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Pigmentos de Calamar Gigante (*Dosidicus gigas*): Estructura Química, Actividad Antioxidante y Antimicrobiana y su Aplicación como Aditivo Alimentario

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M. en C. Jesús Enrique Chan Higuera

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APROBACIÓN

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

CONTENIDO

INTRODUCCIÓN

La producción de alimentos representa una de las principales actividades económicas, ya que de ella depende la nutrición y desarrollo adecuado de las poblaciones. Los alimentos son susceptibles a procesos de degradación tanto por factores endógenos como exógenos. La peroxidación y el desarrollo microbiano son dos reacciones que causan pérdidas de hasta 165 mil millones de dólares anualmente. La industria alimentaria ha combatido esta problemática mediante el uso de antioxidantes y antimicrobianos sintéticos, si bien baratos y altamente efectivos, los cuales han sido asociados al desarrollo de enfermedades crónico-degenerativas.

La investigación en productos naturales ha dedicado su búsqueda de alternativas que puedan actuar como conservadores de alimentos. Se han encontrado compuestos de diversas estructuras químicas capaces de retardar la oxidación lipídica y el desarrollo microbiano. Dichos compuestos han logrado extraerse de hierbas, hongos, partes de plantas, especias secas, por mencionar algunas. Sin embargo, en el lecho marino también se encuentran especies de donde pueden aprovecharse compuestos biológicamente activos.

El calamar gigante (*Dosidicus gigas*) representa una especie de la cual pueden obtenerse moléculas con actividad biológica. Estudios previos han reportado la presencia de proteínas y péptidos bioactivos de regiones anatómicas como las aletas, los tentáculos y los brazos, así como carbohidratos complejos como la quitina y el quitosano, que pueden extraerse del pico y la pluma de este cefalópodo. La piel del calamar gigante es rica en compuestos coloridos llamados omocromos. Estos compuestos han sido sujeto de estudio en recientes años, debido a su actividad antioxidante evaluada tanto *in vitro* como en modelos alimentarios.

La aplicación de los omocromos de calamar gigante se ha enfocado hacia productos como aceite de pescado y en sistemas de hielo para la preservación de peces. Sin embargo, no existe información acerca de la aplicación de los omocromos en matrices alimentarias complejas donde se encuentren los compuestos mezclados con el resto de los componentes alimentarios. Aunado a esto, no se cuenta con una metodología

establecida de extracción, que se enfoque exclusivamente en la maximización de la actividad biológica de los mismos. Además, existe limitada información acerca de la composición química de los compuestos presentes en los extractos de piel de calamar gigante.

En este trabajo se evaluó la optimización del proceso de extracción de los omocromos con actividad biológica de la piel del calamar gigante, así como una evaluación sobre el efecto mutagénico y clastogénico de los extractos como una medida del posible daño genético que podrían producir los extractos en células sanas. La aplicación del extracto de piel de calamar se llevó a cabo en la adición en la formulación de paté de atún aleta amarilla, evaluando las características físicas, químicas, microbiológicas y sensoriales de las muestras a lo largo del muestreo. Por último, se fraccionó el extracto y se caracterizó la fracción de mayor actividad antioxidante para conocer los compuestos de naturaleza omocrómica que contribuyen con la capacidad de actuar como un conservador de alimentos.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

Para probar la hipótesis planteada, el trabajo experimental se dividió en tres etapas, las cuales se describen en los siguientes tres capítulos.

Capítulo 1: **Optimización del proceso de extracción de pigmentos de la piel de calamar gigante y la evaluación de su actividad biológica**

Este capítulo consiste en un manuscrito titulado: “Jumbo squid (*Dosidicus gigas*) skin pigments: Chemical analysis and evaluation of antimicrobial and antimutagenic potential”, aceptado en la revista *Journal of Microbiology, Biotechnology and Food Technology*. El manuscrito contiene información acerca de la optimización de la extracción, así como la actividad antioxidante, antimicrobiana y antimutagénica del extracto obtenido tras la optimización.

Capítulo 2: Efecto de la adición del extracto de piel de calamar gigante sobre la vida de anaquel y la calidad sensorial de paté de atún aleta amarilla

Este capítulo consiste en el manuscrito “Novel additive for quality enhancement of tuna pâté using a *Dosidicus gigas* skin extractl”, sometido a la revista *LWT – Food Science and Technology.* El manuscrito contiene información acerca del cambio de las propiedades físicas, químicas, microbiológicas y sensoriales de muestras de paté de atún, en función de la adición de extractos de piel de calamar gigante.

Capítulo 3: Caracterización química y estructural de los compuestos presentes en el extracto de piel de calamar gigante

Este capítulo consiste en un manuscrito titulado: “Xanthommatin is Behind the Antioxidant Activity of the Skin of *Dosidicus gigas*”, sometido a la revista *Molecules.* El manuscrito contiene información acerca del aislamiento y purificación de los compuestos con actividad antioxidante presentes en el extracto de calamar gigante.

CAPÍTULO 1.

Optimización del proceso de extracción de pigmentos de la piel de calamar gigante y la evaluación de su actividad biológica

REGULAR ARTICLE

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

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Dear *Josafat Marina Ezquerra-Brauer*,

we are pleased to inform you that the above noted manuscript has been accepted for publication in The Journal of Microbiology, Biotechnology and Food Sciences. Your paper will publish in the issue October - November 2019, vol. 9, no. 2. We will publish your article in the 1st October 2020 or the first work day at this month. Then your article will be freely available on JMBFS websites. Abstract and preprint version as HTML form (fulltext without DOI number and pages) will be available after accepting as soon as possible.

