se propone una nueva función interesante para un precursor de omocromo, que va más allá de la coloración y como fotoprotector (Okech *et al*., 2006).

La síntesis de omocromos y productos de excreción también se evaluó en el insecto palo, *Carausius morosus*. Se descubrió que la xantomatina y una omina desconocida estaban presentes en la epidermis, mientras que el ácido quinurénico predominaba en sus heces (Stratakis, 1979). La excreción de un omocromo en *C. morosus* requiere una transformación de los pigmentos más complejos en moléculas más simples para ser eliminados. Un estudio más completo podría aclarar si solo están presentes pequeños metabolitos en las heces de los insectos, para determinar las etapas finales de los omocromos en el tracto digestivo de los insectos.

1.6. Distribución e Importancia Biológica de Omocromos en Cefalópodos

Los cefalópodos marinos han adaptado sus sentidos de muchas maneras para adaptarse al entorno acuático. El tipo y concentración de pigmentos oculares depende de la profundidad en que habitan las especies. Los cefalópodos poseen un sistema bastante único de órganos minúsculos que controlan su coloración y apariencia física. La acción combinada de las estructuras presentes en la piel del cefalópodo permite que estos

cambios sucedan tan rápido como sea necesario. Los cromatóforos están formados por una bolsa elástica central, conectada a las fibras musculares y los nervios. Este tipo de estructura permite que la expansión y contracción del cromatóforo sea regulada por impulsos nerviosos (Van den Branden y Decleir, 1976). Los omocromos actúan en ambos roles, como pigmentos protectores de los rayos UV en los ojos de los cefalópodos, y como pigmentos dinámicos en la piel.

La piel del calamar está compuesta principalmente por dos capas, ambas involucradas en los procesos de camuflaje y señalización. La capa más externa tiene órganos cromatóforos, que contienen pigmentos rojos, amarillos o marrones. Debajo de la capa de cromatóforo, se encuentra otra lámina de piel que contiene a células como los iridóforos y leucóforos que actúan como reflectores de luz (Mathger y Hanlon, 2006). Sus cambios de coloración se deben a la presencia de una miríada de órganos cromatóforos. En la piel del calamar, se organizan según su color: cromatóforos amarillos en la capa más externa, rojos en el medio y, por último, órganos que contienen pigmento marrón en la capa más profunda. Su tamaño aumenta con la profundidad que se encuentran. Otro factor que involucra el tamaño de los cromatóforos es la forma; si están contratados o expandidos (Bell *et al*., 2013).

La apariencia dinámica de los cefalópodos marinos se ha relacionado con funciones como la detección de presas y/o depredadores, navegación, sentido de dirección y mejora del contraste. Se dice que los cefalópodos se comunican dentro de los miembros de su propia especie. Uno de los ejemplos más típicos de comunicación a través de la coloración es la apariencia amenazante que se observa entre los machos de *Sepia officinalis*. El patrón de cebra en sus cuerpos es una señal de intención de lucha. El mecanismo está relacionado con su capacidad visual para reconocer patrones, coloraciones y formas (Mathger et al., 2009).

Los cefalópodos pueden detectar la polarización lineal de la luz entrante. Sus ojos sintetizan pigmentos visuales capaces de absorber la luz presente bajo el agua. Se han encontrado ominas como pigmentos visuales de detección en un calamar de aguas profundas (*Teuthowenia pellucida*). Las capas internas y externas de su ojo tenían ominas, que sirven como compuestos fotoprotectores. Los pigmentos más oscuros estaban presentes en los especímenes más jóvenes en la capa más externa del ojo, mientras que los calamares adultos tenían una menor concentración de pigmentos. La diferencia entre los grupos de edad podría estar asociada a la profundidad a la que se encuentran los

calamares. Los calamares jóvenes se encuentran muy probablemente en aguas relativamente poco profundas, en comparación con los adultos, que habitan en aguas más profundas y oscuras (Evans *et al*., 2015), lo que demuestra la importancia de otro factor exógeno, que son aspectos conductuales en la síntesis de omocromos.

Los omocromos no se encuentran exclusivamente en cefalópodos y artrópodos. Los extractos de metanol acidificado obtenidos de un planario, *Dugesia ryukyuensis*, mostraron las características fisicoquímicas de los omocromos. Los espectros UV-visibles del pigmento purificado mostraron los picos asociados a este tipo de derivados de triptófano. En *D. ryukyuensis*, como se reportó en otras especies, los pigmentos se encontraron en gránulos (Hase *et al*., 2006). Estos hallazgos demuestran que los omocromos son más ubicuos en los invertebrados de lo esperado. Los resultados que establecieron la relación de los omocromos con una función protectora contra la radiación UV guiaron la investigación actual para estudiar su actividad antioxidante, como se discutirá en la siguiente sección.

1.7. Actividad Biológica y Aplicaciones de los Omocromos

Es ampliamente reconocido que la luz desencadena el daño oxidativo, debido al mecanismo de absorción de fotones y la formación de radicales libres. Cuando las especies reactivas buscan estabilidad, oxidan moléculas como los lípidos y las proteínas. La peroxidación lipídica ocurre con mayor frecuencia en las células sensibles a la luz, específicamente en las ubicadas en los ojos. Como la mayoría de las células, las células visuales tienen un sistema antioxidante endógeno (Dontsov *et al*., 1999). Los omocromos pueden actuar como antioxidantes y su mecanismo antioxidante es diverso:

- pueden unir metales y ejercer actividad quelante, y
- pueden actuar como antioxidantes primarios al eliminar los radicales, como el oxígeno singlete y los aniones superóxido (Egorov *et al*., 1987; Ostrovsky *et al*., 1987).

Los omocromos evitan los efectos fotosensibles en los ojos de las especies marinas. El daño oxidativo es causado por numerosas especies reactivas. Los omocromos, junto con otros pigmentos, han ejercido actividad antioxidante contra el oxígeno singlete,

evitando la formación de radicales libres que inician la peroxidación lipídica (Egorov *et al*., 1987).

Sin embargo, 3-HXK es una molécula propensa a oxidarse in vivo, lo que resulta en la formación de especies reactivas de oxígeno. Debido a su naturaleza prooxidante, podría considerarse como un indicador de estrés oxidativo. Cuando el oxígeno molecular y el 3-HXK reaccionan, se generan el superóxido radical y el peróxido de hidrógeno, entre otros productos a través de la reacción de Fenton. Para evitar su descomposición, el 3-HXK se transforma en el ácido xanturénico más estable (Li *et al*., 1999). Esto podría considerarse como un mecanismo antioxidante primario porque se evita la formación de radicales libres al hacer esta conversión.

Se ha demostrado la actividad inhibitoria de los omocromos contra la degradación de la cardiolipina, como una forma de actividad antioxidante. El mecanismo antioxidante está relacionado con el efecto quelante de los omocromos contra la oxidación mediada por hierro, porque su forma ferrosa es un conocido estimulador de la peroxidación lipídica (Dontsov *et al*., 1999).

En un estudio realizado por Dontsov y colaboradores (1999), se compararon las poblaciones de crustáceos de mar y lago (*Mysis relicta*) en función de su estado oxidativo. La formación de TBARS (sustancias reactivas al ácido tiobarbitúrico) fue inversamente proporcional a la concentración de omocromos. Los camarones capturados en el mar tenían un menor valor de TBARS en comparación con el de la población del lago. Los camarones marinos tenían una mayor concentración de omocromo, consistente con su mayor resistencia a la luz brillante. Esta evidencia establece además la relación entre la producción de antioxidantes endógenos y la exposición a la luz.

La actividad antioxidante de los omocromos no siempre se ha relacionado con los órganos fotosensibles, como se describió anteriormente. Un precursor omocromo aislado de *Aldrichina grahami*, 3-HXK, protegió los adipocitos contra la peroxidación lipídica y el daño celular, inducido por el hidroperóxido de ter-butilo. Es importante mencionar que la capacidad antioxidante de 3-HXK fue mayor en comparación con el ácido úrico y la reducción del glutatión, antioxidantes endógenos conocidos. Se predice que el mecanismo interactúa directamente en las reacciones de barrido con radicales libres (Wadano *et al*., 1993).

La actividad biológica de los omocromos contra la oxidación lipídica inducida se ha evaluado, expresada como una disminución de los niveles de malondialdehído en un estudio in vivo con conejos pigmentados y albinos. Las muestras de albino fueron más sensibles a la inducción de peroxidación en comparación con el grupo pigmentado. Este hallazgo marca una conexión directa entre los gránulos que contienen pigmento y la oxidación provocada por la luz, y no con otros antioxidantes endógenos (Ostrovsky *et al*., 1987).

Una de las primeras preguntas en mente cuando se trabaja con actividad antioxidante es el mecanismo por el cual actúan las moléculas. Utilizando métodos *in silico* (dispositivos computacionales y software especializado), se evaluó una estimación de la actividad antioxidante de los omocromos mediante dos mecanismos de eliminación diferentes: transferencia de electrones y transferencia de átomos de hidrógeno. Se encontró que todos los omocromos evaluados eran donantes de electrones, aunque energéticamente posible solo en casos específicos, como KYN, 3-HXK y omatina D. La transferencia de hidrógeno también fue factible, siendo el mejor antioxidante omatina D, como se describe en la **Figura 2**. El átomo de nitrógeno que se muestra como el donante de hidrógeno se eligió en función de la energía de disociación más baja calculada por el software de predicción (Romero y Martínez, 2015). Esto puede considerarse como un trampolín en la investigación de la actividad antioxidante en los omocromos, ya que propone un mecanismo antioxidante para los omocromos.

Los estudios toxicológicos sobre omocromos siguen siendo escasos, pero la información derivada de ellos pone en perspectiva la investigación sobre estos pigmentos naturales. Un estudio in vivo realizado en Neobellieria bullata estableció el efecto toxicológico de algunos omocromos y precursores de omocromos. Los cinco compuestos relacionados con omocromos (Trp, 3-HXK, KYN, ácido antranílico y ácido quinolínico) deterioraron la movilidad de las moscas. Los efectos de Trp, 3-HXK y ácido antranílico era reversible, mientras que los de KYN y el ácido quinolínico eran irreversibles y conducían a la muerte de los insectos. También se evaluó la toxicidad *in vitro* en neuronas de insectos. Se demostró que los ácidos 3-HXK y 3-hidroxi-antranílico eran tóxicos, mientras que KYN y el ácido antranílico fueron negativos en la prueba toxicológica in vitro (Cerstiaens *et al*., 2003).

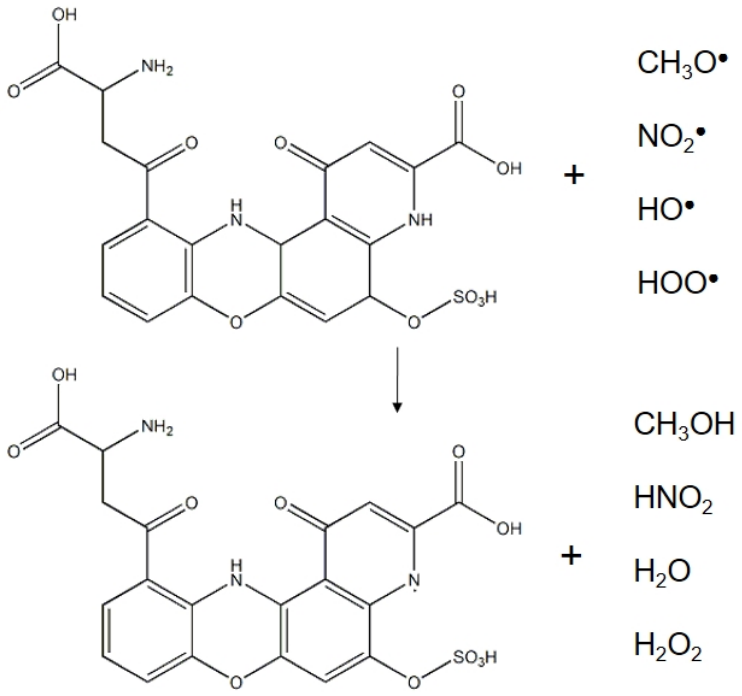

Figura 2. Representación de la reacción entre la xantomatina y algunos radicales libres. Fuente: Elaboración propia

Se ha establecido la relación entre las quinureninas en general y el daño neurológico. No se considera que las quinureninas, aunque están presentes en muestras de tejido, sean el factor causal directo en el desarrollo de enfermedades neurodegenerativas. Sin embargo, se ha demostrado que uno de los derivados de las quinureninas, el ácido quinolínico, causa la muerte neuronal excitotóxica. Paradójicamente, el ácido quinurénico actúa como neuroprotector en los receptores de aspartato (Stone, 2001).

1.8. Prospectos y Aplicaciones

Los pigmentos naturales se usan ampliamente en la formulación de nuevos productos. Los pigmentos de cefalópodos se han aplicado en el campo de la cosmetología. Se evaluó un lápiz labial teñido con extractos de piel de calamar. Cumplió con todas las regulaciones estipuladas para productos de esta naturaleza (físicos, químicos, microbiológicos y organolépticos) (Hassan *et al*., 2015).

Los extractos de pigmentos etanólicos obtenidos de piel de calamar gigante (*Dosidicus gigas*) se usaron como antioxidantes contra la rancidez del aceite de hígado de bacalao inducida por el calor. Como se demostró en este estudio, los pigmentos pudieron retrasar la oxidación de los lípidos durante los primeros d de almacenamiento a 50 °C (Aubourg *et al*., 2016). También se ha demostrado que los pigmentos de calamar gigante ejercen actividad antimicrobiana en la caballa helada y la merluza, expresada como una inhibición de la trimetilamina, la proteólisis microbiana y la lipólisis (Ezquerra-Brauer *et al*., 2016; Ezquerra-Brauer *et al*., 2017). En todos los casos, el uso de pigmentos marinos de piel de calamar transformaría un material desechado en un coproducto comercial y útil.

Como parte de las aplicaciones de los omocromos, en el presente trabajo se evaluó la utilización de estos pigmentos obtenidos a partir de la piel del calamar gigante. Para ello, se establecieron los objetivos que se describen en la próxima sección.

2. OBJETIVOS.

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El **objetivo general** de esta Tesis Doctoral fue establecer la actividad biológica y la estructura química de los pigmentos presentes en la piel de calamar gigante, así como su efecto como aditivo en alimentos.

Para alcanzar dicho objetivo principal se plantearon los siguientes **objetivos específicos**:

- Optimizar las condiciones de extracción de los pigmentos de piel de calamar gigante y evaluar su actividad antioxidante y antimicrobiana.
- Determinar el potencial mutagénico y clastogénico del extracto de piel de calamar gigante.
- Establecer el efecto de la adición del extracto de piel de calamar gigante sobre la vida de anaquel y calidad sensorial de paté de atún.
- Identificar la estructura química de los compuestos omocrómicos responsables de la estabilidad del producto elaborado.

3. RESUMEN DE LA METODOLOGÍA.

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3.1. OBTENCIÓN DE LOS EXTRACTOS Y EVALUACIÓN DE SU ACTIVIDAD BIOLÓGICA

3.1.1. Materia Prima

Diez calamares gigantes (*D. gigas*) se obtuvieron de un establecimiento local en Hermosillo, México (29°05′56″ n 110°57′15″ w), y se evisceraron inmediatamente. La longitud y el peso de los especímenes de calamar variaron de 100 a 150 cm y de 40 a 60 kg, respectivamente. La piel (aproximadamente 50 cm de longitud) se congeló a –80 °C, se liofilizó (Labconco, Kansas City, MO, EE. UU.) durante 2 d y se trituró. Las muestras (100 mg) se colocaron en bolsas de polietileno, se sellaron al vacío y se mantuvieron a –20 °C hasta los análisis posteriores. Todos los productos químicos utilizados fueron de grado reactivo analítico y se compraron a Sigma-Aldrich (St. Louis, MO, EE. UU.).

3.1.2. Extracción de Pigmentos

Brevemente, el proceso de extracción de pigmento consistió en la homogeneización de 20 volúmenes de piel liofilizada (p / v) en metanol acidificado (metanol 99: 1: HCl), seguido de centrifugación (Modelo Biofuge Stratos, Thermo Scientific, Alemania) a 10,000 × *g* durante 15 min. El metanol se eliminó usando un evaporador rotativo (R-100, Büchi, Suiza) y se evaporó adicionalmente usando gas nitrógeno. Los extractos secos se almacenaron en una atmósfera de nitrógeno inerte, a –80 °C, antes de un análisis posterior. Se calculó el rendimiento seco y se prepararon soluciones madre para evaluar la actividad antioxidante.

El rendimiento de extracción se calculó gravimétricamente, utilizando el peso de la muestra de piel como referencia. El rendimiento del pigmento se calculó de la siguiente manera:

Rendimiento de pigmento (%) = [(extracto pigmentado seco (g)) / (piel seca de calamar (g))] × 100

La actividad antioxidante se estableció mediante el método de capacidad de absorbancia de radicales de oxígeno (ORAC). El método ORAC se realizó de acuerdo con la metodología anterior (Garret *et al.,* 2010), pero con ciertas modificaciones. La pérdida de fluorescencia de la fluoresceína se controló durante 90 min a 37 °C en

presencia de diclorhidrato de 2,2'-azobis (2-amidinopropano) (AAPH). Cada muestra (0,5 mg/mL) se analizó por triplicado y se comparó con una curva estándar para expresar los resultados como equivalentes de Trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico).

3.1.3. Actividad Antimicrobiana

Los efectos antimicrobianos de los extractos (10 mg de extracto) se evaluaron después de la prueba de difusión de disco como se reportó anteriormente (Fatrcová-Šramková *et al.,* 2016). La actividad antimicrobiana de los extractos pigmentados de piel de calamar gigante (JSSE) se probó contra tres bacterias Gram negativas (*Haemophilus influenza* CCM 4456, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subs. Enterica CCM 3807), cuatro bacterias Gram positivas (*Bacillus cereus* CCM 2010, *Clostridium perfringens* CCM 4991, *Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subs. Aureus CCM 2461), seis hongos filamentosos microscópicos (*Aspergillus clavatus, A. flavus, A. versicolor, Penicillium chrisogenum, P. griseofulvum, P. expansum*, *Candida albicans* CCM 8186, *C. glabrata* CCM 8270, *C. tropicalis* CCM 8223).

3.1.4. Actividad Antimutagénica

La prueba de Ames se utilizó para evaluar la actividad antimutagénica de los extractos de piel de calamar (Maron y Ames, 1983). El ensayo se realizó usando 100 µL de cepas de *Salmonella typhimurium* T98 y T100 cultivadas durante la noche (1×109 células/mL), 100 µL de extractos de pigmento (0,005, 0,05, 0,5 y 5,0 mg/mL) y el agente mutagénico (Aflatoxina B1, AFB_1) con sistema de activación (500 µL de mezcla S9) en placas por triplicado. Se usó diez DMSO (100 µL) sin AFB1 como control negativo. Después de la incubación durante 48 ha 37 °C, se contó el número de bacterias revertantes por placa. La tasa de inhibición de la actividad mutagénica se calculó utilizando la siguiente ecuación:

$$\textit{Tasa de inhibición (\%)} = [((1-T)) / M] \times 100$$

donde *T* es el número de participantes por muestra de prueba placa en presencia de AFB_1, y *M* es el número de revertantes por placa en el control positivo, después de restar el número de revertantes espontáneos del numerador y denominador. La inhibición de la mutagénesis AFB1 se considera fuerte, moderada o débil cuando los valores son altos en

un 60%, 40-60% o 20-40%, respectivamente, y despreciable cuando el valor es inferior al 20% (Ikke *et al.,* 1999).

3.1.5. Prueba de Clastogenicidad de Punta de Raíz de Cebolla

En este estudio se utilizaron bulbos de cebolla jóvenes y saludables, cultivados en ausencia de herbicidas, pesticidas o fungicidas. La promoción del desarrollo de la raíz se realizó colocando los bulbos en la oscuridad y sumergiéndolos parcialmente en agua. Cuando las raíces tenían 2 cm de largo, las cebollas se transfirieron a placas de Petri con 30 ml de extractos JSSE (0,1, 0,5, 1 y 5 mg cada uno). También se analizaron un control positivo con azida de sodio (10 ng) y un control negativo (agua). Después de 24 h, las raíces se fijaron en etanol / ácido acético glacial 3: 1 (v / v), se aplastaron y se lavaron con agua destilada y se tiñeron con orceína durante 2 h en la oscuridad. Todas las células con alteraciones se contaron (Liman *et al.,* 2015). Durante la evaluación de genotoxicidad, se registró la presencia de células mitóticas con cromosomas irregulares (micronúcleos, estructura cromosómica desorganizada, retraso y cromosomas en barra).

3.1.6. Análisis estadístico

Se usó un diseño de bloques completos al azar factorial 3X3 para obtener una combinación óptima de temperatura y tiempo de sonicación que produjo un alto nivel de extractos pigmentados con una alta actividad antioxidante. La selección de los niveles de temperatura (25, 35 y 45 ° C) y el tiempo de sonicación (5, 10 y 15 min) probados se basaron en estudios preliminares. El diseño del experimento y el análisis estadístico se llevaron a cabo utilizando el software JMP (SAS, Cary, NC, EE. UU.). Las diferencias entre las medias se compararon mediante la prueba de Tukey ($p < 0.05$).

Los datos de la caracterización fisicoquímica del pigmento extraído del calamar gigante, las actividades antimicrobianas y antimutagénicas se basaron en el promedio de tres determinaciones. Para el análisis espectroscópico, se utilizaron estadísticas descriptivas para analizar los datos (Glover y Mitchell, 2015). Para la prueba de solubilidad y las actividades antimicrobianas y antimutagénicas, las variaciones entre las repeticiones fueron <5%. Se calcularon los valores medios de los tres ensayos y las desviaciones estándar.

3.2. APLICACIÓN DEL EXTRACTO EN LA ESTABILIDAD Y CALIDAD SENSORIAL DE ALIMENTOS DE ORIGEN MARINO

3.2.1. Elaboración de Paté de Atún Adicionado con Extracto de Piel de Calamar

Los patés de atún se produjeron en la planta piloto de procesamiento de alimentos en la Universidad Miguel Hernández (Alicante, España). Se obtuvieron filetes de atún aleta amarilla fresco de un mercado local. Los filetes de atún se cortaron en cubos pequeños y se mezclaron durante 15 min en un procesador de alimentos Vorwerk Thermomix (Wuppertal, Alemania). El atún (40 g) se mezcló con hielo (30 g) y sal (1 g) durante 5 min. Posteriormente, se añadieron lentamente caseinato de sodio (4 g) y almidón de maíz (4 g), hasta que se formó una mezcla homogénea. Se añadió suavemente aceite de oliva (5 g) hasta que se formó la emulsión. Finalmente, se añadió vinagre de vino blanco (1 g). Todos los lotes se cocinaron hasta que la temperatura del núcleo del paté alcanzó los 75 °C y luego se enfriaron en un baño de hielo.

