



EL SABER DE MIS HIJOS  
HARA MI GRANDEZA

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**Programa de Posgrado en Ciencias y Tecnología de los Alimentos**

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

**TESIS**

Como requisito parcial para obtener del grado de:

**DOCTOR EN CIENCIAS DE LOS ALIMENTOS**

Presenta:

**M. en C. Jesús Enrique Chan Higuera**

Hermosillo, Sonora

Agosto de 2019

# Universidad de Sonora

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**"El saber de mis hijos  
hará mi grandeza"**



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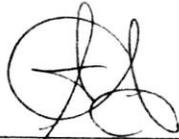
## APROBACIÓN

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

**M. en C. Jesús Enrique Chan Higuera**



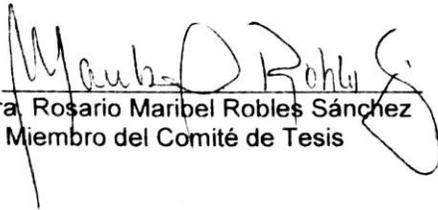
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Agosto de 2019

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## **DEDICATORIA**

*A mis padres; Óscar y María Teresa*

*A mis hermanos; Trina, Óscar y Ana*

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

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## 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 extract”, 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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## ACCEPTANCE LETTER

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Dear *Josafat Marina Ezquerro-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  $\mu\text{mol TE/g}$  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<sub>1</sub> 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 <sup>1</sup>H NMR spectra, and the FT-IR spectra peak at 1742 cm<sup>-1</sup> 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, <sup>1</sup>H NMR; squid skin ommochromes

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## 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 xanthomantins (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 (Ezquerria-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 (Ezquerria-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 Declair, 1976**). Therefore, in this work pigment extraction was prepared with acidified methanol. The extraction method consisted of treatments of different temperatures (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  $10,000 \times g$  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:

$$\text{Pigment yield (\%)} = [(\text{dried pigmented extract (g)}) / (\text{dried squid skin (g)})] \times 100.$$

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. expansum*) 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 ( $1 \times 10^9$  cells/mL), 100 µL of pigment extracts (0.005, 0.05, 0.5, and 5.0 mg/mL) and the mutagenic agent (Aflatoxin B<sub>1</sub>, AFB<sub>1</sub>) with activation system (500 µL S9 mix) in triplicate plates. Ten percent DMSO (100 µl) without AFB<sub>1</sub> 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 AFB<sub>1</sub>, 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 AFB<sub>1</sub> mutagenesis inhibition is considered strong, moderate or weak when the values are high than 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 Declair, 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 an 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<sup>-1</sup>.

Then, <sup>1</sup>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 ( $\mu\text{mol TE/g}$ ). 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<sup>1</sup> from jumbo squid skin.

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

<sup>1</sup>Values are the mean of three repetitions±standard deviation.

<sup>2</sup>Capital letters in columns denote differences by effect of the temperature ( $p < 0.05$ ).

<sup>3</sup>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 evaluate 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-positive 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 tropicalis* scored 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<sup>1</sup>.

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

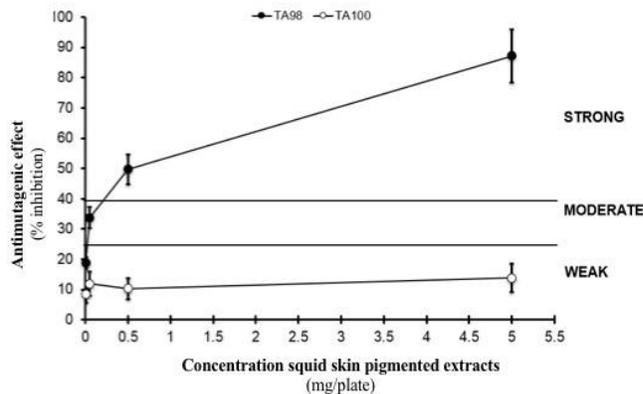
<sup>1</sup>**Data:** mean±standard deviation of three repetitions.

<sup>2</sup>**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<sub>1</sub>, based on *Salmonella typhimurium* 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<sub>1</sub> 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<sup>1</sup>.

