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Fungal stains on paper:

Melanins produced by fungi

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Abstract

Books, prints, drawings, watercolours, engravings as well as all other works of art based on paper comprise a great portion of our cultural heritage. Therefore, its preservation is a matter of great concern.

Paper can be deteriorated due to physical, chemical and biological agents. Fungi are among the most common biodeteriogens affecting paper-based collections, causing severe material and information losses.

This work focused on fungal stains on paper which are often coloured. These interfere with the readability of the artefacts diminishing their artistic and monetary value. Up to now, there is still no definitive answer for this problem. The successful cleaning of fungal stains from paper is a mandatory conservation task, considered a priority by paper conservators. However, most of the authors refer the stain colour and patterns but they do not indicate the colourant or colourants (or chemical compound) responsible for the stain.

Black stains on paper are of major concern because, not only they are very frequent, as well as its dark colour leads to a great loss of visibility. Therefore, this work focused primarily in the extraction and characterization of fungal melanins from three different species: *Aspergillus niger, Chaetomium globosum* and *Cladosporium cladosporioides*, known to be responsible for black staining on paper and melanin production.

UV-Vis, μ-FTIR and μ-Raman analyses were carried out for all three fungi melanin extracts. UV-Vis, μ-FTIR and μ-Raman results show that, after extraction and purification, purified melanin samples were obtained from the three-fungal species. Moreover, SSNMR allowed to characterize *A. niger*'s melanin as a L-DOPA type melanin, and *Cl. cladosporioides* as a DHN type melanin, by comparison with the synthetic L-DOPA melanin (Sigma-Aldrich) and the literature.

This will allow for a colourant-specific testing of newly developed cleaning methods, considering the base structure of the polymer (melanin) to be removed from the paper.

Keywords: fungi, paper, stains, colourant characterization, melanins

Resumo

Livros, impressões, desenhos, aguarelas, gravuras, bem como todas as outras obras de arte em papel compreendem uma grande porção da nossa herança cultural. Sendo assim, a sua preservação é de elevada importância.

O papel pode ser deteriorado devido a agentes físicos, químicos ou biológicos. Os fungos são os principais microrganismos responsáveis pela biodeterioração de coleções em papel, provocando graves perdas de material e de informação.

Este trabalho focou-se em manchas provocadas por fungos em papel. Estas manchas coloridas interferem com a leitura das obras, diminuindo o seu valor artístico e monetário. Até então, não há uma solução definitiva para este problema. A limpeza destas manchas em papel é uma tarefa indispensável, considerada uma prioridade pelos conservadores. No entanto, a maioria dos autores refere a cor da mancha, mas não indica os colorantes (ou compostos químicos) responsáveis pela mancha.

As manchas negras em papel são de maior preocupação, pois, não só são extremamente frequentes, como a sua cor leva a grande perda de visibilidade. Sendo assim, o presente trabalho focou-se primeiramente na extração e caracterização de melaninas produzidas por três espécies de fungos: *Aspergillus niger, Chaetomium globosum* e *Cladosporium cladosporioides,* reconhecidos como responsáveis pela produção de manchas negras em papel, associadas a melaninas.

As melaninas produzidas pelas três espécies de fungos foram extraídas e purificadas. Os resultados das análises de UV-Vis, μ-FTIR e μ-Raman, realizadas para todas as melaninas demonstraram que foi possível obter melaninas purificadas. Além disso, as análises de SSNMR permitiram caracterizar a melanina do *A. niger* como sendo do tipo L-DOPA e a melanina do *Cl. Cladosporioides* como sendo DHN melanina, através de comparações com a melanina sintética L-DOPA (Sigma-Aldrich) e a literatura.

Isto permitirá testes específicos de novos métodos de limpeza desenvolvidos, considerando a estrutura base do polímero (melanina) a ser removido do papel.

Termos chave: fungos, papel, caracterização de colorantes, melaninas

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Symbols and Abbreviations

DCR Department of Conservation and Restoration

FTIR Fourier Transform Infrared Spectroscopy

SSNMR Solid State Nuclear Magnetic Resonance

UV-Vis Ultraviolet-Visible Spectroscopy

DHN type melanin

L-DOPA type melanin

MEA Malt Extract Agar

PDA Potato Dextrose Agar

ME Malt Extract

1. General Introduction

Books, documents, maps and works of art on paper are the carriers of a precious heritage left by our ancestors. These special items are of great significance since they ensure the link between past, present and future by sending a set of values from one generation to another [1]. To ensure the passage of such legacy to future generations, knowing how to preserve paper materials is a matter of the utmost importance.

Paper can be deteriorated due to physical, chemical and biological agents. This material is particularly susceptible to biodeterioration processes due to its organic composition and hygroscopicity [2]. Biodeterioration is any unwanted alteration in a material caused by the vital activities of organisms [3], namely microorganisms, such as bacteria, fungi or lichens [4].

The main microorganisms that deteriorate paper based collections in musea, archives, and libraries all over the world are filamentous fungi [1,5,6]. Filamentous fungi growing on paper induce several chemical and physical decomposing processes, due to the excretion of metabolic substances that react with the substrate, and the development of fungal structures that alter the structure of the paper [7,8]. The excreted metabolites include colourants, enzymes (cellulases and proteases), organic and inorganic acids, chelating agents and other biochemical substances [8]. The excreted substances and the fungal structures themselves are often coloured [9] and interfere with the readability of the artefacts, diminishing their value. Accordingly to the literature, fungal stains on paper present a great variety of colours from black to brown, red, yellow and purple stains and can ultimately deem the document unreadable [7,10]. Figure 1.1 presents examples of different kinds of fungal stains. These stains may migrate through successive pages, causing irreversible damage.

