Purification, characterization and analysis of sepia melanin from commercial sepia ink (*Sepia Officinalis)*

Purificación, caracterización y análisis de la melanina de sepia a partir de la tinta de sepia *(Sepia Officinalis)*

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Abstract

At the moment melanins are still enigmatic bio-pigments with structure and determination methods not clearly understood for the international scientific community. Among biopolymers, melanins are unique in many aspects. Some essential biopolymers (proteins, nucleic acids and carbohydrates) are chemically well characterized and can be determined using well established analytical methodologies. On the contrary, no available methods allow us to accurately determine melanin, mostly due to the intrinsic chemical properties of melanins. These pigments, in fact, are insoluble in a broad range of solvents and pH as well as difficult to purify as a result of the heterogeneity in their structural features. Mammalian melanins exist in two chemically distinct forms: the brown to black Eumelanin and the yellow to reddish-brown Pheomelanin. Sepia melanin obtained from *Sepia Officinalis* consists of more than 98% of Eumelanin and is therefore used as standard material in the analysis of melanic black. Commercial sepia melanin is purified according to an undescribed procedure. In this research, extraction and purification studies were carried out on sepia melanin using a hydrochloric acid (0.5 - 3.0 M) treatment under mechanical or ultrasonic agitation. A high degree purity sepia melanin was obtained and further characterized using Elemental Analysis (EA), Ultraviolet-Visible (UV-VIS) and Infrared (IR) spectroscopy and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for metal ion analysis. Finally, quantitative studies of the chemical degradation of Eu polymers to Pyrrole-2,3,5-tricarboxylic acid (PTCA) and Pyrrole-2,3-dicarboxylic acid (PDCA) by High Performance Liquid Chromatography (HPLC) were performed. The procedure we developed can be used to obtain a considerably less expensive sepia melanin which could prove important as a standard in future determinations of eumelanin and pheomelanin as well as in a vast field of scientific and industrial applications in such fields as human and veterinary medicine, pharmacology and cosmetics.

Key words

Characterization, eumelanin, purification, sepia melanin.

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Resumen

En el momento las melaninas son bio-pigmentos enigmáticos todavía con estructura y métodos de determinación que no son claramente entendidos por la comunidad científica internacional. Entre los biopolímeros, las melaninas son únicas en muchos aspectos. Algunos biopolímeros esenciales (proteínas, ácidos nucléicos y los hidratos de carbono) has sido químicamente bien caracterizados y se pueden caracterizar bien utilizando metodologías analíticas bien establecidas. Por el contrario, no hay métodos disponibles que permitan determinar de una manera precisa la melanina, debido principalmente a las propiedades químicas de la melanina. De hecho, estos pigmentos son insolubles en un amplio rango de solventes y pH, como también difíciles de purificar como resultado de su heterogeneidad estructural. La melanina de mamíferos existe en dos formas distintas: la Eumelanina que va de color marrón a negra, y la Feomelanina que va de amarillo a naranja. La melanina sepia obtenida de la Sepia Officinalis contiene más de 98% de Eumelaina y por tanto es usada como material estándar en el análisis de la melanina negra. La melanina sepia comercial es purificada de acuerdo a un procedimiento no descrito antes. En esta investigación, los estudios de extracción y purificación se llevaron a cabo en la melanina sepia usando un tratamiento de acido hidroclorhídrico (0.5-3.0 M) bajo agitación mecánica y ultrasonográfica. Un alto grado de pureza de la melanina de sepia fue obtenida y luego caracterizada usando Análisis Elemental (EA), Espectroscopia Visible Ultravioleta e Infrarroja (UV-VIS) y Espectrometría de Plasma Inductivamente Acoplada (ICP-MS) para el análisis de iones metálicos. Finalmente, se realizaron estudios cuantitativos en la degradación química de Eumelanina a 2,3,5 triácido carboxílico pirrol (PTCA) y 2,3 diácido carboxílico pirrol por Cromatografía Liquida (HPLC). El procedimiento que desarrollamos puede ser usado para obtener melanina de sepia a mucho menor costo, la cual podría proveer como estándar para determinaciones de Eumelanina o Feomelanina en el futuro, adicionalmente en un amplio campo de aplicaciones industriales, científicas, como también en campos de la medicina, la farmacología y la industria cosmética.

Palabras clave

Caracterización, eumelanina, melanina de sepia, purificación.

