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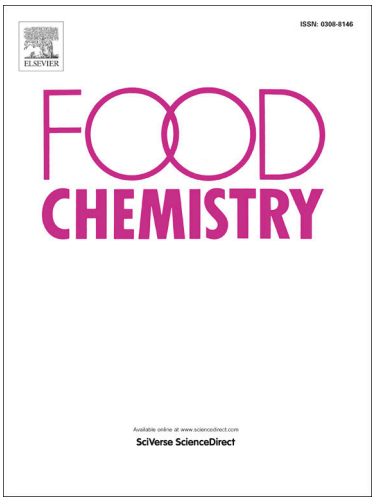

Short communication

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Identification of black sturgeon caviar pigment as eumelanin

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ABSTRACT

Reported herein is the purification of the pigment of black sturgeon caviar and its unambiguous identification as a typical eumelanin by means of chemical degradation coupled with electron paramagnetic resonance (EPR) evidence. HPLC and LC-MS analysis of oxidative degradation mixtures revealed the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA), a specific marker of eumelanin pigments, in yields compatible with a 6.5% w/w pigment content. EPR spectral features and parameters were in close agreement with those reported for a typical natural eumelanin such as *Sepia* melanin from squid ink. The identification for the first time of eumelanin in a fish roe is expected to provide a novel molecular basis for the valorization of black caviar and production wastes thereof in food chemistry and diet.

Keywords: Black caviar; Sturgeon roe; Melanin; Electron paramagnetic resonance (EPR); Pyrrole-2,3,5-tricarboxylic acid (PTCA)

1. Introduction

The black caviar from the roe of sturgeon fish of the *Acipenseridae* family occupies a prominent position in the food market, as a delicacy with high nutritional and commercial value, and in other areas of research, including cosmetics, due to the high content of high quality protein, polyunsaturated fatty acids, and vitamins A, B, C, D (Farag, Abib, Tawfik, Shafik, & Khattab, 2021; Hao et al., 2015; Wang et al., 2012), which are well known to exert beneficial effects on cardiovascular disorders, colon cancer, chronic inflammation, cognitive impairment, and immunomodulation. This is the reason why sturgeon farming is increasing in several countries (Nieminen, Westenius, Halonen, & Mustonen, 2014). Surprisingly, however, very little information is available on the structure and properties of the pigment responsible for the black color of sturgeon roe, despite the obvious and important impact on the overall organoleptic and nutritional characteristics of black caviar.

Melanins are among the major determinants of black or dark colorations in nature and food, being widely distributed across highly divergent phylogenetic branches of the animal kingdom (d'Ischia et al., 2015; Meredith & Sarna, 2006; Panzella, Ebato, Napolitano, & Koike, 2018; Simon, Peles, Wakamatsu, & Ito, 2009; Sugumaran & Barek, 2016). Melanin pigments are generally classified as the dark brown-to-black eumelanins and the yellow-to-reddish brown pheomelanins (Ito & Wakamatsu, 2011; Micillo, Panzella, Koike, Monfrecola, Napolitano, & d'Ischia, 2016; Simon et al., 2009). Eumelanin pigments, in particular, have been attributed with countless properties (Huang, Liu, Huang, Wen, Zhang, & Wei, 2018; Micillo et al., 2016; Panzella et al., 2018b), spanning from photoprotection (Liberti, Alfieri, Monti, Panzella, & Napolitano, 2020) and antioxidant defense (Micillo et al., 2018; Panzella et al., 2013) to metal

binding (Sono, Lye, Moore, Boyd, Gorlin, & Belitsky, 2012), radioprotection (Schweitzer et al., 2009) and modulation of the immune system (Koike & Yamasaki, 2020).