Figure 1.1. Three examples of different stains on paper. A - Black, brown and purple fungal stains on an archival document (AHU-DGLAB, Portugal); B - Foxing stains on an etching (private collection)[11]*; C – Black and brown stains on an etching – CleanART case study (private collection).*

According to a recent survey [6], in the perspective of paper conservators, fungal stain removal is one of the major topics that needs further research in the area of paper biodeterioration. To better achieve a successful removal of the stains, knowing their chemical composition is essential.

1.1. Objectives

Currently, fungal stain removal from paper documents and artworks is still very problematic, since there is no known method that does not damage the substrate. A clear understanding on the composition of such stains is essential for their successful and safe removal; however, information on this area of knowledge is currently very scarce.

In order to create a solution and give a response to this lack of effective methods, the present work is included in a starting project, CleanART, which envisioned a new methodology for cleaning fungal stains in paper. The project activities encompass four phases: identification of the colourants causing paper stains and the respective fungal flora; synthesis of innovative compounds to remove fungal stains; testing the effect of the developed compounds on the properties of different papers and media; and testing their effectiveness on real case studies using antique documents and artworks or *fac símilies*.

The present work is part of the first phase of this project and is mainly focused on the extraction and characterization of colourants produced by fungi, which were formerly identified as stain causing species. This study will contribute to identifying which compounds should be targeted and removed from the paper structure; and will allow for a colourant-specific testing of the newly developed cleaning methods.

To achieve this main goal, it was necessary to:

- Review the literature regarding fungal stains on paper and the respective fungal colourants; select the fungal species to carry out the extractions; analyse and characterize the extracted colourants.

This work focused primarily in fungal melanins, since these are the most damaging colourants for paper documents and artwork, not only due to their dark colour and frequent occurrence, but also because they are the hardest colourant to safely remove from the paper structure.

2. Colourants produced by fungi colonizing paper: an overview

2.1. Filamentous fungi

Filamentous fungi belonging to the Ascomycota phylum are the main microorganisms deteriorating paper-based collections worldwide, being mainly responsible for the appearance of different colour patches with biological origin on paper.

In their vegetative stage, fungi consist of a tangle of slender, thread-like hyphae, whereas the reproductive stage is usually more obvious [8]. In their reproductive stage, both teleomorphic (sexual) and anamorphic (asexual) states may be observed, according to species (Appendix I). In the large majority of the cases, “conidium” is the term used to describe ascomycetes’ asexual spores [12]. Adhesion to the substrate is very important, since it is strictly linked to the ability to transform it. Fungi attach directly by hyphae [13], acting like a root for a better adherence and nutrient absorption, while the reproductive structures like spores allow wide distribution and increase fungal resistance to adversity [8].

Fungal spores can be easily carried by air movements, and eventually settle in the dust [14], which is deposited in all kind of materials, including paper documents. Its high resistance to adverse conditions allows them to remain in a latent state until relative humidity and temperature conditions favours germination [13], putting archival materials at risk.

2.2. Colourants produced by fungi

Fungi can produce organic colourants during their development. These are characteristic of different species, but the colour of fungal stains arises not only from the chemical composition of colourants, but also from many other factors, such as the chemical composition of the substrate; presence of metallic trace elements (e.g. iron); nutrient availability; acidity/alkalinity of the medium; presence of other microbial species; and environmental conditions [13].

Fungal colourants are composed of complex chemical substances that are formed during metabolic processes; these colourants may be encrusted in spores, present in mycelium (the mass of filaments constituting the body of the fungus), or secreted to a substrate such as paper [15]. The release of colourants on the substrate or the presence of coloured microorganisms causes the appearance of different colour stains or patches on many works of art [13].

Some colourants are enzyme inhibitors or antibiotics [13], but the main biological functions of fungal colourants are related to light harvesting and processing, photo-protection, as well as absorption and neutralization of protons that could potentially damage fungal cellular structures [16]. Melanins, for instance, have had several biological functions attributed to them, such as: protection against radiation (e.g. UV), enzymatic lysis, high temperatures, or oxidizing agents; capacity of binding of metals, preventing the entry of toxic metals or concentrating essential

metals; action as a virulence factor; or capacity of increasing resistance to fungicides [17]. Besides, such as in flowers, the striking colouration of fungi can help in their dispersion, since conidia could easily adhere to any animal attracted and touching the mycelial surface [18].

Biosynthetically, most colourants produced by fungi are polyketide-based [19]. Representative classes may include structures such as azaphilones, anthraquinones, hydroxyanthraquinones, naphthoquinones and others, each exhibiting an array of colour hues [20]. Colourants from fungi can be broadly classified chemically as polyketides and carotenoids [20].

2.2.1. Polyketide colourants

Fungal polyketides are natural products that include fungal melanins, and other colourants. These are one of the largest and most structurally diverse classes of naturally occurring compounds, ranging from simple aromatic metabolites to complex macrocyclic lactones [21].

2.2.1.1. Melanins

Melanins are polymers formed by oxidative polymerization of phenolic or indolic compounds [22]. In some instances, the polymer subunits have been discovered; still, the exact arrangement of these subunits in the polymer remains unknown [23]. They have resisted atomic-level structural examination due to their insolubility and amorphous organization [24]. Microscopic studies show an overall granular structure.

In fungi, melanin granules are localized in the cell wall where they are likely cross-linked to polysaccharides [23], being very complex since they also contain intra-granular proteins [25]. Often the resulting colourants are brown or black in colour [22,23]. In fungi, the two most important types are DHN-melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene – 1,8-DHN) and DOPA-melanin (named for one of the precursors, L-3,4- dihydroxyphenylalanine) [22] (Appendix II). In the Ascomycota fungi, where most of the fungi found in archives are represented [8], melanin colourants are generally synthesized from the pentaketide pathway in which 1,8-DHN is the immediate precursor of the polymer [26].

Due to their chemical and physical endurance, the safe removal of melanized fungal stains from paper is very problematic [27], making them the hardest colourants to remove from paper substrates.