We thank you for choosing this journal for publishing your research and hope that you will consider doing so again in the future.

Sincerely yours,

Lukáš Hleba

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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 precent 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:

Inhibition rate (%)=[((1—T))/M]× 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, ^1H 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 μmol 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%).

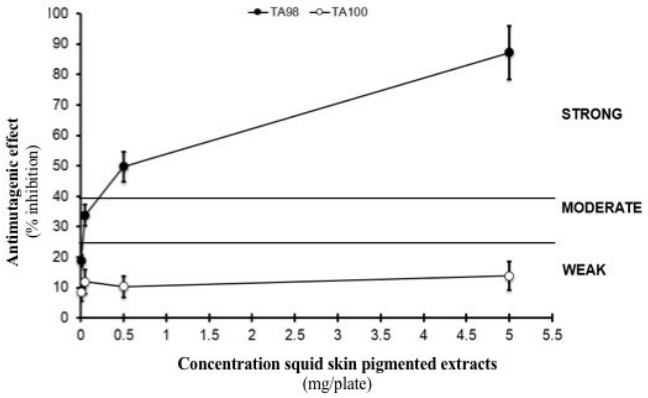

Figure 1. Effect of jumbo squid skin pigments on the mutagenicity induced by aflatoxin B_1, based on *Salmonella typhymurium* TA 98 and TA 100. All values represent mean value of triplicate determination±standard deviation.

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).

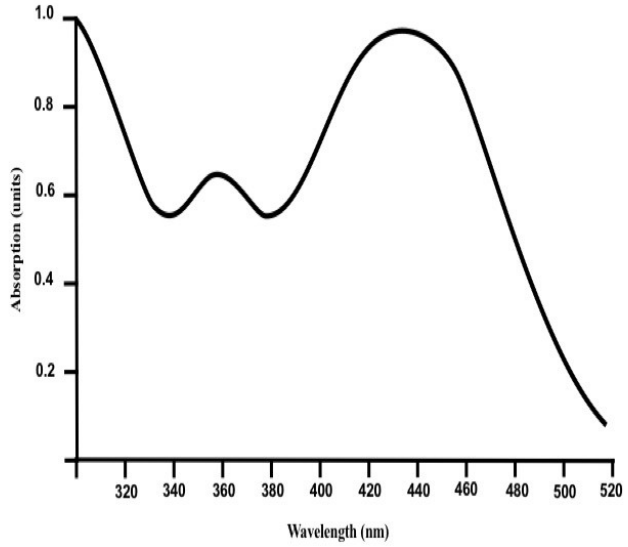

Figure 2 UV-Vis spectrophotometric spectra of jumbo squid skin pigments.

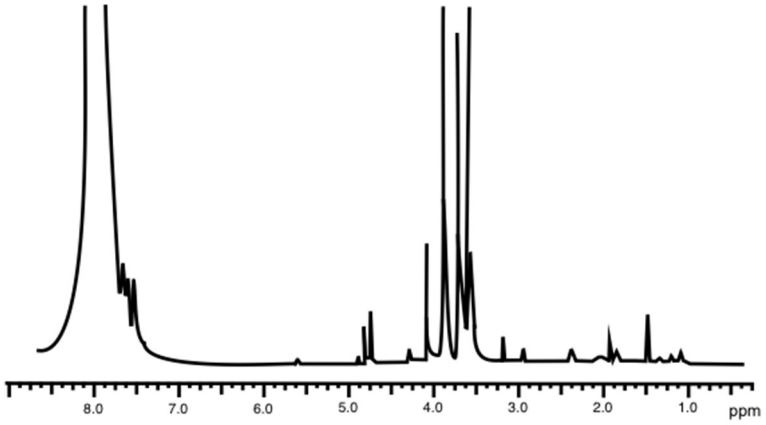

Figure 3 ^{1}H NMR spectrophotometric spectra of jumbo squid skin pigments.

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**).

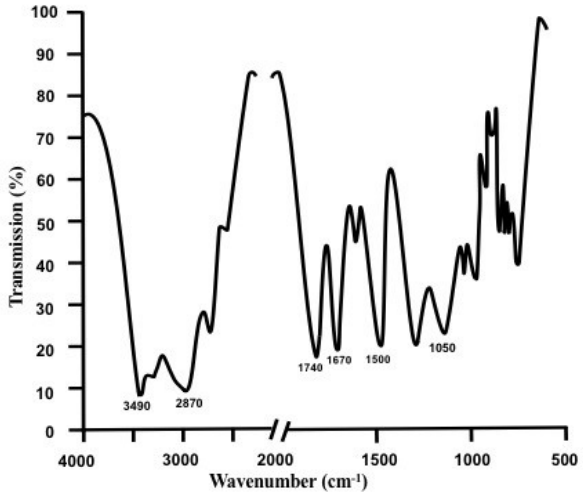

Figure 4 FTIR spectrophotometric spectra of jumbo squid skin pigments.

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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CAPÍTULO 2.