Eumelanins are produced from the oxidative polymerization of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), both deriving from tyrosine or 3,4-dihydroxyphenylalanine (DOPA) oxidation mediated by the enzyme tyrosinase within specialized cells (Micillo et al., 2016; Panzella et al., 2018b). Natural eumelanin pigments are generally distributed in light-exposed sites of the body, although their occurrence in unexposed organs is now well established (Dubey & Roulin, 2014). Eumelanins have been also identified in food products, such as mushrooms (Hou et al., 2019; Ramos et al., 2019; Sun, Zhang, Chen, Zhang, & Zhu, 2016; Sun, Zhang, Sun, Zhang, Shan, & Zhu, 2016), silky fowl meat (Chen, Jiang, Zheng, Xu, Li, & Yang, 2008), and especially squid/cuttlefish ink (*Sepia* melanin) (Palumbo, 2003; Solano, 2017). *Sepia* melanin, in particular, is increasingly appreciated as an added value ingredient in the Mediterranean cuisine including spaghetti-based recipes in Southern Italy. To the best of our knowledge, however, no evidence has been reported so far for the occurrence of eumelanin pigments in fish roe.

Examination of sturgeon roe often reveals an accumulation of dark granules in the spiral part of the larvae intestine, referred to as a “melanin plug”. These granules, which are eliminated when exogenous feeding starts (Gisbert & Williot, 1997), may arise as a waste product from the consumption of the endogenous reserves present in the yolk sac, which is known to contain tyrosine-rich proteins (Gisbert & Sarasquete, 2000; Williot, Nonnotte, & Chebanov, 2018). It is noted, however, that the identification of the sturgeon larvae intestinal pigmented granules as melanin was based only on insolubility and other chemical characteristics revealed by oxidation/reduction assays and histochemical analysis (Gisbert & Sarasquete, 2000). Although a

recent relevant study reported a down-regulation of genes involved in biogenesis of melanin in the white eggs produced by albino Russian sturgeons compared to wild type (Gong et al., 2019), conclusive identification and characterization of melanin in sturgeon species by direct physicochemical methods has not been reported.

Herein, we report the unambiguous identification of the sturgeon roe black pigment as eumelanin based on chemical degradation experiments and electron paramagnetic resonance (EPR) spectroscopy. For comparison, the insoluble, dark-colored protein fraction resulting as a by-product from caviar oil production was also investigated, in an attempt to meet the current need of finding an alternative use for fish processing industry wastes (Ossivipour, Abedian, Motamedzadegan, Rasco, Safari, & Shahiri., 2009; Villamil, Váquiro, & Solanilla, 2017).

2. Material & methods

2.1. Materials

All reagents were purchased from commercial sources and used without further purification. Sturgeon roe and wastes from caviar oil production (insoluble caviar protein powder) came from Acipenser Biotech Ltd (London). Cuttlefish ink sac was provided by a local fish market. DOPA-melanin was prepared following a literature reported procedure (Ito, 1986).

2.2. Methods

X-band (9 GHz) EPR analysis were performed on a Bruker spectrometer. Samples were transferred to flame-sealed glass capillaries which, in turn, were coaxially inserted in a standard 4 mm quartz sample tube. Measurements were performed at room temperature. The instrumental settings were as follows: sweep width: 160 G; time constant: 10.24 sec; modulation frequency: 100.00 kHz; modulation amplitude: 1.0 G, receiver gain: 40 or 60 dB. The amplitude of the field

modulation was preventively checked to be low enough to avoid detectable signal over modulation. EPR spectra were measured with different microwave powers to avoid microwave saturation of resonance absorption curve. Several scans, typically 2, 16 or 32, were accumulated to improve the signal-to-noise ratio. The g value and the spin density were evaluated by means of an internal standard, Mn^{2+}-doped MgO (Micillo et al., 2018).

HPLC analysis were performed on an Agilent 1100 series instrument equipped with a LC-10AD VP pump and a G1314A UV-Vis detector. A Sphereclone C_{18} column (4.6 × 150 mm, 5 μm) was used, with 0.1 % formic acid (eluant A)–methanol (eluant B) under the following gradient conditions: from 5% to 90%, 0-45 min; flow rate: 1 mL/min; detection wavelength: 254 nm. LC-MS analyses were performed on an Agilent ESI-TOF 1260/6230DA instrument in positive ion mode in the following conditions: nebulizer pressure 35 psig; drying gas (nitrogen) 5 L/min, 325 °C; capillary voltage 3500 V; fragmentor voltage 175 V. An Eclipse Plus C18 column, 150 × 4.6 mm, 5 μm, at a flow rate of 0.4 mL/min was used, with the same mobile phase as above.