2.2.1.2. Azaphilones

Azaphilones are polyketide derivates that can be defined as a structurally diverse class of fungal secondary metabolites [19,21], with a highly oxygenated pyranoquinone bicyclic core, usually known as isochromene, and a quaternary carbon centre [21]. Azaphilones can be coloured or uncoloured, and, when coloured, are responsible for the bright yellow, red or green colours of mycelia [21]. These colourants are produced by numerous species of ascomycetes,

including genera *Aspergillus, Penicillium, Chaetomium,* among others [21], which may colonize paper causing the appearance of coloured patches.

2.2.1.3. Quinones

Quinones are a class of organic compounds derived from aromatic compounds by conversion of an even number of –CH= groups into –C(=O)– groups with any necessary rearrangement of double bonds, resulting in a fully conjugated cyclic dione structure [28]. Numerous quinones biosynthesized by the polyketide pathway have been isolated from fungi [29], and display an array of colours, like yellow, orange, or red, according to the position of the keto groups [30].

Hydroxyanthraquinoid (HAQN) colourants, derivatives of quinones, are widespread in nature and have also been found abundantly in microorganisms, particularly in filamentous fungi belonging to the genera *Penicillium* and *Aspergillus* , with different colour hues [31].

Naphthoquinone colourants are also derivatives of quinones, very common in fungi and have been the subject of many studies regarding their chemical structure, biosynthesis and biological significance [30].

2.2.2. Carotenoids

Since fungi are non-photosynthetic organisms, carotenoids are not as widespread as they are in plants, where they play an important role in the photosynthetic process. Nevertheless, carotene hydrocarbons have been found in several fungi [18,29], and are widely accepted as protecting agents against oxidative stress [18]. Containing an aliphatic polyene chain usually composed of eight isoprene units that include light-absorbing conjugated double bonds, carotenoids provide characteristic yellow, orange or reddish colours [18].

2.3. Fungal stains on paper *versus* Colourants produced by fungi

A review of the literature from the paper conservation area dating from 1970 to 2015, was compiled in 26 studies focused on the identification of fungal species from stains on biodeteriorated paper (Appendix III, Table III.1). Accordingly to this review, fungal stains can be divided into three main groups: various colour stains; melanised stains (dark brown or black stains caused by melanin) and foxing (small and round-shaped spots with reddish or yellowish-brown colour resulting from the ageing and/or oxidation of fungal residues).

After gathering all the information from the available literature, 25 different fungal genera were found to cause staining on paper (Appendix III, Table III.2). The fungi that are most frequently identified on paper belong to the genera *Aspergillus* (29%) and *Penicillium* (13%).

The most common stains found on paper and associated with fungal activity were brown stains, followed by black and yellow to greenish tones (Fig. 2.1.A). The brownish colour is most frequently associated with the foxing phenomena described in the literature [32]. Black colour is associated with the presence of melanin from dematiaceous fungi [33,34]. Only a small

percentage (around 13%) of the authors associate a coloured stain with specific colourants produced by fungi. However, no analyses are actually carried out in these studies to confirm the presence of such colourants.

Therefore, a research outside the scope of paper conservation was necessary to understand which chemical structures could be causing this damage, namely the staining of paper by fungi.

There is a great variety of colourants produced by fungi, but not all of them colonize paper. In the literature, several fungal colourants were mentioned and characterized, being used in the food, chemical and pharmaceutical industries. However, not all of them were detected on colonized paper, according to a previously carried out review [11].

From the colourants produced by fungi that colonize paper, circa 80 different compounds were gathered, from literature between 1934 and 2016, and are presented in tables (Appendix III, Tables III.3-III.7) regarding their chemical structure, colour and producing fungal species.

The majority of these colourants were polyketides (about 96%) and only 4% were carotenoids. There were only three different carotenoids identified: Neurosporaxanthin, β-carotene and Sporopollenins. The latter are oxidative polymers of carotenoids and carotenoid esters [35].

From the 96% of polyketides reviewed on the literature, about 20% were azaphilones and 33% were quinones. The remaining 46% were attributed to other types of colourants, that didn't belong to the chemical classes defined initially. Additionally, at least 9 species of fungi identified on paper are known to produce melanins. Melanin was taken into account as a single colourant, although it can be produced by two biosynthetic pathways: DHN and DOPA.

The majority of colourants produced by fungi in the chemical/food/pharmaceutical literature have a yellow colour (44%), followed by red (19%) and orange (14%) (Fig.2.1. B). Many times, the colour is a mixture of hues (*e.g.* red-brown for Tritisporin [36]). In the paper conservation literature, the most common stains produced by fungi on paper substrates are coloured in brown or black (Figure 2.1. A), having no clear correspondence with the bright colours studied in the chemical/food/pharmaceutical areas (Figure 2.1-B).

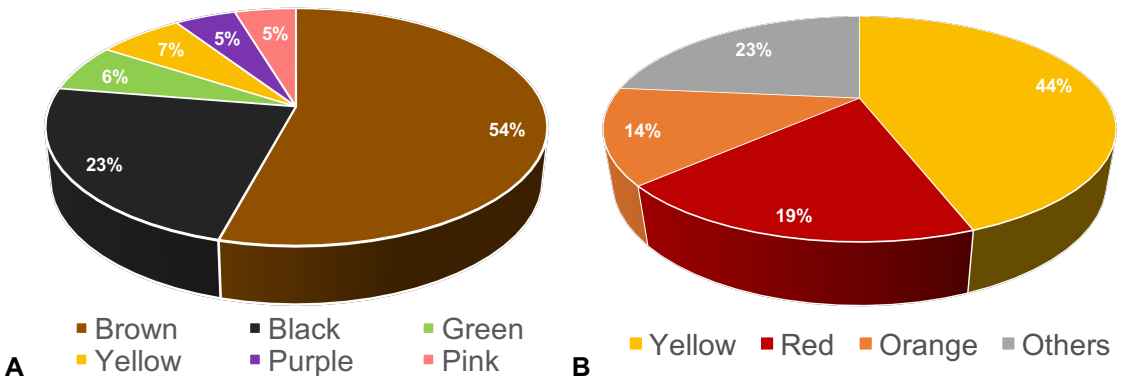

Figure 2.1. A. Percentage of the stains' colours reported in the paper conservation literature. B. Colours of the colourants identified in the chemical/food/pharmaceutical literature.