Según estudios preliminares, JSSE se agregó por separado en dos lotes a concentraciones de 0.05% de paté (P1) y 0.1% de paté (P2). Se añadió butilhidroxianisol (0.1 % de paté) a un tercer lote (BHA), lo que representa un tratamiento diferente. JSSE se mezcló con vinagre antes de mezclarlo con los otros ingredientes. El lote restante se usó como muestra de control (control).

3.2.2. Panel de Aceptación de Consumidores

Las muestras de paté se tomaron del almacenamiento refrigerado y se dejaron a 20 °C durante aproximadamente 15 min, las cuales fueron analizadas por un panel de 70 consumidores. Se pidió que indicaran su preferencia en relación con ocho atributos en una escala (1: extremadamente no me gusta; 5: ni me gusta ni me disgusta; y 9: extremadamente me gusta). Los consumidores entre 20 y 65 años eran estudiantes y miembros del personal del Departamento de Tecnología Agroalimentaria (Universidad Miguel Hernández, Campus Desamparados). Los análisis sensoriales se realizaron después de cocinar el paté.

3.2.3. Análisis Microbiológico

Se tomaron porciones (20 g) de las muestras (para cada uno de los grupos de control y tratamiento, por separado) del paté de atún aleta amarilla de manera aséptica y se homogeneizaron en bolsas esterilizadas (Seward, Reino Unido) con 90 mL de agua con

peptona al 0,1%. Se hicieron diluciones en serie de diez veces en tubos pre-esterilizados que contenían 9 mL de agua de peptona. La preparación de la muestra y la siembre se llevaron a cabo bajo un gabinete de flujo laminar en condiciones estériles. Se analizaron muestras triplicadas de cada ensayo de formulación de paté. Para estimar el recuento total de placas, 1 mL de las diluciones se transfirió asépticamente a las placas de recuento de Petrifilm (3M Corporation, Maplewood, MN, EE. UU.). Los petrifilms se dividieron en dos condiciones de incubación para la determinación de los mesófilos aeróbicos totales (37 °C) y los psicrófilos (7 °C). Las colonias se contaron después de 48 h para mesófilos y 72 h para psicrófilos utilizando un contador de colonias. El recuento promedio se multiplicó por el factor de dilución y se expresó como el log CFU/g de la muestra.

3.2.4. Oxidación de Lípidos

Los dienos conjugados (CD) y los trienos (CT) se midieron mezclando una muestra de paté de 0,5 g con 5 ml de agua destilada y se agitó en vórtex a 1500 rpm durante 1 min. Se mezcló una parte alícuota de 0,5 mL con 2,5 mL de hexano: isopropanol (3: 2 v/v) y se centrifugó a 2000 rpm durante 3 min. El sobrenadante se recuperó y se colocó en cubetas de cuarzo, y las mediciones de CD y CT se realizaron a 232 y 268 nm, respectivamente. La concentración de dienos y trienos se determinó utilizando el coeficiente de extinción molar, específico para ambos tipos de compuestos (Martín-Sánchez *et al.,* 2014).

La cuantificación de los hidroperóxidos, evaluados como productos de peroxidación secundaria, se realizó de acuerdo con informes anteriores (Pateiro *et al.,* 2015), con algunas modificaciones. La extracción de lípidos se realizó con hexano: isopropanol. El extracto (1 mL) se mezcló con 10 ml de cloroformo: metanol (7: 3 v/v) y se agitó en vórtex durante 30 s a 2000 rpm. Más tarde, se añadieron 50 µL de tiocianato de amonio al 30% y 50 µL de cloruro de hierro (II) 20 mM y se centrifugaron a 2500 rpm durante 10 min. La absorbancia del sobrenadante se obtuvo a 480 nm, y la concentración de peróxidos se expresó como el nmol equivalente de hidroperóxido de cumeno por g de paté.

Los productos finales de la peroxidación se determinaron utilizando el ensayo de ácido tiobarbitúrico (TBARS) (Villalobos-Delgado *et al.,* 2017). Se mezcló una muestra de 0,5 g con 2,5 mL de reactivo de ácido tiobarbitúrico (3,75 g de TBA, 150 g de ácido tricloroacético en 1 L de HCl 0,25 N) y se calentó en un baño de agua a 96 °C durante 10

min. Los tubos de ensayo se enfriaron en un baño de hielo y se centrifugaron a 2500 rpm durante 10 min. Se recuperó el sobrenadante y se registró su absorbancia a 532 nm. La curva estándar se preparó con 1,1,3,3-tetrametoxipropano, y los resultados se expresaron como los equivalentes de malondialdehído por g de paté.

Se evaluó la eficacia antioxidante de JSSE en las muestras, con un índice de oxidación a ambas temperaturas de almacenamiento (4 y 8 °C). El porcentaje de inhibición de oxidación [OI (%)] se calculó para PV y TBARS utilizando la ecuación 3 (Frankel *et al.,* 1997):

$$OI\ (\%) = ((c\text{-}s) / c) \times 100$$

donde *c* es el valor obtenido del control el día del valor más alto obtenido, y *s* es el valor para cada condición JSSE en el mismo día.

3.2.5. Calidad sensorial

Para evaluar la calidad sensorial de las muestras durante el almacenamiento, se nombró un panel de siete panelistas calificados y con experiencia en el campo de los productos pesqueros, con el atributo sensorial descrito.

3.2.6. Análisis estadístico

Se utilizaron estadísticas descriptivas para presentar los ensayos antioxidantes (n = 3). Los datos obtenidos del estudio de aceptación del consumidor se evaluaron mediante un análisis de varianza unidireccional (ANOVA) para evaluar la importancia de la adición de JSSE en todas las características estudiadas. Datos del color y la oxidación lipídica de las muestras de paté se evaluaron mediante un análisis de varianza de dos vías (ANOVA). Los datos obtenidos de la prueba de Ames y los análisis microbiológicos y químicos (n =3) fueron sometidos al análisis ANOVA para establecer diferencias que habían resultado de los efectos de la adición JSSE. Se realizó una comparación de los medios mediante el uso honestamente significativo de Tukey prueba (p <0.05). Se analizaron los datos obtenidos de la evaluación sensorial mediante el uso de la prueba no paramétrica de Kruskal-Wallis. En todos los casos, los análisis se llevaron a cabo utilizando el software SAS (SAS Institute, Inc. JMP 5.0.1, Cary, NC, EE. UU.) Y las diferencias entre los tratamientos fueron consideradas significativas con un intervalo de confianza en p <0.05 en todos los casos.

3.3. CARACTERIZACIÓN DE LA FRACCIÓN CON MAYOR ACTIVIDAD BIOLÓGICA PRESENTE EN EL EXTRACTO DE CALAMAR

3.3.1. Fraccionamiento por Cromatografía en Columna Abierta

El extracto de piel de calamar crudo se fraccionó usando la columna de fase líquida para abrir la técnica de columna. Se colocó gel de sílice con un tamaño de partícula de ≤0.063 mm (Merck, Darmstadt, Alemania) como una fase estacionaria en una columna de vidrio, y se usaron una serie de combinaciones de solventes (todas de grado analítico) como la fase móvil.

3.3.2. Cromatografía de Capa Fina

Los compuestos en las fracciones obtenidas previamente se identificaron preliminarmente mediante cromatografía en capa fina (TLC). Se usaron placas de vidrio estático recubiertas con gel de sílice como fase estacionaria, y se usó una combinación de metanol / acetato de etilo / hidróxido de amonio (75: 25: 5) como fase móvil. Las muestras se inyectaron (fracción de 10 µL) y se dejaron correr durante 30 min en una cámara saturada con solventes. Se observó la tasa de flujo (Rf) de las bandas y se calculó para reagrupar a las que exhiben el mismo patrón de separación.

3.3.3. Métodos espectroscópicos

El espectro infrarrojo de la muestra se obtuvo con un espectrómetro Perkin Elmer (Frontier MIR / FIR, Waltham, Massachusetts, EE. UU.). Se realizó una técnica de reflectancia total atenuada (ATR). Los espectros se recogieron a 25 °C entre 4000 y 400 cm-1, acumulando 30 barridos por espectro. Se registró un espectro en blanco para excluir cualquier contaminación cruzada. El espectro se expresó en número de onda (cm-1) versus porcentaje de transmitancia.

El espectro de RMN 1H de la fracción se obtuvo en un espectrómetro de resonancia magnética nuclear Bruker Avance 400 que funciona a 400 MHz. La muestra se disolvió en una mezcla de metanol deuterado (CD3OD) y dimetilsulfóxido, utilizando tetrametilsilano (TMS) como referencia interna. Los cambios químicos se referenciaron a los picos de disolvente, y los valores se registraron en δ. Las multiplicidades de las señales de 1H NMR se indican como *s* (singlete), *d* (doblete) *ym* (multiplete).

3.3.4. Ionización por *electrospray* - espectrometría de masas

El espectro de masas de la fracción se obtuvo usando un espectrómetro de masas (Agilent Technologies 6100 Quadrupole LC / MS, Santa Clara, California, EE. UU.). La muestra disuelta se inyectó en una mezcla de metanol con acetonitrilo. La MS se hizo funcionar en modo negativo para analizar los compuestos presentes en el extracto de piel de calamar. Los datos fueron adquiridos en modo de exploración utilizando un rango *m/z* de 300-650. Se utilizó la técnica ESI porque no es destructiva y, por lo tanto, mantiene la estructura completa de las moléculas en la fracción.

3.3.5. Análisis estadístico

Los datos sobre las actividades antioxidantes de los pigmentos aislados de piel de calamar gigante (JSS) se reportan como promedio de tres determinaciones y analizado utilizando análisis de varianza (ANOVA) con Tukey-Kramer pruebas Los valores de CI50 de las fracciones se obtuvieron mediante un análisis de regresión lineal.

4. PUBLICACIONES.

PUBLICACIÓN 1

New icing media for quality enhancement of chilled hake (*Merluccius merluccius*) using a jumbo squid (*Dosidicus gigas*) skin extract

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New icing media for quality enhancement of chilled hake (*Merluccius merluccius*) using a jumbo squid (*Dosidicus gigas*) skin extract

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Abstract

BACKGROUND: An advanced strategy for chilled fish preservation, based on the inclusion in ice of an extract of jumbo squid (*Dosidicus gigas*) skin (JSS), is proposed. Aqueous solutions including acetic acid–ethanol extracts of JSS were tested at two different concentrations as icing media, with the effects on the quality evolution of chilled hake (*Merluccius merluccius*) being monitored.

RESULTS: A significant inhibition ($P < 0.05$) of microbial activity (aerobes, psychrotrophs, Enterobacteriaceae, proteolytic bacteria; pH, trimethylamine) was obtained in hake corresponding to the icing batch including the highest JSS concentration. Additionally, fish specimens from such icing conditions showed an inhibitory effect ($P < 0.05$) on lipid hydrolysis development, while no effect ($P > 0.05$) was depicted for lipid oxidation. Sensory analysis (skin and mucus development; eyes; gills; texture; external odour; raw and cooked flesh odour; flesh taste) indicated a shelf life extension of chilled hake stored in ice including the highest JSS concentration.

CONCLUSION: A profitable use of JSS, an industrial by-product during jumbo squid commercialisation, has been developed in the present work, which leads to a remarkable microbial inhibition and a significant shelf life extension of chilled hake. In agreement with previous research, ommochrome pigments (i.e. lipophilic-type compounds) would be considered responsible for this preservative effect.

Keywords: Dosidicus gigas; skin; icing media; hake; microbial inhibition; ommochrome pigments.

INTRODUCTION

By-products of marine species are body parts that are usually removed before commercialization to improve preservation qualities, increase the value of the main product or reduce the shipping weight.[1,2] By-products can include heads, blood, bellies, viscera, skin, trimmings, bones and fins. In addition to decreasing waste production, the discards are a valuable source of bio-constituents, such as lipids, proteins and minerals, that could be used for human nutrition, and for their functional properties.[3,4] Squid skin by-product has received considerable attention.[5,6] Although squid skin appears translucent, its coloration comes from yellow, red or brownish –black pigments contained in an elastic sacculus of thousands of neuromuscular organs, called chromatophores, which are located throughout the outer skin layer.[7,8]

Jumbo squid (*Dosidicus gigas*) is one of the biggest known cephalopods and has captured increased technological and com- position interest in a significant number of countries, such as Chile, Peru, Mexico, China and Japan.[5,6] During its processing, up to 60% of its weight is treated as by-products that, nevertheless, may contain biologically active compounds related to antimicrobial[9] and antioxidant[10] activities. However, most previous research has focused on the extraction and characterization of collagen and gelatin.[11,12]

Seafood products undergo a rapid post-mortem quality loss as a result of a wide range of biochemical and microbial degradation mechanisms so that advanced preserving methods are necessary to retain quality.[13-15] This concern is of particular relevance for fish species that are commercialized as fresh products. Among such species *Merluccius merluccius*, commonly known as European hake, is a gadoid fish of considerable commercial acceptance because of its excellent organoleptic properties. Due to the rapid deterioration of European hake, previous research has focused on increasing its shelf life,

including strategies such as slurry ice,[16] controlled and modified atmospheres[17] and the inclusion of natural organic acids in the icing medium.[18] In spite of such technological efforts, quality loss of chilled hake is still a major concern that requires new and complementary strategies.

The present research includes an attempt to use jumbo squid skin (JSS), a by-product of the fishing industry, as a source of natural preservative compounds. Previous research showed that a JSS extract could provide beneficial antioxidant activity when tested in a marine oil system,[19] and displayed effective antimicrobial activity during chilled storage of mackerel (*Scomber scombrus*);[20] preliminary characterization analyses showed that ommochrome pigments present in the JSS extract could be responsible for this preservative effect. In the current study, aqueous solutions containing acetic acid – ethanol extracts of JSS were included into icing media of chilled European hake (*Merluccius merluccius*) at two different levels. The microbial, chemical and sensory quality of hake was evaluated throughout a 13-day chilled storage period.

MATERIALS AND METHODS

Jumbo squid skin: preparation, chemical composition and mutagenicity analysis

Fifty specimens of jumbo squid (*Dosidicus gigas*) were harvested at Guaymas (Sonora, Mexico; 8.75∘ N 112.25∘ W, 15–18 ∘C) in June 2013. Squid specimens were transported to the Seafood Laboratory at the University of Sonora within 8 h. Sizes and weights of specimens were in the 41 –45 cm and 2.2 – 3.1 kg ranges, respectively. The skin, considered a by-product, was separated from other parts such as fins and mantle and excised into portions of ca. 15 cm in length which were freeze-dried (Labonco, Kansas City, MO, USA). Afterwards, portions of 100 mg of the freeze-dried skin were packaged in polyethylene (PE) bags and kept at −25 ± 2 °C. Moisture, crude protein, lipids and ash content of fresh jumbo squid skin (JSS) was determined according to the AOAC procedure.[21] Results are expressed as g kg^{-1}.

The mutagenic potential of JSS extracts was determined according to the procedure described by Maron and Ames.[22] For this assay, Salmonella tester strains TA98 and TA100, with and without bioactivation (S9), were employed, while a nutrient broth (Difco Nutrient Broth, Sparks, MD, USA) was used for bacteria reproduction (12 h, 37

°C). For this, 100 μL of JSS extract were deposited in test tubes (0.1, 0.5, 1 and 5 mg mL^{-1}). Then, each tube was mixed with 2 mL of bacteriologic agar (Sigma-Aldrich, Toluca, Mexico) containing histidine and biotin, bacterial culture (100 μL), and S9 mix (500 μL). The mixture obtained was transferred on minimal glucose agar Petri dishes and incubated for 48 h at 37 °C. All assays were performed in triplicate.

Icing media, hake specimen storage and sampling

Pigment extracts were prepared from portions of 1 g of the freeze-dried skin samples by extraction with 0.5% acetic acid–ethanol solution (v/v) as described elsewhere,[19] the extracts being diluted to 220 mL with such a solvent mixture. Then, 36 and 72 mL of the 220-mL solution were diluted to 6 L with dis- tilled water to obtain C-1 and C-2 ice conditions, respectively. To maintain the same quantity of acid – ethanol solution (namely, 72 mL) in both ice conditions, 36 mL of this solvents mixture were added for the preparation of the C-1 condition solution. Both 6-L solutions were packed in PE bags and kept at −18 ∘C until used as icing media. A control icing medium (C-0 condition) was prepared by diluting 72 mL of the 0.5% acetic acid–ethanol solution to 6 L with distilled water. All solutions were ground into flakes that were used as icing media for hake storage.

Seventy-eight specimens of hake (*Merluccius merluccius*) were caught in the Galician Atlantic coast (north-west Spain), slaughtered onboard and transported on ice to the laboratory in less than 10 h. The length and weight ranges of the specimens were 27 – 34 cm and 175 – 250 g, respectively. Six specimens were grouped into three batches of two specimens per batch and analyzed as initial fish (day 0). The other 72 hake specimens were divided into three batches of 24 units each, and surrounded by C-0, C-1 and C-2 icing media, respectively, with an ice:fish ratio of 1:1. All boxes allowed for the draining of melted ice and were stored in a cold room at 2 ± 1 ∘C. Storage lasted 13 days, with sampling times on days 3, 6, 10 and 13. At each sampling time of the chilled storage, six individuals were taken for analysis from each batch and divided into three groups (two individuals per group) that were studied separately (n = 3). Sensory analysis was conducted on the whole fish and on the fish flesh, while microbiological and chemical analyses were conducted on the white muscle.

Microbiological analyses

Aliquots of fish muscle (10 g) were sampled aseptically, mixed with 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany) in sterile bags (Seward, London, UK) and homogenized in a masticator (AES, Combourg, France), as previously reported.23,24 Serial dilutions (10−1 to 10−8) of these extracts were also prepared in 0.1% peptone water.

Total aerobes and psychrotrophs were enumerated on plate count agar (PCA) (Oxoid Ltd, London, UK) after incubation at 30 ∘C, for 48 h and 7–8 ∘C for 7 days, respectively. In both cases, all colonies were considered to be target bacteria. *Enterobacteriaceae* were enumerated by the pour plate method in violet red bile agar (VRBA) (Merck), after incubation at 37 ± 0.5 ∘C, for 24 h. Only red colonies were counted. In all cases, culture media were prepared according to the manufacturer's instructions. Bacteria exhibiting a proteolytic phenotype were enumerated on casein agar after incubation at 30 ∘C, for 48 h, as previously described.25 Only white or yellow colonies, exhibiting a decolorized halo were counted. Microbial counts were transformed into log CFU g^{-1} muscle, prior to statistical analysis. All analyses were carried out in triplicate.

Chemical analyses

Chemical analyses were performed on hake muscle. All chemicals were reagent grade (Merck).

The pH of hake muscle was determined using a 6-mm diameter insertion electrode (Crison Instruments, Barcelona, Spain). Trimethylamine-nitrogen (TMA-N) formation was determined by the picrate method,[26] using a 5% trichloroacetic acid extract of fish muscle (25 mL per 10 g). Results were expressed as mg TMA-N kg^{-1} muscle.

Lipids were extracted from hake muscle by single-phase solubilization with chloroform/methanol (1:1), as described by Bligh and Dyer.[27] Results were expressed as g lipid kg^{-1} muscle. The free fatty acid (FFA) concentration of the hake lipid extract was determined by colorimetric reaction with cupric acetate –pyridine and absorbance at 715 nm, according to Lowry and Tinsley.[28] Results were expressed as mg FFA kg^{-1} muscle.

The peroxide value (PV) of the hake lipid extract was determined by peroxide reduction with ferric thiocyanate and absorbance at 500 nm (DU 640; Beckman Coulter, London,

UK), as described by Chapman and McKay.[29] Results were expressed as meq active oxygen kg^{-1} lipids.

The thiobarbituric acid index (TBA-i) was determined after the reaction between TBA and a trichloroacetic acid extract of fish muscle, according to Vyncke.[30] The concentration of TBA-reactive substances (TBARS) was determined at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP). Results were expressed as mg malondialdehyde kg^{-1} hake muscle.

Sensory analyses

Sensory analyses were carried out by four experienced judges with more than 15 years of expertise, following the guide of the relevant European Council Regulation31 (descriptors corresponding to the raw state) and previous research32 (descriptors corresponding to the cooked state). Panelists received special training on the evaluation of refrigerated hake specimens exhibiting different qualities. For raw-state descriptors (skin and mucus development, eyes, external odor, texture, gills, consistency and flesh odor), fish specimens were classified in any of the following four categories: highest quality (E), good (A), fair (B) and unacceptable (C). For cooked-state (95 –100 ∘C for 7 min, in a pre-warmed oven with air circulation) descriptors, flesh odor and taste were investigated. Both parameters were evaluated on a scale from 0 (stage of no off-odor or off-taste at all) to 100 (stage where no increase in rancidity is possible); the following categories were ranked: excel- lent (0 – 10, E), good (11 – 29, A), fair (30 –54, B) and rejectable (60 – 100, C).

Specimens were retrieved at each sampling day from each batch. Blind samples in individual trays identified with three-digit random codes were analyzed by the panelists. Sensory evaluation of the raw-state fish was performed first and was followed by the sensory evaluation of the cooked-state fish. Each panelist provided one score for each descriptor in each sample. Results are expressed as mean values among panelists and descriptors in the fresh and cooked states; for it, E, A, B and C scores were given 1, 2,3 and 4 scores, respectively.

Statistical analysis

One-way analysis of variance was employed to explore the significance of the differences among microbiological and chemical data from each batch and sampling time. The least

squares difference (LSD) method was used to compare means while the Kruskal–Wallis test was used to analyze the results of sensory analysis. The PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA) was used. The level of significance between differences was set at 95% ($P < 0.05$). Correlation values among storage time, microbiological data and chemical data were also calculated.