2.3. Sturgeon Roe Black Pigment (SRBP) isolation

Sturgeon roe was mechanically collected from a sturgeon ovary sample and extensively washed with tap water with the aid of a colander. Washing with distilled water was then repeatedly performed in a centrifuge (3000 rpm, 15 min), after that eggs were mechanically broken by the use of a laboratory spatula and re-suspended in water. Centrifugations at increasing rpm (350, 450 and 550, 15 min each) were then performed to enrich the precipitate in the black pigment. This latter was eventually recovered by centrifugation at 3000 rpm for 15 min, followed by lyophilization. For comparison, *Sepia* melanin was prepared by centrifugation of the ink (3000 rpm, 20 min, 4 °C) followed by iterative washings with water and lyophilization.

2.4. Enzymatic digestion

A previously developed procedure was adopted (Panzella et al., 2014). Briefly, insoluble protein waste from caviar oil production or *Sepia* melanin (1 g) was suspended by use of a glass/glass potter in 0.1 M phosphate buffer pH 7.4 (40 mL). The resulting mixture was incubated with proteinase K (3.4 mg, 30 U/mg) and dithiothreitol (120 mg) in an argon atmosphere under vigorous stirring at 37 °C. After 18 h the mixture was centrifuged at 3000 rpm for 20 min at 4 °C; the resulting precipitate was washed with 1% acetic acid (3 × 5 mL), while the supernatant was acidified to pH 3 with 3 M HCl, stored at 4 °C for 1 h, and centrifuged at 3000 rpm for 20 min at 4 °C, to give a precipitate that was washed with 1% acetic acid (3 × 5 mL). The combined precipitates were suspended in 0.1 M phosphate buffer (pH 7.4) and treated again with proteinase K (2.1 mg) and dithiothreitol (72 mg). After 18 h the mixture was treated as above, and the combined precipitates were subjected to two further digestion treatments with proteinase K (1.7 mg for the first treatment and 0.3 mg for the second) and dithiothreitol (1.6 mg for the first treatment and 2.6 mg for the second). The final precipitate was washed with water and lyophilized. The pellet obtained was resuspended at a concentration of 50 mg/mL in 0.1 M phosphate buffer (pH 7.4) containing Triton X-100 (200 μL for 100 mg of pellet) and taken under stirring at 37 °C. After 4 h the mixture was centrifuged at 7000 rpm for 20 min at 4 °C; the resulting precipitate was washed with 1% acetic acid (3 × 5 mL), while the supernatant was acidified to pH 3 with 3 M HCl, stored at 4 °C for 1 h, and centrifuged at 7000 rpm for 20 min at 4 °C, to give a precipitate that was washed with distilled water (3 × 5 mL). The final precipitate was lyophilized.

In the case of SRBP, enzymatic digestion was run on 500 mg of sample and stopped after the fourth treatment with proteinase K/dithiothreitol.

2.5. Chemical degradation

A previously developed procedure was adopted (Panzella, Manini, Monfrecola, d'Ischia, & Napolitano, 2007). Briefly, each sample (10 mg) was suspended in 1 M NaOH (1 mL) and treated with 1.5% H_2O_2 at room temperature, under vigorous stirring. After 24 h the mixture was treated with 5% $Na_2S_2O_5$ (200 μL), taken to pH 3 with 6 M HCl, filtered on Chromafil®PVDF (0.20 μm), and analyzed by HPLC and LC-MS.

3. Results and discussion

To isolate the black caviar pigment, sturgeon roe was mechanically collected by hand from sturgeon ovary (Fig. 1A) and extensively washed with water with the aid of a colander, after that eggs were mechanically broken by the use of a laboratory spatula and resuspended in water. Centrifugations at increasing rpm were then performed to enrich the precipitate in the black pigment (Fig. 1B). This latter, named sturgeon roe black pigment (SRBP), was eventually recovered by lyophilization (Fig. 1C). With the aim of removing most part of the protein matrix and hence further purify the pigment, SRBP was also subjected to a proteolytic enzymatic digestion under conditions previously developed for isolation of hair melanin (Panzella et al., 2014). The same treatment was performed on *Sepia* melanin, as a reference natural melanin pigment, and on the insoluble waste from caviar oil production (Fig. 1D).