Introduction

Melanin is the most widespread natural pigment and can be found in practically all living organisms. Melanins are classified in: Allomelanin (Allo) present in plants and fungi, Neuromelanin (Neu) present in nervous cell, Pheomelanin (Pheo) and Eumelanin (Eu) that can be found in the skin, hair and iris. Studies have determined that eumelanin is more resistant than pheomelanin towards UV-Vis radiation[12,18,21]. Eumelanin is recognized as a protection factor against cell damage caused by ultraviolet radiation. Recent studies show that a higher risk of developing skin tumors is often present in subjects with a lower protection[25]. Furthermore, melanins have other important functions in living organisms such as phenotypic differentiation, camouflage, etc. Their biosynthesis is controlled by genetic, environmental and hormonal factors. In addition, it is important to note that melanin is a radical free polymer presenting a highly-conjugated structure that enables the transformation of UV-Vis radiation into heat[5,6,22].

One of the major problems in studying melanins is the lack of adequate methods for the isolation of pure melanin pigments. The most frequently used source of natural eumelanin is ink sac of cuttlefish from *Sepia Officinalis*. Sepia ink produced by mollusks serves as an alarm substance that both confuses predators and alerts congeners about the presence of danger. Sepia ink is a salt composed by Fe^{3+}, Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}, with different polymeric grade chains whose length depends on age, sex, season, etc. It is extracted as slurry including enzymes and proteins. Sepia melanin

have characterized using a variety of techniques including nuclear magnetic resonance (NMR), scanning electron microscope (SEM)[15], atomic force microscopy (AFM), optical and Mossbauer spectroscopy, mass spectrometry, UV-Vis, FTIR and Raman spectroscopy[1,4,13]. These works have proposed that sepia melanin is a copolymer of eumelanin constituted of approximately 20% of units of 5,6-dihydroxyindole (DHI) and 75% of units of 5,6-dihydroxyindole-2-acid carboxylic (DHICA) (Figure 1) .

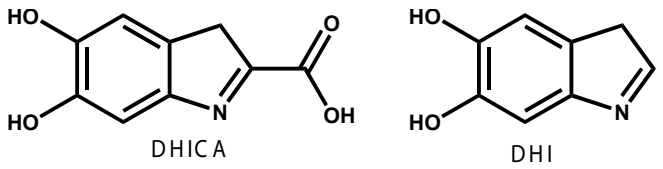


Figure 1. Sepia melanin is a copolymer of eumelanin constituted of approximately 20% of units of 5,6-dihydroxyindole (DHI) and 75% of units of 5,6-dihydroxyindole-2-acid carboxylic (DHICA)

According to the proposed molecular structures for eumelanin, the pigment contains phenolic hydroxyl (OH^-), carboxylic ($COOH^-$) and amino (NH^+) groups as potential binding sites for the metal ions. The binding of the metal to these functional groups involves competition with H^+. The pka`s values of these functional groups in the monomeric units DHICA and DHI have been examined and determined to be 4,2 for carboxylic acid of DHICA and ~9,8 and ~13,2 for the two hydroxyl in both DHI and DHICA[2], unfortunately pka values of these groups in eumelanin are difficult to measure due to the heterogeneity of the pigment. In the case of sepia melanin, the pka of the carboxylic acid group (thought to be derived from DHICA monomer) is reported to be 3,1[3]. The pigment exhibits UV-Vis and IR absorption associated with these functional groups and the frequencies of absorption of such groups are also sensitive to metal coordination. Thus, IR absorption spectroscopy could be informative about the immediate binding environment of metal ion in melanins.

The best known and most used method for the determination of Eumelanin in pigmented tissues consists of the chemical degradation of the polymer into Pyrrole 2,3,5-tricarboxylic acid PTCA and Pyrrole 2,3-dicarboxylic acid PDCA (Figure 2) which are used to quantify the ratio between the two monomers (DHICA and DHI). The first degradation studies were performed using potassium permanganate in an acid environment [8,9,10,11], but recently, an alternative oxidation method of the eumelanin pigment has been developed, involving the use of hydrogen peroxide under alkaline conditions. This new method yields higher PTCA values when compared to the oxidation performed with permanganate, as well as a better linearity in the quantity of PTCA produced and finally a greater stability of PTCA under basic conditions [19,20]. Considering that PDCA is a marker of the DHI units while PTCA indicates the DHICA-derived units in eumelanin, the alkaline H_2O_2 oxidation proves useful in elucidating the structure of sepia melanin [24].