RESULTS AND DISCUSSION

Preliminary analyses of jumbo squid skin

Proximate composition of fresh jumbo squid skin provided the following data (g kg^{-1}): 767.3 ± 26.1 (moisture), 221.4 ± 17.3 (crude protein), 2.2 ± 0.3 (lipids) and 9.1 ± 1.2 (ash). JSS extracts did not induce any mutagenic effect on both Salmonella tester strains (Table 1). Mutagenicity exerted by pigment extracts was considered negative on the basis that the number of revertant colonies counted per plate did not double the number of spontaneous revertants.33 To our knowledge, there is no previous information about the toxicity of squid pigments extracts. The present mutagenicity results related to potential toxicity were similar to those obtained on other natural pigments extracted from several vegetables and fruits by using the Ames test[34] and from an algal heterofucan by employing the *Salmonella* reversion assay.[35]

Table 1. Mutagenic potential of jumbo squid skin (JSS) extracts*

JSS extract (mg mL^{-1})	TA98	TA100
With S9		
SR	23 (2)	251 (1)
0.1	11 (3)	149 (1)
0.5	24 (2)	220 (2)
1.0	25 (2)	233 (8)
5.0	30 (3)	255 (1)
Without S9		
SR	26 (2)	251 (1)
0.1	16 (3)	198 (1)
0.5	17 (4)	201 (9)
1.0	17 (5)	203 (1)
5.0	18 (3)	252 (1)

*Results are given as mean values of three replicates; standard devia- tions are indicated in brackets.
Both Salmonella test strains, TA98 and TA100, with and without bioac- tivation (S9) were exposed to different concentrations of the JSS extracts.
Mutagenicity exerted by pigment extracts was considered negative on the basis that the number of revertant colonies counted per plate did not double the number of spontaneous revertants.
SR, spontaneous revertants.

Assessment of quality evolution during hake chilled storage Determination of microbiological activity by microbial parameters Table 2 displays the aerobic mesophiles and psychrotrophic bacteria counts ound in hake stored with iced media containing various amounts (C-0, C-1 and C-2) of JSS extract. In chilled fish, the growth of psychrotrophic bacteria, such *as Pseudomonas, Shewanella, Acinetobacter, Moraxella and Flavobacterium*, is an important cause of quality loss. Progressive increases ($P < 0.05$) in the aerobic mesophiles ($r^2 = 0.93 – 0.95$) and psychrotrophic bacteria ($r^2 = 0.93 – 0.94$) were observed with storage. However, the icing medium containing the highest amount of JSS extract (namely, the C-2 batch), inhibited both microbes to a greater extent com- pared to the other batches, particularly the control batch. Significant differences among the batches were observed on days 3 (aerobes only), 6 and 10 ($P < 0.05$). The antimicrobial effect of the JSS extract was not significant ($P > 0.05$) for either group of microbes after advanced storage (day 13), at which stage the aerobe counts exceeded 6 log units. The maximum difference between C-2 and C-0 (control batch), 0.64 log units, was observed in the psychrotrophic bacteria on day 10. Thus, a partial antimicrobial effect of JSS extracts on hake muscle was evident.

The *Enterobacteriaceae* and proteolytic bacteria counts found in hake stored (0, 3, 6, 10 and 13 days) with iced media containing various amounts of JSS extract are shown in Table 3. Proteolytic bacteria have a crucial role in the degradation of fish muscle during refrigerated storage. For this reason, the growth inhibition of this microbial group in chilled hake has shown to be of high relevance concerning fish preservation and quality.[36] Both *Enterobacteriaceae* and proteolytic bacteria counts increased ($P < 0.05$) with storage time ($r^2 = 0.88 – 0.92$, quadratic fitting and $r^2 = 0.91 – 0.95$, respectively) in all batches. Lower ($P < 0.05$) counts were assessed for the C-2 batch than in fish corresponding to control condition for *Enterobacteriaceae* (days 3, 6 and 13) and proteolytics (days 6 and 13); differences of 0.99 and 0.50 log CFU g^{-1} for *Enterobacteriaceae* and proteolytic bacteria counts, respectively, were obtained at the end of the study. In contrast, a higher development of both microbial groups was obtained at day 10 in C-2 batch. However, C-2 was the only batch in which *Enterobacteriaceae* and proteolytics counts remained below 6 and 7 log units, respectively, throughout the whole experiment.

Table 2. Assessment of aerobe and psychrotroph counts (log CFU g^{-1} muscle)* in hake muscle stored under different icing conditions (C-0, C-1 and C-2)

	Aerobes			Psychrotrophs		
Chilling time (days)	C-0	C-1	C-2	C-0	C-1	C-2
0	–	3.00 (0.60)	–	–	2.15 (0.15)	–
3	3.71^{ab} (0.24)	3.79^{b} (0.09)	3.38^{a} (0.08)	3.26^{a} (0.21)	3.25^{a} (0.15)	2.79^{a} (0.40)
6	4.76^{c} (0.04)	4.49^{b} (0.03)	4.24^{a} (0.12)	4.21^{b} (0.02)	4.22^{b} (0.14)	3.60^{a} (0.05)
10	5.95^{b} (0.04)	6.01^{b} (0.03)	5.34^{a} (0.48)	5.49^{c} (0.05)	5.27^{b} (0.05)	4.85^{a} (0.35)
13	7.46^{a} (0.05)	7.87^{b} (0.02)	7.19^{a} (0.47)	7.10^{a} (0.06)	7.46^{b} (0.03)	7.27^{a} (0.09)

*Mean values of three replicates ($n = 3$); standard deviations are indicated in brackets.
For each microbial group and chilling time, mean values followed by the same letter are not significantly different ($P > 0.05$).
C-0, C-1 and C-2 denote icing conditions corresponding to 0, 36 and 72 mL of squid skin extract according to the Materials and Methods section.

Table 3. Assessment of enterobacteriaceae and proteolytic counts (log CFU g^{-1} muscle)* in hake muscle stored under different icing conditions (C-0, C-1, C-2)

	Enterobacteriaceae			Proteolytics		
Chilling time (days)	C-0	C-1	C-2	C-0	C-1	C-2
0	–	1.00 (0.00)	–	–	2.45 (0.15)	–
3	3.01^{b} (0.17)	2.66^{a} (0.12)	2.60^{a} (0.04)	3.55^{a} (0.25)	3.33^{a} (0.10)	3.32^{a} (0.22)
6	2.94^{b} (0.01)	2.93^{b} (0.04)	2.19^{a} (0.11)	4.29^{b} (0.06)	4.41^{c} (0.02)	4.11^{a} (0.04)
10	3.57^{a} (0.27)	3.99^{ab} (0.21)	4.32^{b} (0.21)	4.75^{a} (0.27)	5.46^{b} (0.12)	5.34^{b} (0.17)
13	6.49^{b} (0.24)	6.08^{ab} (0.24)	5.50^{a} (0.47)	7.28^{b} (0.17)	7.24^{b} (0.06)	6.78^{a} (0.12)

*Mean values of three replicates ($n = 3$); standard deviations are indicated in brackets.
For each microbial group and chilling time, mean values followed by the same letter are not significantly different ($P > 0.05$). The icing conditions (C-0, C-1 and C-2) are as indicated in Table 2.

The microbiological data showed that the presence of JSS extract in the icing medium (namely, the C-2 batch) had a significant antimicrobial effect during hake storage. The preservative effect (i.e. antioxidant behavior) of an acetic acid – ethanol extract of JSS has been previously reported.[19] Characterization analyses [solubility in various solvents, UV-visible absorption and Fourier transform infrared (FTIR) spectra] showed that ommochrome pigments present in a lipophilic-type JSS extract were responsible for the antioxidant activity. Indeed, xanthommatin (a pyrido[3,2-a]phenoxazine ring system) was found in the JSS extract, in agreement with Ferré et al.,[37] who selectively obtained this compound with dihydroxanthommatin in acidified (i.e. HCl) n-butanol. Additionally, the acidified (i.e. acetic acid) ethanol JSS extract displayed antimicrobial activity during the chilled storage of a pelagic fatty fish species (Atlantic mackerel, *Scomber scombrus*).[20]

Ommochrome compounds are tryptophan metabolites and include several biological pigments that exist in the eyes of crustaceans and insects, as well as in chromatophores of cephalopods.[7,8] The antioxidant and anti-radical activity of ommochrome compounds have been previously reported.[38,39]

However, except for the above-mentioned research on chilled mackerel,[20] to the best of our knowledge no previous information concerning its antimicrobial activity is available. Previous research on the non-lipophilic fractions of JSS includes the isolation and chemical characterization of collagen and gelatin,[12] particularly on their ability to inhibit lipid oxidation.[10] Additionally, enzymatic hydrolysates prepared from JSS have been characterized, showing antimicrobial9 and antioxidant[11] activities. In contrast, the present study focused on a lipophilic extract of JSS that proved to exhibit a substantial antimicrobial activity. However, further research is needed to investigate the role of ommochrome pigments as antimicrobial compounds; as electron donors, ommochrome compounds could be responsible for inducing an imbalance in metabolic pathways of microorganisms.[39]

Determination of microbiological activity by chemical indices

A progressive pH increase with chilling time ($P < 0.05$) was observed in all hake batches ($r^2 = 0.86 – 0.89$) (Fig. 1). Fish specimens corresponding to the C-2 batch exhibited a lower pH value than their control counterparts throughout the 6- to 13-day period. Lower pH values were also found in the C-1 batch for the 10- to 13-day period, compared to its corresponding control. Accordingly, the presence of the JSS extracts helped minimize the pH increase in hake during chilled storage, particularly as the amount of JSS extract in the icing media increased. It has been suggested that at pH > 7 the shelf lives of certain fish species, such as hake, is limited.[40] In the present study, only control samples exceeded pH 7 at the end of the storage time.

An increase in the pH of hake muscle indicates the accumulation of alkaline compounds, such as ammonia and TMA, which are principally derived from microbial activity.[15] High correlation values were obtained between the pH value and microbial counts (i.e. aerobes, psychrotrophs, and proteolytic bacteria; $r^2 = 0.83 – 0.93$) in the present study.

In agreement with the results of this study, JSS treatment led to lower pH values in mackerel (*Scomber scombrus*) muscle during chilled storage[20] compared to the control

(without JSS). Similar observations have been reported in chilled hake when natural organic acids were included in the icing system.[36]

A significant increase ($P < 0.05$) in TMA-N content (Fig. 2) was determined in all batches with chilled storage ($r^2 = 0.86 – 0.89$, quadratic fitting). This increase was particularly apparent at the end of the storage time (day 13). However, TMA-N values remained below 25 mg kg^{-1}, in agreement with previous reports on hake.[18,41] Generally, lower mean TMA values were determined in hake batches stored with icing media containing the most amount of JSS extract (C-2), compared to other batches, and significantly ($P < 0.05$) different than the C-0 batches throughout the entire storage time (3 –13 days). An inhibitory effect on TMA formation was also observed in hake corresponding to the C-1 (6- to 10-day period) batch. Volatile amine compounds, such as TMA, are produced partially as a result of endogenous enzyme activity, but primarily as a result of microbial development.13,15 The TMA con- tents determined in this study showed a moderate correlation with microbial counts (i.e. aerobes, psychrotrophs, and proteolytic bacteria) ($r^2 = 0.79 – 0.87$). In agreement with the microbial data, chemical assessment (i.e. pH and TMA-N) showed an antimicrobial effect in the presence of the JSS extract (namely, C-2 batch) in the icing medium. As mentioned above, ommochrome pigments extracted from JSS by acid – ethanol solutions were probably responsible for this preservative effect.

Chemical assessment of lipid damage development

Lipid hydrolysis was measured as FFA content (Table 4). FFA formation ($P < 0.05$) increased with chilled storage ($r^2 = 0.90 – 0.92$), resulting in 145 – 159 mg FFA kg^{-1} muscle at day 13. A sample comparison showed lower mean FFA values in fish specimens corresponding to the batch with the highest JSS content (C-2 batch), and a significant decrease ($P < 0.05$) compared to the control batch on days 3 and 10. In contrast, the FFA contents of the C-1 batch were similar to the control batch.

FFA is formed during chilled fish storage as a result of endogenous enzyme activity and microbial activity.13,15 Prior to the end of the microbial lag phase (up to 6 – 9 days, depending on several factors), FFA formation is primarily a result of the endogenous enzyme (namely, lipases and phospholipases) activity. Subsequently, however, microbial activity becomes important due to bacterial catabolic processes. Consequently, in the present study, the C-2 icing condition prevented lipid hydrolysis

development by limiting FFA formation due to microbial activity. FFA content showed high correlation with the microbial counts (i.e. aerobes, psychrotrophs and proteolytic bacteria) (r^2 = 0.91 – 0.96) and moderate correlation with other chemical parameters related to microbial development, such as pH (r^2 = 0.80 – 0.93) and TMA-N content (r^2 = 0.68 – 0.85).

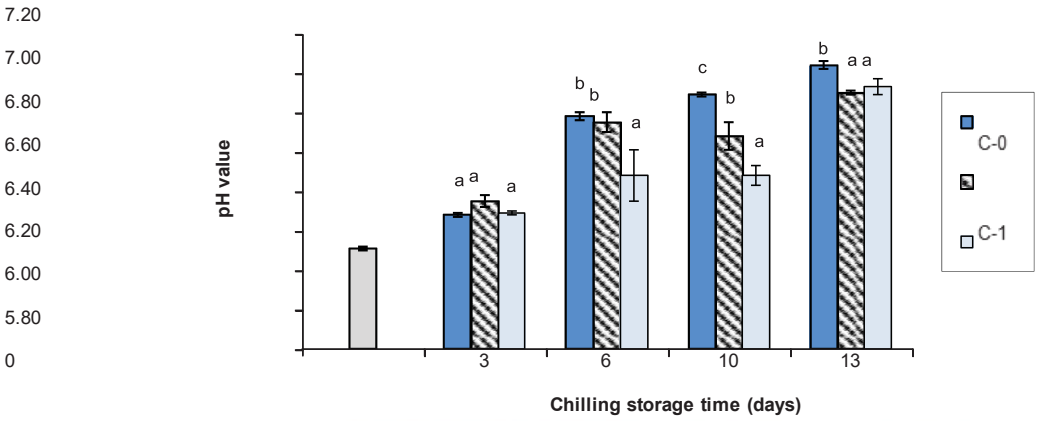

Figure 1. Evolution of the pH value in hake muscle stored under different icing conditions, as indicated in the footnote to Table 2. Results are the mean values of three replicates (n = 3); standard deviations are indicated by bars. For each icing time, values accompanied by the same letter are not significantly different (P > 0.05).

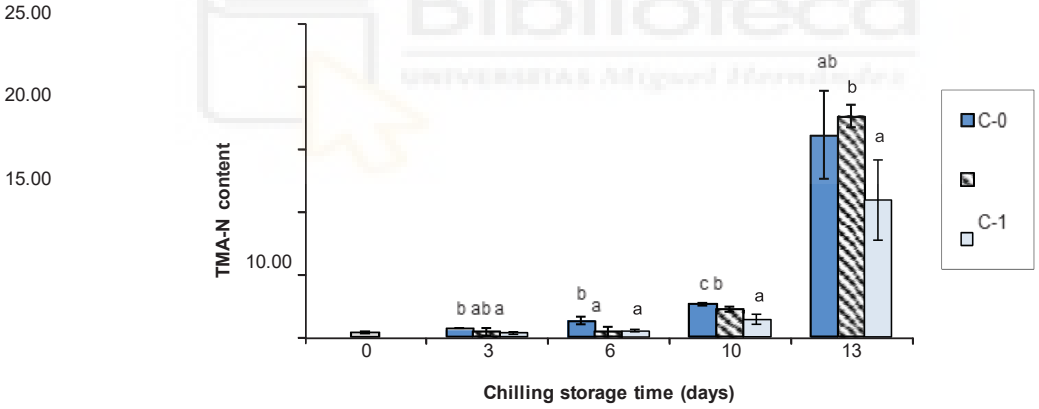

Figure 2. Trimethylamine assessment (mg TMA-N kg^{-1} muscle) in hake muscle stored under different icing conditions, as indicated in the footnote to Table 2. Results are the mean values of three replicates (n = 3); standard deviations are indicated by bars. For each icing time, values accompanied by the same letter are not significantly different (P > 0.05).

The inclusion of natural organic acids (citric and lactic acids) in the icing medium did not lead to an inhibition of FFA formation throughout the chilling storage of hake;18 additionally, no effect was also observed by including a rosemary extract in the icing system during chilled sardine storage.[42] However, an inhibitory effect on FFA formation has been reported by the inclusion of natural compounds on other fish species. This accounts for organic acids (citric, lactic and ascorbic) during megrim and angler storage,43 and rosemary and oregano extract in chilled Chilean jack mackerel.[44]

Lipid oxidation development was evaluated by the peroxide and TBA values. A very low peroxide formation (PV < 4.0) was observed in all hake batches (Table 4). Generally, lower mean PVs were determined in hake specimens corresponding to the C-2 batch, compared to the other batches, and was significantly lower than the control batch at day 10 ($P < 0.05$). TBARS formation showed a slight increase ($P < 0.05$) with storage time ($r^2 = 0.75 – 0.92$, quadratic fitting), particularly at the end of the storage period. A low formation of secondary lipid oxidation compounds was also depicted; TBARS values did not exceed 0.46 in any of the batches. This low oxidation rate in chilled hake[16,43] is expected given that lipid oxidation development is not a crucial degradation pathway during the chilled storage of lean (present lipid content of hake: 4.9 – 6.1 g kg^{-1} muscle) fish species. In the present study, some significant differences in TBARS values were observed among the batches ($P < 0.05$). However, a general trend concerning the effect of the JSS extract in the icing medium on PV and TBARS in the stored hake could not be concluded.

As above mentioned, a previous study also investigated the preservative effect of an acetic acid –ethanol extract of JSS.[19] Such work reported the partial inhibition of lipid oxidation and a better protection of polyunsaturated fatty acids. Based on the solubility behavior in different solvents, as well as on the UV-visible absorption and FTIR spectra, such activity was attributed to the presence of ommochrome compounds, whose antioxidant effect had previously been reported.[38,39]

Table 5. Sensory assessment* in hake fish stored under different icing conditions (C-0, C-1, C-2)

Chilling time (days)	Descriptors corresponding to the raw state			Descriptors corresponding to the cooked state		
	C-0	C-1	C-2	C-0	C-1	C-2
0	–	E	–	–	E	–
3	A	A	A	A	A	A
6	B	B	A	A	A	A
10	B	B	A	B	B	A
13	C	C	B	C	C	B

*Freshness categories: E (excellent), A (good), B (fair) and C (unaccept- able).
The icing conditions (C-0, C-1 and C-2) are as indicated in Table 2.

The antioxidant activity of JSS compounds other than lipids has also been reported. As mentioned above, collagen and gelatine preparations,[10] as well as their enzymatic

hydrolysates,11 showed a marked antioxidant activity. Despite these previous results, the current study did not provide a conclusive antioxidant activity for the JSS extract. As mentioned above, this could be due to the lack of lipid oxidation development in the present study.

Determination of sensory acceptance

Sensory analysis was carried out on descriptors corresponding both to the raw and cooked fish quality (Table 5). The excellent quality described for the initial fish was progressively lost through- out chilled storage in all batches. This quality decrease was found to be faster than in previous research by Ruiz-Capillas and Moral[40] and Baixas-Nogueras et al.,[41] who observed longer shelf life times in non-treated hake (20 –29 days). The difference could be due to the relatively smaller size of the hake specimens considered in the present study, as previously described.[16,18]

Descriptors corresponding to the raw fish showed a higher quality in samples corresponding to the C-2 batch, compared with fish kept under any other condition (6- to 13-day period). Indeed, individuals from such a batch were still acceptable at the end of the storage period, whereas those of other batches were not acceptable. Concerning the descriptors corresponding to the cooked fish, higher quality retention was also observed in the C-2 batch at advanced storage times (10- to 13-day period). Indeed, only the C-2 batch was found acceptable at the end of the storage time. Fish specimens from the C-1 batch did not provide differences with those belonging to the control, according to the raw and cooked fish quality evaluations.

The high sensory acceptance and extended shelf life of fish corresponding to the C-2 batch are in agreement with the above-mentioned results, concerning the microbiological and chemical quality indices. A high sensory acceptance was also observed in chilled mackerel (*Scomber scombrus*) in the presence of JSS extract.[20] Previous studies also reported a sensory quality enhancement and increased shelf life in chilled hake as a result of including natural preservative compounds in the icing medium.[18,43] Concerning other fish species, enhanced sensory quality was observed by using a rosemary extract for the chilled storage of sardine[42] and organic acids for the chilled storage of angler.[43]

CONCLUSIONS

An advanced preservation strategy based on the inclusion of a lipophilic extract of JSS in the icing medium of chilled hake was developed. When employed at the highest concentration, such extract provided a significant ($P < 0.05$) antimicrobial effect on hake muscle during chilled storage up to 13 days; moreover, a significant ($P < 0.05$) inhibitory effect on lipid hydrolysis development could be depicted. The evaluation of sensory quality also confirmed that hake specimens in contact with the highest concentration of JSS extract exhibited extended shelf life as compared to other batches, being acceptable even after 13 days of chilled storage. In agreement with previous research, ommochrome pigments (i.e. lipophilic-type compounds) would be considered responsible for this preservative effect. Thus, the contact of lipophilic ommochrome pigments with the fish surface during ice melting may explain the preservation effect of the ice observed in this study, although further research would be necessary to confirm the nature and action mechanism of the bioactive compounds. This work opens the way to the utilization of an abundant by-product of jumbo squid processing as a natural source susceptible to provide preservative compounds to be employed during the commercialization of marine species.

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PUBLICACIÓN 2

Jumbo squid (*Dosidicus gigas*) skin pigments: Chemical analysis and evaluation of antimicrobial and antimutagenic potential

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PUBLICACIÓN 2: TRANSCRIPCIÓN LITERAL

JUMBO SQUID (*Dosidicus gigas*) SKIN PIGMENTS: CHEMICAL ANALYSIS AND EVALUATION OF ANTIMICROBIAL AND ANTIMUTAGENIC POTENTIAL

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ABSTRACT

There is a great potential to use seafood by-products to create new beneficial products for customers. In a continued exploration of new chemical compounds from seafood by-products, jumbo squid (*Dosidicus gigas*) skin pigmented methanolic extracts (JSSE) were

evaluated for their antimicrobial and antimutagenic activities. Pigments of JSSE were extracted with a yield of 635 mg/g and oxygen radical absorbance capacity-fluorescein (ORAC) with 178 μmol TE/g JSSE using optimal conditions: 25 °C and 5 min of sonication, established by factorial analysis. The antimicrobial activity of JSSE was evaluated using the agar diffusion method. The JSSE showed more than 50% inhibition against *Haemophilys influenza*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Candida albicans*. The high antimicrobial activity of JSSE (<90%) was detected in *Salmonella enterica*. The JSSE also inhibited mutation induced by aflatoxin B_1 in the *Salmonella tryphimurium* strain TA98 (>50%), but not in the TA100 strain (<20%). Data on the solubility behaviour, the maximum absorbance (440 nm), protons observed in the ^{1}H NMR spectra, and the FT-IR spectra peak at 1742 cm^{-1} of JSSE, suggest that the compound responsible for its antimicrobial and antimutagenic activities comes from the ommochrome family. The present study suggests that squid skin ommochromes are pigments of therapeutic value in near future applications in the food or health sector.

