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Purification, Characterization and Photodegradation studies of modified sepia melanin (*Sepia officinalis*).

Determination of Eumelanin content in fibers from Alpaca (*Vicugna pacos*).

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Director:
Prof. Paolo Passamonti

PhD Student Mario Magarelli

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Purification, Characterization and Photodegradation studies in modified sepia melanin (*Sepia officinalis*). Determination of Eumelanin content in Fibers from Alpaca (*Vicugna pacos*)

By

Mario Magarelli

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Committee:

_____: Dr. Moises Canle López, Universidade da Coruña

_____: Dr. Reinaldo Compagnone Universidad Central de Venezuela

_____: Dr. Paolo Passamonti, Università degli studi di Camerino

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DEDICATIONS

To God

This thesis is dedicated to my parents, Geny and Giacomo, who have been a source of encouragement and inspiration throughout my studies.

This thesis is dedicated to my wife Yvette, thank you for providing me strength, courage and support that urge me to strive to achieve my goals in life.

This thesis is dedicated to my lovely sons, Mario and Luigi: there were many times when this thesis took me away from precious moments with them. They teach me about unconditional love.

This thesis is dedicated to other family members and great friends who have contributed in thought, work and support to this project.

This thesis is dedicated to the people who have done so much for me.

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A la casa que vence la sombras Universidad Central de Venezuela y todos sus buenos recuerdos.

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LIST OF ABBREVIATIONS AND SYMBOLS

Eu Eumelanin, **Pheo** Pheomelanin, **Allo** Allomelanin, **NM** Neuromelanin

DHI Dihydroxyindole, **DHICA** Dihydroxyindolecarboxylic acid

PTCA Pyrrole 2,3**,5-**tricarboxylic acid, **PDCA** Pyrrole 2,3-dicarboxylic acid

AHP (4-amino-3-hydroxyphenylalaline)

Com sepia ink commercial unmodified

BTCA 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole

TTCA 1,3-thiazole-2,4,5-tricarboxylic acid

0.5 M sepia melanin sample purified with 0.5 M HCl mechanical agitation

Ultra sepia melanin purified with 0.5 M HCl ultrasound agitation

1.0 M sepia melanin purified with1.0 M HCl

2.0 M sepia melanin purified with 2.0 M HCl

3.0 M sepia melanin purified with 3.0 M HCl

Stand. Mel. Standard sepia melanin from Sigma-Aldrich company

Na, K, Ca, Mg, Fe sepia melanin modified with metal ion Na, K, Ca, Mg, Fe

ABSTRACT

Currently melanins are still enigmatic bio-pigments bearing a structure as well as methods of determination not clearly fixed by the international scientific community. In many respects melanins are unique among biopolymers. The other essential biopolymers, that is proteins, nucleic acids and carbohydrates are chemically well characterized and can be determined using well established methodologies. On the other hand, we still do not have a method of accurately determination of melanin. Basically, the classes of mammalian melanins exist in two chemically distinct forms: the brown to black Eumelanin (Eu) and the yellow to reddish-brown Pheomelanin (Pheo). It is believed that melanin has a photo-protective role in animals. Although melanin has been postulated to act as a cellular antioxidant, little is known about the molecular mechanism of melanin photo-protection. An oxidative insult can produce modifications in the physiochemical characteristics of melanin and compromise its photo protective function. Sepia melanin (*Sepia officcinalis*) represents 98 % of Eumelanin pigment, and is thus used as standard material for the analysis of melanic black. Commercial sepia melanin (very expensive) is purified according to an unknown procedure.

In our research, we carried out studies on the extraction and purification of sepia melanin by acid treatment using different concentrations of hydrochloric acid, under mechanic or ultrasonic agitation. This proved effective in producing a high degree purity sepia melanin then fully characterized by Elemental Analysis (EA), Ultraviolet-Visible (UV-VIS) and Infrared (IR) spectroscopy, Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) for metal ion analysis and quantitative studies of Eu polymers for chemical degradation to PTCA and PDCA by High Performance Liquid Chromatography (HPLC). A first modification of sepia melanin using hydrochloric acid afforded the hydrolysis of the sepia melanin while a second modification, obtained adding different salts, resulted in melanin in the salt form. Sepia melanin stability was studied by photodegradation measurements, UV-Vis absorbance spectra of different melanin solutions were measured before and after ultraviolet exposure. The difference spectra reveal that following ultraviolet exposure, a photo-bleaching effect can be observed in this range. The irradiation with ultraviolet

radiation induced significant photochemical alterations in the sepia melanin. The obtained results can be used to extrapolate melanin degradation rates in vivo conditions, including the potential limits of its protective effect in humans.

Key words: Sepia melanin, Purification, Determination, Photodegradation

AIMS OF THE STUDY

The aims of the present study were:

1. The extraction and purification of sepia melanin (*Sepia officinalis*) from a commercial sepia ink product and in particular the:

    1.1 Study of the effect of the concentration of hydrochloric acid HCl (from 0 to 3.0 Molar) in the purification process.

    1.2 Study of the effects of the type of agitation (mechanical and ultrasound) in the purification process.

    1.3 Modification of sepia melanin by introducing ion metals (Na, K, Mg, Ca and Fe^{3+}).

2. The characterization of sepia melanin samples for the technicians: Quantitative elemental analysis, Absorption spectroscopy (UV-Vis and Infrared), Inductive coupling plasma mass spectrometry ICP-MS and chemical degradation of sepia melanin.

3. Photodegradation studies of purified and modified sepia melanin samples.

4. Quantification of Eumelanin content in sepia melanin and in fibers of Alpaca (*Vicugna pacos*).

The results of this study will provide us the necessary knowledge on the sepia melanin structure and help us establish a purification methodology. Studies of photodegradation were also carried in order to evaluate the photo-stability of the modified sepia melanin. Finally, the content in Eumelanin pigments present in the black-brown coloured fibers of Alpaca derived textiles was a further field of application of the developed methodology.

INTRODUCTION

Melanins are highly heterogeneous polymers of pigment which give color to hair, skin, iris, and nervous cells. The structures of these biopolymers are still unknown (including determination method) at this time. The other essential biopolymers, proteins, nucleic acids, and carbohydrates, are chemically well characterized and their precursors (monomeric units) and connectivity are well known, and the sequences of their connection can be determined with well-established methodologies. On the contrary, no available methods allow us to accurately to determinate melanins. This is largely because of the 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. On the other hand, we still do not have a method to accurately determine the ratio of the various units present in melanins.

The fact that melanin has an interesting chemical complexity has been studied for a long time and different studies have identified some of its physical and chemical properties. Melanin is known to have the capacity to absorb a wide range of electromagnetic radiation ranging from visible light (hence its color is dark) and UV radiation up to the X-ray region. On the other hand, melanin is reported to have the ability to bind different metallic ions. Melanin can also conduct electricity and is thus considered a semiconductor material. It is believed that melanin has a photo-protective role in animals. Although it has been postulated to act as a cellular antioxidant, little is known about the molecular mechanism of photo-protection, but an oxidative process could produce chemical modifications on the structure of melanin and compromise its photo protective functions. Finally, melanins have shown antiviral activity. All these properties of melanin make it an attractive material to use in different applications. Nowadays, many commercial products contain melanins as active ingredient, including creams that act as filters for single-response protection against UV radiation. Finally, melanin is used in cosmetics to fade defects of the skin diseases called ‘vitiligo’.

Moreover, the addition of melanin to plastics has enabled the production of sunglasses with a high ability to block UV radiation. Another very specialized application is its use in the coating of the internal surface of fluorescent lamps. This eliminates entirely the escape of UV light, which usually occurs at a low level in these lamps. This treatment can prevent damage to objects in museums or libraries. In the medical field, it has been shown that melanin can be ingested by patients, functioning as a means of contrast in X-ray studies of the digestive system. Here it is noted that a controlled amount of melanin can be eaten without causing damage as it is not digested or absorbed by the body. Some of us have used melanin to enjoy a good plate of squid in its ink.

It is expected that in the near future new applications and products based on melanin may appear, which will increase the demand for this pigment. There are several natural sources and methods for obtaining them. The extraction from animal tissues or plants is a low cost option for obtaining it. However, a major disadvantage of this method is that the product obtained generally has a low purity and composition may vary in each batch. Moreover, this product can also be generated by chemical methods, which guarantees its purity, but at a high cost.

Eumelanin isolated from the ink sacs of *Sepia officinalis* is a source of melanin with cheaper cost. Commercial sepia melanin is purified according to unknown procedure. Sepia melanin is commonly used as a model to study the spectroscopy, photoreactivity, and morphology of this class of black pigments, because of its high purity as more than 98 % of melanosomes concentration in tissues is Eumelanin.

CHAPTER 1

MELANIN PIGMENTS

1.1 Melanin Characteristics

Melanin is a biopolymer predominantly bearing an indolic structure which is present as a pigment in most vertebrates, plants, bacteria and some fungi. The origin of the name is not quite clear but is historically attributed to the Swedish chemist Berzelius (1840).

The basic units present in the melanins, which can be found in vertebrates and also in some fungi, form a covalently bonded system that lead to the formation of polymeric chains containing varying amounts of indolic monomers in different degrees of polymerization, as can be seen in Figure 1.

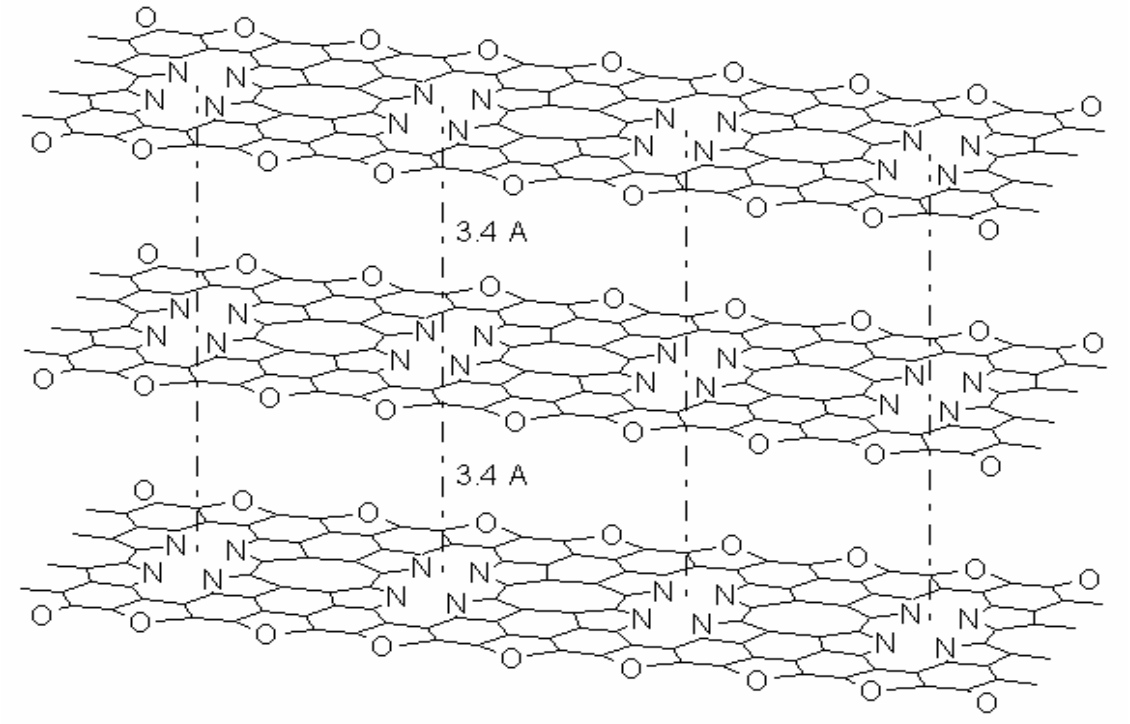


Figure 1. Melanin Polymer

The melanosomes which contain polymeric melanin can be divided into three groups according to their structure [1] : lamellar melanosomes, spherical melanosomes and melanin granules. Lamellar or elongated melanosomes deriving from the skin and hair present a protein skeleton running roughly parallel to the long axis of the melanosomes (0.5-1 μm in length) with an aspect ratio of about 1:2. Spherical or granular melanosomes form optic cells whose protein matrix is organized in a three-dimensional network with a length of 0.5-0.8 μm and an aspect ratio of about 1:1.2-1:1.5. The third group [2,3] comprises melanin granules from the ink sacs of cephalopods: they are spherical and homogeneous without any signs of detailed structure, presenting a length of 150-250 mm and an aspect ratio <1:1.1.

Properties

The melanin polymer has interesting properties such as a considerable spectral absorbance width due to the high degree of conjugation of the molecule. This polymer is capable of absorbing light quantum, both at low and high energies ranging from the infrared to the UV region. The lowest energy transition corresponds to the transition from the nonbonding orbital ***n*** to the antibonding orbital $\boldsymbol{\pi}$ ($n \rightarrow \pi^*$), which occurs predominantly in carbonyl (C = O) bonds that are very abundant in melanin. Melanin has another strong absorption transition involving the orbital energy of the antibonding π^* and the bonding π ($\pi \rightarrow \pi^*$), which occur in the aromatic - unsaturated carbon bonds. The transitions to the antibonding π (pi) orbitals are facilitated by the high degree of conjugation and by the movement of delocalized electrons in the structure of melanin.

The high degree of conjugation can absorb a large amount of visible energy and transform it into heat. Eumelanin which presents many indolic groups shows a strong absorption, mainly due to the carbonyl groups in the red part of the visible region, thus giving it a black color, whereas the pheomelanin which has fewer carbonyl groups absorpts light differently and thus presents a yellowish or reddish appearance.

Melanin in vertebrates is mainly eumelanin, which exhibits a very important feature: it has redox properties and as such melanin can take part in reactions involving 1 or 2 electrons under the effect of light absorption, producing a photo-oxidation of the pigment by

increasing the amount of carbonyl groups. Studies have shown that pheomelanin and eumelanin are degradated by exposure to ultraviolet radiation. Under radiation, the formation of superoxide and hydrogen peroxide [4,5,6] is observed and this in greater proportion in pheomelanin than in eumelanin, Clinical studies have shown a high incidence of skin cancer and increased photosensitivity in people with red hair and fair skin compared to people with dark hair and skin [7,8,9].

Eumelanin is chemically different from pheomelanin; the biggest difference is that pheomelanin has a higher amount of sulfur in the polymer. Exposure to UV radiation produces a decrease in absorbance of both melanins but in greater proportion in pheomelanin and this might be the cause of the poor protection of pheomelanin and therefore of the dermatological disorders and/or skin cancer deriving from exposure to UV radiation [10].

Another important property of melanin is the presence of free radicals: melanin is a stable free radical. All previous studies on melanin, neuromelanin, allomelanin, eumelanin and pheomelanin have something in common: a signal is present in Electronic Paramagnetic Resonance (EPR). In fact, aqueous solutions have a signal of about 4-6 G. The origin of this paramagnetic signal correlates with the nature of the free radicals of melanin and with its biological functions. Many studies show that in every 200-1000 melanin monomers there is a free radical, highly stable. In some breeds of cattle and people with albinism EPR signal is completely absent in hair and skin. This test is characteristic and helps distinguish people with differential albinism from those with false albinism. The chemistry of free radicals is associated with the processes of polymerization and oxidation [11,12].

Functions of melanin in mammals

Melanin is a pigment found in all realms of life in different structures and shapes. This could have an important role in the differentiation of species or phenotypes and the evolution of these as part of a process of adaptation. There are many hypotheses, but none is completely satisfactory [13,14,15,16,17]. Firstly, melanin is used for camouflage and embellishment as melanins are responsible for variation in hair color and it is well known that colors have a role in camouflage and play an important role in the mating of some

species. In the cephalopods, for instance, they play a very important role because the substance may confuse visual predators and alert other cephalopods of the presence of danger.

Melanin also intervenes in other structural functions such as the thermoregulatory system and sunscreen protection. It has, in fact, many biological functions, one of the most important being the protection against the degradation of proteins, through its network of polymeric protective layers that strongly absorbs UV-Visible and infrared radiations [18]. The polymer constitutes a photo-shield since it acts as a shield photoreceptor during light absorption. Furthermore, examples of melanin helping thermoregulation can be observed in reptiles where melanin acts as heat sink. The pigment also has an important role in the photoprotection of human cells preventing DNA damage which could generate mutagenesis [19,20,21]. Finally, melanin has been investigated for its the activity in fungi and its potential use as an antibiotic.

1.2 Classification of Melanins

Melanins are classified according to their natural source:

1.2.1 - Neuromelanin → Neurons, gray matter and central nervous cell

1.2.2 - Pheomelanin and Eumelanin → Skin, hair and eyes

1.2.3 - Allomelanin → plants and fungi

1.2.1 - Neuromelanin (NM)

This brownish-black granular shaped pigment is soluble in alkaline solutions and acts like a semiconductor, like other types of melanins. NM is a complex polymer that tends to accumulate in human brain during the ageing process: it can be found in neurons in the gray matter in the central nervous system. Histological studies show that other mammals phylogenetically close to humans, including chimpanzees and more distant ones as horses and sheep, exhibit this type of pigment. Concentration of NM in humans reaches its maximum at the age of 60 and then begins to decrease. The NM content of human brain is also race dependent.