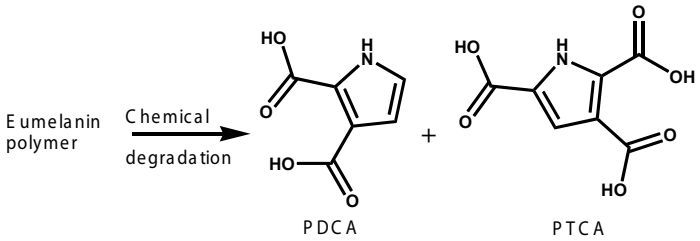


Figure 2. Typical products of the chemical degradation of sepia melanin.

Materials and methods

Sepia ink (concentrated and sterilized foodstuff) is obtained from Nortindal Sea Products Ltd. (Guipúzcoa, Spain). Sepia melanin (standard) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol, acetone (99 %), and formic acid (98 %), as well as hydrochloric acid (25-38 %), hydrogen peroxide (30 %), KBr (99.99 %) were from Fluka (Germany). H_3PO_4 (85 %), $NaHCO_3$ (99.9 %), Na_2CO_3 (99.9 %), $Na_2S_2O_5$ (98.0 %), HNO_3 (65 %) and $HClO_4$ (75 %) were from J.T. Baker (Netherland). $Ca(NO_3)_2$ x $6H_2O$ (99.99 %), $Mg(NO_3)_2$ x $6H_2O$ (99.99 %) from Panreact (Barcelona, Spain) and $Fe(NO_3)_3$ x $9H_2O$ (99.99 %) from Aldrich (St. Louis, MO, USA). PTCA (Pyrrole 2,3,5-tricarboxylic acid) and PDCA (Pyrrole 2,3-dicarboxylic acid) were supplied by Professor Shosuke Ito, Fujita Health University School of Health Sciences, Toyoake, Japan. Water was obtained from a model MilliQ 185 Plus (Dionex, Sunnyvale, CA) apparatus using a resistivity of 18 MΩ x cm.

Extraction and Purification of sepia melanin samples

The extraction and purification were performed in an acid medium. To 50 g of commercial cuttlefish ink were added 100 ml of HCl (in a range of molarities varying between 0, 0.5, 1.0, 2.0 and 3.0 M for each sample) in a dark recipient. The slurry was stirred for 30 min (magnetic or ultrasonic stirring) and then kept for 24 hr at 10 oC. Solid is separated from the supernatant fluid by centrifugation (10000 rpm at 5 oC for 15 min), washed-suspended three times with a 0.5 M HCl solution, water, acetone and finally water. Following a 24 hr lyophilization to remove all solvent, a very thin black product was obtained at the end of the procedure.

Abbreviations for purified melanin sample

Com. Sepia melanin sample obtained from sepia ink which was washed three times with distilled water, acetone and distilled water and then lyophilized.

0,5 M. Sample obtained by treatment with 0,5 M HCl under magnetic stirring.

Ultra. Sample obtained by treatment with 0,5 M HCl under the action of an ultrasonic bath.

1 M. Sample obtained by treatment with 1,0 M HCl.

2 M. Sample obtained by treatment with 2,0 M HCl.

3 M. Sample obtained by treatment with 3,0 M HCl.

Multi. Sample obtained by treating 1,0 g of 0,5 M previously purified sample with addition of saline solution of calcium, magnesium, potassium, sodium and iron. The concentrations of the salts in the saline solution were the same as the one observed in the melanin standard.

Na, K, Mg, Ca and Fe; Samples obtained by treating 1,0 g of 0,5 M previously purified sample with addition of saline solution of calcium, magnesium, potassium, sodium and iron respectively.

Melanin standard. Sample unmodified from Sigma-Aldrich Company.

Instruments and methods for characterization

Quantitative elemental analysis. Appropriate melanin samples were analyzed for sulfur, carbon, nitrogen and hydrogen using a model EA 1108 (Fisons Instr.) of the Microanalysis Laboratory of the Department of Chemical Sciences at the University of Camerino.