Efecto de la adición del extracto de piel de calamar gigante sobre la vida de anaquel y la calidad sensorial de paté de atún aleta amarilla

Novel additive for quality enhancement of tuna pâté using a *Dosidicus gigas* skin extract

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ABSTRACT

An innovative strategy for food preservation, based on a methanol-HCl jumbo squid skin extract (*Dosidicus gigas*) (JSSE), was evaluated at two different concentrations in yellowfin tuna fish pâtés stored at 4 and 8°C for 20 days. JSSE was characterized by determining the antioxidant and mutagenic activities. Tuna pâté was elaborated, with and without the addition of JSSE. During a 20 days storage period conjugated dienes (CD), peroxide value (PV), TBARS and sensory quality attributes were evaluated; results were compared against BHA and control treatments. Affective sensory analysis was performed to establish consumers' preference. JSSE showed antioxidant activity against the DPPH and ABTS radicals, and did not induce neither mutations, nor chromosomal abnormalities. The consumer analysis demonstrated a higher preference for the JSSE-added pâté in seven out of the eight evaluated attributes. CD, PV and TBARS values were lower in comparison with the control pâté. The sensory quality attributes were longer maintained by the JSSE-added pâtés in comparison with the BHA and control treatments. During storage, a significant inhibition of microbial activity was observed in pâté stored at 8°C treated with 0.05 % of JSSE. This study showed that JSSE has potential as an antioxidant and antimicrobial in fish products.

Keywords: antioxidant activity, antimicrobial activity, squid pigments, sensory analysis.

1. INTRODUCTION

Fatty fish-based products, such as fish pâté, constitute a food product of great economic and nutritional importance because of their composition and health benefits (Rangel-Huerta & Gil, 2018). 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 (Kuuliala et al., 2018). 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 (de Almeida et al., 2015).

Fish pâté is widely regarded as a product with an important gastronomic tradition and has a high nutritional value, as well as appreciated sensory attributes. Pâté is more vulnerable to microbial growth and peroxidation given the disruption of the cellular components, induced by the grinding process (de Carli, Moraes-Lovison, & Pinho, 2018). 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 (Geeta & Yadav, 2017). 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 go from 6 to 11°C, in comparison with the recommended temperature of 4°C, the use of additives in refrigerated products is still necessary to prevent microbial growth and lipid oxidation (Brown, Hipps, Easteal, Parry, & Evans, 2014). Even if synthetic antioxidants and antimicrobials are highly effective, there is uncertainty of their negative impact on human health. Replacing synthetic additives with natural compounds that can

exert the same function satisfies the popular demand of clean labels, as well as creating safer products (Kumar, Kaur, Shahi, Kairam, & Tyagi, 2017).

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, among others (Sanches-Silva et al., 2014). One type of biologically active compounds are ommochromes. They 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 via the kynurenine pathway (Daniels & Reed, 2012). The functional groups that vary from molecule to molecule give ommochromes a distinctive reddish to violet coloration, in addition to antiradical *in silico* activity (Romero & Martinez, 2015). These compounds act as antioxidants against UV radiation (Feldman et al., 2008). Recent studies of jumbo squid (*Dosidicus gigas*) skin have demonstrated their antioxidant activity applied in fish oil at different storage temperatures (Aubourg, Torres-Arreola, Trigo, & Ezquerra-Brauer, 2016), and their antimicrobial activity during chilled storage of fresh mackerel and hake (Ezquerra-Brauer, Miranda, Cepeda, Barros-Velazquez, & Aubourg, 2016; Ezquerra-Brauer, Miranda, Chan-Higuera, Barros-Velázquez, & Aubourg, 2017). All these results suggest that ommochromes can be used as additives in the food industry. Notwithstanding, there is still no information regarding the use of JSSE on food matrixes like fish pâté, which tend to be more complex and more susceptible to oxidation reactions.

In the present work, the main objectives were to evaluate the biological activity of jumbo squid skin extract on the lipid oxidative stability and the loss of sensory quality of yellowfin tuna pâtés stored at two different temperatures (4 and 8°C), and on the microbial count of aerobic and anaerobic mesophiles and psychrophiles was established. In order to carry out the addition, the *in vitro* antioxidant activity, the mutagenic activityof the extracts were also evaluated.

2. MATERIALS AND METHODS

2.1. 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 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}, 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 removing the methanol using a rotary evaporator (R-100, Büchi, Switzerland). Solvent was further evaporated using nitrogen gas. The dry extracts stored in an inert nitrogen atmosphere, at -80°C prior to further analysis.

2.1.1. In vitro antioxidant activity

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, previously described (Brand-Williams, Cuvelier, & Berset, 1995; Re et al., 1999). Samples of 1 mg per mL JSSE were dissolved in aqueous methanol (80 %). Inhibition percentage of the extracts was calculated for both methods.

2.1.2. Salmonella mutagenic assay

Mutagenic activity was tested, using the *Salmonella typhimurium* tester strains TA98 and TA100, (MolTox; Annapolis MD, USA), with and without metabolic activation (presence of S9 mix, MolTox) (Anjum, Krakat, Reza, & Klocke, 2014). Four different doses of JSSE were evaluated (0.1, 0.5, 1 and 5 mg per milliliter). All of them were diluted in 0.2 M phosphate buffer (pH 7.4). The concentrations were selected based on a preliminary toxicity test.

2.1.3. Onion root-tip clastogenicity test

Clastogenicity of the samples was evaluated, as previously described by Liman, Cigerci & Ozturk (2015). 0.1, 0.5, 1 and 5 mg of JSSE were used in the assay. A positive control with sodium

azide (10 ng) and a negative control (water) were also analyzed. The number of mitotic cells with irregular chromosomal appearance (i.e. micronuclei, disorganized chromosomal structure, lag and stick chromosomes) was recorded.