The importance of NM has not been entirely understood; many questions remain unanswered about the role of neuromelanin in the gray matter, its physiological function and the development of Parkinson's disease, since there is evidence that the disappearance of this pigment is involved in the death of the cells causing the disease. In patients with Parkinson's disease, the NM values in the brain cells are: from 1.2-1.5 mg/g, this value being less than 50% of the amount of a normal person.

NM is presumed to originate from the oxidation of dopamine, but the synthetic process is still being studied. Analysis of the degradation products resulting from the hydrolysis with $KMnO_4$ and HI show that NM shares similar properties with eumelanin and pheomelanin. For example it has been proved that neuromelanin has high sulfur content (2.5-2.8%), a molar carbon-hydrogen ratio lower than synthetic melanins, a benzothiazinic ring and aliphatic groups. The precursor in the synthesis of neuromelanin has been suggested to be the pair cystenil-dopamine, suggesting that NM is a copolymer composed of derivatives of dopamine: in eumelanin there are up to 25-50% of such derivatives while a lower percentage of units can be found in pheomelanin [22,23,24]. The formation of NM is difficult to understand, but some researchers have proposed a synthetic process containing a step similar to that present in the biosynthesis of eumelanin where a tyrosinase catalyzes the conversion of L-Dopa to Dopaquinone.

It has also been reported that the NM extracted from human gray matter has no melatonin components as proteins and lipids [25,26,27]. NM shows a high content of peptides (about 15-20 %) and a high percentage of polyunsaturated fats with high molecular weight, low volatility and low oxygen content while cholesterol is a minoritarian component in the mixture of lipids.

1.2.2 Eumelanin (Eu) and Pheomelanin (Pheo)

These types of melanins are the most studied and most common in the realm of vertebrates. The degree of pigmentation in the skin, hair and eyes is determined by the ability of certain cells to synthesize certain pigments. There are two types: eumelanin (the most abundant pigment) and pheomelanin and each has a different color: black-brown for eumelanin and yellow-red for pheomelanin. It is the combination of both pigments that produces different

skin and eye color in humans and in most vertebrates, and the proportion of each of the pigments depends on each individual's genetic traits and species. Eumelanin is composed of units of monomers: 5,6-dihydroxyindol (DHI) and 5,6-dihydroxyindol-2-carboxylic acid (DHICA) as shown in Figure 2. It is insoluble in acids and slightly soluble in alkaline solutions. Polymeric Eu contains nitrogen but no sulfur in its structure [28].

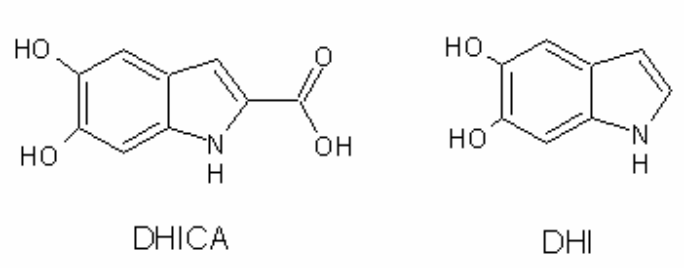


Figure 2. Eumelanin monomers

Pheomelanin is composed of monomeric units of deriving from cysteinyldopa (Figure 3). Pheomelanin polymer is soluble in alkaline solution and has both nitrogen and sulfur in their structures.

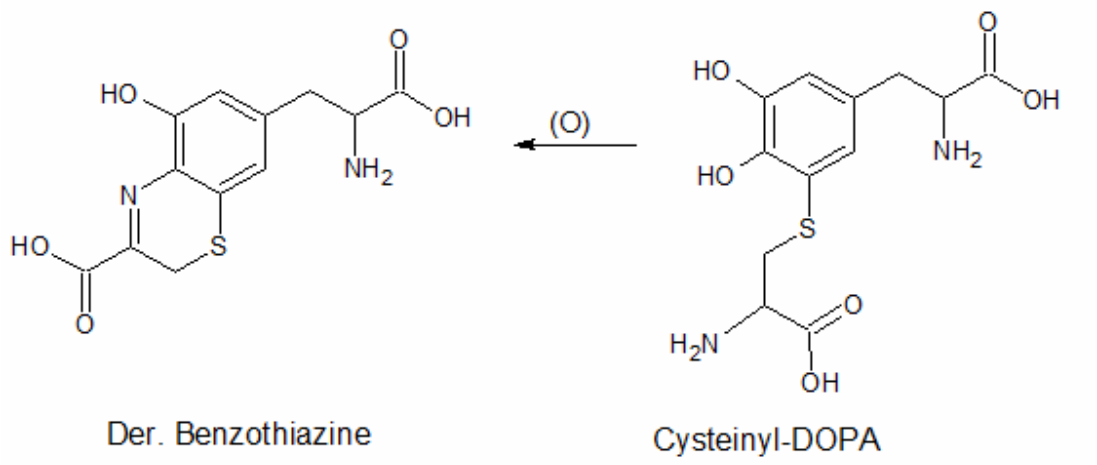


Figure 3. Pheomelanin monomers

Both Eumelanin and Pheomelanin polymer (Figure 4), derived from the common precursor Dopaquinone (see Fig. 9), are formed during the cyclization of tyrosine operated by tyrosinase. Dopaquinone is a highly reactive intermediate which, in absence of thiolic compounds produces Eumelanin while in the case of Pheo, the presence of thiolic groups deriving from cysteine and glutathione leads to the Pheo pigment[29].

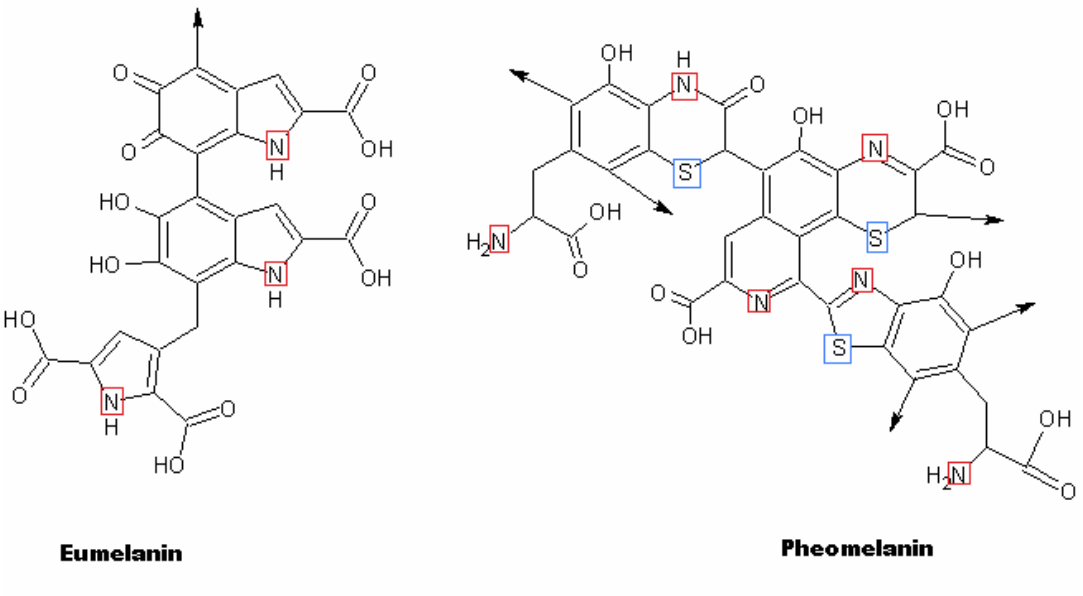


Figure 4. Structural Pheomelanin with possible union to polymer network, in the figure → represent the possible bind to polymer network.

1.2.3 Allomelanin (Allo)

Melanins deriving from plants fungi and bacteria (a limited number of species of *Bacillus*, *Pseudomonas* and *Azotobacter*) are amorphous pigments of different colors including black, dark red, yellow (also shades of the above colors). Allomelanin presents similar characteristics to other melanins, such as: insobility in all solvents and only soluble in alkaline solutions, the presence of an EPR signal due to free radical groups, semiconductor properties, etc.

Microorganisms that produce this type of melanin under aerobic conditions do so through a process that involves oxidation of phenolic substances in the presence of polyphenol-oxidases and other enzymes, which are influenced by radiation, temperature and the presence of some metals. In the future, studying this kind of melaninic pigments can prove of great interest to determine both the structure and the biosynthesis of the melanin polymer.

Fungi also produce melanin to protect their cells against some types of radiation. Melanins produced by these organisms have chemical and physical properties very similar to humic acids. Some examples are microscopic fungi with black and brown pigments (*hyphe, spore, conidia, perithecia* etc.) as well as a large amount of melanin-producing fungi: *Pullularia plullulans, Cladosporium mansonii, Phialanphora joanselmii, Nadsoniella nigra var. besuelica, Neurospora crassa, Aspergillus nidulans, Daldinia concentric, Aspargillus niger, Cephalosporium sp Mollisia caesia, Ustilago maydis DC,* etc. Since some allomelanins have shown antibiotic activity, there is an urge to better characterize these pigments.

Melanins in plants

The formation of brown, red-brown and black pigments is a phenomenon which can be also observed in the leaves and petals of plants. Until today, few physical-chemical studies of this type on the melanins in plants have been conducted because it has been almost impossible to obtain them in pure form. The oxidative degradation of these pigments produced: polyacid carboxylic benzene, benzoic acid and catechol derivatives (1,2-diphenolbenzene).

Melanogenesis in plants often contains non-nitrogenous substrates such as catechol and 1,8-dihydroxynaphthalene. It is known that in contact with atmospheric oxygen in the cells (in the presence of certain enzymes), phenols and polyphenols produce dark brown pigments. Allomelanin comes through oxidation/polymerization of di-(DHN) or 1,8-tetrahydroxynaphtalene, following the formation of colored polymeric Flovioline and DHN-melanin [30,31,32], see figures 5 and 6.

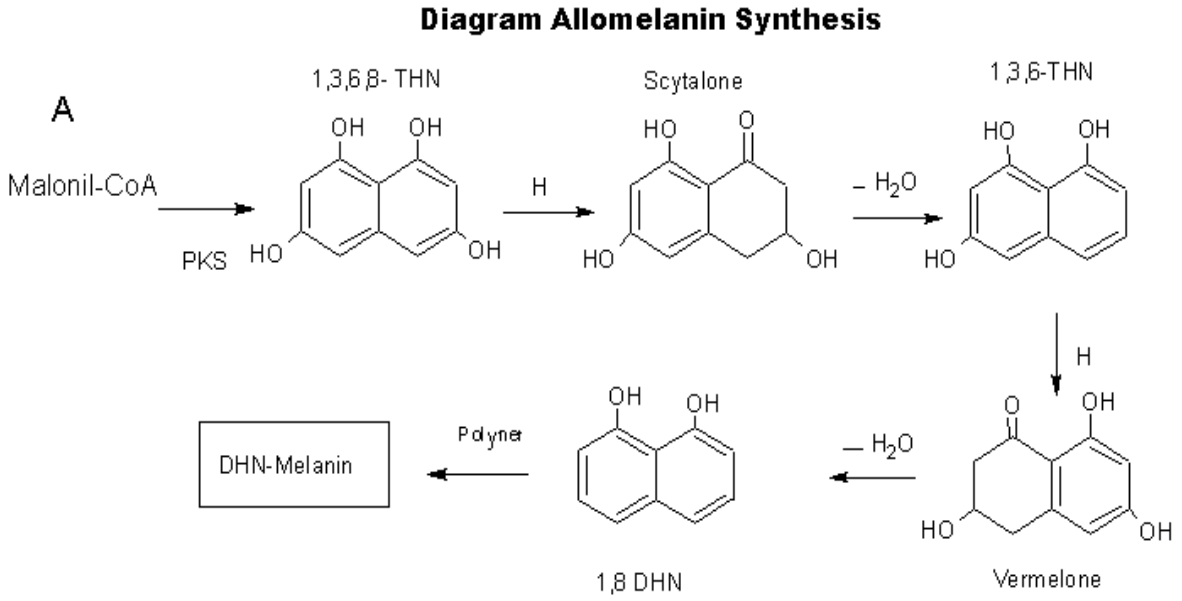


Figure 5. Diagram of the Synthesis of Allomelanin

The synthesis of (DHN)-melanin shown in Figure 5 begins with the formation of 1,3,6,8-tetrahydroxinaphthalene by polyketide synthase PKS. This product is reduced by the action of scytalone reductase. The Scytalone is then reduced by the scytalone dehydratase to 1,3,8-trihydroxynaphtalene, which is then reduced to vermelone dehydratase. Vermelone loses water to obtain 1,8-dihydroxynaphtalene. Finally 1,8- dihydroxynaphtalene-DHN polymerizes to allomelanin.

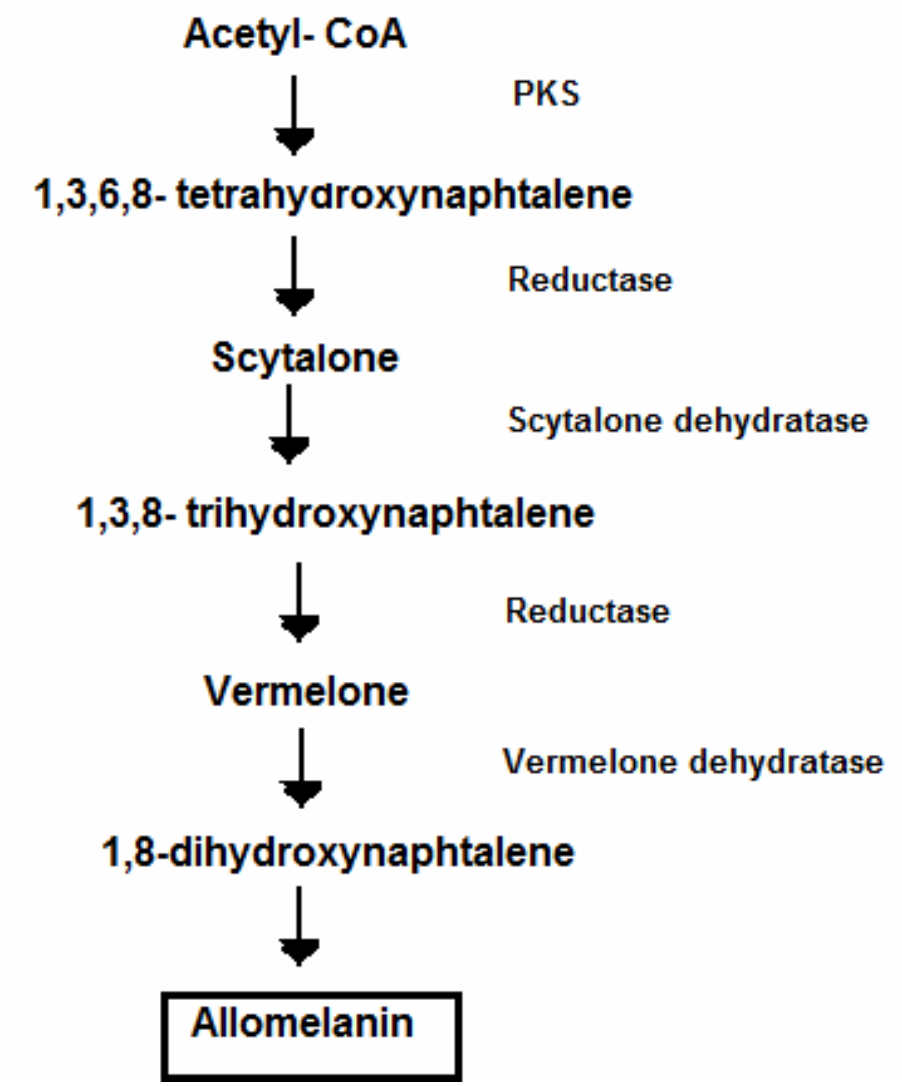
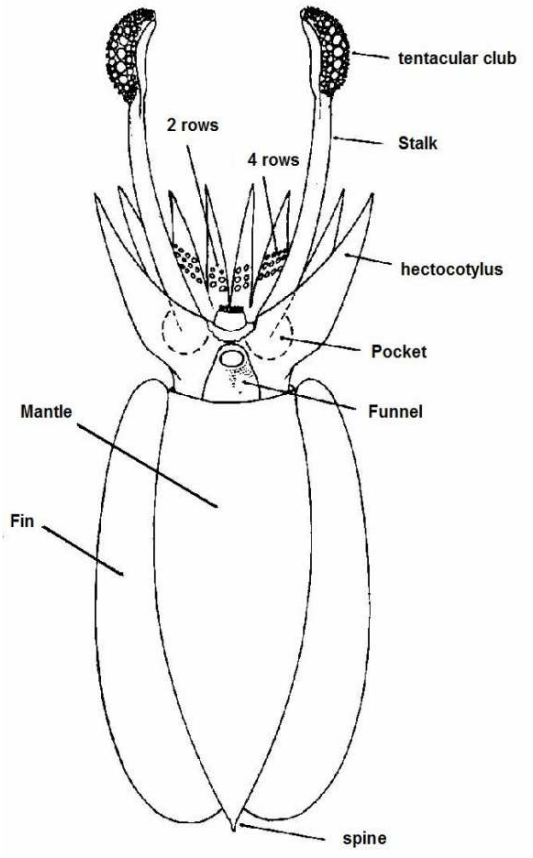


Figure 6. Enzymatic synthesis of Allomelanin

1.3 Sepia melanin from *Sepia officinalis*: General characteristics

Sepia melanin is insoluble in organic solvents, acids, aqueous solutions, and only partially dissolves in alkaline solutions. Sepia ink from *Sepia officinalis* contains $CaCO_3$, $MgCO_3$, NaCl and Na_2SO_4, enzymes and other substances. The pigment can be found in it two different forms: a salt form with Na, K, Ca, Mg and Fe and an acid form obtained following reaction in a dilute acid medium[33]. Purified sepia melanin is a black powder, hygroscopic that should be refrigerated at -20 ° C to avoid decomposition. When the cuttlefish is subjected to heating or higher temperature, it is observed a loss of CO_2 and H_2O by decomposition. Sepia melanin is also sensitive to oxygen, pressure and pulses of radiation which produce a fragmentation of melanosomes similar to what happens in the skin. The pigment is a mixture of particles of different sizes as there may found in different degrees of polymerization [34].