UV-Vis and IR Absorption spectra. Samples were prepared by dissolving the different melanins in a 0,1 M sodium carbonate buffer (pH 10,3) obtaining a concentration of 0,03 g/L. The fresh melanin solutions were stirred for 15 minutes in an ultrasonic bath and then centrifuged at 10000 rpm at 5 oC for 10 min. The spectra of the supernatant solutions were measured using a Model DU 640i (Beckman, Fulterton, CA, USA) at 250-900 nm. For the IR investigation, about 2 mg of melanin and 700 mg of KBr were thoroughly mixed to form pellets and the IR spectra of the melanin samples were recorded using an FTIR spectrometer model Paraguan (Perkin Elmer, Wellesley, MA, USA).

ICP-MS analysis. To the Melanin digestion procedure it was used a solution of 30 mg of sepia melanin in 10 mL of deionized water, 1 mL of nitric acid (65%) and 1 mL of perchloric acid (70%) were added. After heating at 100 oC for 5 min, deionized water was added to reach a volume of 100 mL and the samples were thus ready for ICP analysis. ICP-MS model Element (Thermo, San Jose, CA, USA) was used for elemental analysis.

Chemical degradation of sepia melanin samples and chromatography analysis. An appropriate sample was prepared by suspending 10 mg of sepia melanin sample in 1,0 M aqueous NaOH (2,0 mL) and treating it with 1,5% H_2O_2 (final concentration) at room temperature under vigorous stirring. After 48 hr, the mixture was treated with 5% $Na_2S_2O_5$ (400 µL), taken to pH 4 with 85% H_3PO_4 then filtered through 0,45 µm FP30 (Scheleiche&Schuell) and analyzed by HPLC. A 1200 Agilent pump with DAD detector was employed (Palo Alto, California, USA). The DAD was set at a wavelength of 270 and 285 nm for PTCA and PDCA respectively. The analytical column used was a Adsorbosphere HS C_{18} 250 x 4,6 mm I.D., packed with 5 µm (Grace,

Deerfield, Illinois, USA). The mobile phase was prepared mixing a 1% aqueous formic acid solution up to a pH of 2,8 (with aqueous solution of NaOH) and methanol $(97:3)_{v/v}$, at a flow rate of 0,8 mL/min. A water/methanol $(80:20)_{v/v}$ mixture was used for column washing.

Results

In order to obtain pure sepia melanin starting from commercial cuttlefish ink a new procedure was developed. Starting from a high degree purity sepia melanin that was obtained by a definite methodology, our next goal was to fully characterize the polymer and perform degradation studies that would allow us to quantitative PDCA and PTCA. A hydrochloric acid treatment was used in the extraction process. This purification procedure produced samples in medium acid with a certain acid character because of the hydrolysis of sepia melanin under these conditions. A second modification was operated in order to replace the H^+ protons by metal ions present in the original sepia melanin including: Na^+, K^+, Ca^{2+}, Mg^{2+} and Fe^{3+}.

Elemental analysis of melanin samples

The C: N: H ratios were calculated by dividing the percentage of each element in the sample by their respective atomic weights. Carbon, nitrogen, hydrogen and sulfur analysis on the lyophilized sepia melanin samples were performed for duplicate analysis. The C: N: H ratios were calculated by dividing the percentage of each element in the sample by their respective atomic weights. The empirical formula was calculated to the relative nitrogen value. The mean values were used for the calculation of the empirical formula, taking N as the lowest integral number, instead of S, because the amount of S present was too low to enable an accurate calculation of the formula (Table 1).

ICP-MS characterization Sepia melanin

ICP-MS characterization Sepia melanin isolated from their native environment is associated with many metal ions bound to various functional groups. Such metals include Mg(II), Ca(II), Na(I), K(I) and almost all the first transition metal in which Fe(III) is the most abundant. ICP-MS were realized to analyze the metal ion presents in the purified sample and metal ion modified sepia melanin samples. ICP-MS analysis of sepia melanin samples are presented in the table 2.

Table 1. Elemental composition Carbon, nitrogen, hydrogen and sulfur on the purified sepia melanin samples were performed for duplicate analysis.