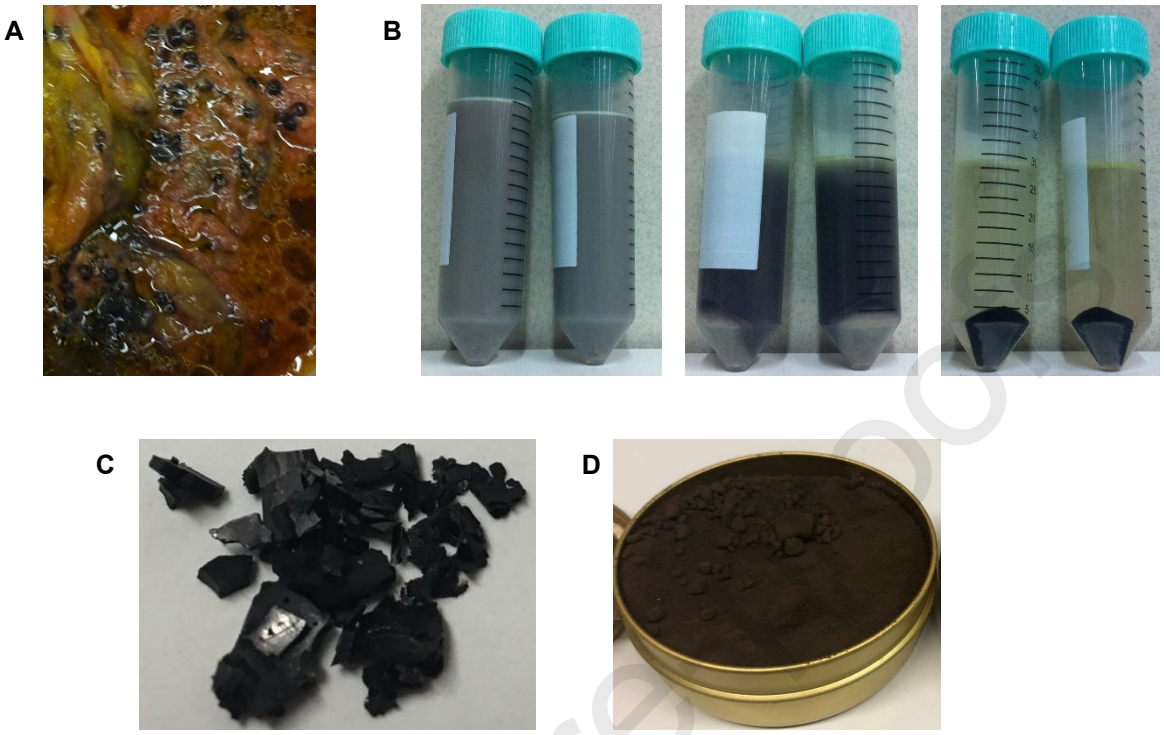

Fig. 1. Sturgeon roe samples used in this study. (A) Starting material: sturgeon roe in ovary tissue. (B) Broken sturgeon eggs suspended in water before (left) and after centrifugation at 350 (middle) and 3000 rpm (right). (C) Lyophilized SRBP. (D) Insoluble waste from caviar oil production.

Two different approaches were adopted to obtain structural information on the black caviar pigment, that is direct EPR analysis of the different samples (SRBP, insoluble waste and *Sepia* melanin) before and after enzymatic digestion, and HPLC and LC-MS analysis of chemical degradation mixtures allowing for identification and quantitation of specific melanin markers (d'Ischia et al., 2013).

Among the unusual physicochemical properties of melanin pigments is a persistent EPR signal due to the presence of stable organic free radicals, which makes EPR one of the elective

techniques for identifying natural melanin samples in complex matrices (d'Ischia et al., 2013; Meredith & Sarna, 2006). EPR experiments indicated a well-defined signal for SRBP and the insoluble waste (Fig. 2). In addition, EPR parameters for the pigment before and after purification by enzymatic digestion (Table 1) were similar to those widely documented for eumelanins (Panzella et al., 2013), and closely matched those of *Sepia* melanin. In general, eumelanin EPR signal derives from a major component assigned to carbon-centered radicals localized at the center of the stacked aromatic moieties, and a minor component likely attributed to semiquinone-type species. In this case a g value compatible with carbon-centered radicals (Mostert, Hanson, Sarna, Gentle, Powell, & Meredith, 2013; Panzella et al., 2013; Panzella et al., 2018a) was determined for all the samples. In addition, the shape of the EPR signal (Fig. 2) allowed to rule out for all the samples the presence of significant amounts of pheomelanins, being the EPR spectra of these latter pigments characterized by a clear hyperfine coupling of unpaired electrons with nitrogen nuclei (d'Ischia et al., 2013).