Sepia officinalis is a mollusk of the class of Cephalopods; it is an invertebrate 30 to 60 cm long. It has an elliptically shaped body; brown, with an internal calcareous structure (cuttlefish) in the back, while the remnant forms the external skeleton. This bone is oval and fluffy. Around the mouth it has 10 arms, 8 wide and short and two longer with suckers only at the ends (see figure 7.) The eyes are bright red and possess melanin and a small internal gland with a considerable amount of melanin.



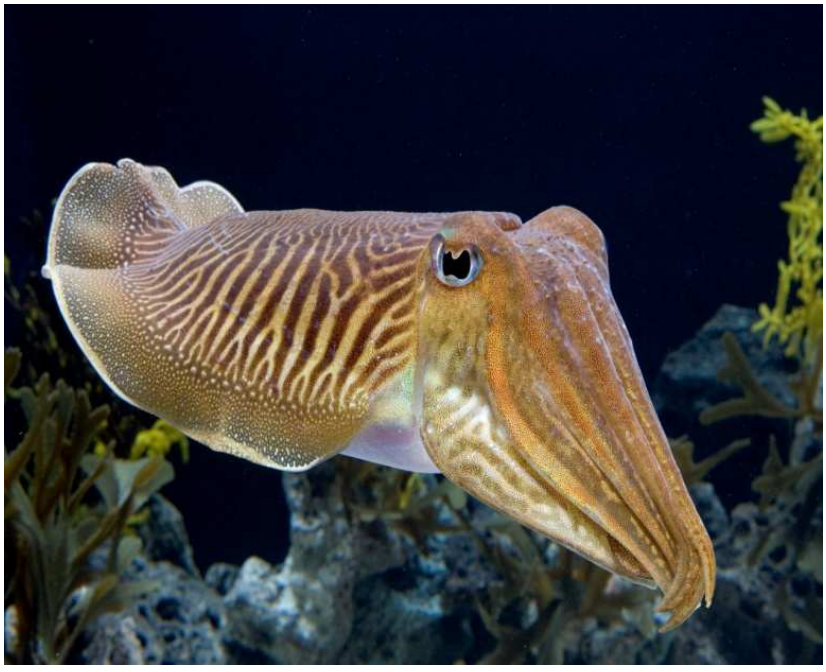


Figure 7. *Sepia officinalis*

1.3.1 Melanogenesis in Sepia officinalis

The study of the excretory ink gland of *Sepia officinalis* has been of great interest for a long time not only for its importance on a biological level (the only defense mechanism of cephalopods), but has also been the starting point in providing important and useful information in different studies on melanogenesis in mammals as well as on the biochemical process of melanin formation in the cell [(35)]. The excretory gland is an organ specialized in the formation of melanin. The excretory gland is anatomically simple, when compared to more complex structures, such as those present in mammals.

In this sense, the study of melanogenesis involves the study of different biological and biochemical processes, including regulatory systems and enzymes involved in multiple steps that not always appear to have any real connection to the pigment. This sepia melanin pigment is used as a natural pattern of eumelanin, and is commercially available. Sepia melanin form *Sepia officinalis* depending on age, size and season can be obtained in large quantities of pigments and different degrees of polymerization [(36)].

In 1982 Girod [(37)] has reported the first histological characteristics of the gland. In the study it is shown that the gland consists of cylindrical cells with a basal nucleus and an apical secretor zone containing granules of melanin. These granules are available in small packages which are filled with the pigment in the apical vacuoles and emptied into the lumen. In Figure 8 you can observe a diagram of the formation of sepia ink in *Sepia officinalis*.

In a second moment, it was shown that the gland is composed of two zones [(38)] each having different biochemical and histological features. The cylindrical cells are located neatly in a single layer on the membrane. In these cells there is a large oval nucleus near the highly developed rough endoplasmic reticulum (RER). The apical pole of the cells is filled with particles of melanosomes, which have different types of pigments in various degrees of melanization and are contained in large vacuoles. However, in mammals the pigments are contained in fibrillar melanosomes.

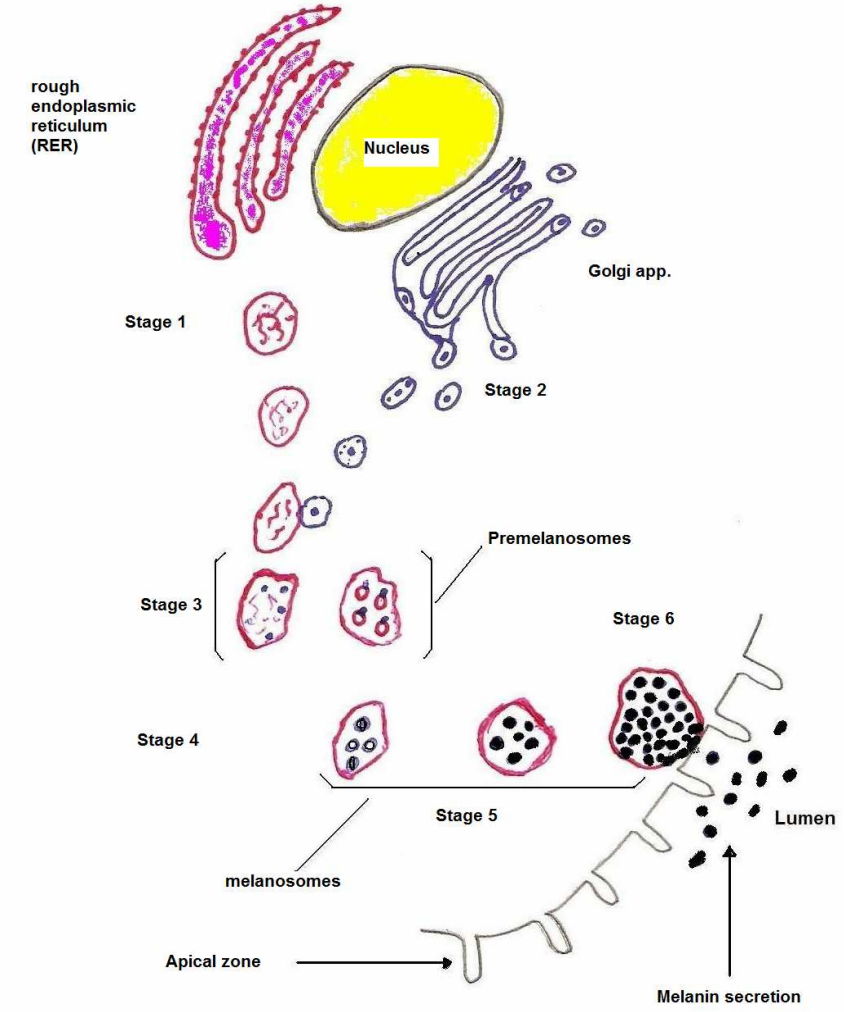


Figure 8. Diagram of the formation of sepia melanin in *Sepia officinalis*

1.3.2 Melanogenic enzyme

Tyrosinase is a copper-containing enzyme widely distributed in nature. The enzyme is responsible for melanization in animals and browning in plants and fungi. Tyrosinase is involved in the melanin pathway and is responsible, in particular, for the first steps of the synthesis of melanin which starting from L-tyrosine leads to the formation of L-DOPA quinone and L-DOPA chrome. The particularity of the tyrosinase enzyme is that it

catalyzes the *o*-hydroxylation of monophenols and the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones, in both cases using molecular oxygen. Subsequently, the *o*-quinone produces different intermediates through non-enzymatic reactions with various nucleophiles and such intermediates will then spontaneously associate into dark brown pigments.

The existence of the tyrosinase enzyme in the gland was demonstrated by enzymatic assays [(39)]; large amount of this enzyme is also found in the excreted ink [(40)]. One ml of sepia melanin contains about 1 mg of the tyrosinase enzyme. As regards with the enzymatic properties of the *Sepia officinalis* tyrosinase, it has a similar behavior to the tyrosinase enzyme deriving from other biological sources, as for example the human tyrosinase responsible for the hydroxylation of tyrosine and the subsequent DOPA oxidation[(37,41).]

The cuttlefish ink excreted contains other melanogenic tyrosinase enzymes which are involved in the catalytic rearrangement of 5,6-dihydroxyindol DOPAchrome (DHI) and this enzyme seems to behave differently from the mammalian tyrosinase[(42,43)] which mainly produces 5,6-dihydroxyindol-2-carboxylic acid (DHICA) starting from DOPAchrome. All these reactions are shown in figure 9.

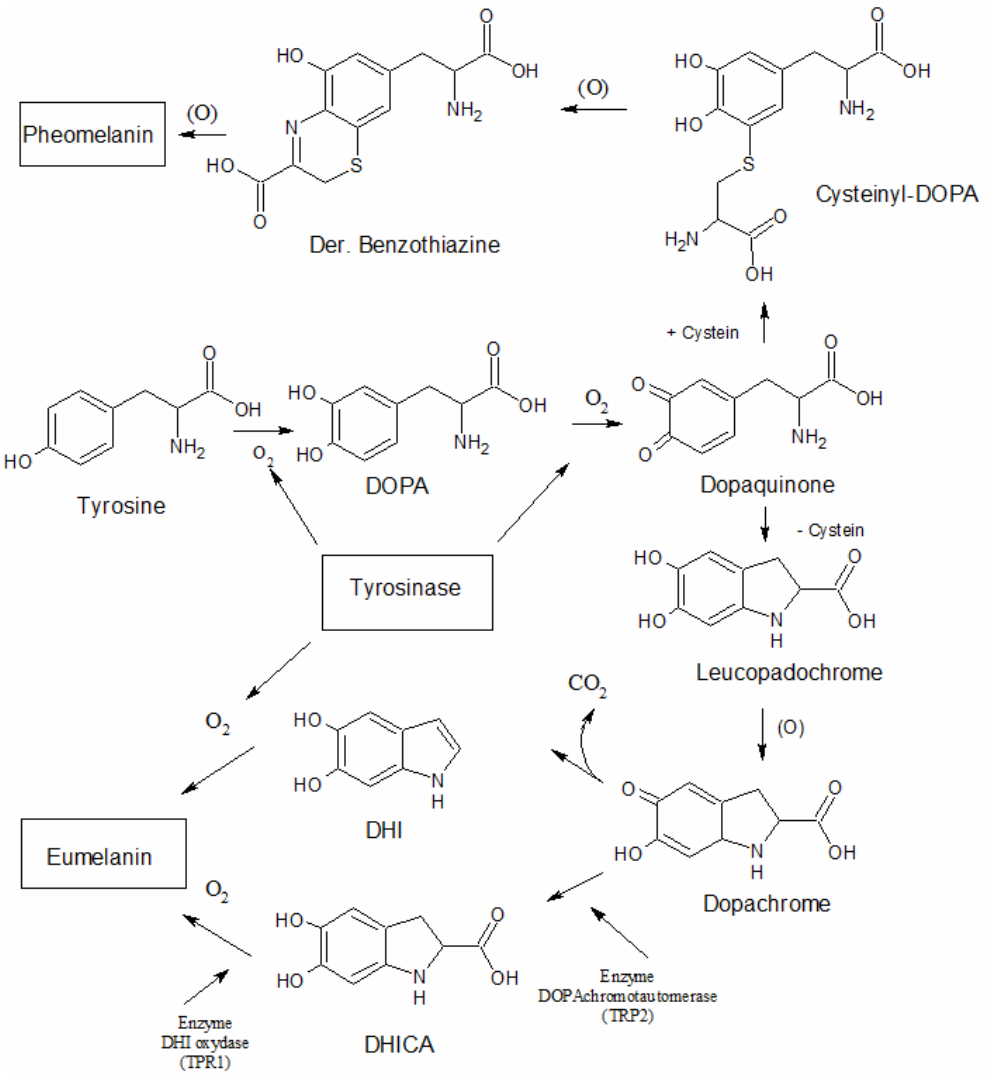


Figure 9. Chemical reactions involved in the formation of Eumelanin and Pheomelanin

1.4 Quantitative analysis of melanins through chemical degradation

A methodology involving chemical degradation is used to determine the quantity and quality of melanins present in pigmented tissues such as hair, wool, feathers, skin, eyes as well as melanoma tumor. Previous methods for the quantification of melanins in pigmented tissues required isolation of melanins. Moreover, none of these methods were suitable for distinguishing between Eumelanin and Pheomelanin. A new, relatively simple methodology [(44)] was introduced by Ito which should provide a highly sensitive and rapid method for quantitatively analysis of Eu and Pheo in tissue samples which makes the

isolation of melanin pigments unnecessary. This method is based on the formation of markers: PTCA by oxidation in acid medium of Eumelanin and AHP (4-amino-3-hydroxyphenylalanine) by hydrolysis, for hydriodic acid HI of Pheomelanin, respectively.

The products of this quite specific degradation are determined by HPLC (where PTCA was quantified by UV detection and AHP by electrochemical detection). These degradation products were chosen not only as they are the major products deriving from Eumelanin and Pheomelanin but also because they are detected at an early stage and with a high sensitivity. The yields of PTCA and AHP are approximately 2% from Eu and 20% from Pheo, respectively, and the tissue contents of Eu and Pheo can therefore be estimate by multiplying the PTCA and AHP contents by a factor of 50 and 5 respectively [45]. Formation of PTCA was interpreted in terms of the oxidative breakdown of the indolic DHICA-unit, either linked through the 2-position or bearing a carboxyl group at the same position. While PDCA could arise from the degradation of the DHI-unit[46], PTCA and PDCA are specific products arising from the DHICA-derivated unit and the DHI-derivated unit structures of Eumelanin.

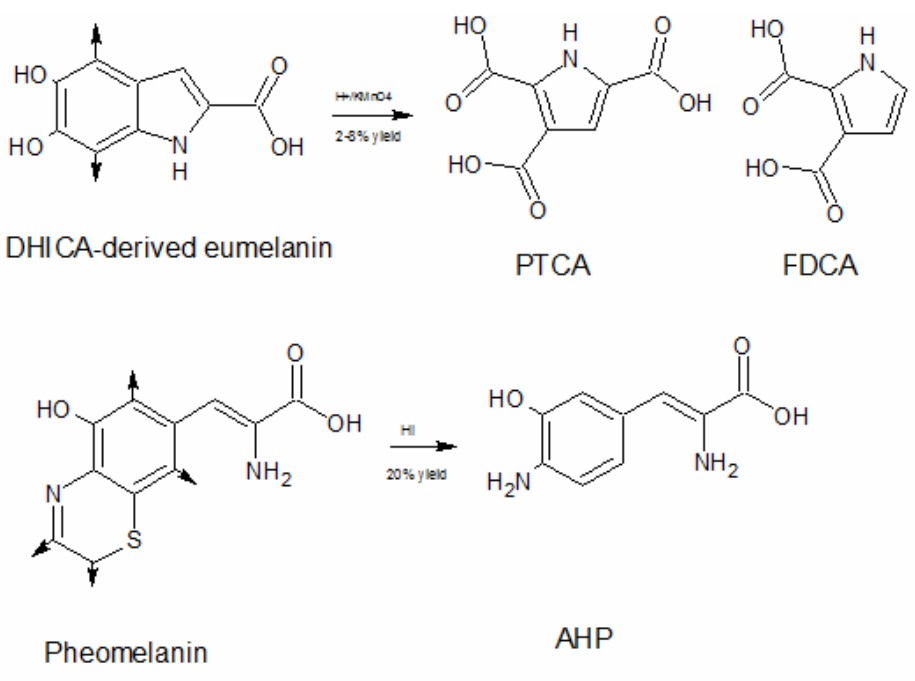


Figure 10. Products of the chemical degradation of Eumelanin and Pheomelanin

The PTCA: PDCA ratio is used as a marker in the estimation of the DHICA:DHI ratio in the Eumelanin polymer. At present, only PDCA is available as a specific indicator for the DHI-derived units in eumelanins. In sepia melanin the analysis of the chemical degradation determined the yields of PDCA and PTCA to be 5.7 % and 14.0 %, respectively. It could be argued that sepia melanin pigment contains 75% of DHICA-derived units and 20% of DHI units, in a molar ratio of 3:1[47].