2.2. Pâté elaboration

Fillets of fresh yellow fin tuna fish was obtained from a local market in Orihuela (Alicante, Spain). Tuna fish fillets were chopped in 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. Sodium caseinate (2 g) and corn starch (2 g) were slowly added until a homogenous mix was formed. Olive oil (5 g) was smoothly added, until the emulsion was formed. Other 2 g of caseinate and starch were added and mixed along with 5 g of oil. Finally, white wine vinegar (1 g) and the rest of the olive oil (5 g) were added. All batches were cooked until the pâté core temperature reached 75°C and cooled in an ice bath.

Based on preliminary studies, JSSE were added separately in two of them, at concentrations of 0.05 g *per* 100 g of pâté (P1), and 0.1 g *per* 100 g of pâté (P2); butyl hydroxy anisole was added to a third batch (BHA) representing different treatments. JSSE were mixed with vinegar before being mixed with other ingredients. The remaining batch was used as control sample (control).

2.2.1. Consumer acceptance panel

Pâté samples were taken from refrigerated storage and left to cool down to approximately 20 ° C and analyzed by a panel of 70 consumers. Consumers were asked their preference of eight attributes, in a scale (1: extremely dislike it, 5: neither like nor dislike it, to 9: extremely like it), as well as the intensity of such attribute (1: too weak, 5: just about right, to 9: too strong). Consumers between 20 and 65 years old were students and staff members of the Department of Agro-Food Technology (Miguel Hernández University, Desamparados Campus).

2.3. Cooling storage and pâté shelf life

All samples were packed in polyethylene plastic bags and divided into bags with and without vacuum and stored at 4 and 8 ± 1°C, under dark conditions, for 20 days. Pâté samples were taken at 0, 4, 8, 12, 16 and 20 d of storage. Analyses were performed the sampling day.

2.3.1. Physical parameters

Water activity was determined in polyethylene capsules with ca. 5 g of pâté and placed in a Labmaster-aw water activity equipment (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. Determinations were done using a pH Basic 20 equipment (Crison, Barcelona, Spain). The color of pâtés was measured by 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. Total color difference between samples was evaluated with the ΔE_{ab}^* parameter, following the equation previously reported (Hunt, Sorheim, & Slinde, 1999):

$$\Delta E_{ab}^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

2.3.2. 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 laminar flux cabinet in sterile conditions. Triplicate samples were analyzed from each trial of pâté formulation. For estimating 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 total aerobic mesophiles (37°C) and psychrophiles (7°C). Colonies were counted under after 48 h for mesophiles and 72 h for

psychrophiles, with a colony counter. The average count was multiplied by the dilution factor and expressed as log CFU g^{-1} of the sample.

2.3.3. Lipid oxidation

Conjugate dienes (CD) and trienes (CT) were measured by mixing 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, placed in quartz cuvettes and measurements of CD and CT were done at 232 and 268 nm, respectively. The concentration of dienes and trienes was determined by using the molar extinction coefficient specific for both types of compounds (Martin-Sanchez et al., 2014).

Quantification of hydroperoxides evaluated as secondary peroxidation products was carried out according to previous reports (Pateiro, Lorenzo, Vazquez, & Franco, 2015) with some modifications. Lipid extraction was done with 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 equivalent nmol of cumene hydroperoxide per g of pâté.

The end-products of peroxidation were determined using the thiobarbituric acid assay (TBARS) (Villalobos-Delgado, Gonzalez-Mondragon, Govea, Andrade, & Santiago-Castro, 2017). 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 malondialdehyde equivalents per g of pâté.

Antioxidant efficacy of JSSE was assessed in the samples with an oxidation index at both storage temperature (4 and 8°C). The percentage of oxidation inhibition [OI (%)] was calculated for PV and TBARS using the following formula (Frankel, Huang, & Aeschbach, 1997):

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

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

2.3.4. Sensory quality

To evaluate the sensory quality of the samples throughout the storage time, a panel of seven qualified and experienced panelists in the field of fish products was appointed, with the sensory attribute descriptors of coded samples. They performed the analysis in a standard sensory laboratory (under white light, 25°C, 50-55 % relative humidity). The same panel evaluated all the samples. The analysis was performed through scoring sensory properties by assigning a scale from 1 to 5 points, where a higher score means a lesser quality value. 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**.

2.4. Statistical analysis

Descriptive statistics was used to present the antioxidant assays (n=3). Data obtained from the consumer acceptance study was evaluated through a two-way analysis of variance (ANOVA) was carried out in order to assess the significance of JSSE addition on all the characteristics studied. Data obtained from the Ames' test, microbiological and chemical analyses (n=3) were subjected to the ANOVA method to establish differences resulting from the effects of the JSSE addition; the comparison of means was performed using the Tukey test ($p<0.05$). Data obtained from the sensory evaluation and the onion root tip assay were analyzed by the non-parametric Kruskal-Wallis test. In all cases, analyses were carried out using the SAS Institute, Inc. (JMP 5.0.1,

USA); differences among treatments were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

3. RESULTS AND DISCUSSION

3.1. Preliminary analysis of jumbo squid skin pigmented extracts

The obtained extract showed a reddish-violet color and the recovery yield (expressed as g of dry JSSE per 100 g of skin) was of 0.65%; these pigmented extracts presented antioxidant activity, measured in terms of DPPH and ABTS radical scavenging activities. The antioxidant compounds present in 1 mg mL^{-1} of JSSE generated an inhibition percentage of 68 against the DPPH radical and 79 in the $ABTS^{\bullet+}$ test. The antioxidant activity values of JSSE were considered effective because they were higher than the results reported previously for DPPH (30 %) and for ABTS (50 %) (Wojdylo, Oszmianski, & Czemerys, 2007).