An estimation of the signal linewidth (ΔB) was obtained by determining the maximum-to-minimum distance in Gauss. Notably, SRBP exhibited a ΔB comparable to the reference pigment *Sepia* melanin, whereas the insoluble waste sample was characterized by a significantly higher value. Moreover, the insoluble waste sample also showed a lower spin density.

As expected, an increase in spin density was observed for both SRBP and insoluble waste after enzymatic digestion. A lower ΔB value, more similar to that exhibited by SRBP and *Sepia* melanin, was also determined for the insoluble waste pigment after purification. On the other hand, enzymatic digestion of *Sepia* melanin did not produce an increase of spin density, possibly because the limited protein component (estimated as much as 14.0 $\pm$ 0.8% w/w) (Ito, 1986) was

not degraded appreciably under the proteolytic conditions adopted (Liu, Hong, Kempf, Wakamatsu, Ito, & Simon, 2004).

These results give additional information on the pigment structure and on the sample supramolecular organization. In general, signal broadening results from dipolar interaction of unpaired magnetic moments of free radicals located at short distance. Thus, the broad and weak signal of the insoluble waste sample could suggest a specific organization, in which a low number of paramagnetic melanin particles are clustered and surrounded by, for example, a protein diamagnetic matrix, which was in part removed by the enzymatic digestion treatment, as shown by the EPR signal changes. An additional/alternative source of signal broadening is given by the melanin dipolar interactions with paramagnetic metal ions, such as iron(III), copper(II) and manganese(II) (Ghiani et al., 2008; Sarna, Hyde, & Swartz, 1976). Indeed, the presence of these elements in the insoluble waste sample was confirmed by inductively coupled plasma (ICP)-MS analysis. However, the signal narrowing upon enzymatic digestion, with no need of EDTA treatment, would suggest a limited contribution of dipolar interactions with these ions.

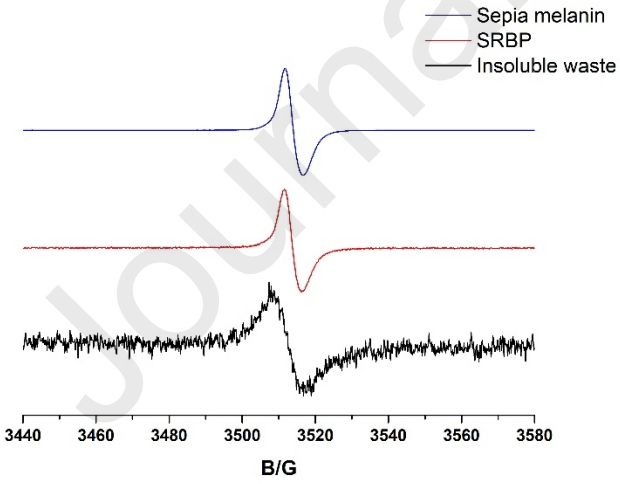

Fig. 2. EPR spectra (microbridge power: 0.6 mW) of *Sepia* melanin (top trace), SRBP (middle trace), and insoluble waste (bottom trace).

Table 1

EPR parameters of SRBP, insoluble waste and *Sepia* melanin before and after enzymatic digestion.

Sample	g-factor	ΔB (Gauss)	Spin density (spin/g)
SRBP	2.0037	5.2	7.1×10^{15}
SRBP after enzymatic digestion	2.0035	5.4	5.1×10^{16}
Insoluble waste	2.0034	9.3	1.1×10^{14}
Insoluble waste after enzymatic digestion	2.0038	5.3	7.0×10^{14}
Sepia melanin	2.0039	4.8	5.9×10^{18}
Sepia melanin after enzymatic digestion	2.0031	4.8	2.7×10^{18}

Experimental uncertainties are ± 0.0003 on g-factor, ± 0.2 G on ΔB, ± 10% on spin density.