An alternative chemical degradation procedure was introduced, for the simultaneous determination of Eumelanin and Pheomelanin based on an alkaline hydrogen peroxide treatment of pigmented tissues. HPLC-UV was used to determine PTCA as well as two new structural markers of Pheomelanin, namely 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole (BTCA) and 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA). The structure of these two new markers is shown in figure 11.

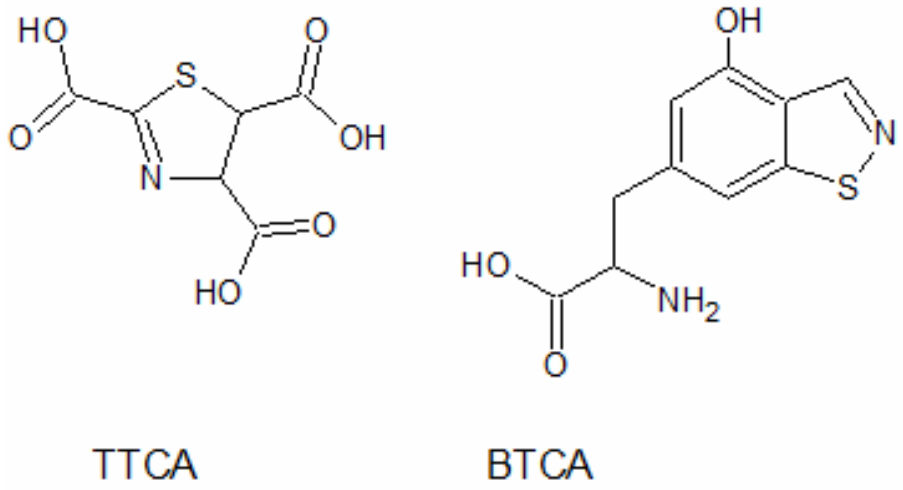


Figure 11. New markers for the analysis of Pheomelanin

1.5 Photodegradation of melanin

Natural melanins are important biological pigments with several well-established biological functions. It is believed that melanin has a photo-protective role in animals[48]. Native human epidermal melanin includes eumelanin and pheomelanin. The exact chemical structure of these two types of melanin is unknown, probably due to the copolymerization and numerous post polymerization modifications which complicated the structure elucidation process. Melanin is a heterogeneous polymer consisting of different monomeric units linked by a variety of bond[49]. However, melanin has been postulated to act as a cellular antioxidant although the molecular and cellular mechanisms of photoprotection offered by melanin are not fully understood. The ability of melanin pigment to absorb light is intrinsically protective proving that the energy of the absorbed photons is rapidly utilized in non-photochemical processes. Melanin is a system in which a very efficient thermal relaxation occurs, that is, the energy absorbed through the photons is rapidly converted to heat *via* internal conversion. As a result, the risk of potentially damaging photochemical reactions mediated by melanin is significantly reduced.

This pigment also contains both *in vitro* and *in vivo* persistent free radicals that can easily be detected by electron paramagnetic resonance (EPR)[50]. Melanin exhibits a surprising degree of physicochemical reactivity [51]. It has been shown that melanin can efficiently interact with reactive species such as hydroxyl radical and hydrated electron [52], singlet oxygen [53-54] and superoxide anion [55]. But an oxidative insult that substantially modifies the physical-chemical properties of melanin may compromise its photo-protective function. Oxidation of melanin and its irreversible bleaching also follows the experimental aerobic irradiation of melanin with UV-Vis radiation which leads to the formation of superoxide anion and hydrogen peroxide. One of the most efficient chemical systems that is known to bleach melanin pigments consists of an alkaline solution of hydrogen peroxide. This process is greatly accelerated by light [56].

Important studies have been performed with both *in vitro* and *in vivo* models of the human epidermis where UV-Vis absorbance spectra of different melanin solutions were measured before and after UV A exposure. The difference spectra reveal that following ultraviolet A exposure, a photo-bleaching effect can be observed in this range, and these changes depend

on both ultraviolet doses exposed and melanin concentrations. Photo-oxidation of pigments gives similar results *in vitro* and *in vivo* systems. The irradiation with ultraviolet A induced significant photochemical alteration in the skin witnessed by increased photo-protection in the visible range and reduced protection in the ultraviolet A range [57].Important studies realized[58] for the photodegradation of RPE melanosomes obtained from human and bovine eyes, these results have confirmed that when a Fe(III)-ADP is added to RPE melanosomes, the photodegradation of this pigment is reduced considerably.

Korytowski and Sarna [59] studied the photodegradation and bleaching phenomena for synthetic Dopa-melanin as a model for eumelanin where the degradation was induced by H_2O_2, O_2 and light at neutral or alkaline pH. These results confirmed that probably more than one mechanism operates the pre-oxidation of hydroquinone (monomer present in melanin) with subsequent nucleophilic attack of OOH^- ions which seem to play an important role in the $H_2O_2/^-OH$ mediated photodegradation reaction of synthetic Dopa-melanin. Free radical mechanisms of melanin bleaching with $OH^\cdot$ radicals being produced via site-specific Fenton-type reaction is probably present. The proposed mechanism included:

1.- The formation of hydroxyl radical during photobleaching of melanin: the radical or other strongly oxidizing species may be involved in melanin degradation.

2.- If the formation of hydroxyl radicals in melanins samples was homogeneous, then the interaction of OH with melanin could simply be described by the following equation:

$$\text{Melanin} + OH^\cdot \xrightarrow{km} \text{Melanin}_{ox}$$

where km = 1.5 x10^9 $M^{-1}s^{-1}$ is a bimolecular rate constant. For this concentration range (0.33mM) of melanin, a pseudo-first order constant for the interaction of $OH^\cdot$ with melanin was observed. This indicates a possible involvement of hydroxyl radicals in the degradation of melanin, but it is not quite clear what is the exact role of the oxidizing species.

3.- For the oxidation of melanin with H_2O_2 and other oxidants (ferrycyanide, persulfate, or periodate) the phenomena taking place can be interpreted as the result of two processes: the reversible oxidation of the functional groups present in melanin, associated with the

transient darkening of the pigment, and the irreversible bleaching of melanin caused by degradative oxidation. The data indicate that the pre-oxidation of melanin facilitates its degradative bleaching (see figure 12).

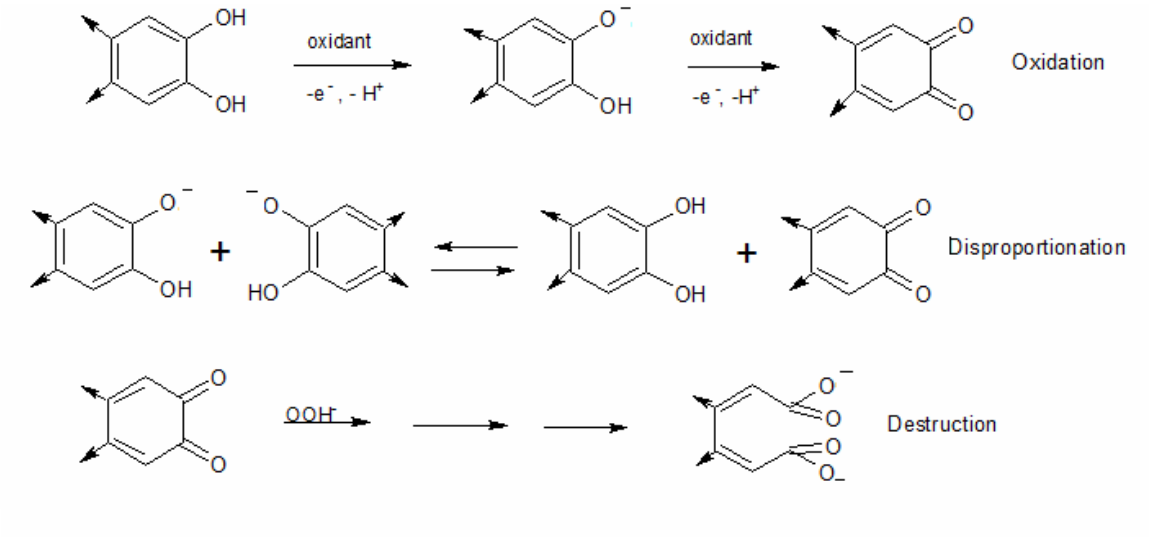


Figure 12. Photodegradation process proposed by Korytowski and Sarna.

1.6 Color characterization using the Munsell system [60]

The Munsell color system is a color space that specifies colors based on three color dimensions: hue, value (lightness), and chroma (color purity). It was created by Professor Albert H. Munsell in the first decade of the 20th century and adopted by the United States of America, Department of Agriculture as the official color system in soil research starting from the 1930s. Several earlier color order systems had placed colors into a three dimensional system of one form or another, but Munsell was the first to separate hue, value, and chroma into perceptually uniform and independent dimensions, and was the first to systematically illustrate the colors in three dimensional space. Munsell's system, and particularly the later renovations, is based on rigorous measurements of human visual responses to color, based on human visual perception.

The system consists of three independent dimensions which can be represented cylindrically in three dimensions as an irregular color solid: *hue*, measured by degrees around horizontal circles; *chroma*, measured radially outward from the neutral (gray) vertical axis; and *value*, measured vertically from 0 (black) to 10 (white). Munsell determined the spacing of colors along these dimensions by taking measurements of human visual responses. In each dimension, Munsell colors are as close to perceptually uniform as he could make them, which makes the resulting shape quite irregular. A color is fully specified by listing the three numbers for hue, value, and chroma.

1- Hue: Each horizontal circle Munsell divided into five principal *hues*: Red, Yellow, Green, Blue, and Purple, along with 5 intermediate hues halfway between adjacent principal hues. Each of these 10 steps is then broken into 10 sub-steps, so that 100 hues are given integer values. Two colors of equal value and chroma, on opposite sides of a hue circle, are complementary colors, and mix additively to the neutral gray of the same value. The diagram below shows 40 evenly-spaced Munsell hues, with complements vertically aligned.

2- Value or lightness: varies vertically along the color solid, from black (value 0) at the bottom, to white (value 10) at the top. Neutral grays lie along the vertical axis between black and white. Several color solids before Munsell's plotted luminosity from black on the bottom to white on the top, with a gray gradient between them, but these systems neglected to keep perceptual lightness constant across horizontal slices. Instead, they plotted fully-saturated yellow (light), and fully saturated blue and purple (dark) along the equator.

3- Chroma: measured radially from the center of each slice, represents the "purity" of a color, with lower chroma being less pure (more washed out, as in pastels). Note that there is no intrinsic upper limit to chroma. Different areas of the color space have different maximal chroma coordinates. For instance light yellow colors have considerably more potential chroma than light purples, due to the nature of the eye and the physics of color stimuli. This led to a wide range of possible chroma levels up to the high 30s for some hue–value combinations (though it is difficult or impossible to make physical objects in colors

of such high chroma, and they cannot be reproduced on current computer displays). Vivid soil colors are in the range of approximately 8.

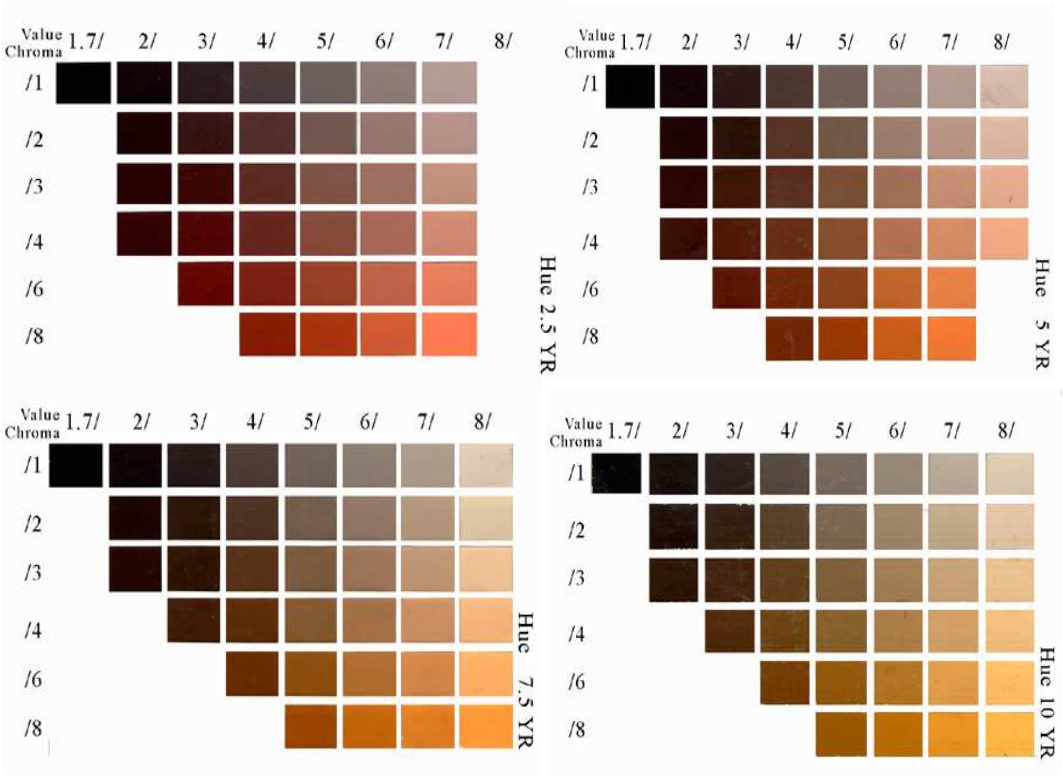


Figure 13. Standard soil color charts YR in the characterization of color by Munsell's system

1.7 Alpaca fiber and importance [61][62]

The Alpaca (*Vicugna pacos* Family: *Camelidae*, Order: *Artiodactyla*, Class *Mammalia*) is a domesticated species of South America. It resembles a sheep in appearance, but is, in fact, larger and possesses a long erect neck as well as a fleece which comes in many colors, see Figure 14, whereas sheep are generally bred to be white. Alpacas are kept in herds that graze on the level heights of the Andes of Ecuador, southern Peru, northern Bolivia, and northern Chile at an altitude of 3500 to 5000 meters above sea-level, throughout the year.

Figure 14. Alpaca (*Vicugna pacos*)

Alpaca fleece is the natural fiber harvested from an alpaca. It is light weighted or heavy weighted, depending on how it is spun. It is a soft, durable, luxurious and silky natural fiber. While similar to sheep's wool, it is warmer, not prickly, and has no lanolin which makes it hypoallergenic; Alpaca is naturally water-repellent and difficult to ignite. Huacaya, an alpaca that grows soft spongy fibers, has natural crimp, thus making it a naturally elastic yarn, perfect for knits. Suri has far less crimp and thus is best suited for woven goods, but is wonderfully luxurious as well. The designer Armani has used Suri alpaca to fashion Men's and Women's suits. Alpaca fleece is made into various products, from very simple and inexpensive garments made by the aboriginal communities to sophisticated, industrially made and expensive products such as suits. In the EEUU, groups of smaller alpaca breeders have banded together to create "fiber co-ops," in order to make the manufacture of alpaca fiber products less expensive.

The Amerindians of Peru used this fiber in the manufacture of many styles of fabrics for thousands of years before its introduction into Europe as a commercial product. The alpaca was a crucial component of ancient life in the Andes, as it provided not only warm clothing but also meat. The first European importations of alpaca fiber were into Spain. Spain transferred the fiber to Germany and France. Apparently alpaca yarn was spun in England for the first time about the year 1808 but the fiber was condemned as an unworkable material. In recent years, interest in alpaca fiber clothing has surged, perhaps partly because

alpaca ranching has a reasonably low impact on the environment. Individual EEUU farms are producing finished alpaca products like hats, scarves, and footwarmers. Outdoor sports enthusiasts recognize that its lighter weight and better warmth provides them more comfort in colder weather, so outfitters such as R.E.I.® and others are beginning to stock more alpaca products. Using an alpaca and wool blend such as merino is common to the alpaca fiber industry in order to improve processing and the qualities of the final product. Good quality alpaca fiber is approximately 18 to 25 micrometres in diameter. Whilst breeders report fibers can sell for 2 to 4 dollars per ounce, the world wholesale price for processed pre-spun alpaca "tops" is only between about $10 to $24 US/kg (according to quality), i.e. about $0.28 to $0.68 per oz. Finer fleeces, ones with a smaller diameter, are preferred, and thus are more expensive. As an alpaca gets older the width of the fibers gets thicker, at between 1 μm and 5 μm per year. This is often caused by over nutrition; if fed too much nutritious food the animal doesn't get fat, instead the fiber gets thicker. As with all fleece-producing animals, quality varies from animal to animal, and some alpacas produce fiber which is less than ideal. Fiber and conformation are the two most important factors in determining an alpaca's value.