Keywords: antimutagenic activity; antimicrobial activity, extraction optimization; FT-IR, ^{1}H NMR; squid skin ommochromes

INTRODUCTION

Due to the development of infectious diseases and degenerative processes associated with reactive oxygen species, the interest in finding natural compounds that can replace synthetic drugs, and which are safe and wholesome is fuelling one of the fastest expanding fields across several industries. In response to this trend, natural dyes and pigments from several food by-products are being used as food and cosmetic additives, among others things (**Helkar *et al*., 2016**). Among seafood by-products, jumbo squid skin is a valuable, underutilized source of pigments (**Aubourg *et al*., 2016**).

Marine organisms develop an extraordinary range of diverse compounds, including pigments with antioxidant, antimicrobial, and antimutagenic activities (**Aquil *et al*., 2011**). The pigments found in marine organisms, mostly distributed in the fatty tissues of marine fish and invertebrates, are usually synthesized within the tissues of photosynthetic bacteria, algae and higher plants, being the phycobilins, melanins, and carotenoids being the most studied pigments from seafood (**Alasalvar and Taylor, 2002**). Among the

compounds responsible for the colour in the cephalopods are ommochromes, which are mainly synthesized in the skin of marine molluscs (**Shamim *et al.*, 2014**). These chromatophores appear as small dots and contain red, yellow or brownish-black pigments. By controlling the size of the cells, they can vary their colour and even create changing patterns. Chromatophores are connected to the nervous system, and their size is determined by muscle contractions (**Deravi *et al.*, 2014).** The metabolic precursor of these pigments is the amino acid tryptophan, from which compounds of varied shades are derived, such as ommatins (low molecular weight, thermolabile and of a faint colour) and ommins (high molecular weight, thermostable and which are related to intense colorations) (**Sahmim *et al.*, 2014**).

Ommochromes, like other pigments, produce colour in the biological system, preventing peroxidation in cellular liposomes caused by UV radiation (**Dontsov *et al.*, 1999; Sahmim *et al.*, 2014**) as well as functioning in the tryptophan detoxification process (**Figon and Casas, 2019**). The potential mechanism of action and reactivity of these molecules, established through theoretical studies, could exist by transferring electrons or transferring the hydrogen atom or both, depending on the chemical structure of the ommochrome (**Romero and Martinez, 2015**). In some cephalopod species, like *Loligo vulgaris*, *Seppia officinalis*, *Octopus vulgaris* and, *Doryteuthis pealeii* the main ommochrome identified is xanthommantin (**Bolognese and Scherillo, 1974; Willimas *et al.*, 2016**).

Another species who synthesizes ommochromes is jumbo squid (*Dosidicus gigas*), and they have been mainly found in its skin. Normally, this anatomical region is discarded. The information on jumbo squid skin mostly comprises collagen and its products (**Ezquerra-Brauer and Aubourg, 2019**). Based on the available scientific literature, there is little information about the functional properties of skin ommochromes. Recently, it has been discovered that ommochromes from jumbo squid skin retarded the oxidation of fish oil (**Aubourg *et al.*, 2016**) and prolonged the shelf life of two stored fish species in ice, linked to antioxidant and antimicrobial activities of these extracts (**Ezquerra-Brauer *et al.*, 2016, 2017)**. These pigments showed a yellow colour and absorbance peaks in the 300—450 nm region, and had an FT-IR spectrum that showed the presence of functional groups associated with the presence of ommochromes (**Aubourg *et al.*, 2016**).

To explore a novel source of compounds with multiple potentials, the aim of this study was to document the antimicrobial and antimutagenic potential and chemical structure of

pigmented compounds extracted from jumbo squid skin. This is the first study of ommochromes' antimicrobial activity against specific strains of bacteria and fungi, as well as their antimutagenic activity. The chemical characteristics of the extracted bioactive pigments was studied by analysing their physical and chemical characteristics. The results of this study provide a more information for the use of jumbo squid skin as another alternative source of bioactive pigments with biological activity.

MATERIAL AND METHODS

Materials

Ten jumbo squids (*D. gigas*) were purchased from a local establishment in Hermosillo, Mexico (29°05′56″n 110°57′15″w), and immediately skinned. The length and weight of the squid specimens ranged from 100 to 150 cm and from 40 to 60 kg, respectively. The skin (about 50 cm length) was frozen at –80 °C, freeze-dried (Labconco, Kansas City, MO, USA) for 2 days and grinded. Samples (100 mg) were put in polyethylene bags, vacuum sealed and kept at –20 °C until analyses. All chemicals used were of analytical reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pigment Extraction

Freeze-dried skin is a mixture of mainly protein and pigments; acidified methanol does not dissolve collagenous and stromal proteins and, at the same time, it is recommended as an ommochrome remover (**Van den Branden and Decleir, 1976**). Therefore, in this work pigment extraction was prepared with acidified methanol. The extraction method consisted of treatments of different temperatures temperature (25, 35, and 45 °C) and sonication times (5, 10, and 15 min). Suitable conditions for obtaining pigmented extracts were established by factorial design in which the dependent variables were yield and antioxidant activity and the independent variables were temperature and sonication time.

Briefly, the pigment extraction process consisted of the homogenization of 20 volumes of freeze-dried skin (w/v) in acidified methanol (99:1 methanol:HCl), followed by centrifugation (Model Biofuge Stratos, Thermo Scientific, Germany) at 10,000 × *g* for 15 min. The methanol was removed using a rotary evaporator (R-100, Büchi, Switzerland) and further evaporated using nitrogen gas. The dry extracts were stored in an inert nitrogen atmosphere, at –80 °C, prior to further analysis. The dried yield was calculated, and stock solutions were prepared to assess antioxidant activity.

Extraction yield was calculated gravimetrically, using the weight of the skin sample as a reference. Pigment yield was calculated as follows:

Pigment yield (%)= [(dried pigmented extract (g))/(dried squid skin (g))]×100.

The antioxidant activity was established by the oxygen radical absorbance capacity (ORAC) method. The ORAC method was carried out according to previous methodology (**Garret *et al.*, 2010**) but with modifications. The fluorescence loss of fluorescein was monitored during 90 min at 37 °c in the presence of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Each sample (0.5 mg/ml) was tested in triplicate and compared with a standard curve to express results as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents.

Extraction conditions for measuring the antimicrobial and antimutagenic activities were selected as the better conditions (temperature and sonication time) for yield and antioxidant activity.

Antimicrobial Activity

The antimicrobial effects of the extracts (10 mg of extract) were assessed following the disc diffusion test as reported previously (**Fatrcová-Šramková *et al.*, 2016**). Antimicrobial activity of the jumbo squid skin pigmented extracts (JSSE) were tested against three Gram-negative bacteria (*Haemophilus influenza* CCM 4456, *Klebsiella pneumoniae* CCM 2318, *Salmonella enterica* subs. enterica CCM 3807), four Gram-positive bacteria (*Bacillus cereus* CCM 2010, *Clostridium perfringens* CCM 4991, *Listeria monocytogenes* CCM 4699, *Staphylococcus aureus* subs. aureus CCM 2461), six microscopic filamentous fungi (*Aspergillus clavatus*, *A. flavus*, *A. versicolor*, *Penicillium chrisogenum*, *P.* griseofulvum, *P. expansusm*) and three yeasts (*Candida albicans* CCM 8186, *C. glabrata* CCM 8270, *C. tropicalis* CCM 8223). Bacteria were collected from the czech collection of microorganisms and microscopic filamentous fungi were collected from the Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Slovakia. The inhibition of microbial growth was measured around the impregnated discs. Antimicrobial activity is considered high, moderate, or trace/zero when the zone diameter is > 10 mm, 5–10 mm or 2–5 mm, respectively, and negligible effect when the value is less than 2 mm (**Boo *et al.*, 2012**).

Antimutagenic Activity

The Ames test was used to evaluate the antimutagenic activity of the squid skin extracts (**Maron and Ames, 1983**). The assay was performed using 100 µL of *Salmonella typhimurium* strains T98 and T100 grown overnight (1x109 cells/mL), 100 µL of pigment extracts (0.005, 0.05, 0.5, and 5.0 mg/mL) and the mutagenic agent (Aflatoxin B_1, AFB_1) with activation system (500 µL S9 mix) in triplicate plates. Ten percent DMSO (100 µL) without AFB_1 was used as negative control. After incubation for 48 h at 37°C, the number of revertant bacterial per plate were counted. The inhibition rate for mutagenic activity was calculated using the following equation:

$$\text{Inhibition rate (\%)}=[((1—T))/M]\times 100,$$

where T is the number of revertants per test sample plate in the presence of AFB1, and M is the number of revertants per plate in the positive control, after subtracting the number of spontaneous revertants from the numerator and denominator. The AFB1 mutagenesis inhibition is considered strong, moderate or weak when the values are high tan 60%, 40–60% or 20–40%, respectively, and negligible when the value is lower than 20% (**Ikke *et al.*, 1999**).

Chemical Structure Analysis

For the analysis the JSSE were freeze-dried and then evaluated.

The solubility test was performed using 5 mL of the following solvents: acetone, ethyl ether, chloroform, 77% aqueous sulfuric acid, and methanol—2% HCl. In each solvent, 5 mg of freeze-dried extracts was dissolved and stirred for 5 min at 24°C (**Van den Branden and Decleir, 1976**).

The absorbance of the extracted pigments was measured using a Cary 50 spectrophotometer (Agilent Technologists, Ciudad de México, México) over the wavelength range of 200–600 nm. The blank solution was methanol.

Fourier transform-infrared spectrum of extracted pigments was obtained from pellets, prepared with 1 mg sample and 100 mg of dry potassium bromide (KBr). The spectra were recorded using and infrared spectrophotometer, Perkin Elmer FT-IR Spectrum GX (Waltham, MA, USA). The FT-IR spectrum (16 scans) was analysed in transmittance mode between 400 and 4000 cm^{-1}.

Then, ^{1}H NMR analysis was measured at 25 °C on a Bruker Avance 400 nuclear magnetic spectrometer (Billerica, MA, USA) operating at 400 MHz. For the experiments, approximately 1 mg of freeze-dried pigments was dissolved in 0.5 ml of a 1 % (v/v) deuterated potassium hydroxide 40% solution with deuterated water. Dimethylsilapentane-5-sulfonic acid (DSS) was used as a reference. The spectral window was 20 ppm.

Statistical Analysis

A 3X3 factorial randomized complete block design was used to obtain an optimal combination of temperature and sonication time that yielded a high level of pigmented extracts with the high antioxidant activity. The selection of the levels of temperature (25, 35 and 45°C) and sonication time (5, 10, and 15 min) tested was based on preliminary studies. The experiment design and statistical analysis were carried out using JMP software (SAS, Cary, NC, USA). Differences between the means were compared using Tukey´s test ($p<0.05$).

Data of the jumbo squid extracted pigment´s physicochemical characterization, antimicrobial and antimutagenic activities were based on the average of three determinations. For spectroscopic analysis, descriptive statistics were used to analyse the data (**Glover and Mitchell, 2015**). For solubility test and antimicrobial and antimutagenic activities, the variations among replicates was <5%. The mean values of the three trials and standard deviations were calculated.

RESULTS AND DISCUSSION

Pigment Extraction

The results of yield and antioxidant activity (Tab 1) indicated that a high yield and high antioxidant activity were obtained when applying a combination of 25°C and 5 min of sonication time. The optimal combination of temperature and sonication time was established by factorial analysis. When the effect of both factors was evaluated, it was observed that the levels of temperature and sonication time affected both positively and negatively the dependent variables ($p < 0.05$). Additionally, an interaction between both factors ($p < 0.05$) was found. The yield of all treatments ranged between 580 and 690 mg of pigment extract per 100 g of fresh squid skin, whereas the antioxidant activity was between 80 and 178 (µmol TE/g). The extraction yield of pigments increased because

the sonication time was longer (Tab 1). However, the prolonged exposure of samples to ultrasonic sounds can render antioxidant compounds inactive (Tab 1).

Table 1 Yield and antioxidant activity of pigmented extracts with different temperature-sonication treatments[1] from jumbo squid skin.

Temperature[2] (°C)	**Sonication Time**[3] (min)					
	5		10		15	
	Yield (mg/100 g skin)	**Antioxidant Activity** (μmol TE/g)	**Yield** (mg/100 g skin)	**Antioxidant Activity** (μmol TE/g)	**Yield** (mg/100 g skin)	**Antioxidan Activity** (μmol TE/g)
25	650 ± 5.5^{Bb}	178 ± 2.1^{Aa}	650 ± 5.5^{Bb}	168 ± 3.5^{Ab}	638 ± 6.2^{Ca}	128 ± 2.8^{Ac}
35	659 ± 4.1^{Bb}	150 ± 4.0^{Ba}	659 ± 4.1^{Bb}	135 ± 1.1^{Bb}	685 ± 8.9^{Ba}	115 ± 1.7^{Bc}
45	679 ± 6.1^{Ab}	130 ± 5.0^{Ca}	679 ± 6.1^{Ab}	90 ± 7.5^{Cb}	690 ± 4.1^{Aa}	80 ± 5.0^{Cc}

[1]Values are the mean of three repetitions±standard deviation.
[2]Capital letters in columns denote differences by effect of the temperature ($p < 0.05$).
[3]Small letters in rows denote differences by effect of sonication time ($p < 0.05$).

The two variables used in this study have been previously reported as relevant to the extraction and antioxidant activity of several biological compounds, including pigments (**Maran *et al.*, 2015**; **Belwal *et al.*, 2016; Mokrani and Madani, 2016**). It has been reported extensively that temperatures above 30 °C help with the extraction of biologically active compounds (**Maran *et al.*, 2015**; **Belwal *et al.*, 2016; Mokrani and Madani, 2016**). As can be observed in table 1, for JSSE pigmented extracts, temperature had a significant effect on the extraction yield; however, when temperature increased above 35 °C, antioxidant activity decreased. This type of behaviour has been observed in other studies that dealt with the extraction of antioxidant compounds (**Michiels *et al.*, 2012**). Maintaining 25 °C makes the extraction both cheaper and safer, avoiding the generation of vapours and the usage of heat plates or heat sources. Another advantage is assuring the preservation of the antioxidant activity of the pigmented extract.

The use of sonication in the extraction of compounds has been extensively reported. Sonication facilitated the lysis of the cells in which the pigments are occluded. Its effectiveness in squid skin relies on the formation of vacuum bubbles in the solvent because of low-pressure and high-pressure cycles mediated by the ultrasonic waves. When the bubbles implode, the saccules that contain pigments and other compounds soluble in methanol are released. The mechanical forces eject the compounds, which are later recuperated. Similar patterns to the results obtained in this work, were observed in

other foodstuffs from different origins (**Altermimi *et al.*, 2015**). The energy release from sonic waves is not completely efficient; some of it is liberated to the environment and eventually ends up generating free radicals via sonolysis in water and aqueous solutions (**Castellanos *et al.*, 2001**). Evidence has been found of the ultrasound-mediated formation of free radicals in red wine, specifically hydroxyethyl radicals (**Zhang *et al.*, 2015**). It is theorized that the antioxidants exert their function with these molecules, thus resulting in a decrease of functionality (**Zhang *et al.*, 2015**).

The best combinations of temperature and sonication conditions yielded 635 mg/100 g JSS and 178 μmol TE/g JSSE hydrogen atom transfer capacity (ORAC test). Previously it was detected that jumbo squid pigmented extracted with ethanol-acetic acid (**Aubourg *et al.*, 2016**) measured using the ORAC assay showed a value of 15.4 μ mol TE/g. Therefore, JSSE contains redox components which are ten times more active than those previously reported. Under these conditions, JSSE pigments were extracted to evaluated their potential antimicrobial and antimutagenic activities.

Antimicrobial Activity

The analysis results of antimicrobial activity of JSSE against selected microbes are shown in table 2. *Haemophilus influenza*, *Salmonella enterica* of Gram–negative bacteria, *Listeria monocytogenes*, *Satphlococcus aureus*, of Gram–positve bacteria, *Aspergillus clavatus*, *Penicillium expanssum*, of fungi, and *Candida albicans* of yeast showed a clear zone formation of growth inhibition. Antimicrobial activity in *Bacillus cereus*, *Klebsiela pneumoniae*, from microscopic fungi *Penicillium chrisogenum* and, from candida *Candida tropicalisscore*d was relative low compared to other strains. The JSSE in the case of *S. enterica* showed the high antimicrobial activity.

Table 2 Antimicrobial effect of the squid skin extract on bacteria, yeasts, and fungi[1].

Microorganism	Inhibition zone size (mm)[2]	Inhibition (%)
Bacteria		
Bacillus cereus	T	39.4 ± 0.3
Clostridium perfringens	T	45.5 ± 0.8
Haemophilus influenza	M	54.5 ± 0.4
Klebsiella pneumoniae	T	39.4 ± 0.4
Listeria monocytogenes	M	60.7 ± 0.1
Staphylococcus aureus subs. Aureus	M	57.8 ± 1.3
Salmonella enterica subs. Enterica	H	93.9 ± 0.3
Fungi		
Aspergillus flavus	T	42.4 ± 2.1
Aspergillus versicolor	T	42.4 ± 1.7
Aspergillus clavatus	M	48.4 ± 0.8
Penicillium chrisogenum	T	39.4 ± 3.2
Penicillium griseofulvum	T	42.4 ± 2.4
Penicillium expansum	M	48.5 ± 1.1
Yeast		
Candida albicans	M	66.7 ± 1.5
Candida tropicalis	T	33.3 ± 2.3
Candida glabrata	M	42.4 ± 0.2

[1]**Data:** mean±standard deviation of three repetitions.
[2]**Legend**: H – > than 10 mm, M – > 5–10 mm, T– > 2–5 mm.

The antimicrobial activity detected in JSSE pigments could be due to the amphipathic nature of the ommochromes that gives them the ability to interact with cell membrane components, as well as other bacterial protection factors. At this time a widespread range of natural substances are recognized as having antimicrobial activity, but few studies related to antimicrobial efficacy of squid skin pigments have been done, and some are not made up. Some mechanisms of antibacterial activity, of similar compounds to those reported in the squid skin, are (i) the ability to form pores in cells and (ii) breaking cell walls (**Senan, 2015**). As to the antifungal activity, the main mechanisms recognized for this are attacks on the membrane, microtubules, RNA, and synthesis of ergosterol, among others. However, in the case of the compounds present in the sepia ink, the antifungal activity was related to an imbalance in the redox balance of the fungus (**Fahmy et al., 2014**).

Antimutagenic Activity

Antimutagenic activity of squid skin pigments has not been previously reported. Although, the pigments decreased the revertants/plate in a dose-response relationship in both *S. typhimurium* TA98 and TA100 strains (Fig 1), only in TA98 was the percentage of inhibition considered effective, from strong (49–87 %) to moderate (38 %), and a very low inhibition percentage was observed in *S. typhimurium* TA 100 (<14%).

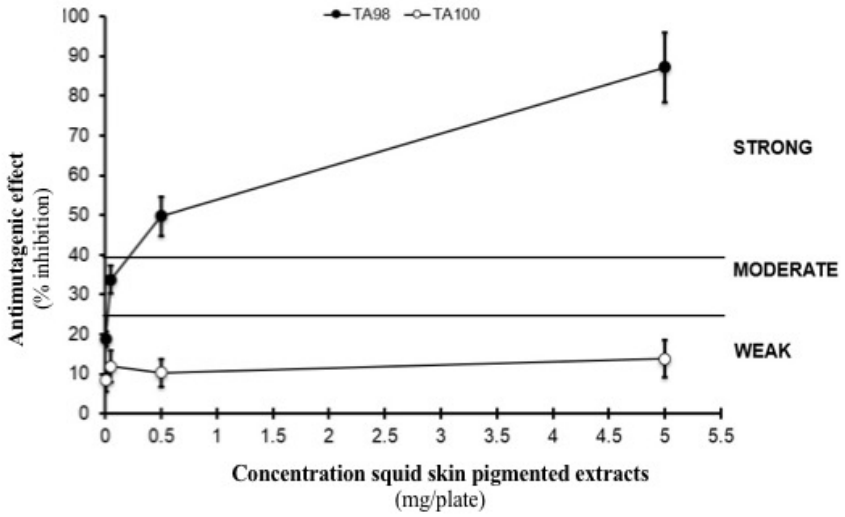

Figure 1. Effect of jumbo squid skin pigments on the mutagenicity induced by aflatoxin B_1, based on *Salmonella typhymurium* TA 98 and TA 100. All values represent mean value of triplicate determination±standard deviation.

The very low inhibition percentage observed in *S. typhimurium* could be due to the complexity of the sample. Therefore, these results suggested that the extracted pigments only protect the genetic material against only one type of mutation, a frameshift mutation detected by TA98 strain, and not a base pair substitution, because the pigments were not capable of producing at least a moderate inhibition of mutation induced by AFB_1 in TA100 strains (**Jurado *et al.*, 1993**).

It is known that mutations induced by numerous mutagens were reduced by active oxygen scavengers (**Osuna *et al.*, 2016**). Furthermore, it was reported that some antioxidant compounds could prevent mutations because they can induce the synthesis of antioxidant enzymes (**Alasalvar and Taylor, 2002**). In the case of ommochromes, which are the main class of pigments in cephalopods, they have been reported to act as electron accepting or donating systems, as well as tryptophan detoxification products (**Shamim *et al.*, 2014**).

Chemical Structure Analysis

The reddish colour of JSSE suggests that certain types of ommochromes compounds exist in the obtained extract (**Van den Branden and Decleir, 1976**). To corroborate the nature of the components in the JSSE, solubility tests were performed (Tab 3), and the behaviour detected was similar to that expected for ommochrome (**Van den Branden and Decleir,**

1976). Therefore, the JSSE solubility behavior of the compounds present in the obtained extract can be associated with the presence of ommochromes

Table 3 Solubility tests of the squid skin extract[1].