Sample	***Elemental Composition***				***Empirical formula***
	C%	***N%***	***H%***	***S%***	***C: N:H***
Com	29,125	2,696	2,771	<0,001	13:1:14
0,5 M	53,069	8,693	4,030	<0,001	7:1:6
Ultra	53,165	9,326	4,140	<0,001	7:1:6
1M	53,714	8,864	4,119	<0,001	7:1:7
2M	55,102	8,883	4,319	<0,001	7:1:7
3M	53,260	8,754	4,036	<0,001	7:1:6
Std. Mel.	33,859	6,068	3,362	<0,001	7:1:8
Multi	8,43	50,31	3,67	<0,001	7:1:6
Na	8,48	51,22	3,82	<0,001	7:1:6
K	8,27	49,36	3,90	<0,001	7:1:7
Ca	8,07	48,01	3,80	<0,001	7:1:7
Mg	8,58	51,03	3,96	<0,001	7:1:6
Fe	8,45	50,51	3,64	<0,001	7:1:6
Synth.[*12]	46,79	6,82	3,67	<0,001	8:1:8
Acid Hydrol.[**17]	51,69	7,70	3,07	<0,001	8:1:6

Synth. = eumelanin synthesized from enzymatic reaction of DHI+DHICA; Acid Hydrol.**= Sepia melanin obtained from an acid procedure purification.*

Table 2. ICP-MS analysis for sepia melanin samples (values are in mg/gr and (mol/gr)).

Sample	*Na*	*K*	*Mg*	*Ca*	*Fe*
Com	14.63 (0.138)	0.633 (0.016)	18.183 (0.742)	20.931 (0.522)	0.036 (0.003)
0,5M	0.459 (0.019)	<0.001	0.099 (0.004)	0.318 (0.008)	0.029 (0.003)
Ultra	0.472	<0.001	0.007	<0.001	<0.001
1M	<0.001	<0.001	<0.001	<0.001	<0.001
2M	<0.001	<0.001	<0.001	<0.001	<0.001
3M	0.003	<0.001	0.020	<0.001	<0.001
Standard Melanin	5.500(0.052)	1.300(0.032)	17.310 (0.710)	47.302 (1.18)	0.100 (0.009)
Literature data (i)	3.500(0.033)	2.300(0.058)	23.600(0.963)	17.200 (0.429)	0.180 (0.017)
Multi	0,301 (0.001)	0,333 (0.009)	0,894 (0.036)	2,453 (0.061)	5,568 (0.522)
Na	3,174 (0.138)	-------	-------	-------	------
K	-------	4,076 (0.104)	-------	------	-------
Mg	-------	-------	3,136 (0.129)	-------	-------
Ca	-------	-------	-------	6,343 (0.158)	------
Fe	-------	-------	-------	-------	19,335 (0.347)

IR spectroscopy

IR spectroscopy is important for the interpretation of the structure, binding capacity, affinity, and sites of metal ions in melanin. These are important factors for a better understanding of the metals-melanin complexation and also of its consequences, which is useful for the study of the purification-modification effect. In the figure 3 and figure 4, are presented IR spectra.

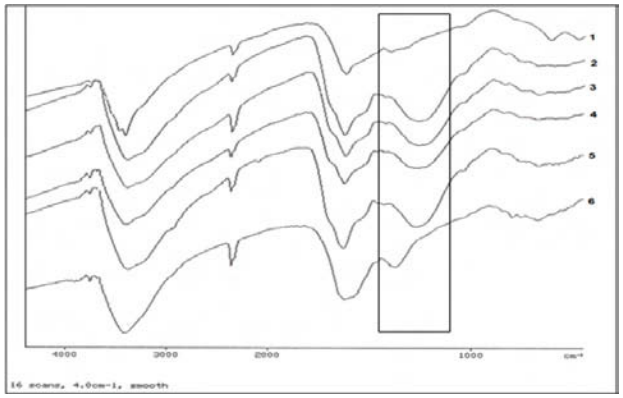


Figure 3. Infrarred absoption spectra, for samples purified at different acid concentration, sepia melanin standard and Com (ink sepia crude). Strong absorption at 3500, 1700 and 1300 cm^{-1}. IR spectra presented variation in ubication and form band in 1200-1300 cm^{-1}: (1) Com: absent (2) 3M: 1245,2 cm^{-1} (3) 2M: 1245,3 cm-1 (4) 1M: 1265,5 cm^{-1} and (5) 0,5M: 1285,7 cm^{-1} (6) Sepia melanin standard: 1357,1 cm^{-1}.