Figure 15. Alpaca fibers

Alpacas come in many shades from a true-blue black through browns-black, browns, fawns, white, silver-greys, and rose-greys. However, white is predominant, because of selective breeding: the white fiber can be dyed in the largest ranges of colours. The demand for darker fibers sprung up in the United States and Europe, however in order to reintroduce the colors, the quality of the darker fiber has decreased slightly. Breeders have been diligently working on breeding dark animals with exceptional fiber, and much progress has been made in these areas over the last 5–7 years.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Sepia ink (concentrate and sterilized foodstuff) is obtained from Nortindal Sea Products Ltd. (Guipúzcoa, Spain). The standard melanin was sepia melanin Sigma-Aldrich (St. Louis, MO, USA). Methanol HPLC grade, acetone (99 %), formic acid HPLC grade (98 %), hydrochloric acid (25-38 %) and hydrogen peroxide (30 %) KBr (99.99 %) 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 %), $HClO_4$ (75 %), from J.T. Baker (Netherland), K_2CO_3 (97 %) from Meck (Germany), $Ca(NO_3)_2$ 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).Water was obtained from apparatus mod. MilliQ 185 Plus (Dionex) with resistivity of 18 $M\Omega \times cm$. 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.

2. Purification and modification of sepia melanin samples

The extraction and purification were performed in an acid medium which results in smaller structural changes. To 50 g of commercial cuttlefish ink were added 100.0 ml of hydrochloric acid (in a range of molarity varying between 0, 0.5, 1.0, 2.0 and 3.0 Molar 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 °C. Solid is separated from the supernatant fluid by centrifugation (10000 rpm at 5 °C for 15 min), washed three times with a 0.5 M HCl solution, water, acetone and finally water. Following a 24 hour lyophilization to remove all solvent, a very thin black product was obtained at the end of the procedure.

Figure 16. Commercial sepia ink.

Abbreviations for melanin sample

Com: Sepia melanin sample obtained by treatment of the sepia ink through washes (in triplicate) with distilled water-acetone-distilled water and then lyophilized. **0.5 M:** Sample of sepia melanin, obtained by treatment with 0.5 M HCl under magnetic stirring. **Ultra:** Sample of sepia melanin, obtained by treatment with 0.5 M HCl under the action of an ultrasonic bath. **1.0 M:** Sample of sepia melanin, obtained by treatment with 1.0 M HCl. **2.0 M:** Sepia melanin sample, obtained by treatment with 2.0 M HCl. **3.0 M:** Sepia melanin sample, obtained by treatment with 3.0 M HCl. **Multi:** Displays of sepia melanin, obtained by treating a sample size 0.5 M with a saline solution of calcium, magnesium, potassium, sodium and iron cations. **Na, K, Mg, Ca and Fe:** samples produced by adding appropriate amounts of respective cations in solution, 3 hr of mechanical agitation, then were lyophilized**. Melanin standard:** Sample of melanin from Sigma-Aldrich Company.

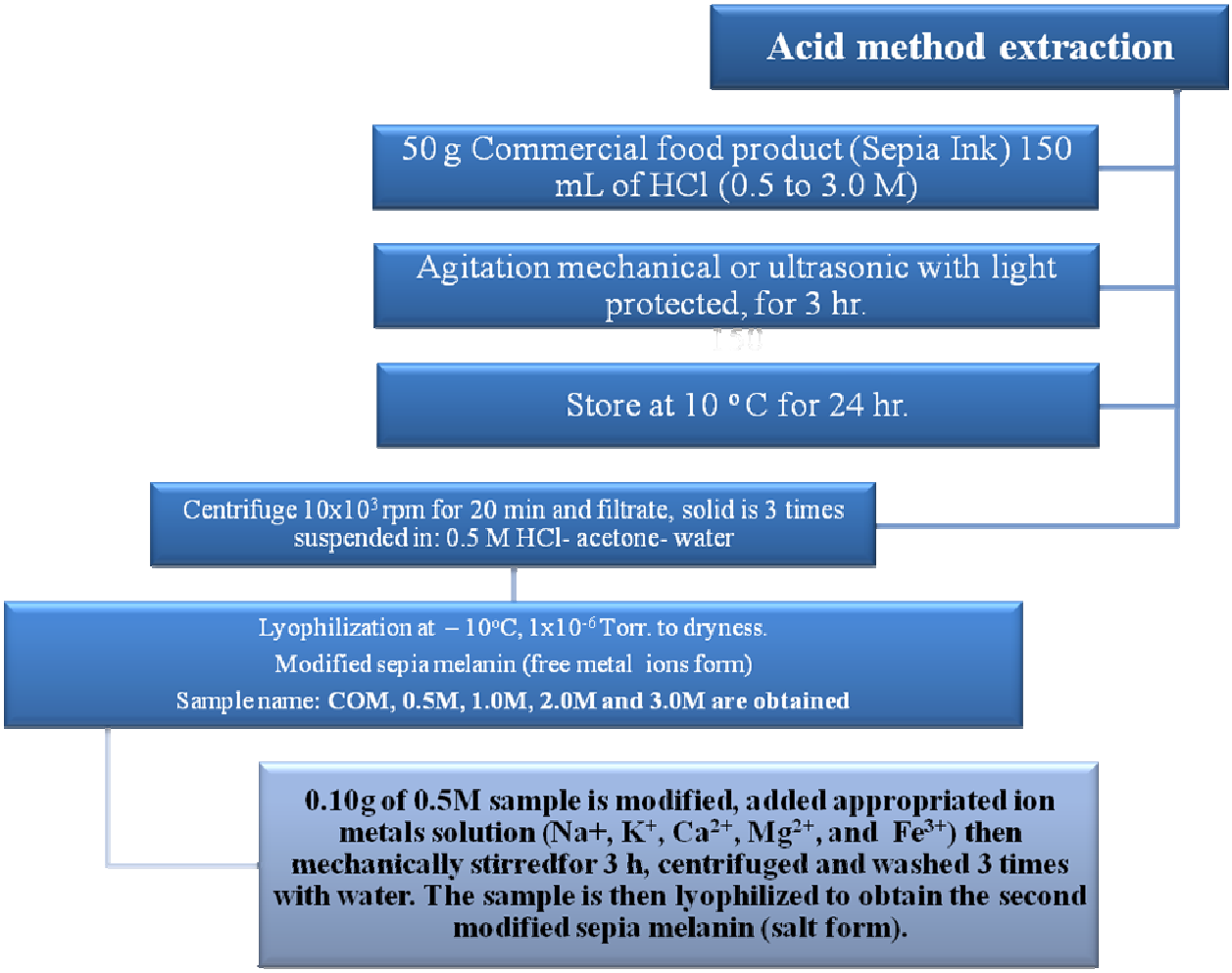


Figure 17. Diagram of the purification and modification of sepia melanin

3. Quantitative elemental analysis of melanins

Appropriate melanin samples were analyzed for Sulfur, Carbon, nitrogen and hydrogen using a model EA 1108 (Fisons Instr.) model EA 1108 in the Microanalysis Laboratory of Chemical Sciences, Department University of Camerino and at SAI Servizos de Apoio à Investigacion University of A Coruña, Spain.

4. 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 0.03g/L. The fresh melanin solutions were stirred for 15 min in ultrasonic bath and then centrifuged at 10000 rpm at 5 ^{o}C 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, approximately 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).

5. ICP-MS analysis

Melanin digestion procedure: to a solution of 30 mg of sepia melanin in 10mL of deionized water, 1ml of nitric acid (65 %) and 1 ml of perchloric acid (70 %) were added. After heating at 100 ^{o}C for 5 min, deionized water was added until a volume of 100.0 ml and the samples were thus ready for ICP analysis. ICP-MS model Element (Thermo, San Jose, CA, USA) was used for elemental analysis.

6. Chemical degradation of sepia melanin samples and chromatography analysis

An appropriate sample was prepared by suspending 10 mg of sepia melanin sample or 20 mg of Alpaca fiber, 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 h the mixture was treated with 5 % $Na_2S_2O_5$ (400 μl), taken to pH 4.0 with 85 % H_3PO_4 then filtered through 0.45 μm FP30 (Scheleiche&Schuell) and analyzed by HPLC. A 1200 Agilent with DAD

detector was employed. The DAD was set at a wavelength of 270 and 285 nm for PTCA and PDCA respectively. The analytical column used was an Adsorbosphere HS C18, 250 x 4.6 mm I.D., packed with 5 µm particles (Grace, Deerfield, IL 60015, USA). The mobile phase was prepared mixing aqueous formic acid 1 % up to pH 2.8 and methanol (97:3)v/v, at a flow rate of 0.8 ml/min. Water/methanol (80:20)v/v mixture was used for column washing.

7. Fibers cleaning and treatment for analysis

Fiber or hair of Alpaca before being analyzed by chemical degradation should be cut and washed in order to avoid interferences in the respective melanin analysis. The procedure for washing aims at removing the natural fasts in this type of fiber and inorganic material deposited on the surface of the fiber. The washing procedure is as follows (figure 18):

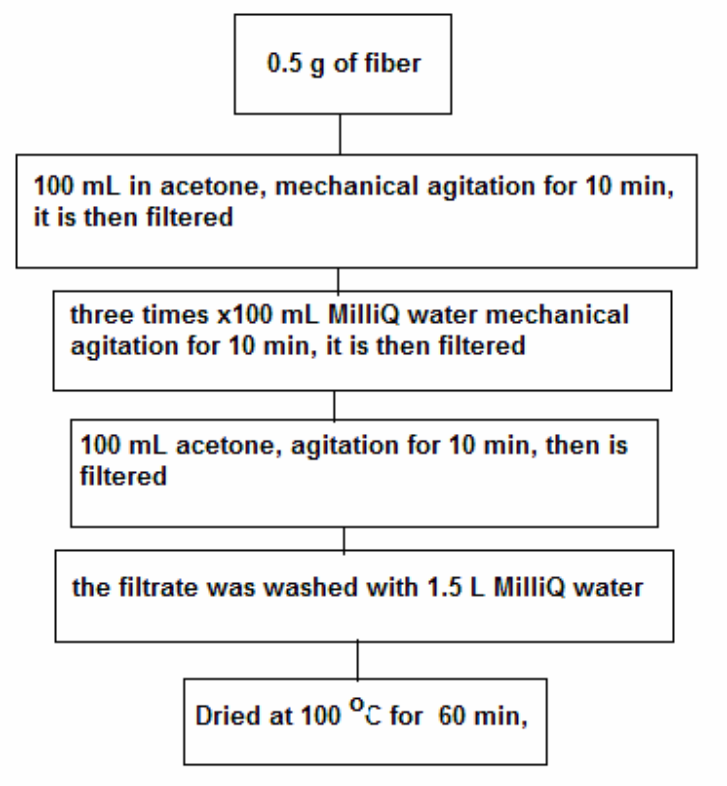


Figure 18. Washing procedure for hair and fibers of Alpaca

8. Kinetic of photodegradation

Photochemical degradation was carried out in a specially designed[63] double walled reaction vessel (volume 500 ml) with a UV chamber equipped with a Hg low pressure lamp at a wavelength of 254 nm (Figure 19). Constant stirring of the solution (approximately 0.5 g of sepia melanin in 1L of 0.1 M buffer carbonate pH 9.0) was ensured by using magnetic stirring. The temperature was maintained constant at 25 ^{o}C throughout the reaction time by circulating the water in the jacketed wall reactor with a thermostat. At various times small amounts of sample were taken; the absorbance was measured from 900-220 nm in a spectrophotometer UV-Vis.

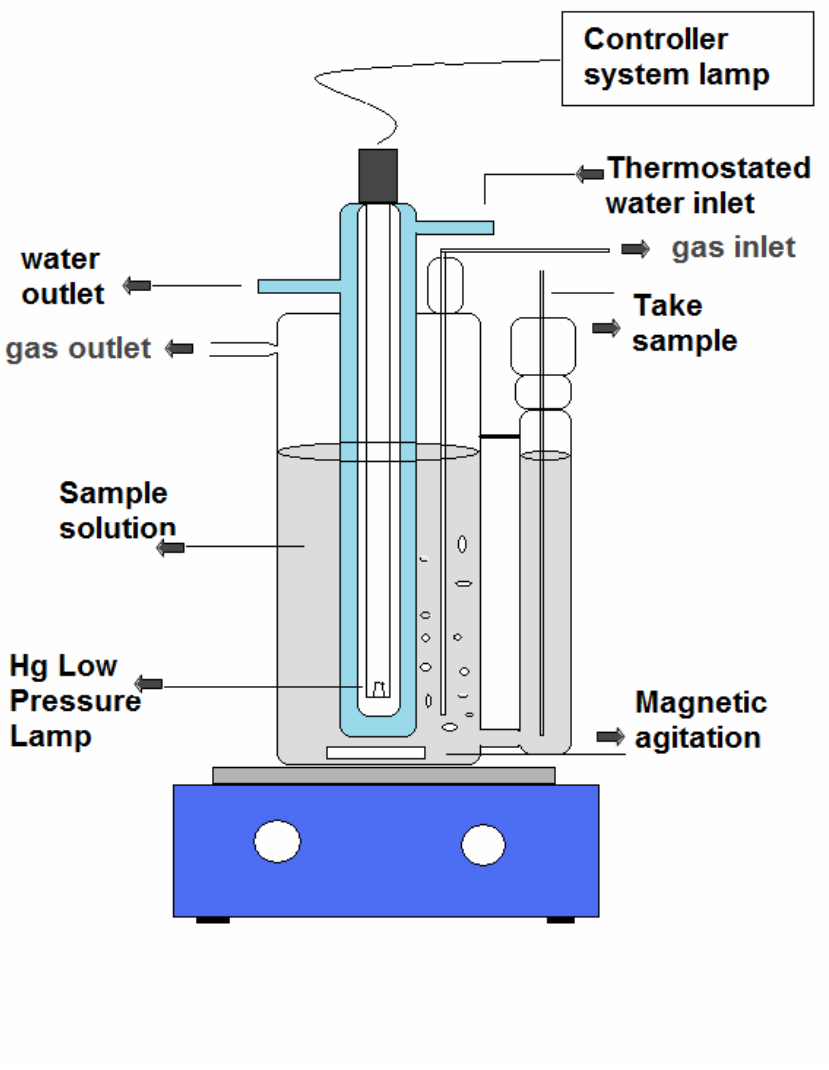


Figure 19. Photodegradation Instrument

CHAPTER 3

RESULTS AND DISCUSSION

The purification of sepia melanin samples was carried out in an acid medium, varying the concentration of hydrochloric acid, which has been used in previous references cited. Studies were also performed with regards to the type of agitation used during the purification (mechanical or ultrasound). 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 (I), K (I), Ca (II), Mg (II) and Fe (III). According to the proposed molecular structures for eumelanin, the pigment contains phenolic hydroxyl groups (- OH), carboxylic (-COOH) and amino ($-NH_2$) groups as potential binding sites for metal ions. The pka`s values of these functional groups in the monomeric units DHI and DHICA have seen examined and determined to be 4.2 for carboxylic acid of DHICA and ~9.8 and ~13.2 for two hydroxyl in the both DHI and DHICA, 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 group is reported to be ~3.1.

Characterization of melanin

Melanins are difficult to characterize because of their intractable chemical properties and the heterogeneity in their structural features. Melanin pigments, in fact, are composed of many different types of monomeric units that are connected through strong carbon-carbon bonds. Its high insolubility and undefined chemical entities are two obstacles in its complete characterization. The samples were characterized and evaluated by different techniques: elemental analysis of carbon, nitrogen, sulfur and hydrogen, analysis of metal content by ICP-MS and IR and UV-Vis spectroscopy to observe changes in the structure of sepia melanin[64].

Elemental analysis of melanin samples

Carbon, nitrogen, hydrogen and sulfur analysis on the lyophilized sepia melanin samples (see Figure 20) 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 permit an accurate calculation of the formula.