However, these data must be considered at light of the research area they belong to, since red and yellow hues are appealing colours and have been the most extensively used food colourants [20], for instance. Besides, the colours found on stained paper may be a combination of the colourants produced by fungi present on paper. When extracting the colourant from *Aspergillus melleus*, it was verified that the brown solid contained viomellein – reddish-brown (11%), xanthomegnin – orange (50%), and viopurpurin – purple-black (1%) [37].

Also, stains' colour produced by fungi on paper may as well be a result of the colourants' degradation in the given medium, causing its darkening. For instance, sporopollenin is a brown product of oxidative polymerization of yellow β-carotene [31].

Besides, many of the identified colourants are biosynthetic precursors of others, so depending on the development stage, a different colour may be observed. This is the case of '4C-labeled red averufin, the orange versiconal hemiacetal acetate, and the pink-orange versicolourin A, which are converted to the yellow sterigmatocystin by *Aspergillus versicolour* [38]. This is also the case of dopachrome and melanochrome, the pink and purple intermediates in the formation of the melanin colourant in *Aspergillus nidulans* by the L-DOPA pathway [26].

Finally, different colours may often be due to the modification of the same colourant, depending on different reactions with the substrate. For instance, variation in copper levels among commercial fungal culture media (*e.g.* potato dextrose agar) correspond to variable colouration in multiple fungal species, including *Stachybotrys atra* and *Cladosporium herbarum* [39].

Overall, there is no doubt that colourants produced by fungi are a serious problem to conservators, especially as the dominant species that produce these colourants belong to the most common genera identified on paper substrates, namely *Aspergillus* and *Penicillium*. Each colourant, with different properties, may require diverse removal methods. Therefore, it is of the utmost importance that developments are carried out in this field of knowledge, so that targeted removal techniques can be developed without damaging our cultural records on paper.

The present study is an important advance in terms of knowledge, since almost no information was published about the colourants produced by fungi on paper. By relating the information acquired in different areas, such as the food industry, with the information already acquired in the conservation area, we are one step closer to define which colourants may be present in specific stains on the paper substrate, which will have an impact on the conservation strategy. By knowing which colourants to target, new and safer methods can be developed. Likewise, it is also relevant to consider whether the fungi produce colourants within their structure or as excreted coloured products, which determines the extent of staining – whether a simple removal of fungi is sufficient, or its products are linked to the paper structure, which carries major extent.

3. Extraction and characterization of melanins produced by fungi

According to the litterature, there are virtually no studies regarding the chemical structures of the colourants that fungi produce on paper substrates. To get enlightenment on this subject, melanins produced by fungi were selected as primary colourants for the initial phase of the CleanART project, where the extraction and characterization of colourants produced by fungi is intended. Melanins were chosen because they cause much of the black and brown staining on paper objects, existing either as components of the fungal structures or as excreted polymers attached to the paper fibres [27]. Removal of melanins without damaging works of art is a problematic issue, since these polymers have a great chemical endurance [17].

After decades of investigation, the exact molecular structure of these colourants remains a mistery. This is due to several challenging features of the system, including almost complete insolubility in all solvents, an amorphous particulate character, and extreme molecular heterogeneity. A proposed chemical structure for these polymers' (DHN-melanin and L-DOPA-melanin) molecular arrangement is given in Fig. 3.1, based on the literature [52–55, for e.g.], where several models have been proposed. The main, if not the only, similarity between the various models is the presence of the monomers linked by covalent bonds, where these primary units are in different states of oxidation. The spatial arrangement of these units in the biopolymer structure may be quite variable, providing the material with increased heterogeneity.

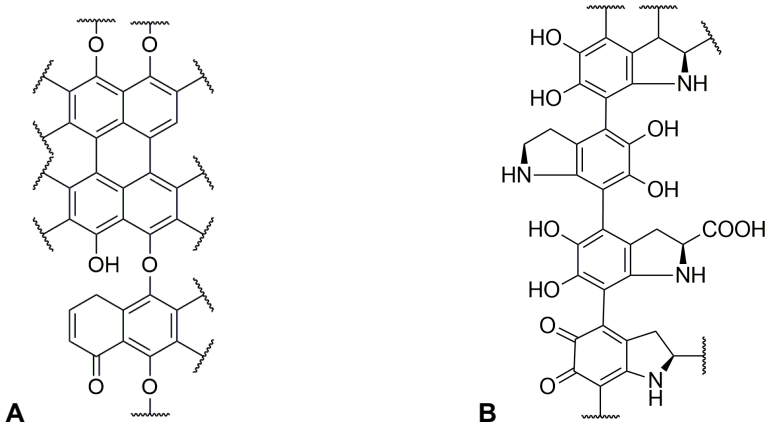

Figure 3.1. Proposed chemical structure for the DHN-melanin polymer (A) and for the L-DOPA-melanin polymer (B).

For the characterization of fungal melanins, three species known to produce this colourant on paper [11,15,22,23,30,33,44] were selected as case studies: *Aspergillus niger, Chaetomium globosum and Cladosporium cladosporioides*. The characteristics of the three-selected species are presented in greater detail.

Aspergillus niger

Aspergillus niger is a very common member of the Aspergilli [29]. It's a filamentous ascomycete [45], that produces a white mycelium before forming black conidia. Mature colonies appear grey/ greenish-black (Fig.3.2.A) [29]. Its reverse is usually yellow (Fig.3.2.B) [46].