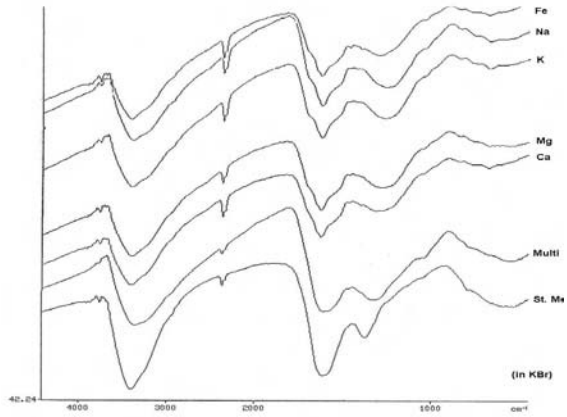
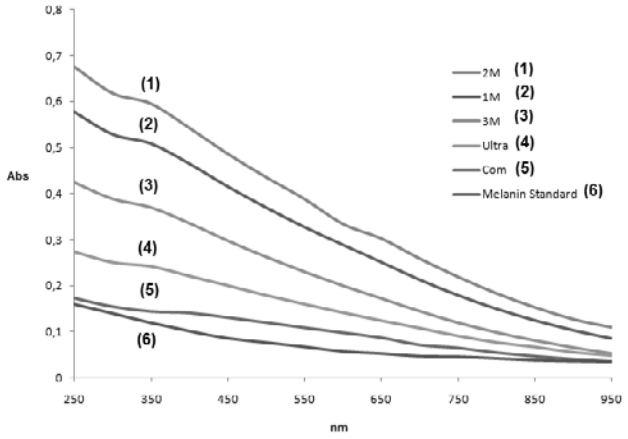
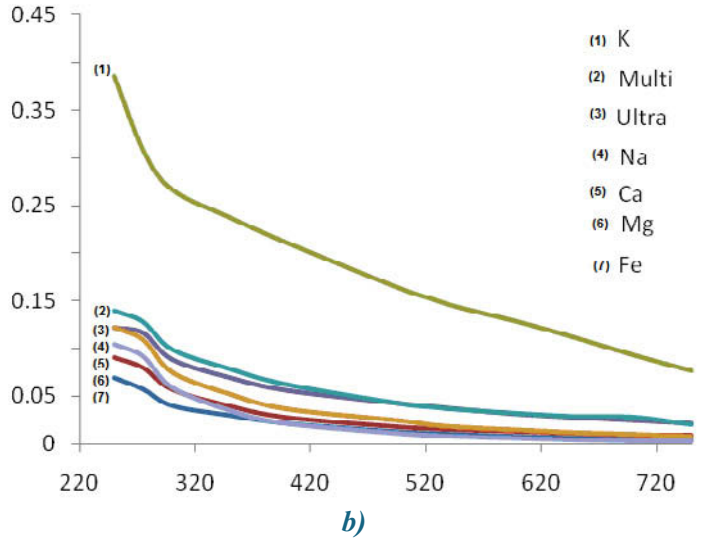


Figure 4. Infrarred absoption spectra, for samples modified with differents saline solution Multi, Na, K, Ca, Mg and Fe.

UV-Visible spectroscopy was used to analize the structural of sepia melanin sample, the results are presented in the figure 5a and figure 5b.



a)



b)

Figure 5. ***a)*** UV-VIS Absorption spectra of the different purified melanin samples: 2M, 1M, 3M and Ultra identified as (1,2,3,4), Com (5) and melanin standard (6). ***b)*** UV-VIS Absorption spectra for samples modified with differents saline solution Multi, Na, K, Ca, Mg and Fe.

Degradation chemical of sepia melanin samples

For the analysis of sepia melanin samples, two markers of sepia melanin, namely, Pyrrole-2,3,5-tricarboxylic acid (PTCA) and Pyrrole-2,3-dicarboxylic acid (PDCA) were used in the quantification of sepia melanin. PDCA is an indicator of DHI-derived units and PTCA is an indicator of DHICA-derived units and thus, of the presence of the two monomers in the melanin polymer.

By chromatographic analysis, HPLC chromatograms measured at λ_{max} 270 nm for PTCA and 285 nm for PDCA (a high molar absorption coefficient), eluted at retention times of 13,52 min and 27,67 min respectively, at a flow of 0,7 mL/min. In order to reduce analysis time, the flow was increased from 0,7 to 1,0 mL/min, obtaining an excellent chromatographic separation with retention times of 4,63 min and 7,74 min, at a flow of 1,0 mL/min, for PDCA and PTCA respectively. In the linear regression analysis for PTCA and PDCA the data presented a linear behavior with a correlation coefficient of R^2=0,9989 and 0,9988, respectively, in relation to the appropriate concentration range.