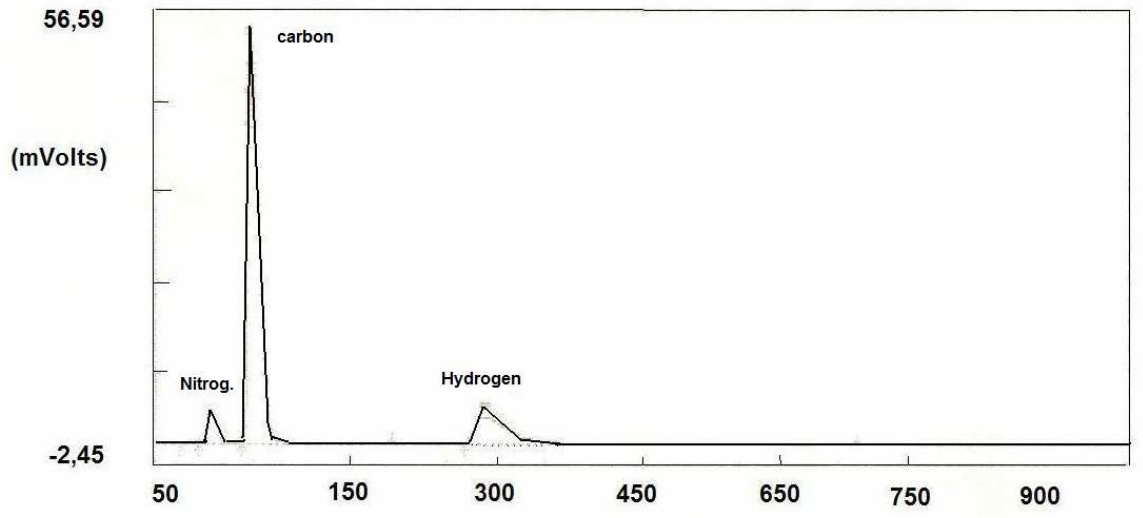


Figure 20. Example of quantitative elemental analysis of sepia melanin

Results of elemental analysis confirmed the effects of the purification: evidences of this effect were the increase in the percentage of C, H and N for all the samples when compared to the sample obtained from commercial ink (COM). Results are consistent with values reported in different previous references (see 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 0.5 M to 3.0 M. Percentage results do not

show considerable variations between the samples. We performed a study on the effect of the method of agitation, mechanical or ultrasound, of the purified sample at a concentration of 0.5 M hydrochloric acid. The results confirmed that there is no variation with respect 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 ($C_7N_1H_7$).

With regards to modified sepia melanin where metal ions had been added, (see Table 2), similar values in percentages and empirical formula were obtained, not showing any variation in the samples when the metal ion, bound to the melanin through the modification process, changed.

Sample	**Elemental Composition**				**Empirical formula C: N:H**
	C%	**N%**	**H%**	**S%**	
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
1.0 M	53.714	8.864	4.119	<0.001	7:1:7
2.0 M	55.102	8.883	4.319	<0.001	7:1:7
3.0 M	53.260	8.754	4.036	<0.001	7:1:6
Std. Mel.	33.859	6.068	3.362	<0.001	7:1:8
Synth.* [65]	46.79	6.82	3.67	<0.001	8:1:8
Acid Hydrol.**[66]	51.69	7.70	3.07	<0.001	8:1:6

Table 1. Quantitative elemental composition of purified sepia melanin and sepia melanin Synth* = eumelanin synthesized from enzymatic reaction of DHI+DHICA; Acid Hydrol.**= Sepia melanin obtained from an acid procedure purification

Samples	Elemental Composition				Empirical formula C: N:H
	C%	N%	H%	S%	
Multi	50.31	8.43	3.67	<0.001	7:1:6
Na	51.22	8.48	3.82	<0.001	7:1:6
K	49.36	8.27	3.90	<0.001	7:1:7
Ca	48.01	8.07	3.80	<0.001	7:1:7
Mg	51.03	8.58	3.96	<0.001	7:1:6
Fe	50.51	8.45	3.64	<0.001	7:1:6

Table 2. Quantitative elemental analysis for metal ion modified sepia melanin

2. Inductive Couple Plasma Mass Spectrometry ICP-MS

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. This pigment can serve as a reservoir of metal ions, such as Ca(II) and as a trap of heavy metal ion, such as Cu(II) and Fe(III). It has also been suggested that the integrity of molecular structure of the pigment could be impaired by high metal concentration.

Through ICP-MS analysis (Table 3) of Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} and Fe^{3+} contents it was confirmed that:

- We have verified the presence of metal ions, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} and Fe^{3+} in the samples Com and Standard Melanin: these two sample were not subjected to extraction-purification effect. Content variation (Com and Stand. Mel) is observed with respect to values of literature data cited in the table 3. Com and Standard melanin samples are in a saline form.

- Extraction-Purification effect produced loss of ion metals: this is verified in the analysis of metal ions content for 0.5, 1.0, 2.0, 3.0 M and Ultra samples. The hydrolysis of sepia melanin salt (Com sample) to sepia melanin acid form (0.5, 1.0, 2.0, 3.0 M and Ultra samples) is supported by literature data [45]. Metal ions are replaced by H^+ protons as supported by the results of quantitative elemental analysis, previously analyzed.

- When we 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 (0.5M) through the extraction-purification procedure.

- Standard Melanin showed a greater content of Ca^{2+} with respect to the other analyzed samples.

- Purification effect produced K^+ and Mg^{2+} losses more consistently than the observed loss of Fe^{3+} which is more strongly bound to the polymeric network.

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
1.0 M	<0.001	<0.001	<0.001	<0.001	<0.001
2.0 M	<0.001	<0.001	<0.001	<0.001	<0.001
3.0 M	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 [67]	3.500 (0.033)	2.300 (0.058)	23.600 (0.963)	17.200 (0.429)	0.180 (0.017)

Table 3. ICP-MS analysis for sepia melanin samples purified (values are in mg/g and (mmol/g))

For modified sample (Multi, Na, K, Ca, Mg and Fe sample) we observed that (see Table 4):

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

- In the study of the modification of sepia melanin with metallic ions, we assume that no changes in the morphology of sepia melanin happen and this is supported by different studies of the Liu and Simon group[67] that indicate that no changes are observed in melanin morphology when different metallic ions are associated with melanin. This is because, once the granule is assembled, melanin is able to absorb or release metals without significant structural change.

Sample	Na	K	Mg	Ca	Fe
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)

Table 4. ICP-MS for samples of modified sepia melanin with metal ions (values are in mg/g and (mmol/g))

3. UV-Vis Absorption spectra

The UV and visible spectra of the melanin samples are given in Figure 4. 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. [68][69][70]. 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 [71].

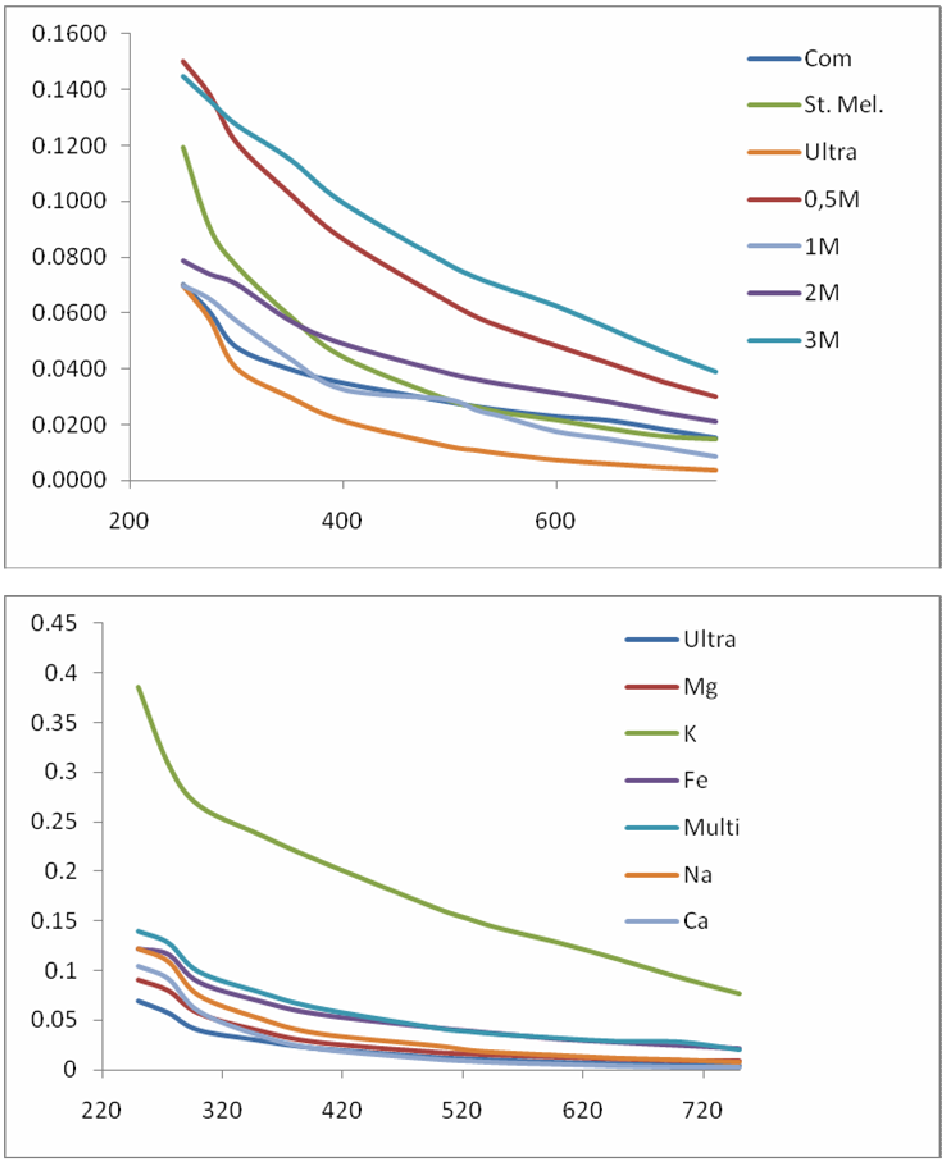


Figure 21 and Figure 22. UV-Vis Absorption spectra of sepia melanin samples

4. Infrared absorption IR spectra analysis

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 better understanding the metals-melanin complexation and its consequences. It is useful for the study of the purification-modification effect.

IR studies (Fig. 23 and Fig. 24), we have verified that:

- Samples present IR absorption spectra similar to the literature data [72,73], IR analysis presented typical band and stretching of functional groups (presents in sepia melanin structure). 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, 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. Finally, 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) present a bending vibration in the 1400-1300 cm^{-1} area: all samples presented similar characteristics in form and position.

- After the extraction and purification process, the spectra presented a variation in the ubication and band form in the 1200-1300 cm^{-1} region: (1) with the Com sample the variation was very small (2) 3.0 M: 1245.2 cm^{-1} (3) 2.0 M: 1245.3 cm^{-1} (4) 1.0 M: 1265.5 cm^{-1} and (5) 0.5 M: 1285.7 cm^{-1} (6) Standard melanin: 1357.1 cm^{-1}. The bending in standard melanin and Com melanin (very small) are less intense in comparation 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, to the purpose, the absorption in the 3500 cm^{-1} region (O-H and N-H peaks) was unaffected or slightly increased in intensity following the metal binding, indicating that OH and NH groups are not directly involved in the binding of metal ions. The decrease in

intensity of the 1300 cm^{-1} band, which reflects the deprotonation of the OH (overlapping absorption of the phenolic and carboxyl OH), also fits in with the above conclusion [74].

In the IR spectra of the samples submitted to the modification effect, we obseved:

- That the spectra presented similar form and position, only small variation in form of the bending at 1700 cm^{-1}.
- A great simility between the spectra of the Multi modified sample and standard melanin.

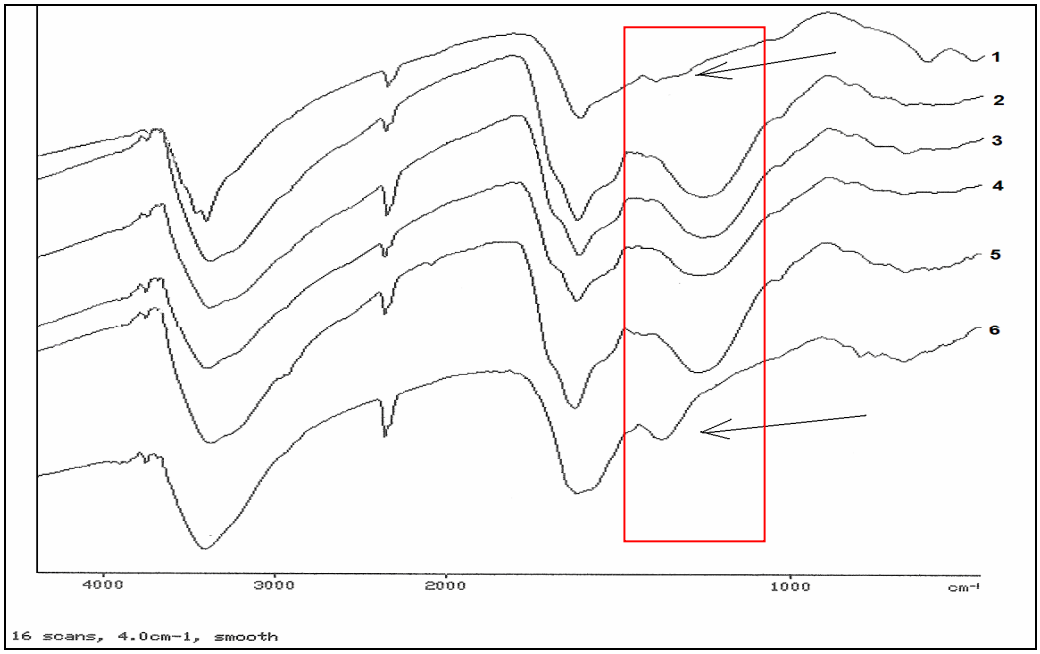


Figure 23. Infrarred absoption spectra of the purified samples at different acid concentration, Standard melanin and Com (sepia ink crude). Strong absorption at 3500, 1700 and 1300 cm^{-1} .

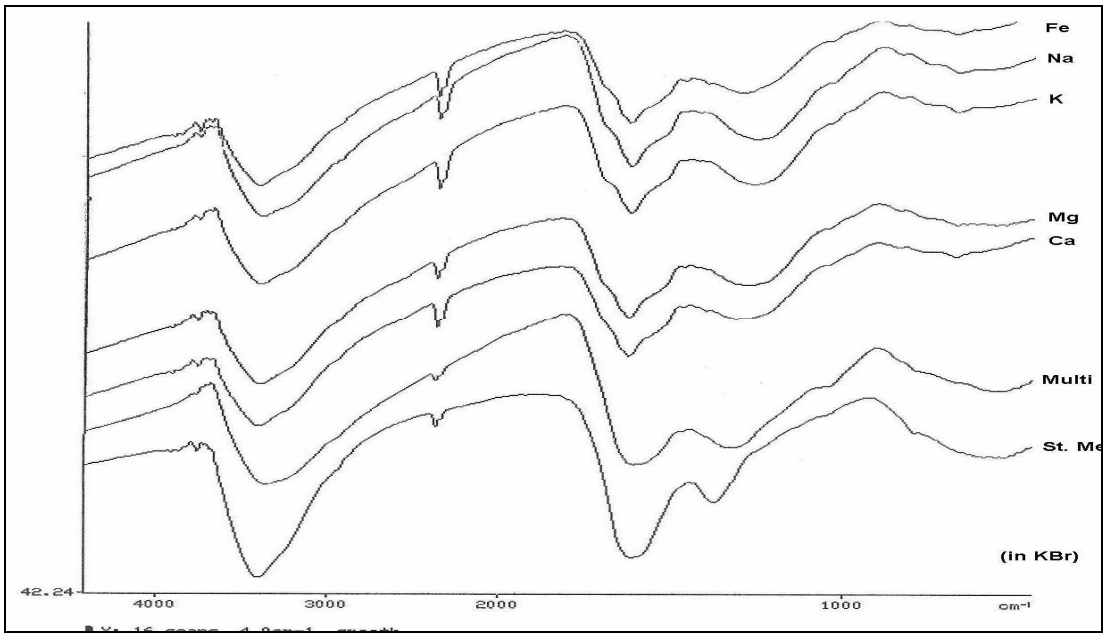


Figure 24. Infrared absorption spectra of modified sample with metal ions.

5. Photodegration studies of sepia melanin samples

In order to examine the photodegradation kinetics (Tables 5 and Table 6) of sepia melanin samples, the evolution of the absorbance as a function of wavelength was monitored during the photodegradation reaction through measurements at different reaction times. was monitored the evolution from the absorbance as a function of wavelength, measured at different reaction times. The evolution is represented by the curve: Absorbance= $f(\lambda)$ at different times of reaction, for example see figure 25.

We observed that:

The absorbance decreases in an exponential manner in function of the reaction time progression, as a consequence of the photodegradation of sepia melanin and an evident destruction of the melanic network produced by the photo-oxidation reaction[(75)].