Mutagenicity test with *Salmonella* strains and the onion root-tip clastogenicity tests are used for evaluating the potential toxic effect of a sample on the genetic material in prokaryotic and eukaryotic cells. In the *Salmonella* test, the mutagenicity is considered negative when the number of revertant colonies counted per plate did not double the number of spontaneous revertants (Mortelmans & Zeiger, 2000). Based on this, the mutagenicity of pigmented extracts was considered negative (**Fig. 1**). Similarly, purple natural pigments (Gutierrez-Zuniga, Arriaga-Alba, Ordaz-Pichardo, Gutierrez-Macias, & Barragan-Huerta, 2014), reported no mutagenic effect on the same *Salmonella* strains.

The JSSE clastogenicity test showed its degree of chromosomal damage by the onion root-tip assay (**Table 2**). The method was validated by the percentage of observed cells (over 150), and mitotic cells (over 100). Genotoxic activity is considered when the percentage of cells with damaged chromosomes is over 50 %, in comparison with 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 by the results of this study.

3.2. Consumer acceptance

The evaluation of consumer acceptance showed the effect of JSSE addition in the sensory attributes of tuna pâtés (**Table 3**). Results demonstrated that JSSE-treated samples (0.05 and 0.1 %) had scores significantly higher ($p < 0.05$) when compared with 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, in comparison with the control samples.

3.3. Assessment of quality evolution during fish pâté cooling storage

3.3.1. Physical index

All tuna pâtés water activity ranged from 0.93 to 0.95 during all samplings. JSSE addition did not affect directly the proportion of free water in the samples, when compared with control treatments ($p>0.05$). These results suggested that JSSE does not have an impact on water activity, as an indicator of possible microbial development (Maqsood, Benjakul, & Balange, 2012).

Results concerning the pH levels showed no differences among the samples ($p>0.05$). Nevertheless, a significant pH drop was detected at storage day 4 for all pâté treatments. This can be due to the microbial development, resulting in formation and accumulation of acidic compounds such as lactic acid among other acidic metabolites (Fan, Chen, Sun, & Zhang, 2014).

The color of the samples became more intense when the amount of JSSE added to the tuna pâté increased. The changes in ΔE_{ab}^* values during storage days were statistically significant, as shown in **Fig. 2**. Values greater than 5 are recognized as perceptible to the human eye (Hunt et al., 1999). In both storage temperatures (4 and 8 ° C), for the control and BHA treatments, the values of delta e were greater than 5 from day four. JSSE-pâtés (P1 and P2) maintained ΔE_{ab}^* values of less than 5 until day 16 of storage. The results demonstrated that samples that were added with JSSE maintained their color throughout all the experiment.

3.3.2. Microbiological analysis

The changes with time of the aerobic mesophiles in the four types of pâté are shown in **Table 4**. The inclusion of JSSE in the pâté exerted 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} (NOM-210-SSA1, 2014). In the period of 16-20 days, the mean aerobic concentrations were greater than 250 logarithmic units in control and BHA.

The examination of psychrotrophs in the types of pâté is also presented in Table 4. Species-specific spoilage bacteria, including members of the genera *Pseudomonas, Shewanella, Acinetobacter, Moraxella* or *Flavobacterium*, are being considered in the psychrotrophic group. It should be noted that the treatments with JSSE, the concentrations of psychotrophs were less than 250 logarithmic units at 12 days and 8 days at 4ºC and 8ºC, respectively.

The results presented in this work demonstrated a bacteriostatic effect on tuna pâté because of the inclusion of a squid skin extract (JSSE) in the formulation, at both storage temperatures 4°C and 8°C and in both addition concentrations. Previous studies also evaluated the preservative effect of an acetic acid-ethanol extracts of jumbo squid skin in chilled fish (Ezquerra-Brauer et al., 2016; Ezquerra-Brauer et al., 2017). In these studies, an antimicrobial effect (lower aerobic mesophiles and psychrotrophs counts) was observed during the chilled storage in fresh fish. This behavior may be due to the electron donor capacity of the ommochromes (Romero & Martinez, 2015), inducing an imbalance in metabolic pathways in microorganisms. However, further research is needed to establish the possible mechanism of compounds present in the extract.

3.3.3. Lipid oxidation index

The primary oxidation products, conjugated dienes (CD) and trienes (CT) evaluated throughout the storage time, at both temperatures (4 and 8°C) showed that all treatments had no statistically significant differences between them (p>0.05) and presented the trend typical of peroxidation. Over time, the amount of CD and CT decreased (data not shown). Therefore, the

addition of JSSE did not improve or promote the formation of primary peroxidation products. At both storage temperatures (4 and 8ºC), 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; they tended to associate with other components of the food or to decompose. Similar results have been reported in products such as vegetable oils (Redondo-Cuevas, Castellano, & Raikos, 2017).

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 (Nielsen & Jacobsen, 2013). It was established that the PV maximum tolerance value should be 0.5 mM cumene hydroperoxide equivalents (Pateiro et al., 2015). The maximum of hydroperoxide values at 4ºC was detected at 4–day for all treatments (**Fig. 3**), whereas at 8ºC was observed only in 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 and inhibitory effect (p<0.05). At 4–day of the experiment at both temperatures, the following inhibitor effect on hydroperoxide formation was demonstrated: Control=BHA=P1<P2.