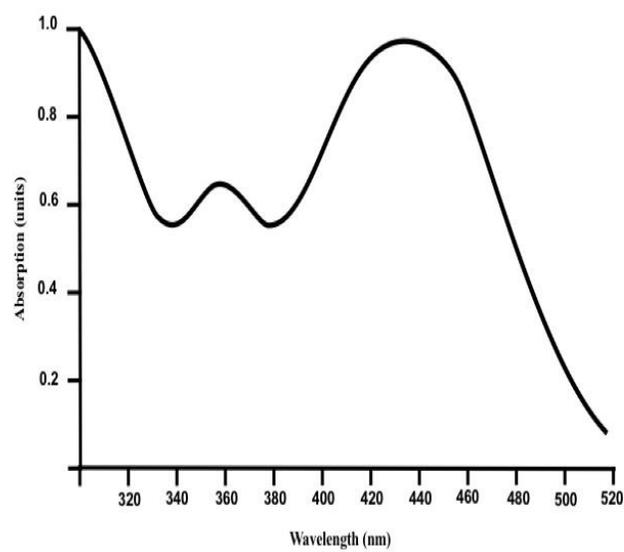
Solvent	Squid Skin Extract <sup>2</sup>	Ommochromes Reported Behavior <sup>3</sup>
Distilled water	NS	NS
Hydrochloric acid 5 M	CS	CS
Acetone	NS	NS
Potassium hydroxide 20%	CS	CS
Acetic acid	PS	PS
Methanol	NS	NS
Acidified methanol	CS	CS
Sulfuric acid 0.25 M	CS	CS
Chloroform	NS	NS

<sup>1</sup> **Data:** all analyses were run in triplicate.

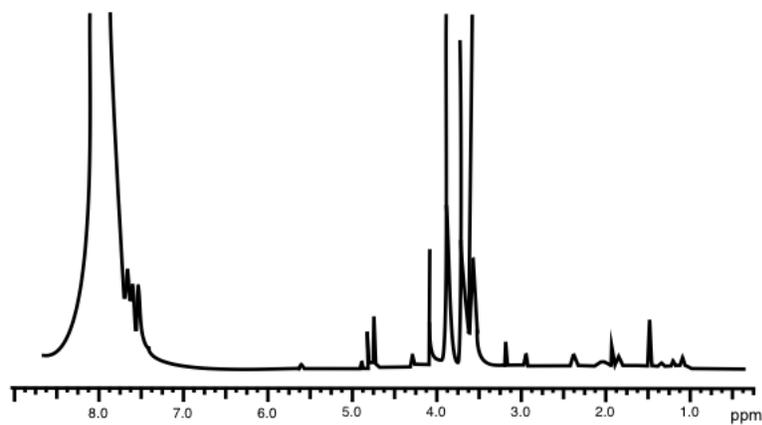
<sup>2</sup> **Legend:** NS — no solubility, PS — poor solubility, CS— complete solubility.

<sup>3</sup> **Reference:** Van den Branden and Decleir (1976).

To confirm whether the pigments extracted from jumbo squid skin contained ommochromes UV-Vis, FT-IR, and <sup>1</sup>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 <sup>1</sup>H NMR spectrum (Fig 3) was similar to those of ommins (**Kumar et al., 2018**). The <sup>1</sup>H NMR spectrum indicated aromatic protons at  $\delta$  7.4 ppm (singlet) and at 7.2 ppm (singlet) and, functional group adjacent to a methyl carbon at  $\delta$  3.8 (triplet) and at 3.0 ppm (multiplet).

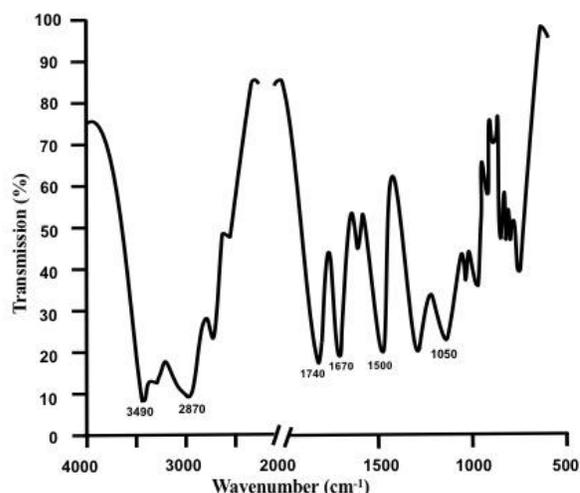


**Figure 2** UV-Vis spectrophotometric spectra of jumbo squid skin pigments.



**Figure 3**  $^1\text{H}$  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 ( $4000\text{--}400\text{ cm}^{-1}$ ) of the pigments (Fig 4) represented those reported for ommochromes (**Bolognese and Scherillo, 1974**). The main

signals observed were at 3550–3100  $\text{cm}^{-1}$  (N–H), 3000–2700  $\text{cm}^{-1}$  (C–H stretching vibrations), 1500–1425  $\text{cm}^{-1}$  (N–H and C–H bending vibrations), 1240–1050  $\text{cm}^{-1}$  (C–O and C–N stretching vibrations) (Dyer, 1965). Furthermore, wave numbers for carbomethoxy C=O (1740  $\text{cm}^{-1}$ ) and quinonic C=O (1670  $\text{cm}^{-1}$ ) 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.