Sample time	Com	Ultra	0.5M	1.0 M	2.0 M	3.0 M	St. Mel.
0	0.0585	0.0699	0.1500	-------	-------	0.1448	0.1193
60	0.0485	0.0613	-------	0.2307	0.0785	0.1213	-------
90	-------	-------	0.1172	------	-------	-------	-------
120	-------	0.0595	-------	0.1875	-------	-------	-------
150	-------	-------	0.1044	-------	-------	-------	-------
180	0.0391	0.0528	------	0.1363	0.0534	------	0.0857
240	0.0382	0.0505	-------	-------	-------	-------	-------
270	-------	-------	0.0725	0.1217	------	------	-------
300	-------	-------	-------	0.1158	0.0424	0.0962	0.0771
310	------	-------	0.0651	-------	-------	-------	-------
360	-------	0.084	-------	-------	-------	-------	-------

Table 5. Values of Absorbance and time observed in the photodegradation studies

Sample time	Multi	Na	K	Mg	Ca	Fe
0	0.1401	0.1226	0.3860	0.0901	0.1043	0.1225
60	-------	0.0875	0.3207	0.0777	0.0423	0.1017
120	0.1250	0.0731	0.2707	0.0510	0.0338	0.0862
240	0.1235	-------	-------	0.0404	-------	0.0732
360	-------	-------	-------	0.0307	-------	-------
420	-------	-------	0.0935	-------	-------	-------

Table 6 . Values of Absorbance and time in the photodegradation studies of the modified samples

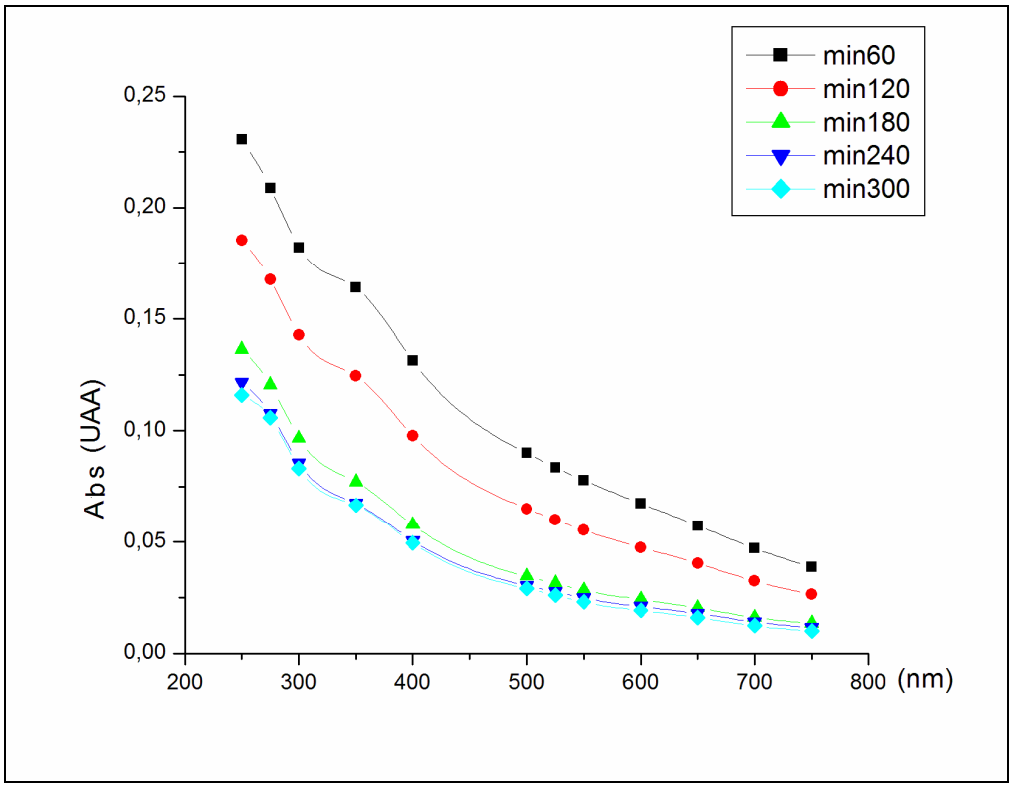


Figure 25. Typical study of photodegradation (1.0 M sample) Absorbance= $f(\lambda)$ to 60 at 300 min

To transform an absorbance unit into a concentration unit, the concentration of the melanin sample was determined at each time, using absorbance values and the respective calibration curves (Absorbance=*f* (concentration)). Furthermore, the obtained values were used in the linear regression analysis to obtain the respective linear equation (which have excellent correlation coefficient $R^2 > 0.98$) as can be seen in Table 7.

With the absorbance values and the respective equation for each sample, the concentration was estimated for each photodegradation kinetic.

Sample	Regression Equation	Sample	Regression Equation
COM	Y=12.876X-0.0014	Multi	Y=19.688X+0.0339
0.5 M	Y=21.558X+0.0212	Fe	Y=30.681X-0.0208
Ultra	Y= 25.378X-0.0454	Mg	Y=13.032X+0.0208
1.0 M	Y=21.456X+0.0126	Ca	Y=17.159X-0.0109
2.0 M	Y=27.439X-0.0278	K	Y=20.571X-0.0488
3.0 M	Y=15.726+0.0748	Na	Y=24.300X-0.1268
Std. Mel.	Y=5.7066X-0.0087		

Table 7. Regression equation for curves Absorbance=*f* (concentration)

The photobleaching of the solutions of sepia melanin (from deep black to light brown color) was monitored and the change in absorption at 250 nm (at a high molar absorption coefficient) was converted into a concentration value. With concentration results and time data the kinetic study of the behavior was conducted and the obtained data were plotted as a Concentration of melanin=*f* (t) as can be observed in concerned Figure 26.

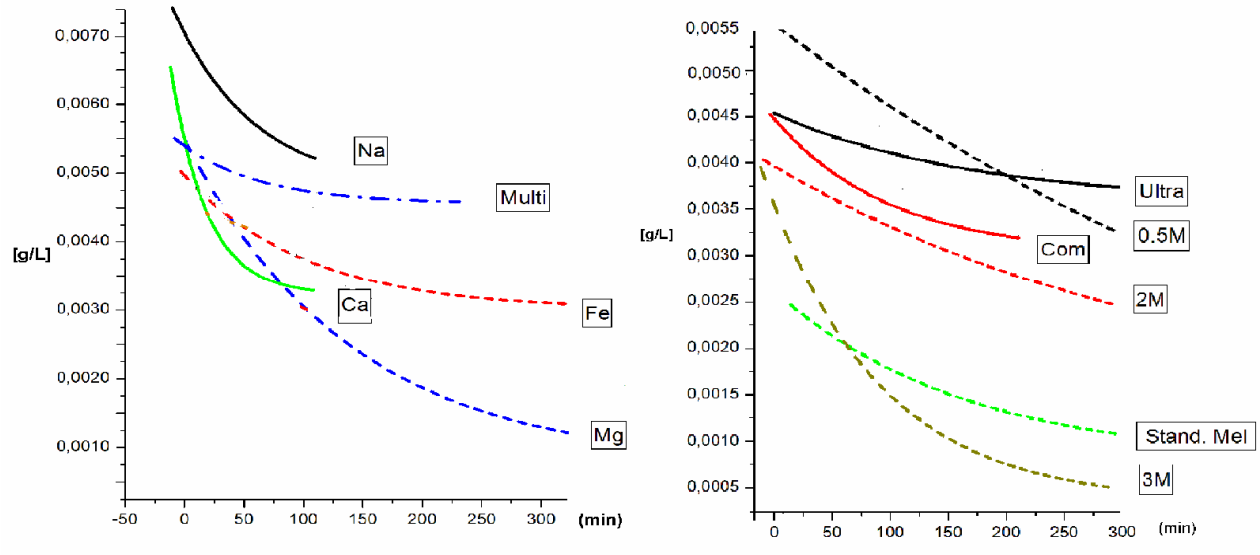


Figure 26. Concentration of sepia melanin (g/L) vs Time (min) for sepia melanin samples at 250 nm.

To determine the rate constant (k) and half-life ($t_{1/2}$), we considered melanin absorption as altered by photodegration and yielding photoproducts according to the chemical equation:

$$\text{Melanin} + h\nu\ (\text{oxidant}) \rightarrow \text{Photoproduct(s)}$$

Where $V = k[\text{Mel}]^a$ (1) and $V = -d[\text{Mel}]/dt$ (2) where a=1 for the first order reaction, if (1) = (2) then $k[\text{Mel}] = -d[\text{Mel}]/dt$ (3), to apply Integral method kinetic to (3) as result, we have obtained the linear equation (4):

$$Ln[\text{Mel}]_t = -k\,t + \text{Ln}[\text{Mel}]_o \quad (4)$$

For this reaction, it is assumed that the photoproducts absorbance did not occur in the same areas and this was, in fact, verified in each study. For the first order reaction studies, the plotted Ln[melanin concentration]= f(t), we have verified that:

- Results showed a linear correlation in all studies of the melanin samples, with correlation coefficient R^2 greater than 0.95 (Table 8 and Figure 27 show some examples).
- The linearity of the graphs, to check if there is a behavior of a first order or pseudo-first order reaction. These results are very similar to those obtained[(76)] through the photodegradation of simple molecules such as: indole-2-carboxylic acid indole-5-carboxylic acid and 5-methoxy indole-2-carboxylic acid which present similar indolic groups to the DHI and DHICA monomeric units present in melanin.

Sample	Linear Coefficient correlation R^2	Sample	Linear Coefficient correlation R^2
Ultra	0.948	**Na**	0.978
Mg	0.958	**0.5 M**	0.989
K	0.971	**Com**	0.971
Fe	0.950	**2.0 M**	0.998
Multi	0.949	**Stand. Mel.**	0.984
Ca	0.948	**3.0 M**	0.995

Table 8. Linear correlation coefficient R^2 , for kinetic studies -Ln[Mel] vs. time

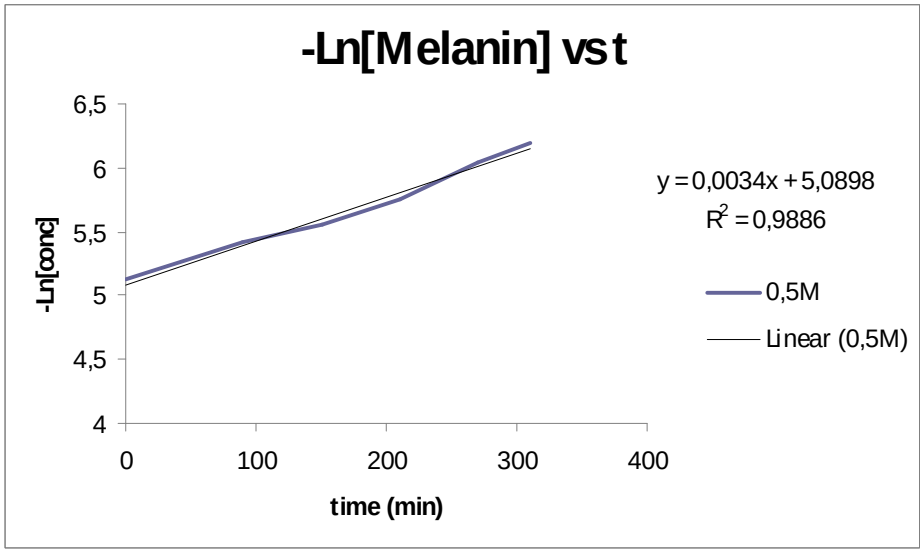


Figure 27. Kinetic studies: *Ln* [concentration melanin] vs. time in minutes (sample 0.5 M).

The values (see Table 9) of the pseudo-first order constant (k`) and half-life ($t_{1/2}$) were determined.

We observed that:

- Some sepia melanin samples are more easily degraded than other. The *k*' of pseudo-first order reaction range from 5.7 to 78.9 $L.g^{-1}.min^{-1}$ and $t_{½}$ from 0.122 to 0.009 min.
- The photodegradacion sequence is: Ca > 3.0 M > Mg > 0.5 M >K > Com > Na > 2.0 M > Stand.Mel > Fe > Multi >Ultra.
- The sepia melanin samples Fe, Multi and Ultra are more resistant to UV photodegradation, under these reaction conditions.

Sample	**k`L.g^{-1}.min^{-1}**	**T ½ (min)**	**Sample**	**k`L.g^{-1}.min^{-1}**	**T ½ (min)**
Com	17.2	0.040	**Multi**	**7.1**	**0.098**
Ultra	**5.7**	**0.122**	Ca	78.9	0.009
Stand. Mel.	13.6	0.051	Mg	42.4	0.016
0.5M	34.4	0.020	Na	18.4	0.038
2M	13.9	0.050	K	28.3	0.025
3M	53.6	0.013	**Fe**	**10.2**	**0.068**

Table 9. Values of the pseudo-first order constant (k') and $t_{1/2}$

6. Chemical degradation of melanin

In 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) are 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.

Through chromatographic analysis, we have observed that:

- HPLC chromatograms measured at λ_{max}= 270 nm for PTCA and 285 nm for PDCA (at a high molar absorption coefficient, see Figure 28), eluted at retention times: 13.52 and 27.67 (at a flow of 0.7 mL/min); in order to reduce analysis time, we increased the flow from 0.7 to 1.0 mL/min, and obtained an excellent chromatographic separation with retention times of 4.63 and 7.74 min (at flow 1.0 mL/min) for PDCA and PTCA respectively, see Figures 29 and 32.

- In the linear regression analysis for PTCA and PDCA (Figures 30 and 31) the data presented a linear behavior with an acceptable correlation coefficient of R^2=0.9989 and 0.9988, respectively, to the appropriate concentration range.

- During the structural characterization of the sepia melanin samples, we chose PTCA and PDCA as standards because both were proposed markers for the sepia melanin polymer, PDCA of the DHI-derived units and PTCA of the DHICA-derived units. The PTCA:PDCA ratio could be an important parameter for the structural characterization of melanin. In the results obtained (Table # 10), we 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 for Liu and Simon [68] where the acid form samples showed a higher reactivity towards chemical degradation when compared to salt form samples.

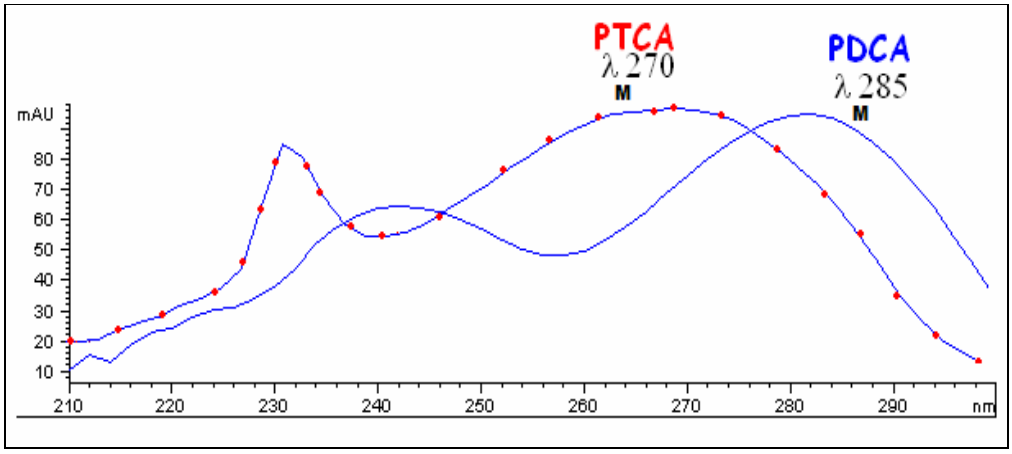


Figure 28. UV absorption spectra of markers of sepia melanin: PTCA and PDCA

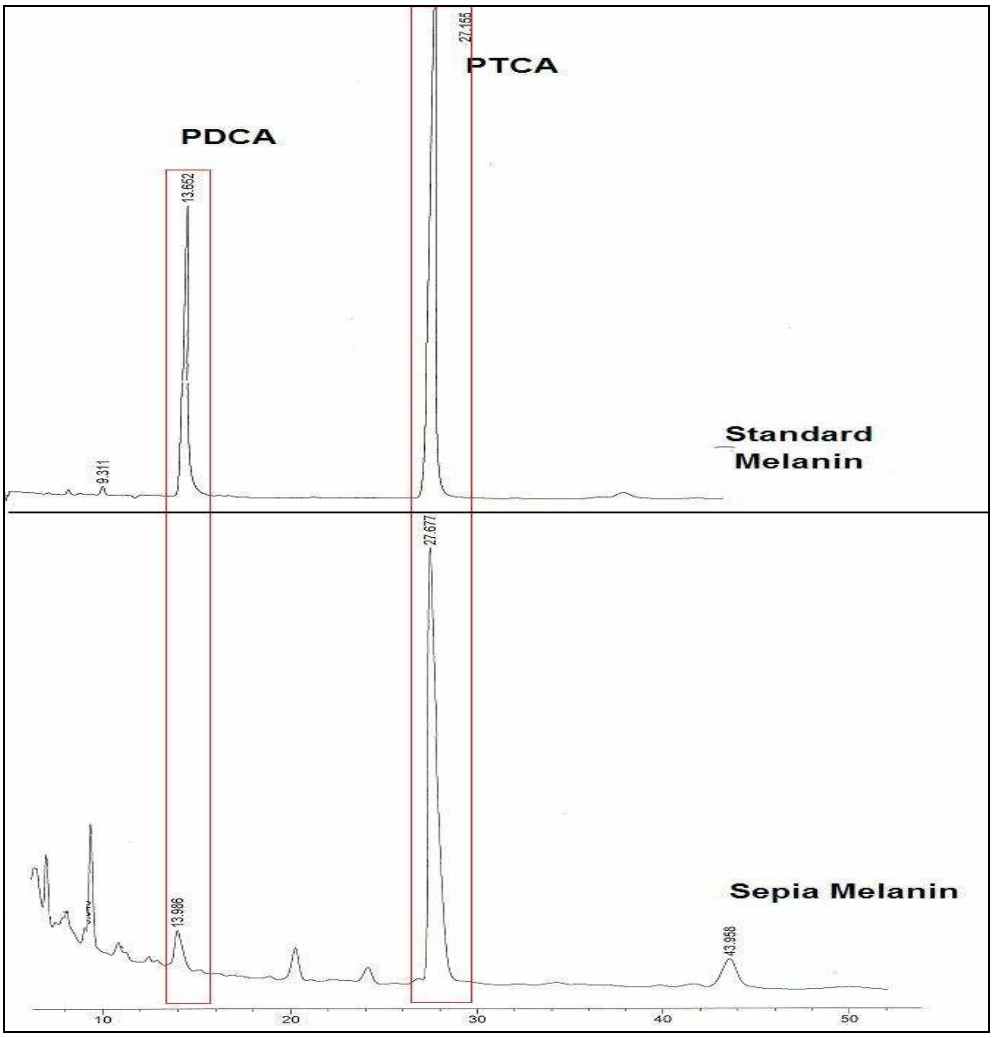


Figure 29. Comparisons between chromatograms of standard PTCA, PDCA with chemical degradation products of sepia melanin (at flow 0.7 mL/min with DAD detection λ 270 nm)