The end products of lipid peroxidation found in the pâté samples were evaluated using the TBARS method (**Fig. 4**). The increase in TBARS denotes aldehyde compound formation. The decrease in this indicator implied that the volatile aldehydes were transformed into other compounds (Nielsen & Jacobsen, 2013), as was detected for control, P1 and P2 treatments at 4ºC, and in control and P1 treatments at 8ºC. The maximum allowed value for TBARS values is 2 mg MDA kg^{-1} sample (Villalobos et al., 2017). Control samples showed the maximum value at day 16 of storage at 4ºC. An inhibitory effect on TBARS formation was observed for the two JSSE

concentrations tested throughout the entire storage period. 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 inhibitor effect when compared with all treatments (p<0.05).

The inhibition percentage of JSSE in pâté lipid oxidation was calculated for PV and TBARS at 4ºC and 8ºC based on the control conditions scores obtained. Consistent with the results discussed above, the percentage of oxidation inhibition measured using PV index for BHA and JSSE samples, indicated that the pâté added with 0.1 % of JSSE (P2) was the sample that showed the highest inhibition at 4°C. The order of oxidation inhibition values in peroxide formation at 4°C was as follows P2(41.5%)>BHA(14.9%)>P1(6.2%), and at 8°C 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 was BHA(73.9%)>P2(45%)>P1(25.5%). 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 have been reported in other products, such as pork sausages (Balzan et al., 2017and salmon paste (Ortiz-Viedma et al., 2017). These results proved the protective effect of JSSE against lipid oxidation on the pâté samples.

3.3.4. Sensory evaluation

The evolution of sensory quality is depicted in **Table 5**. Initial pâté types were found with the highest quality (E score). During storage time, a gradual quality loss was perceived by the trained panel in the different sensory descriptors for all treatments. This behavior was similar to previous studies (Jimenez-Martin, Perez-Palacios, Carrascal, & Rojas, 2016), who reported an increased quality loss, up until the end of the analysis, when studying minced chicken products. After 8 days of storage at 4ºC, samples corresponding to the P1 (0.05% JSEE) treatments showed a good quality and were considered in the A category. Remarkably, pâté types belonging to control, BHA

and P2 (0.1%) treatments showed a fair or poor quality than P1 counterparts at this storage time. Meanwhile, at 8ºC until 12 days, the sensory quality of P1 treatment was considered good. Whereas the control, BHA and P2 at this period time the quality was considered fair or poor. Previous 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 (Ezquerra-Brauer et al., 2016; Ezquerra-Brauer et al., 2017).

4. Conclusions

A novel food additive obtained from squid skin (JSSE), with antioxidant and antimicrobial activity, as well as no mutagenic nor clastogenic effect, was implemented in the formulation of tuna fish pâté. When used at 0.05% (w/w), such extract provided a significant antioxidant and antimicrobial effect on pâté samples during storage at two different temperatures (4 and 8°C) up to 20 days. The loss of sensory quality also confirmed that samples with 0.05% of JSSE extract exhibited extended shelf life when compared to other pâtés, being acceptable even after 20 days of chilled storage. These results expand the way to the utilization of a jumbo squid by-product as a source of biologically active compounds.

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Figure Legends

Fig. 1. Mutagenic effect of giant squid skin extract* on strains (a) TA98 and (b) TA100 with and without metabolic activation (S9 Mix)**

* Mean values of three replicates (n=3); standard deviations are indicated by bars. Values accompanied by different letters indicate significant differences ($p<0.05$).

**SR = Spontaneous revertants per plate

Fig. 2. Effect of the addition of jumbo squid skin extract on the ΔEab* of the different processed pâtés**

*Mean values of three replicates (n=3); standard deviations are indicated by bars. Values accompanied by different letters indicate significant differences ($p<0.05$)

**Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) as expressed in Table 3.

Fig. 3. Effect of the addition of jumbo squid skin extract on the peroxide value* of the different processed pâtés**

*Mean values of three replicates (n=3); standard deviations are indicated by bars. Values accompanied by different letters indicate significant differences ($p<0.05$)

Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) as expressed in **Table 3.

Fig. 4. Effect of the addition of jumbo squid skin Extract on the TBARS value* of the different processed pâtés**

*Mean values of three replicates (n=3); standard deviations are indicated by bars. Values accompanied by different letters indicate significant differences ($p<0.05$)

Abbreviations of BHA and jumbo squid pigmented extracts (P1 and P2) as expressed in **Table 3.

CAPÍTULO 3.

Caracterización química y estructural de los compuestos presentes en el extracto de piel de calamar gigante

Type of the Paper Article

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

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Abstract: 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 highest percentage of $ABTS^{\bullet+}$ inhibition per milligram, and fractions F3 and F7 showed the highest FRAP power. Finally, fraction F7 exerted 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. Its structure was identified as that of xanthommatin (11-(3-amino-3-carboxypropanoyl)-1-hydroxy-5-oxo-5H-pyrido[3,2-a]phenoxazine-3-carboxylic acid). The results show that JSS pigments contain ommochrome molecules with antioxidant properties, and xanthommatin stands out with particularly high antioxidant activity.

Keywords: Antioxidant activity; Chromatography; Ommochromes; Spectroscopy; Xanthommatin

1. Introduction

Among the different species of squids, one of the most notable for its commercial impact and tonnage of capture is the jumbo squid (*Dosidicus gigas*) [1]. 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 [2].