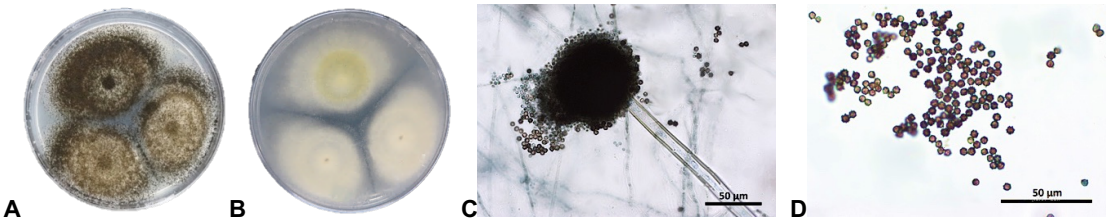

Figure 3.2. A. niger colonies on PDA (Potato Dextrose Agar medium), 14 days, 25°C, (A) front and (B) reverse. Petri dish = 90mm. A. niger (C) conidiophore, (D) conidia, bars = 50 µm.

A. niger's brown Aspergillin has been the subject of biochemical characterization, and was assumed to be a polymer composed of DHN melanin and a hexahydroxyl pentacyclic quinoid (HPQ) compound [47]. Consequently, one of the main characteristics of *A. niger* is the production of black or dark brown conidia resulting from combination of these dark brown melanins with hexahydroxyl pentacyclic quinoid green colourants [44].

Although most Ascomycetes produce melanin through the DHN pathway, recent studies suggest that some *Aspergillus* spp. produce L-DOPA melanin, *A. niger* included. According to the literature [48] melanin synthesis by *A. niger* was inhibited by kojic acid and tropolone, indicating a L-DOPA melanin pathway.

Chaetomium globosum

Chaetomium globosum belongs to the phylum Ascomycota [12]. Ascomata are superficial, greenish olivaceous or slightly dark olivaceous. Terminal hairs are abundant and brown and the ascospores are olivaceous and brown when mature (Fig. 3.3. A) [49]. Orange exudates diffusing into the medium are noticeable on its reverse (Fig. 3.3. B) [49].

Ch. globosum is a known melanin producer and, according to the literature [50], this biosynthesis is via the DHN pathway; as, when tricyclazole, an inhibitor of the DHN pathway, was added to plates, it resulted in an absence of pigmentation.

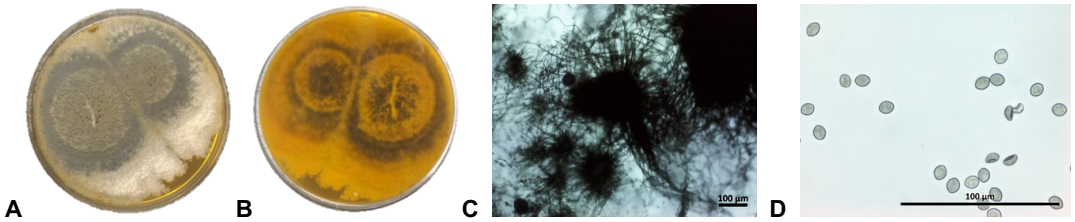

Figure 3.3. Ch. globosum colonies on MEA (Malt Extract Agar medium) (A) front and (B) reverse, 14 days, 25°C. Petri dish = 90mm. Ch. globosum (C) perithecia and (D) ascospores (bars = 100 µm).

Cladosporium cladosporioides

Cladosporium cladosporioides belongs to one of the largest genera of dematiaceous hyphomycetes [51]. Colonies are rapidly growing, olive-gray to olive-brown or black (Fig. 3.4. A, B) [52].

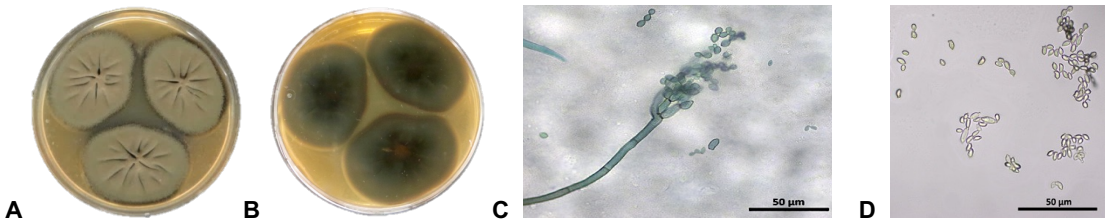

Figure 3.4. Cl. cladosporioides colonies on MEA, (A) front and (B) reverse, 14 days, 25°C. Petri dish = 90mm. Cl. cladosporioides (C) conidiophore and (D) conidia (bars = 50 µm).

This fungus has already been frequently isolated from paper materials [53] and has been pointed out as a DHN-melanin producer [51], since when adding tricyclazole, a specific inhibitor of DHN-melanin synthesis, growth and colouration of colonies were affected.

3.1. Materials and Methods

A. niger was obtained from the mycological collection of Instituto Nacional de Saúde Doutor Ricardo Jorge (Lisbon, Portugal). *Ch. globosum* and *Cl. cladosporioides* were isolated from fungal stains on an engraving and identified by Molecular Biology at Universidade de Coimbra (Coimbra, Portugal).

Fungal cultures were grown in three culture media: a) Potato Dextrose Agar (PDA) - Solid medium, b) Malt Extract Agar (MEA) – Solid medium and c) Malt Extract (ME) – Liquid medium.

PDA was used for maintenance of the cultures. Liquid ME allowed for an easier extraction of colourants from *A. niger* and *Cl. cladosporioides* by filtration. Solid MEA had to be used for *Ch. globosum*, since it favoured its sporulation, and the melanin to be extrated was located in those reproductive structures (spores).