## 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<sup>•</sup>) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>•+</sup>) 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<sup>•+</sup> inhibition per milligram, and fractions F3 and F7 showed the highest FRAP power. Finally, fraction F7 exerted the highest DPPH<sup>•</sup> scavenging activity. The chemical structure of the F7 fraction was characterized by infrared spectroscopy, <sup>1</sup>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 sacculus 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 R<sub>f</sub> 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<sup>+</sup>) 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<sup>•</sup>) 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 <sup>1</sup>.

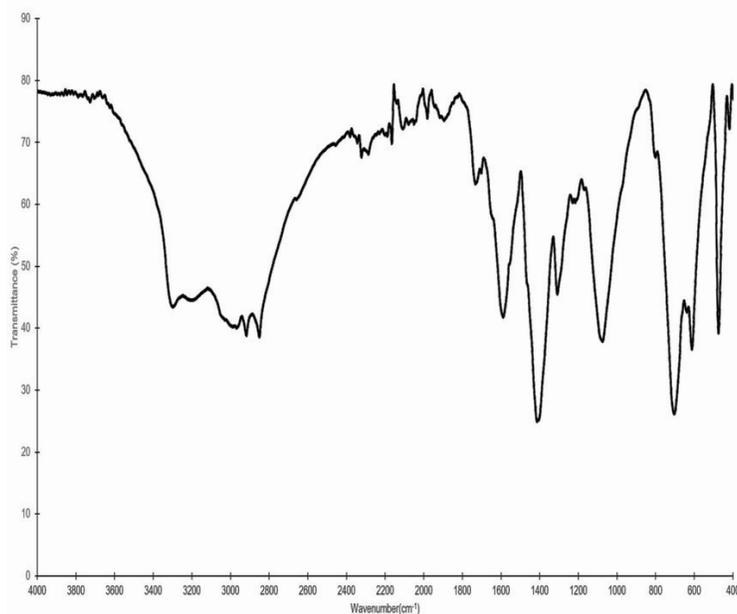
Fraction	DPPH <sup>2</sup> (% Inhibition mg <sup>-1</sup> fraction)	ABTS <sup>2</sup> (% Inhibition mg <sup>-1</sup> fraction)	FRAP <sup>2</sup> (% Inhibition mg <sup>-1</sup> fraction)
F1	15.4 ± 0.2 <sup>e</sup>	47.2 ± 1.0 <sup>a</sup>	15.3 ± 0.7 <sup>c</sup>
F2	21.4 ± 0.2 <sup>c</sup>	36.1 ± 0.6 <sup>c</sup>	24.1 ± 1.0 <sup>b</sup>
F3	28.1 ± 0.6 <sup>b</sup>	48.2 ± 0.7 <sup>a</sup>	39.6 ± 2.1 <sup>a</sup>
F4	11.5 ± 1.1 <sup>e</sup>	9.8 ± 0.2 <sup>e</sup>	8.1 ± 3.3 <sup>d</sup>
F5	10.5 ± 0.9 <sup>e</sup>	16.6 ± 0.5 <sup>d</sup>	14.5 ± 1.8 <sup>c</sup>
F6	18.7 ± 0.4 <sup>d</sup>	44.5 ± 0.2 <sup>b</sup>	25.1 ± 1.7 <sup>b</sup>
F7	38.4 ± 0.4 <sup>a</sup>	48.1 ± 0.2 <sup>a</sup>	44.5 ± 2.0 <sup>a</sup>
F8	29.1 ± 0.8 <sup>b</sup>	17.3 ± 1.2 <sup>d</sup>	13.7 ± 2.5 <sup>c,d</sup>

<sup>1</sup>The values represent the average of three repetitions ± standard deviation.

<sup>2</sup>Different letters in the same column indicate significant differences ( $p < 0.05$ ).