Solvent	Squid Skin Extract[2]	Ommochromes Reported Behavior[3]
Distilled water	NS	NS
Hydrochloric acid 5 M	CS	CS
Acetone	NS	NS
Potassium hydroxide 20%	CS	CS
Acetic acid	PS	PS
Methanol	NS	NS
Acidified methanol	CS	CS
Sulfuric acid 0.25 M	CS	CS
Chloroform	NS	NS

[1] **Data**: all analyses were run in triplicate.
[2] **Legend**: NS — no solubility, PS — poor solubility, CS— complete solubility.
[3] **Reference:** Van den Branden and Decleir (1976).

To confirm whether the pigments extracted from jumbo squid skin contained ommochromes UV-Vis, FT-IR, and ^{1}H NMR spectroscopies were employed. The UV-Vis spectroscopy of extracted pigments had an absorption maximum of 440 nm (Fig 2), which is similar to those red-pigments compounds previously reported in squid *D. pealeii* (**Williams *et al.*, 2016**). Ommochromes are usually distinguished by their specific absorbance spectra; this characteristic implied that the squid pigments contain ommins, one of the two groups of ommochromes (**Shamim *et al.*, 2014**). Moreover, the ^{1}H NMR spectrum (Fig 3) was similar to those of ommins (**Kumar *et al.*, 2018**). The ^{1}H NMR spectrum indicated aromatic protons at δ 7.4 ppm (singlet) and at 7.2 ppm (singlet) and, functional group adjacent to a methyl carbon at δ 3.8 (triplet) and at 3.0 ppm (multiplet).

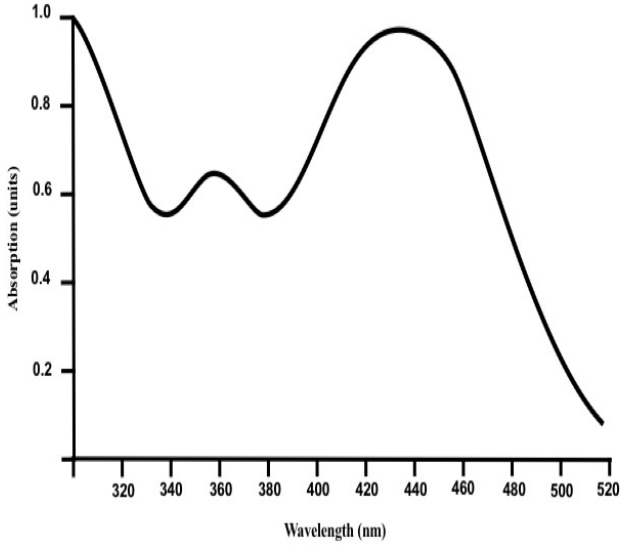

Figure 2 UV-Vis spectrophotometric spectra of jumbo squid skin pigments.

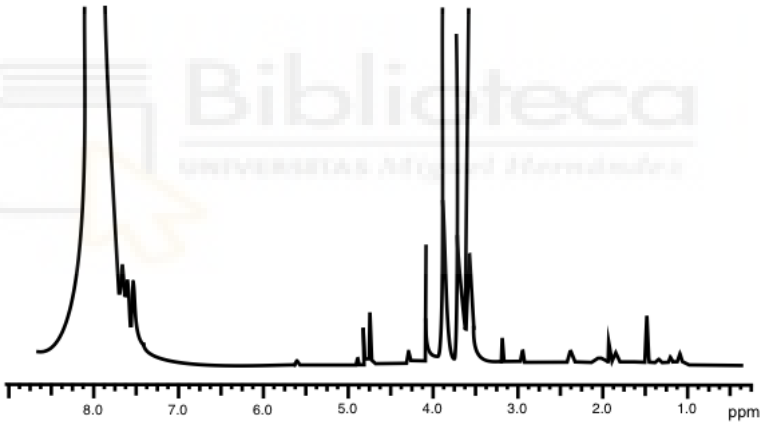

Figure 3 ^{1}H NMR spectrophotometric spectra of jumbo squid skin pigments.

Infrared spectroscopy provides more information regarding the chemical composition and conformation of the obtained pigments. The FT-IR spectra (4000–400 cm^{-1}) of the pigments (Fig 4) represented those reported for ommochromes (**Bolognese and Scherillo, 1974**). The main signals observed were at 3550–3100 cm^{-1} (N–H), 3000–2700 cm^{-1}(C–H stretching vibrations), 1500–1425 cm^{-1} (N–H and C–H bending vibrations), 1240–1050 cm^{-1} (C–O and C–N stretching vibrations) (**Dyer, 1965**). Furthermore, wave numbers for carbomethoxy C=O (1740 cm-1) and quinonic C=O (1670 cm^{-1}) indicated that squid pigments contained ommochromes compounds of the xanthommatin-type (**Bolognese and Scherillo, 1974**).

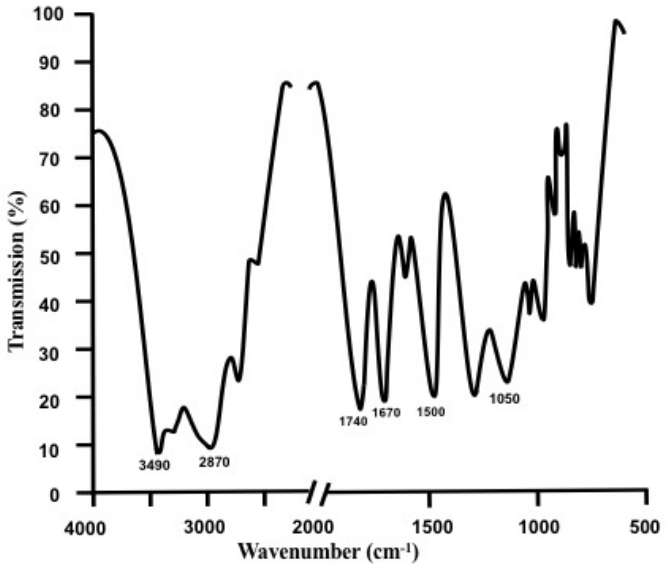

Figure 4 FTIR spectrophotometric spectra of jumbo squid skin pigments.

CONCLUSION

Jumbo squid skin pigments contain antibacterial and antimutagenic compounds, which were detected in the methanol–HCl soluble extracts. The extraction of bioactive pigments from jumbo squid skins was determined by both temperature and sonication time. Additionally, the present study suggests that one of the main compounds that exerted the biological activity in squid skin pigmented extracts were ommatins, specifically of the xanthommatin type. However, future studies need to focus on the identification of the specific antimicrobial and antimutagenic mechanisms of the compounds present in the jumbo squid skin pigmented extract.

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Conflicts of Interest: All authors declare that there are no conflicts of interest regarding the publication of this paper.

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PUBLICACIÓN 3

Xanthommatin is Behind the Antioxidant Activity of the Skin of *Dosidicus gigas*

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PUBLICACIÓN 3: TRANSCRIPCIÓN LITERAL

Xanthommatin is Behind the Antioxidant Activity of the Skin of *Dosidicus gigas*

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Abstract: Marine bioactive compounds have been found in very different sources and exert a very vast array of activities. Squid skin, normally considered a discard, is a source of bioactive compounds such as pigments. Recovering these compounds is a potential means of valorizing seafood byproducts. Until now, the structure and molecular properties of the bioactive pigments in jumbo squid skin (JSS) have not been established. In this study, methanol–HCl (1%) pigment extracts from JSS were fractionated by open column chromatography and grouped by thin-layer chromatography in order to isolate antioxidant pigments. Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging assays and ferric reducing power (FRAP) assay. Fractions 11–34 were separated and grouped according to flow rate values (F1–F8). Fractions F1, F3, and F7 had the lowest IC_{50} against $ABTS^{\bullet+}$ per milligram, and fractions F3 and F7 showed the lowest IC_{50} in the FRAP assay. Finally, fraction F7 had the highest $DPPH^{\bullet}$ scavenging activity. The chemical structure of the F7 fraction was characterized by

infrared spectroscopy, ^{1}H nuclear magnetic resonance, and electrospray ionization–mass spectrometry. One of the compounds identified in the fraction was xanthommatin (11-(3-amino- 3-carboxypropanoyl)- 1-hydroxy- 5-oxo- 5H-pyrido [3,2-a]phenoxazine- 3 carboxylic acid) and their derivatives (hydro- and dihydroxanthommatin). The results show that JSS pigments contain ommochrome molecules like xanthommatin, to which the antioxidant activity can be attributed.
Keywords: antioxidant activity; chromatography; ommochromes; spectroscopy; xanthommatin

Introduction

Marine bioactive compounds show noteworthy and natural properties that support their nutraceutical and pharmaceutical potential and are regarded as more secure options in contrast to synthetic drugs and food additives. Marine bioactive compounds have been isolated and characterized from different sources, like plants, microorganisms, algae and animals (vertebrates and invertebrates) [1]. Among the different invertebrates studied, one of the most notable for its commercial impact and tonnage of capture is the jumbo squid (*Dosidicus gigas*) [2]. As with most marine species, only the squid muscle is of economic importance. Obtaining clean squid filet requires the removal of skin and other anatomical regions. This process creates waste that accounts for over 40% of the total squid weight [3].

Squid skin (normally considered a discard) is particularly rich in biologically active compounds, such as gelatin, collagen, and their peptides, as well as pigments [4–7]. The pigments found in jumbo squid skin are a part of its defense mechanism, which has been perfected through years of evolution. They can instantaneously change their coloration to adapt to the environment. This unique combination of neuromuscular organs present on their skin is formed by an elastic saccule that allows the chromatophores to expand and relax, producing different colors [8]. The pigments in cephalopods have been previously characterized as ommochromes, and they constitute a class of polycyclic aromatic compounds that are synthesized through the metabolic pathway of tryptophan oxidation [9]. Their basic structure is a ring of fenoxazone (ommatins) or phenothiazine (ommins and possibly ommidins). Among the ommatins found in invertebrates, xanthommatin and their derivatives like hydro- and dihydroxanthommatin have been related to biological activities, mainly the oxidative protection against free radicals [10].

Ommochromes can act as antioxidants, and their antioxidative mechanisms can be achieved through chelating activity, and they can also act as primary antioxidants by scavenging radicals such as singlet oxygen and superoxide anions [11]. Ommochromes prevent photodamaging effects in the eyes of marine species [12]. Ethanolic pigment extracts obtained from jumbo squid skin (*Dosidicus gigas*) were used as antioxidants against the heat-induced rancidity of cod liver oil [6]. Jumbo squid pigments have also been proven to exert antimicrobial activity in iced mackerel and hake by inhibiting trimethylamine, microbial proteolysis, and lipolysis [13,14].

Although the antioxidant activity of squid skin extracts has been examined in some studies, most reports have described antioxidant proteins and peptides [15]. Moreover, no reports exist on the identification of the pigments responsible for the antioxidant activity of this important fishery resource. The aim of this work was to isolate and identify the pigments responsible for the antioxidant activity detected in squid skin (*Dosidicus gigas*).

Results

Isolation of the Bioactive Pigments

The liquid-phase column to open column method resulted in the elution of 34 fractions from the raw extract. The obtained fractions were analyzed according to their physical characteristics, as well as the results of solubility tests (data not shown). Fractions 1–10 were excluded from further analysis because no compounds were collected, as determined by the equal weight of the vial before and after evaporating the solvent. The remaining fractions (11–34) were analyzed by identifying their separation pattern in thin-layer chromatography plates. From the obtained results, the fractions were grouped according to the number of bands in each extract, as well as their Rf values. They were reclassified for a total of eight fractions, designated F1–F8.

Antioxidant activity

IC_{50} values showed that the compounds in fractions F1, F3, and F7 had the ability to perform the single electron transference (SET) mechanisms against the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical. In the ferric reducing power ferric reducing power (FRAP) method, the highest electron transference activity was detected in fractions F3 and F7. Lastly, the hydrogen atom transference (HAT) capacity was the highest ($p < 0.05$) in fraction F7, against the 2,2-diphenyl-1-picrylhydrazyl

(DPPH•) radical. Although the three techniques evaluate the ability to stabilize different radical species, the F7 fraction showed the highest activity in all of them. From this information, it was decided to proceed with the chemical characterization of fraction F7.

Table 1. Antioxidant activity of the collected fractions of squid skin extract, evaluated by three methods.[1]

Fraction	ABTS[2] (IC_{50} mg mL^{-1})	FRAP[2] (IC_{50} mg mL^{-1})	DPPH[2] (IC_{50} mg mL^{-1})
F1	2.12 ± 0.11^{a}	6.54 ± 0.06^{c}	6.49 ± 0.04^{e}
F2	2.77 ± 0.08^{c}	4.15 ± 0.11^{b}	4.67 ± 0.03^{c}
F3	2.07 ± 0.11^{a}	2.52 ± 0.20^{a}	3.56 ± 0.08^{b}
F4	10.2 ± 0.02^{e}	12.34 ± 0.33^{d}	8.69 ± 0.09^{e}
F5	6.02 ± 0.05^{d}	6.89 ± 0.07^{c}	9.52 ± 1.01^{e}
F6	2.25 ± 0.02^{b}	3.98 ± 0.10^{b}	5.34 ± 0.05^{d}
F7	2.08 ± 0.02^{a}	2.25 ± 0.09^{a}	2.60 ± 0.04^{a}
F8	5.78 ± 1.02^{d}	7.30 ± 0.03^{c}	3.44 ± 0.09^{b}

[1] The values represent the average of three repetitions ± standard deviation. [2] Different letters in the same column indicate significant differences ($p < 0.05$). ABTS: the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid; FRAP: ferric reducing power; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

Structure Elucidation

FT-IR, 1H NMR and electrospray ionization–mass spectrometry (ESI-MS) spectra were compared against previously published data and spectrum databases.

Figure 1 presents the IR spectrum of fraction F7. The peak associated with the stretching of primary amines is observed at 3298 cm^{-1}. This signal is also associated with the flexure of the primary amine, which is detected as a peak at 705 cm^{-1}. In the region of 3250–3600 cm^{-1}, a characteristic peak of the –OH functional group is observed, which overlaps with the previously described amino group. The presence of aromatic rings is associated with signals between 3000 and 3300 cm^{-1}, which are related to aryl carbons. This is corroborated by the signals located between 1600 and 2000 cm^{-1}, which are related to aromatic overtones.

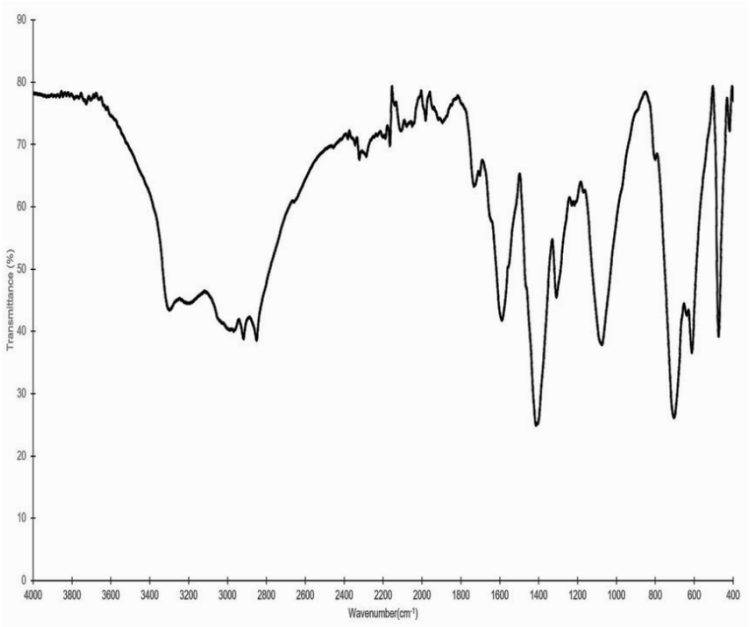

Figure 1. Infrared spectrum of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays.

One of the main compounds present in fraction F7 was established by comparing its ^{1}H NMR spectrum with previously published data [15–17]. The main signals detected by ^{1}H NMR (CD_3OD, 400 MHz) were δ 8.40 (s, 1H), 8.01 (d, 1H, J = 8.0 Hz), 7.96 (t, 1H, J = 8 Hz), 7.92 (s, 1H), 7.45 (d, 1H, J = 8Hz), 3.91 (dd 1H, J =5.03 Hz, 5.52 Hz), 3.56(dd J = 5.03 Hz, 1.92 Hz), and 3.52 ppm (dd, 1H, J = 5.52 Hz, 1.92Hz) (Figure 2). Moreover, the ^{1}H NMR spectrum shows signals due to sp3 carbons at δ 3.87 (Figure 2, letters f and g; m, 2H), aromatic protons at δ 7.88 (Figure 2, letters d and e; d, 2H), and amine group protons at δ 7.70 (s, 1H).

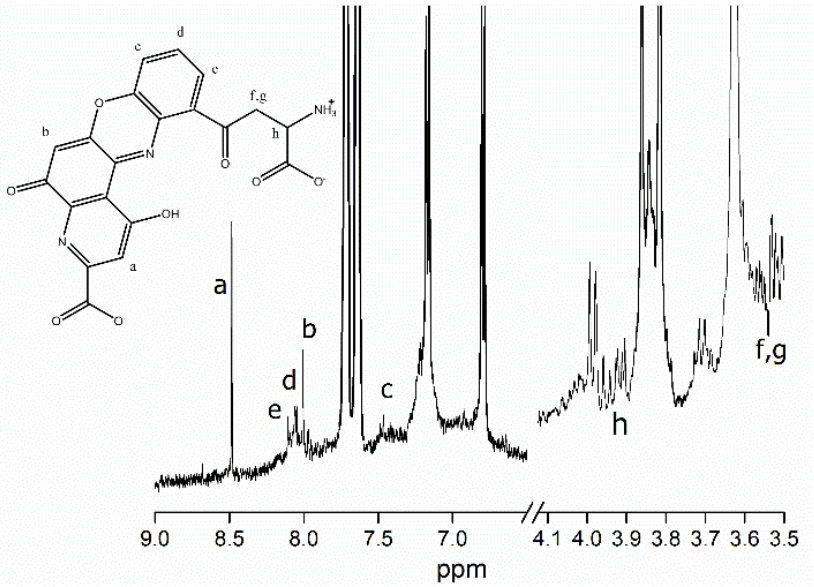

Figure 2. [1]H NMR spectra of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays.

Electrospray Ionization–Mass Spectrometry

The positive ESI-MS exhibited a quasimolecular peak at m/z 424 $(M + H)^+$ in full scan mode (Figure 3). Thus, it was inferred that the relative molecular weight of the compounds found were about 423 to 427.

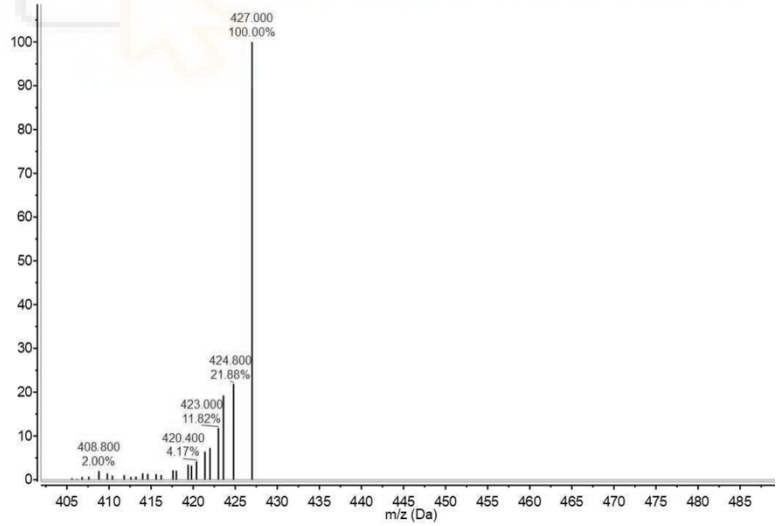

Figure 3. Electrospray ionization–mass spectrum of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays. The data were acquired in scan mode using an m/z range of 300–650.

Discussion

The separation of the extract was achieved using an open column, taking advantage of the characteristics of the pigments in squid skin that have been previously reported [19,21]. A high affinity between the sample and the extract was observed. Additional observations include a delayed elution and more effective recovery as the solvent polarity increased. This behavior could be due to the chemical structure of the silica gel, which contains a large proportion of hydroxyl groups. Ommochromes have hydrophobic parts and polar groups (amino and hydroxyl, particularly), and the latter can form hydrogen bonds and interact strongly with silica. Previous studies have reported that compounds with polar functional groups can be separated using a polar stationary phase, even if there are strong intermolecular interactions [22].

Since oxidation reactions do not all follow a single mechanism, evaluating antioxidant capacity through several assays is widely encouraged to allow the assessment of different modes of antioxidant action. The $DPPH^{\bullet}$, $ABTS^{+}$, and FRAP methods were used to evaluate the electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms of antioxidant activity. The results obtained in the antioxidant part of the study were used to identify the fraction with the highest activity by both mechanisms and thus characterize the compounds responsible for this biological activity. While fractions F1 and F3 showed the lowest IC_{50} values along with F7, fraction F7 had the highest in the DPPH method, and this information indicated that the compound or compounds in this fraction could either donate electrons or hydrogen atoms. The results obtained in this study strongly suggest that the pigments in fraction F7 are able to exert such mechanisms, as suggested by previous in silico studies [23]. These findings are relevant because of the importance of the hydrogen atom transference mechanism is relation to the prevention of peroxidation reactions in foodstuffs, as well as oxidation of biologically important molecules [5]. One of the ommochromes was identified as a potent electron and hydrogen donor, namely, xanthommatin [11-(3-amino-3-carboxypropanoyl)-1-hydroxy-5-oxo-5H-pyrido [3,2-a]phenoxazine-3-carboxylic acid]. The signals of two other similar molecules were also observed: hydro- and dihydroxanthommatin. The structures of rhodommatin, ommatin D, hydroxykynurenine, and xanthommatin have functional groups that are related to antioxidant action, primarily hydroxyl linked to aromatic rings.

Structure elucidation is a complex process that involves the interpretation and comparison of different assays, in order to establish the presence of certain molecules.