3.1.1. Extraction and purification of melanins from fungal biomass

This experimental protocol was adapted from the literature [48]. Twenty Petri dishes with *Ch. globosum* cultures on MEA were incubated for one month in the dark, at 25ºC inside a drying oven, after which the cultures were collected and boiled in distilled water and filtered. One-month old cultures of *A. niger* and *Cl. cladosporioides* in ME were also filtered. The individual fungal masses obtained were separately crushed with 2M NaOH (pH 10.5) and allowed to stand for 48 h. After a second filtration to remove fungal debris, the resulting filtrates were precipitated with 2M HCl to pH 2.5. These were incubated overnight at room temperature and afterwards centrifuged at 180 rpm for 10 min. The precipitates were purified by acid hydrolysis

using 6M HCl at 100ºC for 2h to remove carbohydrates and proteins. They were then treated with organic solvents: chloroform, ethyl acetate and ethanol to remove lipids. The resulting precipitates were dried at room temperature, dissolved in 2M NaOH and filtered. The collected solutions were precipitated with 6M HCl (kept overnight), washed with distilled water and allowed to dry at room temperature (Appendix IV). These precipitates were considered to be purified melanin and were used for further analyses.

3.2. Results and Discussion - Characterization of melanins

UV-Vis analyses

Melanin samples were dissolved in a NaOH 0.1 M solution and scanned from 200 to 600 nm with 2 nm intervals on a T90+ spectrometer from PG Instruments. Before starting all measurements, a baseline was made with the solvent. Since the apparatus has a double beam, all measurements were made against a reference cell which had solvent, thus discounting any possible fluctuations in absorption which may have other origins as physical differences in the cells used.

The UV-Vis absorption spectrum of a given colourant, melanins in this case, is a graphical representation of the absorbed radiation as a function of the wavelength (λ) [16]. The spectra obtained for the purified fungal melanins and synthetic melanin showed a more pronounced band at the UV region (Fig. 3.5). These appear to be atypical in organic chromophores and are probably the result of a structure that presents considerable heterogeneity and disorder.

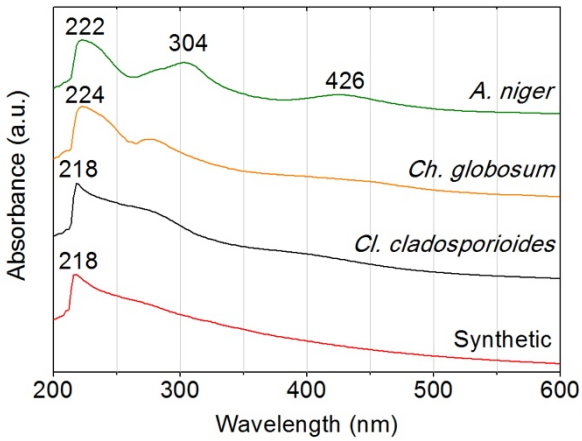

Figure 3.5. UV-Vis spectra of the purified melanins.

In the melanin samples, the monomeric units from DHN-melanin and L-DOPA-melanin, as well as units resulting from the oxidation of these monomers, are predominant in their structure (Fig. 3.1). The presence of this variety of species in the composition of the melanin polymers leads to an overlapping of energy levels, with a greater number of electronic transitions along the electromagnetic spectrum and, consequently, an extension of the absorption spectrum. On the other hand, the aromaticity of the units that make up the melanin structure favours efficient electronic delocalization, which contributes to the stabilization of the polymer and, consequently, to an increase in its photo-protective capacity [54]. The result is typical of the absorption profile of these type of aromatic compounds, such as melanin, which absorb strongly in the UV region and decrease progressively as the wavelength is increased [55]. This is in agreement with the photoprotection function of melanin suggested in fungi [25].

The nature of the purified colourants from *A. niger*, *Ch. globosum and Cl. cladosporioides* were therefore confirmed by their spectral profiles, since among biological colourants, only melanins absorb throughout the entire visible region. Since the colours we observe are the result of the selective light absorption process, if all wavelengths of light are absorbed and none reflected, we recognize it as black. This characteristic is responsible for the dark colour of these colourants, since we perceive no colour [55]. Recent reports mention a characteristic absorption peak was observed at 217 nm with a small shoulder around 260-280 nm, which is similar to synthetic melanin, and suggests the presence of phenol groups [48,56].

For the *Aspergillus niger*'s spectrum, two distinct maxima are observed at 304 and 426 nm, respectively. These maxima can be assigned to absorption of light by the residual protein fragments which are not completely removed during the isolation and purification procedure [25]. Besides, according to the literature [47], *A. niger*'s melanin is linked to a green colourant, which could be the reason of the absorbance in the violet region (426 nm).

μ-FTIR analyses

Micro-FTIR analyses were performed on a Nicolet Nexus spectrophotometer interfaced with a Continuμm microscope with a MCT-A detector cooled by liquid nitrogen. The spectra were collected in transmission mode, with a spatial resolution of 50–100 μm, optical resolution of 4 cm^{-1} and 128 co-added scans, using a thermo-diamond anvil compression cell.

FTIR spectra were carried out and studied to confirm if the extracted and purified colourants were melanins. The obtained spectra are shown in Figs. 3.6-3.8.