Power saturation experiments were run by monitoring the EPR signal intensity as a function of the microwave irradiation power, allowing a deeper insight into the electron relaxation behavior to be obtained. Power saturation profiles (Fig. 3A) presented an evident maximum for SRBP and *Sepia* melanin, indicating the presence of a homogeneous populations of radicals with the same relaxation behavior (Panzella et al., 2013), and confirming the similarity between the two samples. The relatively low power required for saturation indicates a long relaxation time of the free electrons in these systems. In contrast, just a slope change in a monotonously increasing trend was observed for the insoluble waste, suggesting a higher degree of molecular heterogeneity of paramagnetic centers in this latter sample (Panzella et al., 2013), compared to SRBP, with a general decrease of the relaxation times due to dipolar interactions among the

various melanin spins and/or between them and paramagnetic ions present in the sample (Ghiani et al., 2008; Sarna, Hyde, & Swartz, 1976).

Notably, a broad maximum was observed for the power saturation profile of insoluble waste after enzymatic digestion (Fig. 3B), likely as the result of an increase in the degree of purity of the pigment, in agreement with the higher spin density value.

Overall, the results of the EPR experiments showed the eumelanic nature of the black caviar pigment as well as the obtainment of a pigment with a higher degree or purity further to the enzymatic digestion treatment.

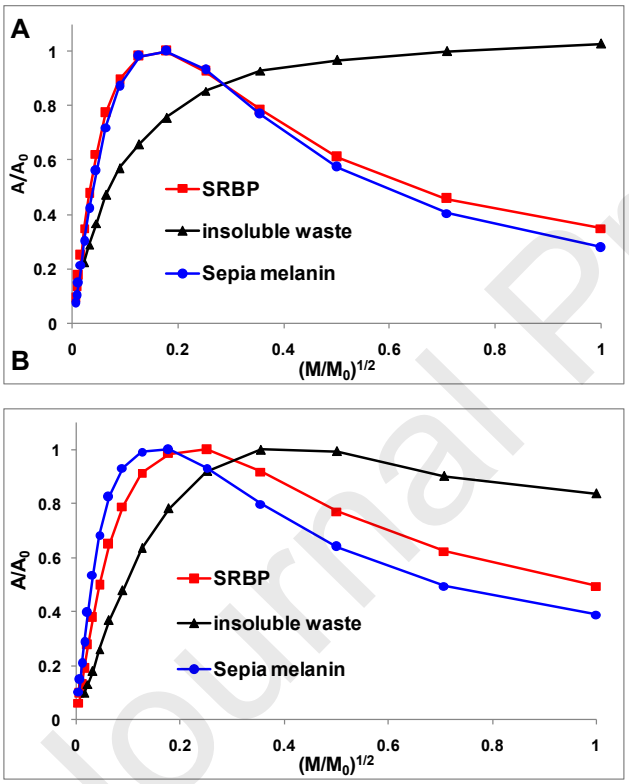

Fig. 3. Power saturation profiles (amplitude *vs* power intensity) of SRBP, insoluble waste and *Sepia* melanin before (A) and after (B) enzymatic digestion.

Given the insolubility of SRBP, the chemical nature of the pigment was further investigated by an oxidative degradation approach commonly used for the qualitative and quantitative

determination of melanin pigments in biological tissues, involving treatment with hydrogen peroxide under alkaline conditions (Panzella et al., 2007). SRBP gave rise to a complex HPLC elutographic profile (Supplementary Fig. S1A), exhibiting in particular a compound eluted at 5.8 min, which was also present in the degradation mixture of *Sepia* melanin. Spiking with an authentic sample (Napolitano, Pezzella, Vincensi, & Prota, 1995) allowed to identify this product as pyrrole-2,3,5-tricarboxylic acid (PTCA), a typical marker of eumelanins (Supplementary Fig. S2), as confirmed also by LC-MS analysis (Supplementary Fig. S1B). Based on comparison with the amount of PTCA formed by alkaline hydrogen peroxide degradation of a reference synthetic eumelanin pigment, DOPA-melanin (Table 2), a melanin content of ca. 65 μg/mg could be determined in SRBP. Notably, PTCA was detectable also in the alkaline hydrogen peroxide degradation mixture of the insoluble waste pigment purified by enzymatic digestion (Table 2). This finding is in agreement with the results of EPR investigations, showing a pigment enrichment in the insoluble waste sample following enzymatic treatment. As expected, an increase (ca. 40%) in PTCA yield was observed also for the SRBP sample following enzymatic digestion.