During the structural characterization of the sepia melanin samples, we chose PTCA and PDCA as standard

assays because both were proposed as markers for the sepia melanin polymer: PDCA of the DHI-derived units and PTCA of the DHICA-derived units. Chemical degradation results are presented in the table 3.

Table 3. Results of the chemical degradation of purified sepia melanin samples and modified samples (with metal ions), in PDCA% [a] and PTCA% [b]. The values obtained were the average from two assays.

Sample	*PDCA%* [a]	*PTCA%* [b]	*PTCA:PDCA*
Com	0.04	3.05	76.25
Ultra	0.28	9.89	35.32
0.5M	0.32	9.75	30.46
1.0 M	0.28	10.05	35.89
2.0 M	0.54	10.06	18.63
3.0 M	0.55	10.10	18.36
Stand. Mel.	0.04	4.64	116
Multi	0.15	6.51	43,40
Na	0.11	5,29	48.09
K	0.08	4,89	61.13
Mg	0.09	6.78	75.33
Ca	0.10	6,66	66.60
Fe	0.02	4,67	233.5

Discusion

Elemental analysis of melanin samples

Results of elemental analysis confirmed the effects of the purification, indeed, evidences of this effect was the increase in the percentage of C, H and N for all the samples when compared to the untreated in acid medium sample obtained from commercial ink (called Com). These results are consistent with values reported in different previous references (Table 1).

To study the effects of the purification process at different concentrations of hydrochloric acid, several tests were performed with acid concentration ranging from 0 to 3,0 M. Percentage results do not show considerable variations between the samples. We performed a study to analyze the effect of the agitation method, (mechanical or ultrasound) of the purified sample at a concentration of 0,5 M hydrochloric acid. The results confirmed that there is no variation in relation to the percentage of analyzed elements (C, H and N). In both cases, the samples obtained from mechanical and ultrasound agitation produced similar empirical formulas ($C_7N_1H_7$). Regarding to modified sepia melanin (Multi, Na, K, Mg, Ca and Fe) with metal ions addition, similar values in percentages and empirical formula were obtained, not showing any variation in this sample when the metal ion bound to the melanin through the modification process changed.

Inductive Couple Plasma Mass Spectrometry ICP-MS

Through ICP-MS analysis (Table 2) it was confirmed the presence of metal ions, Na^+, K^+, Mg^{2+}, Ca^{2+} and Fe^{3+} in the samples of Com untreated acid medium and Standard Melanin. Content variation in Comand Standard Melanin was observed in relation to the reference values. Com and Standard melanin samples are in a saline form. Modification effect produced loss of ion metals, and this fact was verified in the analysis of metal ions content for 0.5, 1.0, 2.0, 3.0 and Ultra samples. The hydrolysis of sepia melanin salt (Com sample) to sepia melanin acid form is supported by literature data [16].

Metal ions are replaced by H^+ protons as supported by the results of quantitative elemental analysis, previously analyzed. When it was increase the acid concentration of 0,5 M to 1,0 M, there was a total loss of metal ions. Ultrasonic (Ultra sample) agitation produced a greater loss of metal ions than mechanical agitation (0,5 M) through the purification procedure. Standard Melanin showed a greater content of Ca^{2+} in comparison with the others analyzed samples. Purification effect produced

greater loss of K^+ and Mg^{2+} than Fe^{3+}, because the latter is more strongly bound to the polymeric network.

For modified sample (Multi, Na^+, K^+, Ca^{++}, Mg^{++} and Fe^{3+} sample) the results showed a competitive process involving the absorption site. In the case where all metal ions were added together during the modification process (Multi sample), the Fe^{3+} ion was absorbed in a major proportion than other lighter ions as Na^+, K^+, Ca^{2+}, and Mg^{2+}. This was verified by sample analysis where each absorbed ion was separately measured, and the data showed that Fe^{3+} was absorbed in a molar proportion of 3:1 in comparison with other metallic ions.

In the assay of the sepia melanin modification with metallic ions, we assume that no changes in the morphology of sepia melanin happened, this fact is supported by different studies from Liu and Simon [16] who described that no changes were observed in melanin morphology when different metallic ions are associated with melanin, maybe because, once the granule is assembled, melanin is able to absorb or release metals without significant structural change.