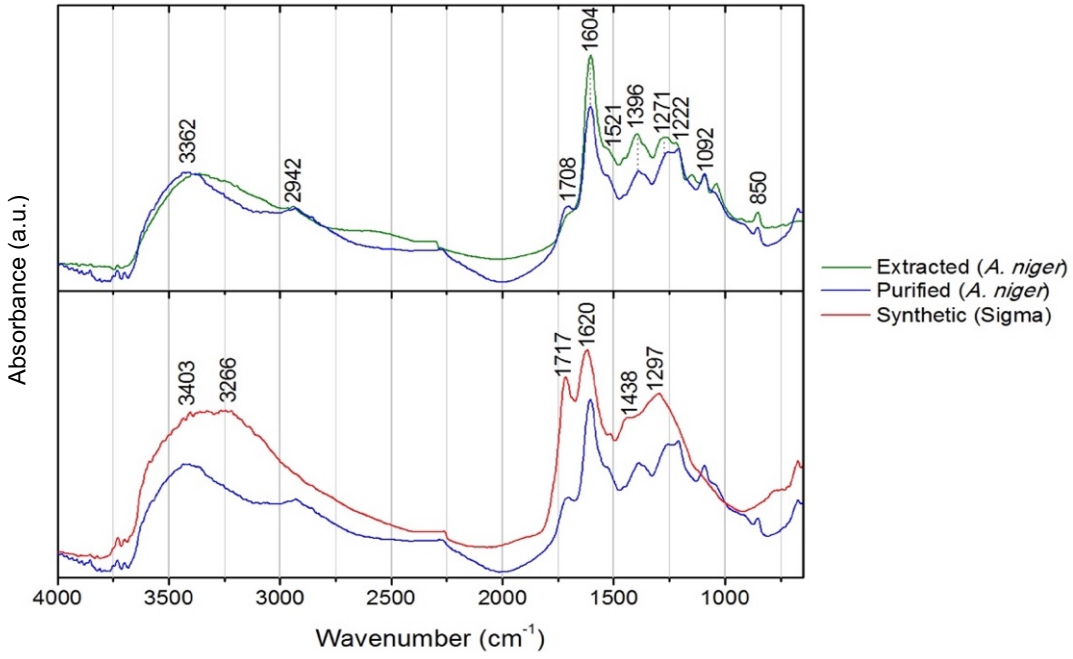

Figure 3.6. μ-FTIR spectra of the extracted and purified melanins from Aspergillus niger in comparison with synthetic melanin from Sigma.

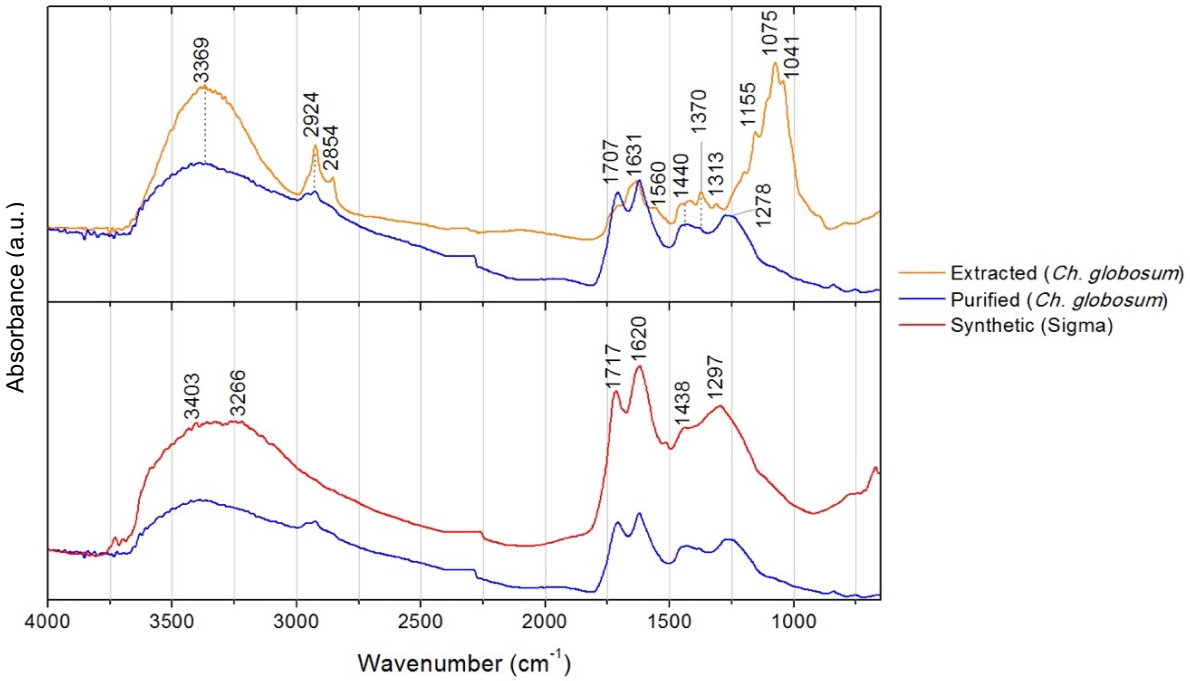

*Figure 3.7. **μ**-FTIR spectra of the extracted and purified melanins from Chaetomium globosum in comparison with synthetic melanin from Sigma.*