IR, ^{1}H NMR, and ESI-MS are techniques regarded as useful tools that help in the identification of molecules present in natural extracts. Previous reports have successfully elucidated the structure of pigments present in natural sources [24,25]. The identification of the functional groups in the molecules was achieved through IR analysis. The signals of certain functional groups associated with both antioxidant activity and compounds in the ommochrome family were detected. The amino group, both primary and secondary, in fraction F7 can act as an antioxidant given its electron transference capacity. A cyclic amine is present in the structure of the compound [26]. The tendency to donate electrons is related to the fact that the amine concentrates its electronic density in the aromatic ring. In addition, the amine forms stable resonance structures with the aromatic ring, which is absent once the amine is protonated. However, the peak attributed to the –OH functional group overlaps with that of the amino group. The characteristics of the sample, combined with the results of other techniques described later, suggest the presence of these groups. The antioxidant capacity of the hydroxyl groups has been widely reported, and phenolic compounds are recognized as being some of the most potent antioxidants in nature. The mechanism is driven by the resonance stabilization of the aromatic ring [25]. The IR results suggest that, in effect, aromatic rings are present in the compounds in fraction F7. In general, ommochromes have a basic structure of phenoxazone, which is derived from the amino acid tryptophan [27]. In addition to these results, Aubourg et al. [6] reported that a peak at 1740 cm^{-1} is characteristic of xanthommatin, an ommochrome present in squid skin extracts obtained with ethanol/acetic acid. Moreover, the data obtained for fraction F7 agree with previous reports on xanthommatin [28].

The ^{1}H NMR spectrum of fraction F7 shows signals that can be attributed to the presence of a phenoxazone core [17]. This kind of compound has been previously detected in the skin of some cephalopods [16,17]. The NMR spectrum, along with the FT-IR spectrum, confirms the presence of functional groups associated with antioxidant activity.

It has been established that phenoxazone cyclizes to dehydroxanthommatin, which oxidizes itself to xanthommatin (molecular weight, 423 g mol^{-1}) [18]. The ion at m/z 427 was assumed to be the corresponding quasimolecular ion of another ommochrome, such as dihydroxanthommatin; whereas hydroxanthommatin has a molecular weight of 425 g mol^{-1} [20]. Therefore, from the FT-IR and IR results, combined with the ESI-MS results, the presence of ommochromes, such as xanthommatin in fraction F7 is supported.

The compounds that showed radical scavenging activity and ferric reduction antioxidant power in *Dosidicus gigas* skin extracts are ommochromes. In this study, the ommochromes xanthommatin, hydroxanthommatin, and dihydroxanthommatin were found as molecular components responsible for the antioxidant activity of the extract, and its antioxidative mechanisms have been described as hydrogen atom transference and single electron transference. The results of this study confirm the presence of ommochromes with biological activity in jumbo squid extracts. This information can help establish that jumbo squid skin pigments have potential use in the food industry as a preventive agent against oxidation. Currently, there is an ongoing study on the application of the fraction with the greatest antioxidant activity for the preservation of a food product and its possible toxicological risk, as well as the contribution of antioxidant activity of the different compounds present in the fraction.

Materials and Methods

Sample Preparation

Jumbo squid (*Dosidicus gigas*) was obtained from a local establishment in Hermosillo, México (29°05′56″N, 110°57′15″W) and immediately skinned. About 10 kg of fresh skin was frozen at −80 °C, freeze-dried (LabConco, Kansas City, MO, USA), and grinded. Samples were kept at −20 °C until further analyses were performed.

Pigment Extraction

Freeze-dried skin was mixed with acidified methanol (1% HCl; 1:20 *w/v* proportion) and sonicated for 5 min. Samples were centrifuged (10,000× *g* for 15 min), the supernatant was collected, and the extraction solvent was removed using a rotary evaporator (R-100, Büchi, Switzerland).

Fractioning by Open Column Chromatography

The raw squid skin extract was fractionated using the liquid-phase column to open column technique. Silica gel with a particle size of ≤0.063 mm (Merck, Darmstadt, Germany) was placed as a stationary phase in a glass column, and a series of solvent combinations (all of analytical grade) were used as the mobile phase; this information is shown in Table 2.

Table 2. Solvents used as the mobile phase during open column chromatography.

Mixture of solvents	Proportion
Ethyl acetate/Methanol	60:40
Ethyl acetate/Methanol	40:60
Ethyl acetate/Methanol	20:80
Methanol	100
Acetic acid/Water	5:95
Acetic acid/Water	10:90
Ammonium hydroxide/Water	4:96
Ammonium hydroxide/Water	8:92

Thin-Layer Chromatography

The compounds in the previously obtained fractions were preliminarily identified through thin-layer chromatography (TLC). Static glass plates coated with silica gel were used as the stationary phase, and a combination of methanol/ethyl acetate/ammonium hydroxide (75:25:5) was used as the mobile phase. The samples were injected (10 µL fraction) and allowed to run for 30 min in a chamber saturated with solvents. The rate of flow (Rf) of the bands was observed and calculated to regroup those exhibiting the same pattern of separation.

Antioxidant Activity

The in vitro antioxidant activity of the collected fractions was evaluated by the three spectrophotometric assays.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was the first assay employed to determine the antioxidant activity, according to the method of Brand-Williams et al. [29]. Aliquots of each collected fraction (1 mg mL^{-1}) were dissolved in 1 mL of methanol, followed by the addition of 4 mL of a DPPH solution (0.004% w/v) in methanol. The samples were placed at 25 °C for 30 min, and the absorbance was read at 517 nm.

The second assay was the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging [30]. The ABTS radical cation ($ABTS^{\bullet+}$) was activated by adding 7 mmol ABTS in water and 0.14 mmol potassium persulfate. The mixture was incubated in the dark at 25 °C for 16 h. After mixing the $ABTS^{\bullet+}$ solution ($Abs_{734}nm$ = 0.70) with the samples, the mixtures were incubated for 30 min. The absorbance was recorded at 734 nm (Cary 50 UV–Vis, Agilent Technologies, Toluca, Mex., Mexico).

The third analysis involved the ferric reducing or antioxidant power of the samples [31]. An aliquot of 100 µL of the samples (1 mg mL^{-1}) was mixed with 1 mL of FRAP

reagent (10 mM tripyridyl triazine prepared in 40 mM HCl, 25 mL acetate buffer, and 2.5 mL of 20 mM $FeCl_3 \bullet H_2O$), and the reaction mixture was incubated at 25 °C for 30 min. The absorbance increase was registered at 593 nm (Cary 50 UV–Vis, Agilent Technologies).

The concentration of the sample required to scavenge 50% of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, or to reduce 50% of the Fe^{3+} atoms was determined using an inhibition curve using different concentrations of the fractions.

Spectroscopic Methods

The infrared spectrum of the sample was obtained with a Perkin Elmer spectrometer (Frontier MIR/FIR, Waltham, Massachusetts, USA). An attenuated total reflectance (ATR) technique was performed. The spectra were collected at 25 °C between 4000 and 400 cm^{-1}, accumulating 30 scans per spectrum. A blank spectrum was recorded to exclude any cross-contamination. The spectrum was expressed in wavenumber (cm^{-1}) versus transmittance percentage.

The ^1H NMR spectrum of the fraction was obtained on a Bruker Avance 400 nuclear magnetic resonance spectrometer operating at 400 MHz. The sample was dissolved in a mixture of deuterated methanol (CD_3OD) and dimethyl sulfoxide, using tetramethylsilane (TMS) as the internal reference. Chemical shifts were referenced to the solvent peaks, and the values were recorded in δ. The multiplicities of the ^1H NMR signals are indicated as s (singlet), d (doublet), and m (multiplet).

Electrospray Ionization–Mass Spectrometry

The mass spectrum of the fraction was obtained using a mass spectrometer (Agilent Technologies 6100 Quadrupole LC/MS, Santa Clara, California, USA). The dissolved sample was injected into a mixture of methanol with acetonitrile. The MS was operated in positive mode to analyze the compounds present in the squid skin extract. The data were acquired in scan mode using an m/z range of 300–650. The ESI technique was used because it is nondestructive and thus maintains the complete structure of the molecules in the fraction.

Statistical Analysis

Data on the antioxidant activities of isolated jumbo squid skin (JSS) pigments are reported as the average of three determinations and analyzed using analysis of variance

(ANOVA) with Tukey–Kramer tests. The IC_{50} values of the fractions were obtained through a linear regression analysis.

Author Contributions: J.E.C.H. developed the formal analysis and the results analysis. J.M.E.B. established the conceptualization of the study; H.C.S.O. and A.A.C.B. helped with the proper establishment and interpretation of the chemical structural analysis; S.G.C.R. supported the establishment conditions of pigment isolation; R.M.R.S. assisted with the appropriate explanation of antioxidant results. All authors contributed to the writing—original draft preparation. The author responsible for project supervision and funding acquisition was J.M.E.B..

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Conflicts of Interest: The authors declare no conflicts of interest.

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PUBLICACIÓN 4

Evaluation of *Dosidicus gigas* Skin Extract as an Antioxidant and Preservative in Tuna Pâté

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PUBLICACIÓN 4: TRANSCRIPCIÓN LITERAL

Evaluation of *Dosidicus gigas* Skin Extract as an Antioxidant and Preservative in Tuna Pâté

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Abstract: A strategy for food preservation, based on a methanol-HCl squid skin extract (*Dosidicus gigas*) (JSSE), was evaluated at two concentrations in yellowfin tuna fish pâtés, which were stored at 4 and 8 °C for 20 d. JSSE was characterized by determining its antioxidant and mutagenic activities. Yellowfin tuna pâté was elaborated, with and without the addition of JSSE. An affective sensory analysis was performed to establish consumers' preferences. During a 20-day storage period, the water activity (aw), pH, color (ΔE_{ab}*), microbiological, lipid oxidation and sensory quality attributes were evaluated, and the results were compared with the results of BHA and control treatments. JSSE showed antioxidant activity against the $DPPH^+$ and ABTS radicals and did not induce mutation, according to the Ames' Salmonella test, nor chromosomal abnormalities, according to the onion root-tip cell assay. The consumer analysis demonstrated a higher preference for the pâté with added JSSE in seven out of the eight evaluated attributes. During storage, JSSE neither had an impact on aw nor pH,

maintained lower ΔE_{ab}* values, inhibited the microbial activity and lipid oxidation, unlike the control pâté, and preserved the sensory quality attributes, unlike the BHA and control treatments. This study showed that JSSE has potential as an antioxidant and antimicrobial in yellowfin tuna fish pâtés.

Introduction

Fatty fish-based products, such as fish pâté, are food items of great economic and nutritional importance because of their composition and health benefits [1]. Nonetheless, during their processing and storage, microbial growth and lipid peroxidation in these fatty fish-based products lead to sensory and nutritional quality losses. Microorganisms can directly affect food through the development of undesirable flavors, odors and colors, rendering products unsuitable for human consumption [2]. Oxidative reactions cause the degradation of nucleic acids, proteins, lipids and pigments. Lipid peroxidation is responsible for the detrimental changes in sensory attributes, as well as the production of toxic compounds [3].

Fish pâté is widely regarded as a product with an important gastronomic tradition and a high nutritional value, as well as appreciated sensory attributes. Pâté is more vulnerable to microbial growth and peroxidation due to the disruption of its cellular components, which is induced by the grinding process [4]. Minced muscle has a lower shelf life because of the increase in nutrient availability and moisture migration, which enable microbial development. Moreover, exposed fatty acids can react with pro-oxidant molecules and interact with oxygen, light and metals, which promote their decomposition [5]. In the formulation of fish pâté, the use of fatty fish filets (specifically from yellowfin tuna, *Thunnus albacares*) is preferred because of their palatable characteristics.

Since domestic refrigeration conditions range from 6 to 11 °C, instead of the recommended temperature of 4°C, the use of additives in refrigerated products is necessary to prevent microbial growth and lipid oxidation [6]. Even if synthetic antioxidants and antimicrobials are highly effective, there is uncertainty about their negative impact on human health. Replacing synthetic additives with natural compounds that can exert the same function satisfies the popular demand for clean labels and contributes to the creation of safer products [7].

Biologically active compounds have been identified and characterized from different natural sources: meat, fish and vegetable peptides, phenolic compounds from spices and herbs, vegetable pigments, etc. [8]. One type of biologically active compounds is

represented by ommochromes, which are pigments found in the eyes of invertebrates, like crustaceans and arthropods, as well as the skin of mollusks. Chemically, ommochromes have a basic structure consisting of a phenoxazine ring, with different substituents. They are considered tryptophan-derived metabolites that come from the kynurenine pathway [9]. Their functional groups, which vary from molecule to molecule, give ommochromes a distinctive reddish to violet coloration, in addition to antiradical *in silico* activity [10]. These compounds act as antioxidants against UV radiation [11]. Studies of jumbo squid (*Dosidicus gigas*) skin have demonstrated their antioxidant activity, applied in fish oil at different storage temperatures [12], and their antimicrobial activity during the chilled storage of fresh mackerel and hake [13, 14]. All these results suggest that ommochromes can be used as additives in the food industry; however, there is still no information regarding the use of JSSE in food matrixes, like fish pâté, which tend to be more complex and more susceptible to oxidation reactions.

In this work, the main objectives were to evaluate the biological activity of giant squid skin extract in the oxidative stability of lipids, microbial growth of mesophiles and psychrophiles and the loss of sensory quality of yellowfin tuna pates, which were stored at two temperatures (4 and 8 °C).In order to carry out the addition of the extract to the tuna pâtés, the *in vitro* antioxidant activity and the mutagenic activity of the extracts were also evaluated.

Materials and Methods

Jumbo squid skin extraction and preliminary analysis

Jumbo squid skin extracts (JSSE) were obtained using acidified methanol (1% HCl). A preliminary study was conducted to determine the effect of the skin:solvent ratio, sonication time and extraction temperature on the recovery of pigmented extracts with antioxidant activity from squid skin. It was found that 20 mL g^{-1} and sonication for 5 min at 25 °C provided the maximum recovery of pigmented extracts with antioxidant activity. The extracts were centrifuged (Model Biofuge Stratos, Thermo Scientific, Germany) at 10,000×*g* for 15 min, before the methanol was removed using a rotary evaporator (R-100, Büchi, Switzerland). The solvent was further evaporated using nitrogen gas. The dry extracts were stored in an inert nitrogen atmosphere at -80 °C, prior to further analysis.

In vitro antioxidant activity

The free radical scavenging activity of JSSE was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, which has previously been described [15, 16]. Samples of 1 mg per mL JSSE were dissolved in aqueous methanol (80 %). The inhibition percentage of the extracts (IP) was calculated for both methods and expressed per mg of the extract using equation 1:

$$IP = 100 - \left(\frac{(A_{sample})(100)}{A_{blank}}\right), \quad (1)$$

where A_{sample} is the absorbance of the aqueous methanol, and A_{blank} is the absorbance of the extract solution.

Salmonella mutagenic assay

The mutagenic activity was tested using the *Salmonella typhimurium* tester strains, TA98 and TA100, purchased from Molecular Toxicology Inc. (MolTox; Annapolis MD, USA), with and without metabolic activation (presence of an S9 mix, MolTox). Four doses of JSSE were evaluated (0.1, 0.5, 1 and 5 mg mL^{-1}). All of them were diluted in a 0.2 M phosphate buffer (pH 7.4). The concentrations were selected based on a preliminary toxicity test. The plates were incubated at 37 °C for 48 h, and the revertant colonies were counted manually. All experiments were analyzed in triplicate. A sample was considered mutagenic when a dose–response relationship was detected, and a two-fold increase in the number of spontaneous mutants (MI ≥ 2) was observed with at least one concentration [17]. The standard mutagens, used as positive controls in the experiments without the S9 mix, were hydrogen peroxide (340 µg $plate^{-1}$) for TA98 and sodium azide (1.25 µg $plate^{-1}$) for TA100. Aflatoxin B_1 (0.5 µg $plate^{-1}$) was used with TA98 and TA100 in the experiments with metabolic activation. A phosphate buffer served as the negative (solvent) control.

Onion root-tip clastogenicity test

Healthy young onion bulbs, grown in the absence of herbicides, pesticides or fungicides, were used in this study. The promotion of root development was performed by placing the bulbs in darkness and partially submerging them in water. When the roots were 2 cm long, the onions were transferred to Petri dishes with 30 mL of JSSE extracts (0.1, 0.5, 1 and 5 mg each). A positive control with sodium azide (10 ng) and a negative

control (water) were also analyzed. After 24 h, the roots were fixed in 3:1 (v/v) ethanol/glacial acetic acid, squashed and washed with distilled water and stained with orcein for 2 h in the dark. All cells with alterations were counted [18]. During the genotoxicity assessment, the presence of mitotic cells with irregular chromosomes (i.e., micronuclei, disorganized chromosomal structure, lag and stick chromosomes) was recorded.

Pâté elaboration

Tuna pâtés were produced in the food processing pilot plant at the Miguel Hernández University (Alicante, Spain). Fillets of fresh yellowfin tuna fish were obtained from a local market in Orihuela (Alicante, Spain). Tuna fish fillets were chopped into small cubes and mixed for 15 min in a Vorwerk Thermomix food processor (Wuppertal, Germany). Tuna (40 g) was mixed with ice (30 g) and salt (1 g) for 5 min. Afterwards, sodium caseinate (4 g) and corn starch (4 g) were slowly added, until a homogenous mix was formed. Olive oil (5 g) was smoothly added, until the emulsion was formed. Finally, white wine vinegar (1 g) was added. All batches were cooked, until the pâté core temperature reached 75 °C, and they were then cooled in an ice bath.

Based on preliminary studies, JSSE was added separately in two batches at concentrations of 0.05 % of pâté (P1) and 0.1 % of pâté (P2). Butyl hydroxy anisole (0.1 g % of pâté) was added to a third batch (BHA), representing a different treatment. JSSE was mixed with vinegar before being mixed with the other ingredients. The remaining batch was used as a control sample (control).

Consumer acceptance panel

Pâté samples were taken from refrigerated storage and left at 20 °C for about 15 min. They were then analyzed by a panel of 70 consumers. Consumers were asked to state their preference in relation to eight attributes on a scale (1: extremely dislike it; 5: neither like nor dislike it; and 9: extremely like it). Consumers between 20 and 65 years old were students and staff members of the Department of Agro-Food Technology (Miguel Hernández University, Desamparados Campus). The sensory analyses were performed, after the pâté was cooked.

Cooling storage and pâté shelf life

All samples were packed in polyethylene plastic bags and divided into vacuum sealed and non-vacuum sealed bags. They were then stored at 4 and 8 ± 1 °C under dark conditions for 20 d. Pâté samples were taken randomly at 0, 4, 8, 12, 16 and 20 d of storage. Analyses were performed on the sampling day. All determinations were performed in triplicate (n = 3).

Physical-chemical parameters

The water activity was determined in polyethylene capsules with ca. 5 g of pâté were placed in a Labmaster-aw instrument (Novasina, Lachen, Switzerland). Analyses were performed at 25 °C. The pH was evaluated by mixing 10 g of pâté with 100 mL of distilled water and stirred in a magnetic plate. pH measurements were made using a pH Basic 20 instrument (Crison, Barcelona, Spain). The color of the pâtés was measured using a CR-400 Chromameter (Konica Minolta, Tokyo, Japan), and the *L**, *a** and *b** parameters were registered, as specified by the International Commission on Illumination. The total color difference over the time between samples was evaluated with the ΔE_{ab}* parameter, following equation 2 [19]:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}\,, \qquad (2)$$

Microbiological analysis

Portions (20 g) of the samples (for each of the control and treatment groups, separately) from yellowfin tuna pâté were taken aseptically and homogenized in sterilized stomacher bags (Seward, United Kingdom) with 90 mL of 0.1 % peptone water. Serial tenfold dilutions were made in pre-sterilized tubes containing 9 mL of peptone water. The sample preparation and plating were carried out under a laminar flux cabinet in sterile conditions. Triplicate samples were analyzed from each trial of pâté formulation. To estimate the total plate count, 1 mL of the dilutions was aseptically transferred to Petrifilm count plates (3M Corporation, Maplewood, MN, USA). Petrifilms were divided into two incubating conditions for the determination of the total aerobic mesophiles (37 °C) and psychrophiles (7 °C). Colonies were counted after 48 h for mesophiles and 72 h for psychrophiles using a colony counter. The average count was multiplied by the dilution factor and expressed as the log CFU g^{-1} of the sample.

Lipid oxidation

Conjugate dienes (CD) and trienes (CT) were measured by mixing a 0.5 g sample of pâté with 5 mL of distilled water and vortexed at 1500 rpm for 1 min. An aliquot of 0.5 mL was mixed with 2.5 mL of hexane:isopropanol (3:2 v/v) and centrifuged at 2000 rpm for 3 min. The supernatant was recovered and placed in quartz cuvettes, and measurements of CD and CT were conducted at 232 and 268 nm, respectively. The concentration of dienes and trienes was determined using the molar extinction coefficient, specific for both types of compounds [20].

The quantification of hydroperoxides, evaluated as secondary peroxidation products, was carried out according to previous reports [21], with some modifications. Lipid extraction was conducted using hexane:isopropanol. The extract (1 mL) was mixed with 10 mL of chloroform:methanol (7:3 v/v) and vortexed for 30 s at 2000 rpm. Later, 50 µL of 30 % ammonium thiocyanate and 50 µL of 20 mM iron (II) chloride were added and centrifuged at 2500 rpm for 10 min. The absorbance of the supernatant was obtained at 480 nm, and the concentration of peroxides was expressed as the equivalent nmol of cumene hydroperoxide per g of pâté.

The end-products of peroxidation were determined using the thiobarbituric acid assay (TBARS) [22]. A sample of 0.5 g was mixed with 2.5 mL of thiobarbituric acid reagent (3.75 g of TBA, 150 g of trichloroacetic acid in 1 L of 0.25 N HCl) and heated in a water bath at 96 °C for 10 min. The test tubes were cooled down in an ice bath and centrifuged at 2500 rpm for 10 min. The supernatant was recovered, and its absorbance was recorded at 532 nm. The standard curve was prepared with 1,1,3,3-tetramethoxypropane, and the results were expressed as the malondialdehyde equivalents per g of pâté.

The antioxidant efficacy of JSSE in the samples was assessed, with an oxidation index at both storage temperatures (4 and 8 °C). The percentage of oxidation inhibition [OI (%)] was calculated for PV and TBARS using equation 3 [23]:

$$OI\ (\%)\ = \left(\frac{c-s}{c}\right) \times\ 100, \qquad (3)$$

where *c* is the value obtained from the control on the day of the highest obtained value, and *s* is the value for each JSSE condition on the same day.