IR absorption spectra analysis

In this assay IR analysis presented typical band and stretching of functional groups presents in sepia melanin structure (Figures 3 and 4); therefore, we could verified that samples showed IR spectra absorption similar to the reported data [17].

The signals in the 3600-2800 cm^{-1} area are attributed to the stretching vibrations (O-H and N-H) of the carboxylic acid, phenolic and aromatic amino functions presents in the indolic and pyrrolic systems. In the spectrum area between 1750 and 1550 cm^{-1} the bending vibrations of the C=O double bond (COOH) can be found as well as the ones of the carbon-carbon double bond, the carbon-nitrogen bond of the aromatic system and of the carbon-oxygen double bond of those carboxylic functions that are interested in the bond formation with the metal ions. The OH bending of the phenolic and carboxylic groups were present in the 1400-1300 cm^{-1} area.

The out-of-plane bending of the aromatic carbon hydrogen bond can be found in the 700-600 cm^{-1} area. Spectra of the purified samples (0.5, 1.0, 2.0 and 3.0 M) presented a bending vibration in the 1400-1300 cm^{-1} area, and all samples presented similar characteristics in form and position. After the extraction and purification process, the spectra presented a variation in the location and band form in the 1200-1300 cm^{-1} region, describe as follows: 1) in the Com sample the variation was very small, 2) 3 M: 1245,2 cm^{-1}, 3) 2 M: 1245,3 cm^{-1}, 4) 1 M: 1265,5 cm^{-1}, 5) 0.5 M: 1285,7 cm^{-1} and 6) Standard melanin: 1357,1 cm^{-1}. The bending in standard melanin and Com melanin which are very small were less intense in comparison with the same bending vibration observed in the purified samples.

When metal ions are present in sepia melanin a concentration-dependent decrease of the signal in the 1700 cm^{-1} region was observed. This change in the spectra reflects the change in the concentration of carboxylic groups not bonded to metal ions, therefore, the decrease in the signal with increasing salt concentrations supports the fact that metal ions bind to the ionized acid group. Furthermore, the absorption in the 3500 cm^{-1} region (O-H and N-H peaks) was unaffected or slightly increased in relation to the intensity following the metal binding, indicating that OH and NH groups are not directly involved in the binding of metal ions.

The intensity decrease of the 1300 cm^{-1} band, which indicates the deprotonation of the OH demonstrated by overlapping absorption of the phenolic and carboxyl OH remarks the above conclusion [7]. In the IR spectra of the samples submitted to the modification effect, we obseved that the spectra presented similar form and position, with only small variation in the way of bending at 1700cm^{-1}. A great simility between the spectra of the Multi modified sample and standard melanin was observed.

UV-Vis Absorption spectra

The UV and visible spectra of the melanin samples are given in Figure 5a and Figure 5b. The spectra of the purified sepia melanin samples were similar to the spectrum of sepia melanin standard and did not show any different

peaks in the whole 250-950 nm region. The UV-Vis spectrum obtained showed the typical absorption profile of melanin [23]. All spectra show a strong UV absorption in the 200-300 nm region that can be attributed to the $\pi \Rightarrow \pi^*$ and $n \Rightarrow \pi^*$ of the amino, carboxylic and aromatic moieties [14].

Chemical degradation analysis

The PTCA: PDCA ratio could be an important parameter for the structural characterization of melanin. In the results (Table 3) it was determined that considering the PTCA:PDCA ratio, the melanin samples may be divided into two types: a type I with a higher ratio range (43,40-233,5) and a type II with a lower ratio range (18,36 - 35,32).

For this purpose the quantitative elemental composition was also considered. In this case we observed that Type I is represented by sepia melanin bonded to metal ions (salt form) Com, Multi, Stand. Mel., Na, K, Ca, Mg, and Fe samples, and Type II was represented by a second group of melanin samples that have low content in metal ions (acid form), namely 0.5, 1.0, 2.0, 3.0 and Ultra. The difference between type I and type II melanin samples was explained by the fact that oligomers of DHICA are solubilized in aqueous solution during the purification process. Similar results were obtained [16], where the acid form samples showed a higher reactivity towards chemical degradation when compared to salt form samples.

We have evolved in the present study, a simple methodology for the extraction and purification of sepia melanin. With this proposed methodology, it is possible to obtain a high degree purity sepia melanin, and this method was verified by different characterizing techniques.

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