A more in-depth analysis of the LC-MS elutographic profiles of the alkaline hydrogen peroxide degradation mixtures of SRBP and insoluble waste further to enzymatic digestion allowed for identification of an additional typical marker of eumelanins, that is pyrrole-2,3-dicarboxylic acid (PDCA), mainly deriving from DHI-related units (d'Ischia et al., 2013) (Supplementary Fig. S2). Based on the yields of PTCA and PDCA determined for the different samples (Table 2), it can be concluded that SRBP is more similar to DOPA-melanin rather than to *Sepia* melanin in terms of DHI/DHICA unit ratio. The relative high yield of PDCA in the insoluble waste-derived sample

compared to that from SRBP is likely a consequence of significant decarboxylation of DHICA-derived units due to the high industrial processing temperature (> 90 °C).

Table 2

Yields of eumelanin markers from alkaline hydrogen peroxide degradation of SRBP, insoluble waste, *Sepia* melanin and DOPA-melanin samples.

Sample	Yields (μg/mg)	
	PTCA	PDCA
SRBP	0.28	<0.10
SRBP after enzymatic digestion	0.51	0.19
Insoluble waste	<0.10	<0.10
Insoluble waste after enzymatic digestion	0.48	0.43
Sepia melanin	6.5	0.22
DOPA-melanin	4.3	3.1

4. Conclusion

We have reported herein the identification of the black caviar pigment as a eumelanin, based on EPR and chemical degradation experiments. This finding, which provides the first unambiguous demonstration of the occurrence of a melanin pigment in a fish roe, points to so far unrecognized biological roles of these pigments, for example as protective agents during embryo development. The demonstration of eumelanin in black caviar is of considerable relevance to the food sector, given the importance of natural melanins as antioxidants and antimicrobial agents, and opens

new perspectives for a possible exploitation of by-products of caviar oil production as functional ingredients in cosmetics and other applications.

CRediT authorship contribution statement

Lucia Panzella: Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing. **Kenneth Benning**: Resources, Writing - review & editing. **Darren N. Nesbeth**: Writing - review & editing. **Brunella Setaro**: Investigation **Gerardino D'Errico**: Methodology, Validation, Writing - review & editing. **Alessandra Napolitano**: Methodology, Writing - review & editing. **Marco d'Ischia**: Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found on line.

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Figure legends

Fig. 1. Sturgeon roe samples used in this study. (A) Starting material: sturgeon roe in ovary tissue. (B) Broken sturgeon eggs suspended in water before (left) and after centrifugation at 350 (middle) and 3000 rpm (right). (C) Lyophilized SRBP. (D) Insoluble waste from caviar oil production.

Fig. 2. EPR spectra (microbridge power: 0.6 mW) of *Sepia* melanin (top trace), SRBP (middle trace), and insoluble waste (bottom trace).

Fig. 3. Power saturation profiles (amplitude *vs* power intensity) of SRBP, insoluble waste and *Sepia* melanin before (A) and after (B) enzymatic digestion.

CRediT authorship contribution statement

Lucia Panzella: Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing. **Kenneth Benning**: Resources, Writing - review & editing. **Darren N. Nesbeth**: Writing - review & editing. **Brunella Setaro**: Investigation **Gerardino D'Errico**: Methodology, Validation, Writing - review & editing. **Alessandra Napolitano**: Methodology, Writing - review & editing. **Marco d'Ischia**: Conceptualization, Methodology, Writing - review & editing.

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

The black pigment of sturgeon roe (SRBP) was purified by sequential centrifugation.

Electron paramagnetic resonance allowed to identify SRBP as a eumelanin.

A specific eumelanin marker was detected in SRBP chemical degradation mixture.

This is the first clear-cut demonstration of the occurrence of melanin in a fish roe.

The presence of melanin was detected also in a caviar oil production by-product.