Sensory quality

To evaluate the sensory quality of the samples during storage, a panel of seven qualified and experienced panelists in the field of fish products was appointed, with the sensory attribute descriptors of the coded samples. They performed the analysis in a

standard sensory laboratory (under white light, 25 °C, and 50–55 % relative humidity). The same panel evaluated all the samples. The analysis was performed through scoring sensory properties by assigning five categories: highest quality (E), good quality (A), fair quality (B), poor quality (C) and unacceptable quality (D). The sensory descriptors of the pâté were odor, presence of off-colors, presence of slime, firmness and emulsion stability. The attribute descriptors are shown in Table 1.

Table 1. Scale used in the evaluation of the sensory quality of yellowfin tuna pâtés.

Descriptor Attribute	Excellent (E)	Good (A)	Fair (B)	Poor (C)	Unacceptable (D)
Odor	Strong seaweed and shellfish	Weak seaweed and shellfish	Imperceptible seaweed and shellfish smell	Initial ammonia and putrid odor	Ammonia and putrid odor
Off-colors	Bright coloration; complete absence of decolorated areas	Dim coloration, small (<5 mm) decolorated areas	Dim coloration, larger (<10 mm) decolorated areas	Brownish coloration; presence of small greenish spots	Brown coloration throughout; greenish spots throughout
Slime	Complete absence of slime in the package and the sample	Droplets of transparent slime in the bags, located only in the corners	Slime present at the bottom of the bag; sample with slime droplets	Whitish slime present throughout package and sample	Opaque slime present throughout package and sample
Firmness	Elastic and spreadable samples; cohesive and moist particles	Firm consistency; less spreadable sample	Presence of thin cracks in the samples; complete loss of elasticity	Presence of wide cracks; separated pâté particles	Complete loss of water, important shape changes throughout
Emulsion stability	Homogeneous appearance throughout; no sign of oil separation	Homogeneous appearance in most of the sample; small droplets of oil	Heterogenous appearance in areas of the sample; droplets of water and oil at the surface	Heterogenous appearance in most of the sample; liquid condensed at the bottom of the package	Heterogeneous appearance throughout; water and oil separated at the bottom of the package

Statistical analysis

Descriptive statistics was used to present the antioxidant assays (n = 3). The data obtained from the consumer acceptance study were evaluated through a two-way analysis of variance (ANOVA) in order to assess the significance of the JSSE addition on all of the studied characteristics. The data obtained from the Ames' test and the microbiological and chemical analyses (n = 3) were subjected to the ANOVA analysis to establish differences resulting from the effects of the JSSE addition. A comparison of the means was performed using the Tukey test ($p < 0.05$). The data obtained from the sensory evaluation and the onion root tip assay were analyzed using the non-parametric Kruskal-

Wallis test. In all cases, the analyses were carried out using SAS (SAS Institute, Inc. JMP 5.0.1, USA), and differences among treatments were considered significant with a confidence interval at $p < 0.05$ in all cases.

Results

Preliminary analysis of jumbo squid skin pigmented extracts

The obtained extract showed a reddish-violet color, and the recovery yield (expressed as g dry JSSE 100 g^{-1} skin) was 0.65 %. The antioxidant compounds present in the 1 mg mL^{-1} of JSSE generated an inhibition percentage of 68 against the DPPH* radical and 79 in the ABTS$^{\bullet}$*test.

The mutagenicity of JSSE against both *Salmonella* tester strains was considered negative, because the pigmented extracts did not double the number of spontaneous revertant colonies, counted *per* plate (Figure 1).

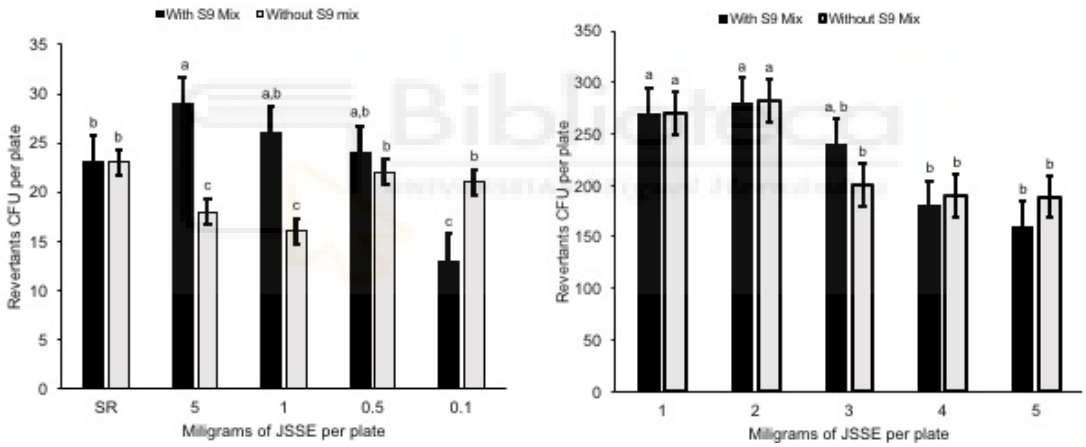


Figure 1. Mutagenic effect of giant squid skin extract on (a) the TA98 and (b) TA100 strains, with and without metabolic activation (S9 Mix). Mean values are of three replicates (n = 3), and standard deviations are indicated by bars. Values with different letters are significantly different ($p < 0.05$). SR = Spontaneous revertants per plate.

The JSSE clastogenicity test showed that the results obtained, determined by the onion root-tip assay, could be considered as non-genotoxic. The percentage of cells with damaged chromosomes was lower than 50 %, as compared with the sodium azide root (Table 2). The method was validated by the percentage of observed cells (over 150) and mitotic cells (over 100).

Table 2. Clastogenic effect of JSSE in the mitotic cells of Allium cepa*.

Treatment	Total Cells	Mitotic Cells	Damaged Cells	Genotoxicity Percentage	Adjusted Percentage**
Negative control	215 ± 10	134	12	8.9	10.9[a]
Sodium azide (10 ng)	195 ± 9	106	87	82.1	100[d]
JSSE 0.1 mg	223 ± 19	125	19	15.2	18.5[b]
JSSE 0.5 mg	208 ± 11	119	21	17.6	21.5[b]
JSSE 1 mg	197 ± 13	109	16	14.7	17.9[b]
JSSE 5 mg	200 ± 13	120	29	24.2	29.5[c]

*The values represent the average of three repetitions ± standard deviation. **Mean values with different letters (a, b, c, d) indicate differences ($p < 0.05$) between samples, compared with the sodium azide control.

Consumer acceptance

The evaluation of consumer acceptance showed that the JSSE addition in tuna pâtés improved their sensory attributes (Table 3).

Table 3. Degree of acceptance* of different pâté treatments by consumers.

Treatment**	Attribute							
	Color	Odor	Tuna Flavor	Saltiness	Spreadability	Cohesiveness	Aftertaste	Global
P2	6.1[a]	5.7[ab]	6.0[a]	5.8[a]	5.2[a]	5.6[a]	6.2[a]	5.8[a]
P1	5.2[a]	5.8[ab]	5.9[a]	5.7[a]	5.3[a]	5.5[ab]	6.0[a]	5.6[a]
Control	3.2[b]	4.7[bc]	4.8[b]	5.1[a]	4.1[b]	4.8[ab]	5.0[b]	4.4[a]
BHA	3.4[b]	4.3[c]	4.6[b]	5.3[a]	4.0[b]	4.5[b]	4.6[b]	4.0[a]

*The values represent the average of the results obtained from 70 panelists. Mean values with different letters (a, b, c) are significantly different ($p < 0.05$) due to the addition of jumbo squid skin extract. **The BHA abbreviation denotes butylated hydroxyanisole, and P1 and P2 denote the concentration of jumbo squid skin extracts, corresponding to 0.05 % and 0.1 %, respectively, according to the Materials and Methods section.

Assessment of quality evolution during fish pâté cooling storage

Physical-chemical parameters

The water activity of all of the tuna pâtés ranged from 0.93 to 0.95 during all samplings. The JSSE addition did not directly affect the proportion of free water in the

samples, when compared with the control treatments ($p > 0.05$). The results concerning the pH levels showed no differences among the samples ($p > 0.05$). Nevertheless, a significant pH drop, from about 6.4 to 5.7, was detected at storage day 4 for all pâté treatments.

The color of the samples became more intense, when the amount of JSSE added to the tuna pâté increased (Table 4). The changes in the ΔE_{ab}* values during the storage days were statistically significant, as shown in Table 5. In the period of 12–20 d at 4 °C, the mean value changes were greater than 5 units in the control and BHA treatments, whereas at 8 °C, they were less than 5 units on the 8th day in the JSSE and BHA pâtés.

Table 4. Effect of the addition of jumbo squid skin extract on the L, a, and b color parameters*.

Treatment**	L*	a*	b*
P1	65.5^{d}	1.7^{b}	12.9^{ab}
P2	67.2^{c}	2.8^{a}	13.6^{a}
Control	75.4^{a}	-0.2^{c}	12.4^{b}
BHA	70.6^{b}	-0.5^{c}	13.7^{a}

*Mean values are of three replicates (n=3). Values with different letters (a, b, c, d) indicate significant differences ($p < 0.05$) among the treatments. **Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) are the same as those expressed in Table 3.

Table 5. Effect of the addition of jumbo squid skin extract on the ΔEab^{*} of the different processed pâté samples at 4 and 8 °C*.

Treatment**	**4 °C**						**8 °C**					
	Day						Day					
	0	4	8	12	16	20	0	4	12	8	16	20
P1	0	1.2^{cC}	3.0^{bB}	3.9^{cB}	6.9^{cA}	7.2^{dA}	0	2.7^{bD}	2.8^{bD}	3.6^{cC}	6.2^{cB}	8.1^{cA}
P2	0	2.5^{bC}	3.4^{bB}	3.6^{cB}	8.7^{bA}	9.1^{cA}	0	1.0^{cD}	1.7^{cD}	3.1^{cC}	7.8^{bB}	9.3^{bA}
Control	0	5.3^{aD}	6.5^{aC}	7.3^{aC}	8.9^{bB}	16.2^{aA}	0	7.6^{aD}	7.9^{aD}	8.7^{aC}	11.1^{aB}	15.3^{aA}
BHA	0	3.0^{bC}	3.2^{bC}	5.6^{bB}	9.9^{aA}	10.1^{bA}	0	0.9^{cE}	1.6^{cD}	2.5^{bC}	6.9^{cB}	8.2^{cA}

*Mean values are of three replicates (n = 3). Values with different lowercase letters (a, b, c, d) indicate significant differences ($p < 0.05$) among the treatments, and uppercase letters (A, B, C, D, E) indicate significant differences during storage time. **Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) are the same as those shown in Table 3.

Microbiological analysis

The changes with time of the aerobic mesophiles and psychrotrophs in the four types of pâté are shown in Table 6. In the period of 12–20 d, the mean of the aerobic concentrations of mesophiles was greater than 250 logarithmic units in the control and BHA, whereas the concentrations of psychotrophs were less than 250 logarithmic units on the 8th day and 4th day at 4 °C and 8 °C, respectively, in the JSSE-pâté.

Table 6. Total microbial count of mesophiles and psychrophiles* in the pâté samples at 4 and 8 °C.

	Mesophiles (4 °C)						Mesophiles (8 °C)					
	Day						Day					
Treatment**	0	4	8	12	16	20	0	4	8	12	16	20
Control	BDL*	10	258	3617	8365	8700	BDL	26	68	560	5400	UNC**
BHA	BDL	57	100	570	6050	8500	BDL	25	32	120	1200	UNC
P1	BDL	10	14	17	28	35	BDL	3	4	6	10	310
P2	BDL	3	4	6	7	10	BDL	2	3	5	20	190
	Psycrophiles (4 °C)						**Psycrophiles (8 °C)**					
	Day0						**Day**					
Treatment**	0	4	8	12	16	20	0	4	8	12	16	20
Control	BDL	540	910	UNC	UNC	UNC	BDL	620	1230	7600	UNC	UNC
BHA	BDL	30	100	1500	UNC	UNC	BDL	760	1020	1580	UNC	UNC
P1	BDL	10	120	1200	2500	UNC	BDL	240	310	3100	9700	UNC
P2	BDL	10	110	1000	2900	UNC	BDL	190	280	3000	8100	UNC

*Mean value of three replicates (n = 3). **Abbreviations of BHA and concentrations of jumbo squid pigmented extracts (P1, P2) are the same as those expressed in Table 3. BDL = Below detection limits. UNC = The value could not be determined due to an excessive number of colonies.

Lipid oxidation index

Comparative studies on the level of the primary oxidation products, conjugated dienes (CD) and trienes (CT) are shown in Table 7. At both storage temperatures (4 and 8 °C), the concentration of CD and CT increased from day 16 to day 20. A definitive trend due to the presence of JSSE in tuna pâté cannot be demonstrated.

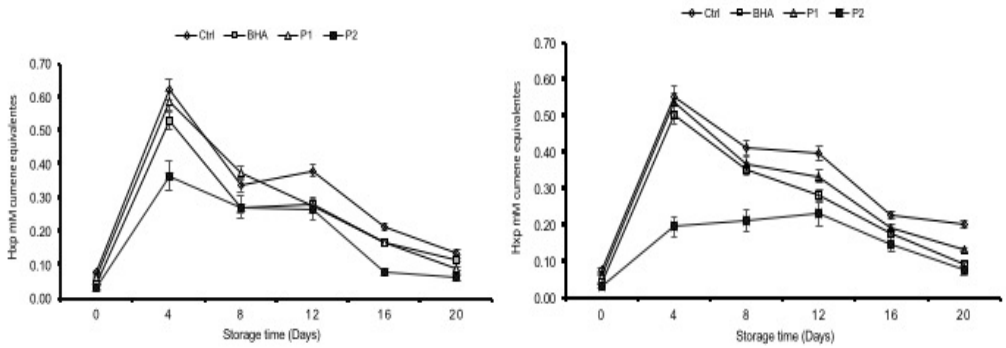
Table 7. Conjugated dienes and trienes formation* in pâté samples at 4 and 8 °C.

Treatment**	Conjugated dienes (mmol g^{-1})									
	4 °C					8 °C				
	Day					Day				
	4	8	12	16	20	4	8	12	16	20
Control	1.8^{a}	1.1^{b}	1.9^{a}	3.7^{a}	5.2^{a}	2.8^{a}	2.3^{a}	2.7^{a}	2.0^{a}	4.9^{b}
BHA	2.3^{a}	0.5^{b}	1.7^{a}	2.1^{b}	2.3^{b}	1.5^{a}	2.3^{b}	1.6^{b}	2.1^{c}	3.4^{b}
P1	2.8^{a}	1.0^{b}	1.7^{a}	2.8^{ab}	2.2^{b}	2.0^{a}	1.3^{b}	4.1^{a}	4.3^{b}	2.8^{b}
P2	3.3^{a}	2.0^{a}	0.6^{b}	2.3^{b}	5.3^{a}	2.3^{a}	1.7^{b}	2.2^{b}	5.3^{b}	9.6^{a}
Treatment**	**Conjugated trienes (mmol g^{-1})**									
	4 °C					**8 °C**				
	Day					**Day**				
	4	8	12	16	20	4	8	12	16	20
Control	0.5^{a}	0.6^{b}	1.3^{a}	2.5^{b}	2.1^{b}	1.8^{a}	2.4^{a}	1.5^{b}	3.8^{a}	3.6^{b}
BHA	0.5^{a}	0.6^{b}	1.2^{a}	1.6^{c}	3.1^{a}	0.9^{b}	1.6^{b}	1.9^{ab}	1.5^{b}	3.3^{b}
P1	1.0^{a}	0.7^{b}	1.5^{a}	1.9^{c}	2.2^{b}	1.3^{b}	1.0^{b}	2.6^{a}	3.5^{a}	2.5^{c}
P2	0.9^{a}	1.7^{a}	0.8^{a}	3.4^{a}	3.7^{a}	2.2^{a}	1.2^{b}	1.9^{ab}	4.2^{a}	7.6^{a}

*Mean value of three replicates (n = 3). For each lipid parameter, mean values followed by the same letter are not significantly different ($P > 0.05$). **Abbreviations of BHA and concentrations of jumbo squid pigmented extracts (P1, P2) are the same as those expressed in Table 3.

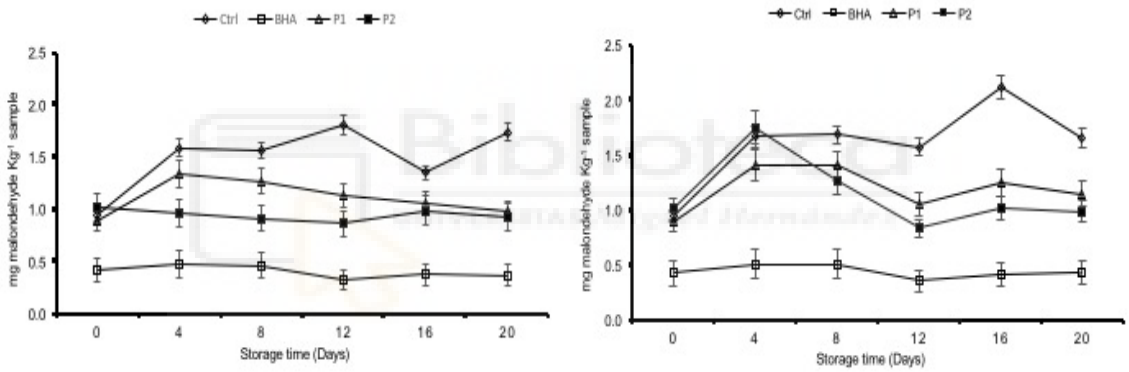
The maximum of the hydroperoxide values at 4 °C was detected on day 4 for all treatments (Figure 2), whereas at 8 °C, it was observed only in the control, BHA and P1. At both temperatures, the sample treated with the higher concentration of JSSE (P2) exhibited period values under 0.5 mM equivalents of PV, indicating an inhibitory effect ($p < 0.05$). On day 4 of the experiment, at both temperatures, the following hydroperoxide formation was detected: Control = BHA = P1 < P2.

The end products of lipid peroxidation found in the pâté samples were evaluated using the TBARS method (Figure 3). As a general trend, this inhibitory effect ($p < 0.05$) was higher in P2 throughout the entire experiment, at both storage temperatures, and was also higher ($p < 0.05$) than the control pâtés. As expected, BHA exerted the highest inhibitive effect, when compared with all the other treatments ($p < 0.05$).



(a) (b)

Figure 2. Hydroperoxide formation (Hxp mM cumene equivalents) * in the different processed pâtés ** during storage at: (a) 4 °C; and (b) 8 °C. *Mean values are of three replicates (n = 3); and standard deviations are indicated by bars. **Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) are the same as those expressed in Table 3.



(a) (b)

Figure 3. Formation of thiobarbituric acid reactive substances* in the different processed d pâtés** during storage at: (a) 4 °C; and (b) 8 °C. *Mean values are of three replicates (n = 3); and standard deviations are indicated by bars. **Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) are the same as those expressed in Table 3.

The inhibition percentage of JSSE in the pâté lipid oxidation was calculated for PV and TBARS at 4 °C and 8 °C, based on the control condition scores obtained. The percentage of oxidation inhibition, measured using the PV index for the BHA and JSSE samples, indicated that the pâté with 0.1 % of JSSE (P2) was the sample that showed the highest inhibition at 4 °C. The order of oxidation inhibition values in the peroxide formation at 4 °C was as follows P2 (41.5 %) > BHA (14.9 %) > P1 (6.2 %), and at 8 °C, it was P2 (57.8 %) > BHA (9.0 %) > P1 (2.5 %). The TBARS content inhibition at 4 °C

was BHA (76 %) > P1 (33.9 %) > P2 (17.9%), and at 8 °C, it was BHA (73.9 %) > P2 (45 %) > P1 (25.5 %).

Sensory evaluation

The evolution of the sensory qualities of the pâtés is depicted in Table 8. The initial pâté types were found to have the highest quality (E score). After 8 d of storage at 4 °C, samples corresponding to the P1 (0.05 % JSEE) treatments showed a good quality and were considered to be in the A category. Remarkably, the pâté types belonging to the control, BHA and P2 (0.1 %) treatments showed a fair or poor quality, compared with their P1 counterparts at this storage time. Meanwhile, at 8 °C, until day 12, the sensory quality of the P1 treatment was considered good, whereas the control, BHA and P2 at this period of time had a fair or poor quality.

Table 8. Sensory quality[a] evaluation during the storage of pâté samples[b] at 4 and 8 °C.

Storage Conditions Temperature	**Day**	**Treatment**			
		Control	BHA	P1	P2
4 °C	0		E		
	4	E	E	E	E
	8	B	C	A	B
	12	B	C	B	C
	16	D	D	C	D
	20	D	D	C	D
8 °C	0		E		
	4	E	E	E	E
	8	B	C	A	B
	12	B	C	A	B
	16	D	D	C	D
	20	D	D	C	D

[a]Quality codification: Excellent (E), Good (A), Fair (B), Poor (C), Unacceptable (D). [b]Abbreviations of BHA and concentrations of jumbo squid pigmented extracts (P1, P2) are the same as those expressed in Table 3.

Discussion

The main objective of the first part of the study was to characterize the obtained jumbo squid pigment extracts, mainly their antioxidant activity and potential toxic effect. The antioxidant activity of the pigmented extracts was measured in terms of their DPPH* and ABTS$^{\bullet}$* radical scavenging activities, whereas the mutagenicity test with *Salmonella* strains and the onion root-tip clastogenicity tests were used to evaluate the potential toxic effect of a sample on the genetic material in prokaryotic and eukaryotic cells. The antioxidant activity of the JSSE was considered effective, because it was higher than the

results reported for DPPH* (30 %) and for ABTS$^{\bullet}$* (50 %) [24]. The *Salmonella* test indicated that the mutagenicity was considered negative, when the number of revertant colonies counted per plate did not double the number of spontaneous revertants [25]. Based on this test, the mutagenicity of the pigmented extracts was considered negative. Similarly, for plant-based products and purple natural pigments, no mutagenic effect on the same *Salmonella* strains was detected [26, 27]. Concerning the onion root-tip test, there is genotoxic activity when the percentage of cells with damaged chromosomes is over 50 %, as compared to the sodium azide roots. None of the JSSE treatments evaluated (0.1, 0.5, 1 and 5 mg mL^{-1}) can be considered as clastogenic, according to the results of this study.