Squid skin (normally considered a discard) is particularly rich in biologically active compounds, such as gelatin, collagen, and their peptides, as well as pigments [3–6]. 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 [7]. 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 [8]. Their basic structure is a ring of fenoxazone (ommatins) or phenothiazine (ommins and possibly ommidins) [9].

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 [10]. Ommochromes prevent photodamaging effects in the eyes of marine species [11]. 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 [12, 13].

Although the antioxidant activity of squid skin extracts has been examined in some studies, most reports have described antioxidant proteins and peptides [14]. 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*).

2. Results

2.1. Isolation and Purification

The liquid-phase column to open column method resulted in the elution of thirty-four 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 8 fractions, designated F1–F8.

2.2. Antioxidant activity

The antioxidant activity of fractions F1–F8 are shown in Table 1. As seen in Table 1, three fractions (F1, F3, and F7) had the highest percentage of inhibition per milligram of sample in the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+}$) assay. In the ferric reducing power (FRAP) assay, two fractions (F3 and F7) had the highest activity, while the activity of the F7 fraction was significantly higher ($p < 0.05$) than that of the other fractions in the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay. 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	DPPH[2] (% Inhibition mg^{-1} fraction)	ABTS[2] (% Inhibition mg^{-1} fraction)	FRAP[2] (% Inhibition mg^{-1} fraction)
F1	15.4 ± 0.2^{e}	47.2 ± 1.0^{a}	15.3 ± 0.7^{c}
F2	21.4 ± 0.2^{c}	36.1 ± 0.6^{c}	24.1 ± 1.0^{b}
F3	28.1 ± 0.6^{b}	48.2 ± 0.7^{a}	39.6 ± 2.1^{a}
F4	11.5 ± 1.1^{e}	9.8 ± 0.2^{e}	8.1 ± 3.3^{d}
F5	10.5 ± 0.9^{e}	16.6 ± 0.5^{d}	14.5 ± 1.8^{c}
F6	18.7 ± 0.4^{d}	44.5 ± 0.2^{b}	25.1 ± 1.7^{b}
F7	38.4 ± 0.4^{a}	48.1 ± 0.2^{a}	44.5 ± 2.0^{a}
F8	29.1 ± 0.8^{b}	17.3 ± 1.2^{d}	$13.7 \pm 2.5^{c,d}$

[1]The values represent the average of three repetitions ± standard deviation.

[2]Different letters in the same column indicate significant differences ($p < 0.05$).

2.3. Structure elucidation

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.

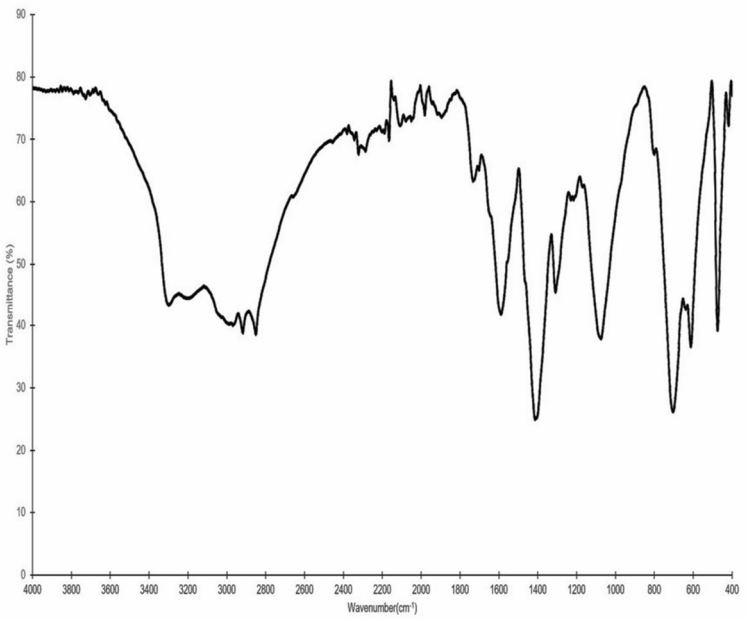

Figure 1. Infrared spectrum of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays.

The main compound 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 8.41 (d, 3H), 8.15 (t, 1H), 7.88 (d, 2H), 7.70 (s, 1H), 6.68 (s, 1H), 4.51 (d, 1H), and 3.87 ppm (m, 2H) (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).

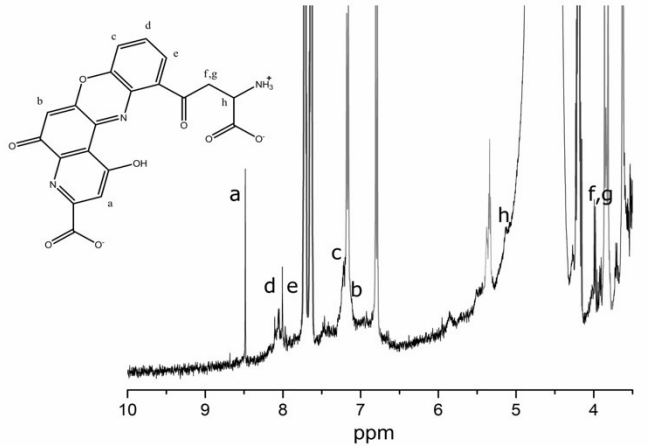

Figure 2. ^{1}H NMR spectra of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays.

2.4. Electrospray ionization–mass spectrometry

The negative 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 ommochrome was about 424.