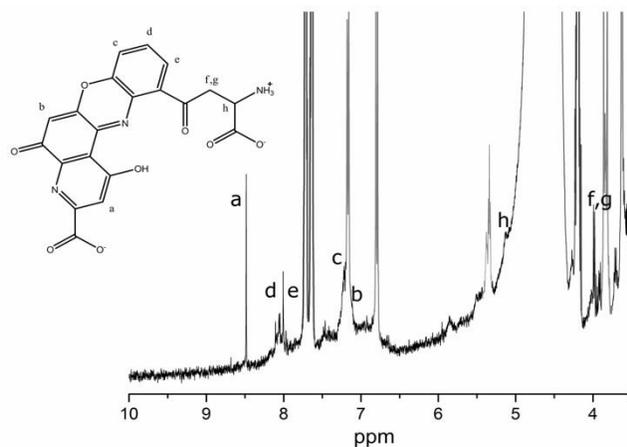
### 2.3. Structure elucidation

Figure 1 presents the IR spectrum of fraction F7. The peak associated with the stretching of primary amines is observed at 3298 cm<sup>-1</sup>. This signal is also associated with the flexure of the primary amine, which is detected as a peak at 705 cm<sup>-1</sup>. In the region of 3250–3600 cm<sup>-1</sup>, 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<sup>-1</sup>, which are related to aryl carbons. This is corroborated by the signals located between 1600 and 2000 cm<sup>-1</sup>, 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.

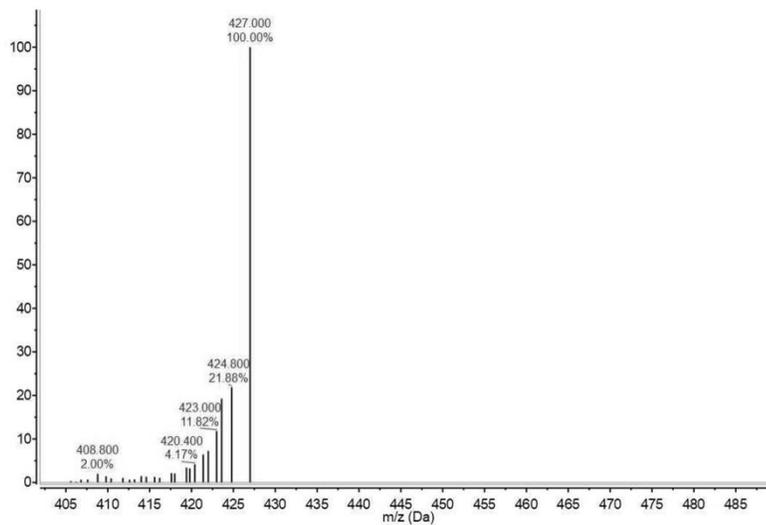
The main compound in fraction F7 was established by comparing its  $^1\text{H}$  NMR spectrum with previously published data [15–17]. The main signals detected by  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400 MHz) were  $\delta$  8.41 (d, 3H), 8.15 (t, 1H), 7.88 (d, 2H), 7.70 (s, 1H), 6.68 (s, 1H), 4.51 (d, 1H), and 3.87 ppm (m, 2H) (Figure 2). Moreover, the  $^1\text{H}$  NMR spectrum shows signals due to  $\text{sp}^3$  carbons at  $\delta$  3.87 (Figure 2, letters f and g; m, 2H), aromatic protons at  $\delta$  7.88 (Figure 2, letters d and e; d, 2H), and amine group protons at  $\delta$  7.70 (s, 1H).



**Figure 2.**  $^1\text{H}$  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  $[\text{M} + \text{H}]^+$  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<sup>•</sup>, ABTS<sup>•+</sup>, 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<sup>-1</sup> 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 <sup>1</sup>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  $\leq 0.063$  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  $\mu$ L 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<sup>-1</sup>) 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  $\text{Abs}_{517\text{nm}}$  (% inhibition mg<sup>-1</sup> fraction).

The second assay was the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging [27]. The ABTS radical cation (ABTS<sup>•+</sup>) 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<sup>•+</sup> solution ( $\text{Abs}_{734\text{nm}} = 0.70$ ) 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  $\text{Abs}_{734\text{nm}}$  (% inhibition mg<sup>-1</sup> fraction).

The third analysis involved the ferric reducing or antioxidant power of the samples [28]. An aliquot of 100  $\mu\text{L}$  of the samples ( $1 \text{ mg mL}^{-1}$ ) 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  $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ ), 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  $\text{mg}^{-1}$  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  $\text{cm}^{-1}$ , accumulating 30 scans per spectrum. A blank spectrum was recorded to exclude any cross-contamination. The spectrum was expressed in wavenumber ( $\text{cm}^{-1}$ ) versus transmittance percentage.

The  $^1\text{H}$  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 ( $\text{CD}_3\text{OD}$ ) and dimethyl sulfoxide, using tetramethylsilane (TMS) as the internal reference. Chemical shifts were referenced to the solvent peaks, and the values were recorded in  $\delta$ . The multiplicities of the  $^1\text{H}$  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. Ezquerro-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 Ezquerro-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