The objective of the second part of the study was to establish if the addition of JSSE would affect the consumer acceptability of the tuna pâtés. Consumers' sensory results demonstrated that JSSE-treated samples (0.05 and 0.1 %) had scores significantly higher ($p < 0.05$), as compared to the control and BHA samples, in 7 out of the 8 evaluated attributes (color, odor, tuna flavor, spreadability, cohesiveness, aftertaste and overall acceptance). Therefore, the evaluated JSSE concentrations were accepted to a higher degree, as compared to the control samples.

The third phase of the study was designed to establish the effect of the JSSE on the tuna pâtés' storage life at domestic refrigeration conditions (8 °C) and recommended temperature (4 °C). Concerning the physical-chemical results, color was the only parameter of the pâtés that was affected by the JSSE. The decrease in the pH values on day 4 may have been due to the microbial development, resulting in the formation and accumulation of acidic compounds, such as lactic acid, among other acidic metabolites [28]. Regarding the color, values greater than 5 are considered to be perceptible to the human eye [18]. At the storage temperature of 4 °C for the control treatment, the values of ΔE were greater than 5 from day 4 onwards, and for the BHT treatment, they were greater than 5 from day 8. JSSE-pâtés (P1 and P2) maintained ΔE_{ab}* values of less than 5 until day 12 of storage, whereas at 8 °C, samples with JSSE and BHT maintained their color up until day 8 of the experiment. Therefore, the results demonstrated that samples with JSSE maintained their color for at least 8 d, depending on the storage temperature.

The inclusion of JSSE in the pâté caused a better microbial growth control, compared to the control and BHA treatments. The maximum allowed levels of microorganisms established for meat products, cured and cooked meat products, and emulsified and cooked cured products are 250 log CFU g^{-1} [29]. Species-specific spoilage psychrotrophs

bacteria, including members of the genera, *Pseudomonas, Shewanella, Acinetobacter, Moraxella* or *Flavobacterium*, are considered to be in the psychrotrophic group. The results of this experiment demonstrated a bacteriostatic effect on tuna pâté due to the inclusion of a squid skin extract (JSSE) in the formulation, at both 4 °C and 8 °C storage temperatures and at both addition concentrations. Previous studies also evaluated the preservative effect of acetic acid-ethanol extracts of jumbo squid skin in chilled fish [13, 14]. In those studies, an antimicrobial effect (lower aerobic mesophiles and psychrotrophs counts) was observed during the chilled storage of fresh fish. This behavior may be due to the electron donor capacity of the ommochromes [10], inducing an imbalance in the metabolic pathways in microorganisms. However, further research is needed to establish the possible mechanism of the compounds present in the extract.

Regarding lipid oxidation, the addition of JSSE did not improve or promote the formation of primary peroxidation products. At both storage temperatures (4 and 8 °C), the concentration of CD and CT increased from day 16 and 20. This behavior indicated the isomerization of new fatty acids in the product, mediated by free radicals. CD and CT did not have enough stability to maintain their structure, and they tended to associate with other components of the food or to decompose. Similar results were reported in products, such as vegetable oils [30]. Through the oxidation process, the quantity of the different molecules generated in these phases sequentially increased and then decreased over time. Peroxide formation was measured, as an estimation of the propagation phase of rancidity. At this stage, molecular oxygen compounded with unsaturated fatty acids, generating hydroperoxides and free radicals, which, at the same time, react with further lipid molecules to develop other reactive chemical species [31]. It was established that the PV maximum tolerance value should be a 0.5 mM cumene hydroperoxide equivalent [21]. Then, the inclusion of 0.1 % JSSE in tuna pâté did lead to an inhibition of PV at both temperatures.

The increase in TBARS denotes an aldehyde compound formation. The decrease in this indicator implied that the volatile aldehydes were transformed into other compounds [31], as was detected for the control and the P1 and P2 treatments at 4 °C, and in the control and P1 treatments at 8 °C. The maximum allowed value for the TBARS values is a 2 mg MDA kg^{-1} sample [22]. Control samples showed the maximum value at day 16 of storage at 4 °C. An inhibitory effect on the TBARS formation was observed for the two JSSE concentrations tested throughout the entire storage period.

Peroxides are the most reactive compounds generated during lipid oxidation. These findings strongly suggested that JSSE acts as a secondary antioxidant, interacting directly with the radicals derived from fatty acids. Similar results were reported in other products, such as pork sausages [32], chicken fillets [33], and salmon paste [34]. These results proved the protective effect of JSSE against lipid oxidation on the pâté samples.

During storage, a gradual quality loss was perceived by the trained panel in the different sensory descriptors for all treatments. However, after day 8 of storage at 4 °C, and after day 12 at 8 °C, samples with 0.05% JSEE showed a good quality. This behavior was similar to that shown in other studies [35], which reported an increased quality loss up until the end of the analysis, when studying minced chicken products. Other reports described a sensory quality improvement and increased shelf life in chilled mackerel and hake, as a result of including squid skin extracts in the icing medium [12, 13].

This work demonstrated that squid skin pigments extracts, with antioxidant and antimicrobial activity, as well as no mutagenic nor clastogenic effect, can be used in the formulation of tuna fish pâté. When used at 0.05% (w/w), such an extract provided a significant antioxidant and antimicrobial effect on pâté samples during storage at two different temperatures (4 and 8 °C) for 12 days. The loss of sensory quality also confirmed that samples with 0.05 % of JSSE (P1) extract exhibited an extended shelf life, when compared to other pâtés, being acceptable even after 12 d of chilled storage. These results provide a way in which the jumbo squid by-product can be used as a source of biologically active compounds.

Author Contributions: Chan-Higuera developed the formal analysis and the results analysis; Carbonell-Barrachina and Ezquerra-Brauer established the conceptualization of the study; and Cano-Lamadrid, Rizzitano and Lipan helped in the proper establishment and interpretation of the microbiological, lipid oxidation and sensory analysis. All authors contributed to the writing—original draft preparation. The authors in charge of project supervision and funding acquisition were Carbonell-Barrachina and Ezquerra-Brauer.

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5. RESUMEN DE RESULTADOS, DISCUSIÓN Y CONCLUSIONES.

5.1. New icing media for quality enhancement of chilled hake (*Merluccius merluccius*) using a jumbo squid (*Dosidicus gigas*) skin extract

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5.1.1. Objetivo: Este artículo presenta un primer acercamiento al aprovechamiento de la piel de calamar gigante, un subproducto de la industria pesquera, como fuente de compuestos conservantes naturales. Se incluyeron soluciones acuosas que contenían extractos obtenidos con etanol – ácido acético, en medios de hielo de merluza europea (*Merluccius merluccius*) en dos niveles diferentes.

5.1.2. Resumen de Resultados y Discusión

Se propuso una estrategia avanzada para la conservación del pescado refrigerado, basada en la inclusión en el hielo de un extracto de piel de calamar gigante (*Dosidicus gigas*).

Se obtuvo una inhibición significativa ($P<0.05$) de la actividad microbiana (aerobios, psicrotrofos, *Enterobacteriaceae*, bacterias proteolíticas; pH, trimetilamina) en la merluza correspondiente al lote de formación de hielo incluyendo la concentración más alta de extracto. Además, las muestras de pescado a las cuales se añadió el extracto mostraron un efecto inhibidor ($P<0.05$) sobre el desarrollo de la hidrólisis lipídica, mientras que no se describió ningún efecto ($P>0.05$) para la oxidación lipídica. El análisis sensorial (desarrollo de piel y moco; ojos; branquias; textura; olor externo; olor a carne cruda y cocida; sabor a carne) indicó una extensión de la vida útil de la merluza fría almacenada en hielo, incluida la concentración más alta de extracto.

En lo que respecta a la actividad mutagénica del extracto de piel de calamar se logró demostrar que no es capaz de causar mutaciones ni de errores en el marco de lectura ni sustitución de bases, evaluado en dos cepas de *Salmonella typhimurium* Sub. Enterica (TA 98 y 100). Las concentraciones utilizadas en el ensayo resultaron no mutagénicas, aún siendo las máximas posibles por placa.

En el presente trabajo se ha desarrollado un uso rentable de extracto de piel de calamar gigante, un subproducto industrial durante la comercialización del calamar, que conduce a una notable inhibición microbiana y una prolongación significativa de la vida

útil de la merluza refrigerada. De acuerdo con investigaciones anteriores, los omocromos (compuestos de tipo lipofílico) se considerarían responsables de este efecto conservante.

5.1.3. Resumen de Conclusiones

Cuando se empleó a la concentración más alta, dicho extracto proporcionó un efecto antimicrobiano significativo ($P < 0.05$) en el músculo de la merluza durante el almacenamiento refrigerado hasta 13 d; además, se podría representar un efecto inhibitorio significativo ($P < 0.05$) en el desarrollo de la hidrólisis lipídica.

La evaluación de la calidad sensorial también confirmó que las muestras de merluza en contacto con la concentración más alta de extracto de calamar exhibieron una vida útil prolongada en comparación con otros lotes, siendo aceptable incluso después de 13 d de almacenamiento refrigerado.

La nula toxicidad encontrada para los extractos en células de *Salmonella* sugiere la inocuidad de los mismos; sin embargo, es necesario realizar estudios donde se confirme que el extracto no ejerce ningún tipo de efecto mutagénico ni clastogénico en células eucariotas.

Este trabajo abre el camino a la utilización de un subproducto abundante del procesamiento del calamar gigante como fuente natural susceptible de proporcionar compuestos conservadores para ser empleados durante la comercialización de especies marinas.

5.2. Jumbo Squid (*Dosidicus Gigas*) Skin Pigments: Chemical Analysis and Evaluation of Antimicrobial and Antimutagenic Potential

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5.2.1. Objetivo: El objetivo de este estudio fue establecer el potencial antimicrobiano y antimutagénico y la estructura química de los compuestos pigmentados extraídos de la piel de calamar gigante. Este es el primer estudio de la actividad antimicrobiana de los omocromos contra cepas específicas de bacterias y hongos, así como su actividad antimutagénica.

5.2.2. Resumen de Resultados y Discusión

Mediante la manipulación de tres variables independientes se logró obtener las condiciones de extracción donde se obtuvo un mayor rendimiento de extracción y actividad antioxidante. Las variables evaluadas fueron tiempo de sonicación, temperatura de extracción y cantidad de solvente. Se extrajeron pigmentos de la piel de calamar gigante con un rendimiento de 635 mg/g y capacidad de absorbancia de radicales de oxígeno (ORAC) con 178 µmol de TE/g usando condiciones óptimas: 25 °C y 5 min de sonicación, establecido por análisis factorial.

La actividad antimicrobiana de los extractos se evaluó utilizando el método de difusión en agar. Se realizó utilizando cepas de bacterias Gram positivas y negativas, mohos y levaduras. El extracto mostró más del 50% de inhibición contra *Haemophilys influenza, Listeria monocytogenes, Staphylococcus aureus* y *Candida albicans*. La alta actividad antimicrobiana de JSSE (<90%) se detectó en *Salmonella enterica*.

El extracto obtenido inhibió la mutación inducida por aflatoxina B_1 en la cepa TA98 de *Salmonella tryphimurium* (> 50%), pero no en la cepa TA100 (<20%). Esto habla de que, además de no ser mutágenico en el ensayo de Ames, el extracto logró ser antimutagénico, previniendo el daño causado por la aflatoxina B_1.

Los datos sobre el comportamiento de solubilidad, la absorbancia máxima (440 nm), los protones observados en los espectros de RMN 1H y el pico de espectros FT-IR a 1742 cm^{-1} de JSSE, sugieren que el compuesto responsable de sus actividades antimicrobianas y antimutagénicas proviene de la familia de los omocromos. La compilación de los resultados derivados de las técnicas espectroscópicas permitió llegar a la conclusión de que estaba presente en el extracto de piel de calamar gigante compuestos pertenecientes a los omocromos. Uno de los componentes que se presupone presente en el extracto es la xantomatina, omocromo característicos de cefalópodos marinos como el calamar, la sepia y el pulpo.

5.2.3. Resumen de Conclusiones

Los pigmentos de piel de calamar gigante contienen compuestos antibacterianos y antimutagénicos, que se detectaron en los extractos solubles en metanol-HCl. La extracción de pigmentos bioactivos de pieles de calamar gigante se optimizó tanto por temperatura como por tiempo de sonicación. La actividad antioxidante y antimicrobiana se vio afectada negativamente a medida que se aumentaba el tiempo de sonicación y la temperatura; la cantidad de solvente utilizado durante la extracción resultó ser proporcional a la actividad antioxidante, es decir, a mayor cantidad de solvente, mayor actividad antioxidante. Esto se debe posiblemente al efecto de saturación del solvente que evitaría la extracción completa de los omocromos a partir de los cromatóforos de la piel del calamar gigante.

El presente estudio sugiere que uno de los principales compuestos que ejerció la actividad biológica en los extractos pigmentados de piel de calamar fueron las omatinas, específicamente del tipo de xantomatina. Sin embargo, los estudios futuros deben centrarse en la identificación de los mecanismos antimicrobianos y antimutagénicos específicos de los compuestos presentes en el extracto pigmentado de piel de calamar gigante.

5.3. Xanthommatin is Behind the Antioxidant Activity of the Skin of *Dosidicus gigas*.

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Molecules; https://doi.org/10.3390/molecules24193420

5.3.1. Objetivo: Aislar e identificar los pigmentos responsables de la actividad antioxidante detectada en la piel del calamar (*Dosidicus gigas*).

5.3.2. Resumen de Resultados y Conclusión: Los extractos de pigmento metanol-HCl (1%) de JSS se fraccionaron por cromatografía en columna abierta. Se utilizó como fase estacionaria sílica gel y como fase móvil la combinación de los siguientes solventes: metanol, acetato de etilo, ácido acético e hidróxido de amonio. Las fracciones colectadas (32) se agruparon por cromatografía en capa fina para aislar los pigmentos antioxidantes. Las condiciones fueron las siguientes: como fase estacionaria se utilizaron placas de vidrio con sílica gel y como fase móvil la combinación de metanol, acetato de etilo e hidróxido de amonio.

La actividad antioxidante se determinó mediante los ensayos de estabilización de radicales DPPH, ABTS y el poder reductor férrico (FRAP). En cada uno de los ensayos se evalúa por métodos distintos, la actividad antioxidante de una muestra. En DPPH se mide si los compuestos son capaces de donar un átomo de hidrógeno a este radical; en ABTS se evalúa la capacidad de donar un electrón a este radical obtenido de la oxidación mediada por el persulfato de potasio. En FRAP se mide la capacidad de donar un electrón a un átomo de hierro, transformándolo de ion férrico a ferroso.

Las fracciones 11–34 se separaron y agruparon de acuerdo con los valores del caudal (F1 – F8). Las fracciones F1, F3 y F7 tuvieron la IC_{50} más baja contra ABTS por miligramo, y las fracciones F3 y F7 mostraron la IC_{50} más baja en el ensayo FRAP. Finalmente, la fracción F7 tuvo la mayor actividad de eliminación de DPPH. La estructura química de la fracción F7 se caracterizó por espectroscopía infrarroja, resonancia magnética nuclear 1H y ionización por electrospray-espectrometría de masas. Uno de los

compuestos identificados en la fracción fue xantomomatina (ácido 11- (3-amino-3-carboxipropanoil) -1-hidroxi-5-oxo-5H-pirido [ácido 3,2-a] fenoxazina-3-carboxílico) y sus derivados (hidro y dihidroxantomatina).

5.3.3. Resumen de Conclusiones

Las fracciones obtenidas por la separación en columna abierta fueron obtenidas dadas las características de los compuestos presentes en el extracto. El carácter anfipático de los omcoromos permitió la obtención de las moléculas separadas. La técnica de cromatografía de capa fina logró agrupar las fracciones que, según el patrón de migración, eran iguales. Se pasó de 32 a 8 fracciones, de las cuales se evaluó la capacidad antioxidante mediante tres métodos distintos.

En este estudio, los omocromos xantomomatina, hidroxantomatina y dihidroxantomomatina se encontraron como componentes moleculares responsables de la actividad antioxidante del extracto, y sus mecanismos antioxidantes se han descrito como transferencia de átomos de hidrógeno y transferencia de electrones individuales. Los resultados de este estudio confirman la presencia de omocromos con actividad biológica en extractos de calamar gigante.

Esta información puede ayudar a establecer que los pigmentos de piel de calamar gigante tienen un uso potencial en la industria alimentaria como agente preventivo contra la oxidación. Actualmente, hay un estudio en curso sobre la aplicación de la fracción con la mayor actividad antioxidante para la preservación de un producto alimenticio y su posible riesgo toxicológico, así como la contribución de la actividad antioxidante de los diferentes compuestos presentes en la fracción.

5.4 Evaluation of *Dosidicus gigas* Skin Extract as an Antioxidant and Preservative in Tuna Pâté

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5.4.1. Objetivo. Evaluar la actividad biológica de los extractos de piel de calamar gigante en la estabilidad oxidativa de los lípidos, el crecimiento microbiano de los mesófilos y psicrófilos, y la pérdida de la calidad sensorial de los patés de atún aleta amarilla, que se almacenaron a dos temperaturas (4 y 8 °C).

5.4.2. Resumen de Resultados y Discusión. Se evaluó una estrategia para la conservación de alimentos, basada en un extracto de piel de calamar de metanol-HCl (Dosidicus gigas) (JSSE), a dos concentraciones en patés de atún aleta amarilla, que se almacenaron a 4 y 8 °C durante 20 d. El JSSE se caracterizó por determinar sus actividades antioxidantes y mutagénicas. Se elaboró un paté de atún aleta amarilla, con y sin la adición de JSSE. Se realizó un análisis sensorial afectivo para establecer las preferencias de los consumidores. Durante un período de almacenamiento de 20 d, se evaluaron la actividad del agua (aw), el pH, la diferencia de color (ΔE*ab), el análisis microbiológico, la oxidación lipídica y los atributos de calidad sensorial, y los resultados se compararon con los resultados del hidroxianisol butilado (BHA) y tratamientos de control. El JSSE mostró actividad antioxidante contra los radicales 2,2-difenil-1-picrilhidrazilo (DPPH+) y 2,2′-azino-bis (ácido 3-etilbenzotiazolina-6-sulfónico) (ABTS) y no indujo mutación, de acuerdo con la prueba de Salmonella de Ames, ni las anomalías cromosómicas, de acuerdo con el ensayo de células con punta de raíz de cebolla. El análisis del consumidor demostró una mayor preferencia por el paté con el JSSE agregado en siete de los ocho atributos evaluados. Durante el almacenamiento, el JSSE no tuvo impacto en aw ni pH, mantuvo valores más bajos de ΔE*ab, inhibió la actividad microbiana y la oxidación de los lípidos (a diferencia del paté de control), y preservó los atributos de calidad sensorial, a diferencia del BHA y los tratamientos de control. Este estudio mostró que el JSSE tiene pigmentos biológicamente activos que pueden actuar como antioxidantes y antimicrobianos en los patés de atún aleta amarilla.

5.4.3. Resumen de Conclusiones. Este trabajo demostró que los pigmentos extraídos de la piel del calamar con actividad antioxidante y antimicrobiana, así como sin efectos mutagénicos o clastogénicos, pueden usarse en la formulación del paté de atún. Cuando se usó al 0.05% (p / p), dicho extracto proporcionó efectos antioxidantes y antimicrobianos significativos en las muestras de paté durante el almacenamiento a dos temperaturas diferentes (4 y 8 °C) durante 12 d. La pérdida de calidad sensorial también confirmó que las muestras con 0.05% del extracto de JSSE (P1) exhibieron una vida útil prolongada en comparación con otros patés, siendo aceptable incluso después de 12 d de almacenamiento refrigerado. Estos resultados proporcionan una forma en que el subproducto del calamar gigante se puede utilizar como fuente de compuestos biológicamente activos.

6. CONCLUSIONES E INVESTIGACIONES FUTURAS

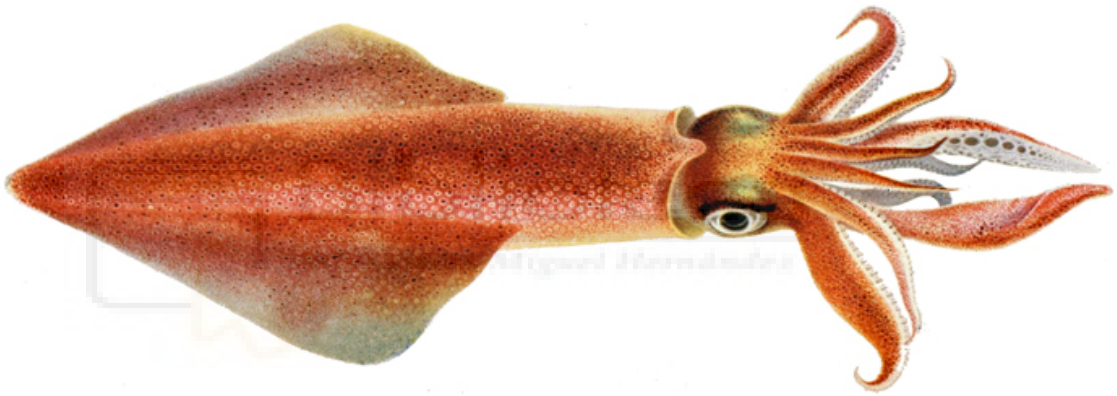
6.1 CONCLUSIONES

1. El proceso de extracción de pigmentos antioxidantes de piel de calamar gigante fue optimizado (25 °C, 5 min de sonicación, proporción 1:20 de solvente).
2. Los pigmentos de calamar gigante presentan actividad antimicrobiana frente a bacterias y hongos patógenos y de importancia en alimentos.
3. Los extractos de pigmentos de piel de calamar no poseen actividad mutagénica ni clastogénica en modelos de células procariotas y eucariotas.
4. La adición de los pigmentos de calamar gigante mejora las propiedades sensoriales de paté de atún y prolongan su vida de anaquel y su calidad sensorial.
5. La xantomatina es el compuesto omocrómico encontrado por métodos espectrofotométricos y espectrométricos en el extracto de piel de calamar

6.2 INVESTIGACIONES FUTURAS

- Establecimiento de los límites posibles de adición de extracto de piel de calamar gigante a productos elaborados para consumo humano.
- Evaluación de la actividad como conservador de alimentos del extracto de piel de calamar gigante en otras matrices alimentarias de diversos orígenes.
- Esclarecimiento del mecanismo de actividad antioxidante y antimicrobiano de la xantomatina de manera experimental.
- Contribución de la xantomatina a la actividad antioxidante y antimicrobiana del extracto crudo.

7. REFERENCIAS



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