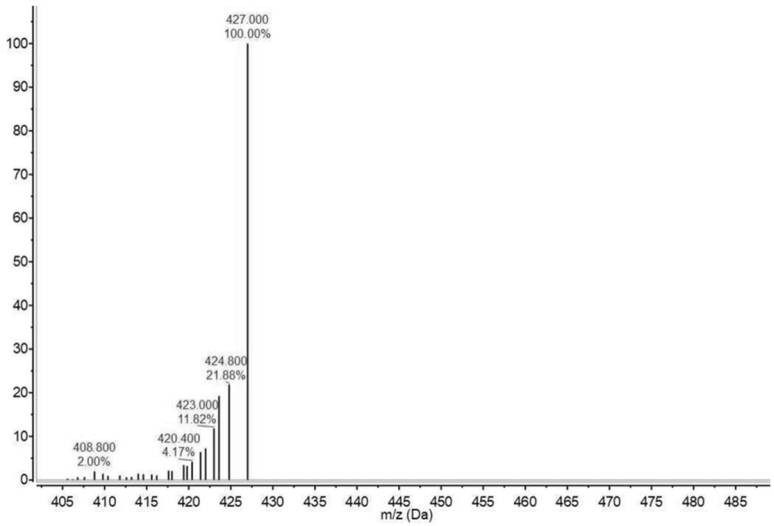

Figure 3. Electrospray ionization–mass spectrum of fraction F7, which showed the highest activity in the DPPH, ABTS, and FRAP antioxidant assays.
The data were acquired in scan mode using an m/z range of 300–650.

3. 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 [18, 19]. 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 [20].

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. Fraction F7 had the highest detected activity in the three methods, 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 the squid skin extract are able to exert such mechanisms, as suggested by previous *in silico* studies [21]. 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 structures of rhodommatin, ommatin D, hydroxykynurenine, and xanthommatin have functional groups that are related to antioxidant action, primarily hydroxyl linked to aromatic rings.

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 [22]. 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 [23]. 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 [24]. 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 [25].

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 [15, 16]. 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 [16]. The ion at m/z 427 was assumed to be the corresponding quasimolecular ion of another ommochrome, such as dihydroxanthommatin [24]. Therefore, from the FTIR and IR results, combined with the ESI-MS results, the presence of xanthommatin in F7 is confirmed.

The compounds that showed radical scavenging activity and ferric reduction antioxidant power in *Dosidicus gigas* skin extracts are ommochromes. In this study, the ommochrome "xanthommatin" was proved to be one of the main molecular components responsible for the antioxidant activity of the extract, and its antioxidative mechanisms are hydrogen atom transference and single electron transference. These results confirm the presence of ommochromes with biological activity in jumbo squid extracts. This information can help establish that giant 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.

4. Materials and Methods

4.1. 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.

2.2. Pigment extraction

Freeze-dried skin was mixed with acidified methanol (1% HCl; 1:20 w/v proportion) and sonicated for 5 minutes. 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).

2.3. 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

2.4. 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.

2.5. 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. [26]. 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 percentage inhibition of the samples was calculated Abs_{517nm} (% inhibition mg^{-1} fraction).

The second assay was the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging [27]. 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). Percentage inhibition of the fractions was calculated Abs_{734nm} (% inhibition mg^{-1} fraction).

The third analysis involved the ferric reducing or antioxidant power of the samples [28]. 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 FRAP values are expressed as % Activity mg^{-1} fraction.

2.6. 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).

2.6. 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 negative 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.

2.7. Statistical analysis

Data on the antioxidant activities of isolated JSS pigments are reported as the average of three determinations and analyzed using analysis of variance (ANOVA) with Tukey–Kramer tests.

Author Contributions: Chan-Higuera developed the formal analysis and the results analysis. Ezquerra-Brauer established the conceptualization of the study; Santacruz-Ortega and Carbonell-Barrachina helped with the proper establishment and interpretation of the chemical structural analysis; Cruz-Ramírez supported the establishment conditions of pigment isolation; Robles-Sánchez 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 Ezquerra-Brauer.

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Conflicts of Interest: "The authors declare no conflict of interest."

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CONCLUSIONES

Se logró optimizar del proceso de extracción de pigmentos antioxidantes de piel de calamar gigante, así como la determinación de su actividad antimicrobiana frente a bacterias y hongos

Se demostró que el extracto de pigmentos de piel de calamar no poseía actividad mutagénica ni clastogénica, lo cual indica que el material genético no se ve afectado por su presencia en células procariotas y eucariotas

Se estableció que los atributos sensoriales de paté de atún fueron mejor evaluados en las muestras adicionadas con el extracto de piel de calamar. Además, se determinó su efectividad en la prolongación de la vida de anaquel y la calidad sensorial del paté contra el control

La xantomatina fue el compuesto omocrómico encontrado en el extracto de piel de calamar, el cual fue recuperado tras la obtención de fracciones con actividad antirradical y su posterior caracterización química por métodos espectrofotométricos y espectrométricos

RECOMENDACIONES

Determinar los límites posibles de adición a productos elaborados para consumo humano, a través del cálculo de parámetros como la LD50 y la LDLo para el extracto de piel de calamar gigante xantomatina

Evaluar la actividad como conservador de alimentos del extracto de piel de calamar gigante en otras matrices alimentarias de diversos orígenes, estableciendo la versatilidad de aplicaciones y usos

Establecer el mecanismo de actividad antioxidante de la xantomatina de manera experimental a través de estudios químico-estructurales, así como su mecanismo de internación microbiana

Aislar y purificar la xantomatina de piel de calamar gigante para evaluar su aportación a la actividad antioxidante y antimicrobiana del extracto crudo