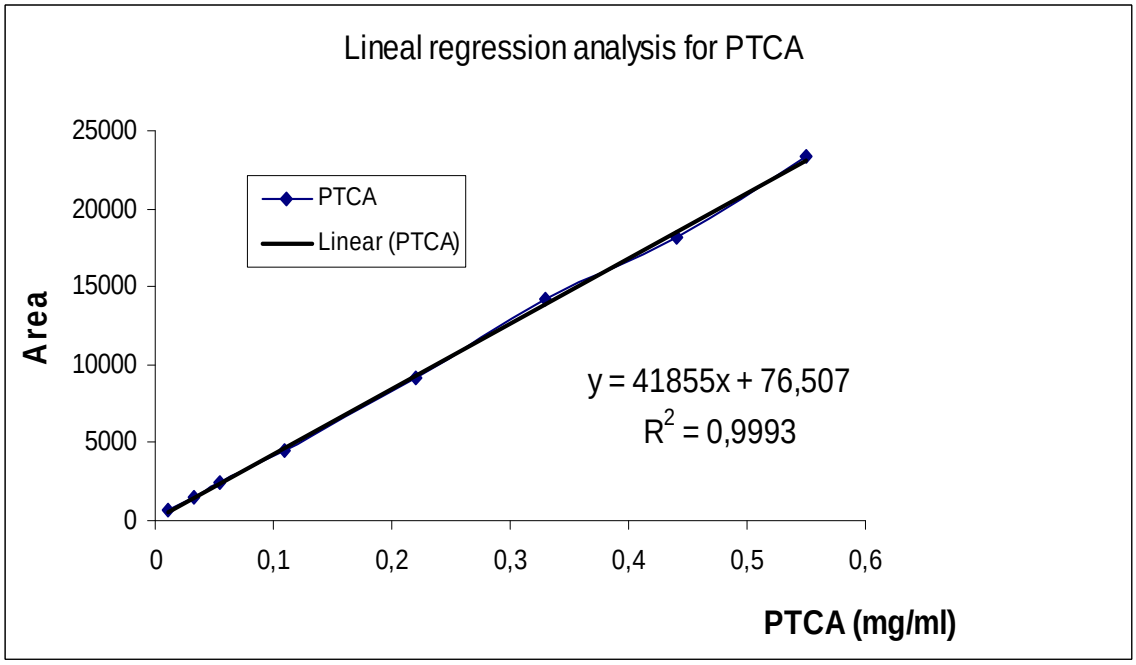


Figure 30. Linear regression analysis for PDCA

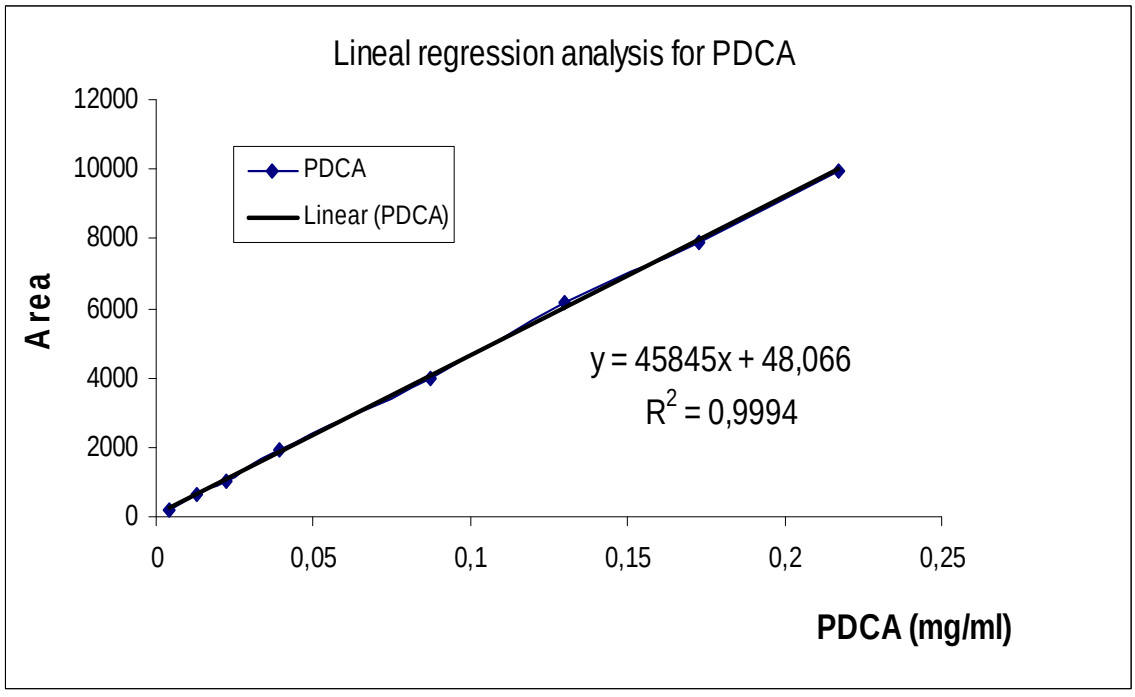


Figure 31. Linear regression analysis for PTCA

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

Table 10. 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.

For the characterization of Alpaca fibers or hair (*Vicugna pacos*)

- The first characterization was realized through the identification of the fiber color through the Munsell system, the visual comparison of colors of Alpaca fibers (Figure 33) with standard soil color charts Hue YR (Yellow-Red range).

- The second characterization involved the determination of eumelanin content in fiber samples. This was realized through chemical degradation and determination through HPLC with DAD detection (Figure 32). We used the methodology proposed by Napolitano et al[77] and PTCA as a eumelanin marker. The PTCA% range was from 0.0056 (light color) to 0.5762 % (dark color).

- We established an "eumelanin" scale based on the yield of PTCA, the pigment marker. This scale comprises four groups:

    - **Type I** PTCA range (0.0056-0.0170) Color (Hue 10YR7/2, Hue 10YR 8/1, Hue 10YR7/3) Light blonde – blonde hair.

    - **Type II** PTCA range (0.0280-0.0390) Color (Hue 10YR4/4, Hue 7.5YR5/6, Hue 7.5YR5/4, Hue 5YR5/4) Light brown- brown.

    - **Type III** PTCA range (0.1101-0.1130) Color (Hue 7.5YR4/3, Hue 7.5YR4/3, Hue 5YR3/3) Brown orange - dark brown.

    - **Type IV** PTCA range (0.2789-< 0.5762) Color (Hue 5YR3/1, Hue 7.5YR2/1) Dark brown- Black

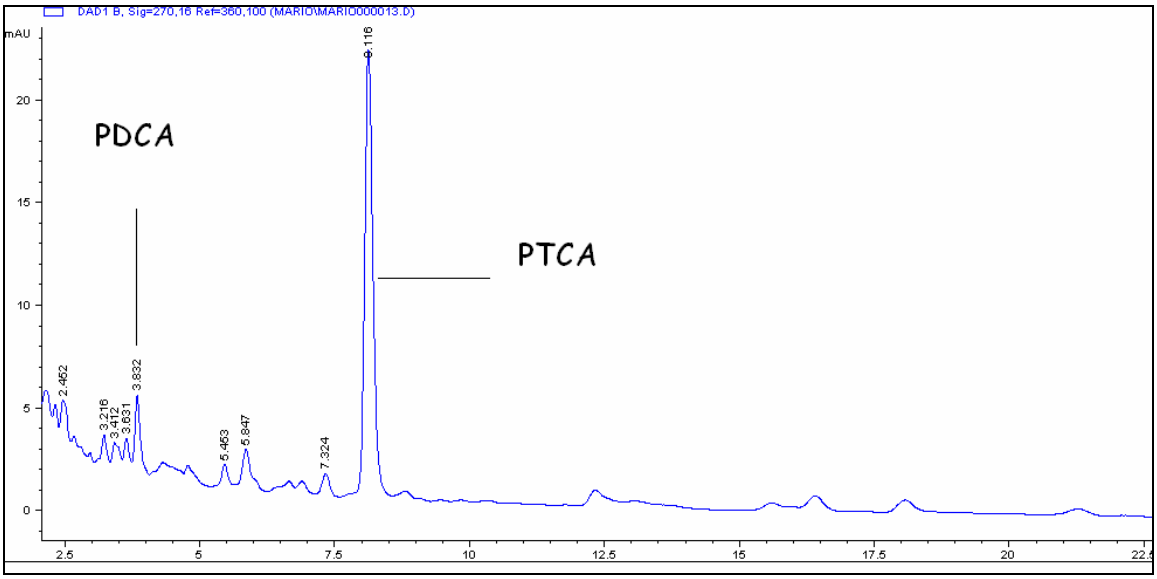


Figure 32. Example of Chromatogram of PDCA and PTCA analysis of hair sample (Alpaca 529)

Hair sample	Color Munsell	PTCA %[a]	Classification
Alpaca 023	Hue 10YR7/2	0.0056	Type I
Alpaca 197	Hue 10YR 8/1	0.0056	Type I
Alpaca 828	Hue 10YR7/3	0.0176	Type I
Alpaca 029	Hue 10YR4/4	0.0283	Type II
Alpaca 275	Hue 7.5YR5/6	0.0367	Type II
Alpaca 529	Hue 7.5YR5/4	0.0359	Type II
Alpaca 179	Hue 5YR5/4	0.0389	Type II
Alpaca 248	Hue 7.5YR4/3	0.1101	Type III
Alpaca 576	Hue 7.5YR4/4	0.1120	Type III
Alpaca 247	Hue 5YR3/3	0.1130	Type III
Alpaca 616	Hue 5YR3/1	0.2789	Type IV
Alpaca 134	Hue 7.5YR2/1	0.5762	Type IV

Table 11. Characterizations through Munsell system, PTCA content and classification of Alpaca hair sample [a] average of two assays.

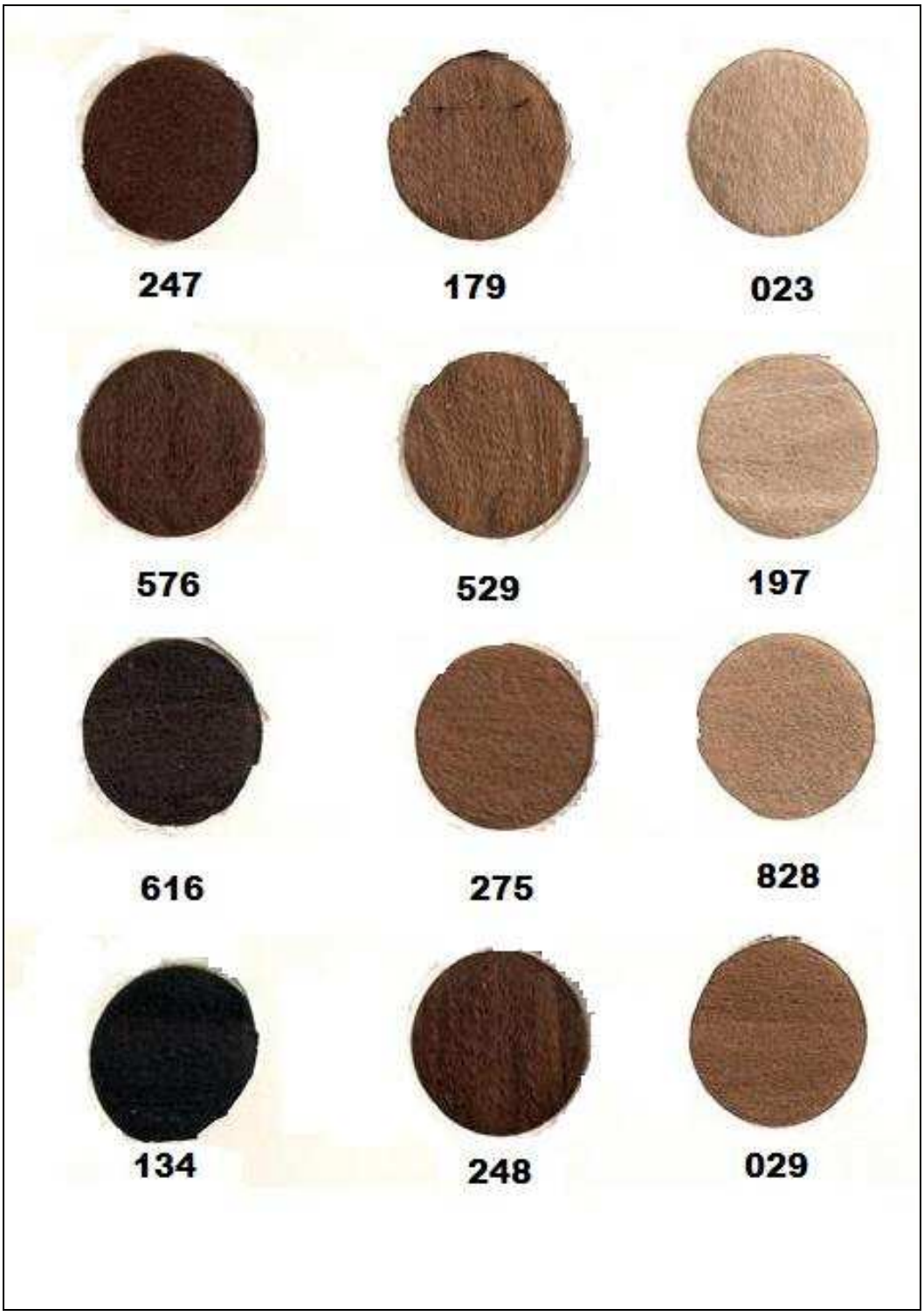


Figure 33. Alpaca fibers analyzed

CONCLUSIONS

- The work of this PhD focused on the direct analysis and study of the structural properties of melanin pigments and in particular the study of eumelanin obtained from sepia melanin. The establishment of a methology for the purification of melanin was one of the principal aims of this work.
- This work shows the succeful purification of sepia melanin (from *Sepia officinalis*) obtained from a commercial sepia ink, whose high degree of purity was verified by full characterization through Elemental Analysis (EA), Ultraviolet-Visible (UV-VIS) and Infrared (IR) spectroscopy, Inductively Coupled Plasma-Mass **S**pectrometry (ICP-MS) and studies of chemical degradation.
- Purification process showed very effective, confirmed by the results of elemental analysis, which verifies a significant increase in the percentage of C, N, H, takes place a process of hydrolysis of sepia melanin, which could explain the structure and behavior of sepia melanin during the purification process in acid medium. This could explain the possibility that there may be a chemical equilibrium between the acid form and salt form of the sepia melanin.
- Could also determine the melanin in sepia the character of heavy metal absorbing substance, since the results the heaviest element Fe was absorbed in greater proportion than other metal ions in the structure of sepia melanin, an important result set that could open new applications in the industry.
- Important results were obtained from studies of photodegradation of modified and unmodified sepia melanin samples. We were able to determine a major stability of modified sepia melanin when exposed to severe UV radiation, when the samples are subjected to purification-modification process.

- The classification based on the level of content of PTCA, used as a marker of eumelanin content, represents a good criterion for the differentiation among different coat colours in Alpaca and can be used in the marketing of Alpaca fibres as an objetive parameter in colour classification.

- Finally, in the course of this doctoral research, a series of studies on the chemistry of eumelanin pigments and its oligomers was carried out. They represent an important step forward in defining the struture of melanin and may prove of great interest to the cosmetic industry, the production of semicondutor materials and in potential uses of melanin as photovoltaic cells, solar filters, etc.

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