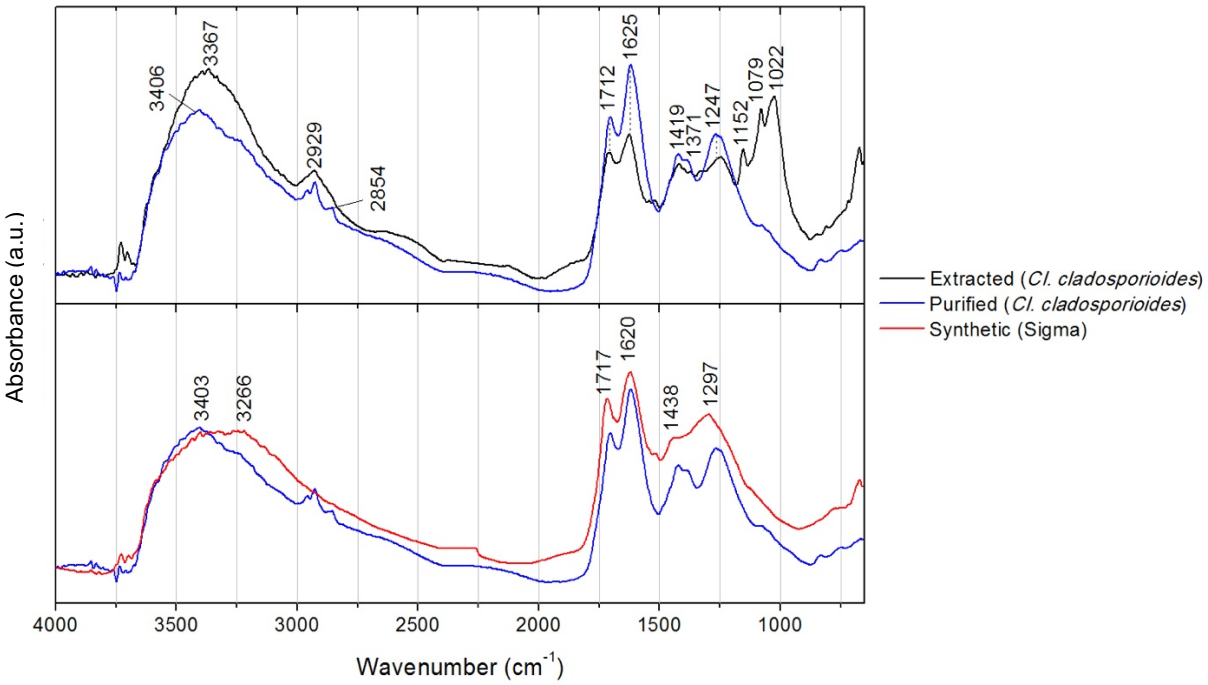

*Figure 3.8. **μ**-FTIR spectra of the extracted and purified melanins from Cladosporium cladosporioides in comparison with synthetic melanin from Sigma.*

Firstly, spectra were acquired for the extracted melanins, prior purification, to assess if this would be sufficient. Melanins are typically localized in cell walls where they are most likely cross-linked to polysaccharides, mainly chitin [56] (Appendix V, Fig. V.2) which is a major constituent of the fungal cell wall [29]. Accordingly, many of the characteristics bands of fungal chitin were observed in the acquired spectra (Appendix V, Fig. V.1), showing that the extraction

procedure alone is not sufficient for the isolation from other cell wall constituents, especially for *Ch. globosum* and *Cl. cladosporioides*, for which the FTIR spectra are almost coincident with their mycelia's FTIR spectrum (Appendix V, Fig. V.2). A proposed assignment for the chitin bands present in the spectra of the extracted melanins is given in Table V.1 (Appendix V). Following purification of the extracted melanins, new FTIR spectra were acquired. These were compared with Sigma's synthetic melanin spectrum in order to verify their nature, and also with the previously obtained spectra of the extracted melanins, to confirm their differences and a successful purification (Fig. 3.6, 3.7 and 3.8).

Although early researchers on melanin structure used UV and visible spectroscopy, as well as IR spectra, to distinguish between melanins from different sources, these methods are now generally regarded to be of dubious value [17]. However, it is possible to access if the extracted and purified samples are in fact from melanin, by comparison with the synthetic melanin from Sigma-Aldrich (DOPA-melanin). As previously mentioned, there are two distinct pathways for fungal melanin formation. *Ch. globosum* and *Cl. cladosporioides* are reported to produce DHN-type melanins (Fig. 3.1.A), whereas *A. niger* is reported to produce L-DOPA-melanin (Fig. 3.1.B), each of which having different precursors (1,8-DHN) for DHN-melanin and dihydroxyindoles for L-DOPA-melanin).

The presence of carboxyl groups in the melanins' structures, as well as aromatic rings and phenolic groups, is evidenced by bands in the obtained spectra, characteristic of these polymers' molecular arrangement (Fig.3.1). Purified melanin spectra are very similar to the spectrum of synthetic melanin, having common absorption peaks: the broad bands in the 3600-3200 cm^{-1} region, attributed to OH stretching, further indicating the presence of phenolic and carboxylic groups in the melanin structure [51,56,57]; the clear peaks at 1720-1700 cm^{-1}, corresponding to the oscillations of C-O groups from acids, esters or ketones [56]; a strong absorption at 1650–1620 cm^{-1} attributed to stretching vibrations of aromatic C=C or C=O groups [48,51], which is typical of a conjugated quinoid structure and is believed to be important for the identification of melanin [48]; in case of L-DOPA melanins, such as the synthetic melanin from Sigma, it may be indicative of the compounds containing carbonyl groups conjugated with a benzene ring, corresponding to the typical quinone structure of DOPA-melanin [51]; an absorption band at 1440-1370 cm^{-1}, indicating nitrogen content (C-N bending) [56], and indolic NH stretches [57], as DOPA melanins, in the case of synthetic DOPA melanin, and phenolic COH bends in both melanins; and the peak around 1250 cm^{-1} that corresponds to C-OH stretching or angular deformation of O-H [51,56].

Comparing the purified melanins from *Ch. globosum* and *Cl. cladosporioides* with the extracted melanin prior purification, a great difference is confirmed between the different samples. Chitin bands are no longer noticeable, except for a region around 2930-2860 cm^{-1}, assigned to

aliphatic νCH_2; νCH_3, which could indicate a remaining contamination of these cell wall carbohydrates [48,56,57].

A proposed assignment for the specific peaks of these melanins (*Ch. globosum*/*Cl. cladosporioides*) is given (Table 3.1), regarding the chemical structure from dihydroxynaphthalene (DHN)-melanin, formed by oxidative polymerization of 1,8-DHN (Fig. 3.1. A). The similarity between these melanins' spectra supports the literature, since both these fungi are reported to produce DHN-type-melanins.

Table 3.1. FTIR band wavenumbers (cm^{-1}) and assignments for Ch. globosum and Cl. cladosporioides; ν=streching mode; δ=*bending mode.*

FTIR band wavenumber (cm^{-1})		**Proposed vibrational assignments**
Ch. globosum	*Cl. cladosporioides*	
3369	3406	νO-H
2924	2929; 2854	aliphatic νCH_2; νCH_3 (Contamination of cell wall carbohydrates)
1707	1712	Oscillations of C-O groups
1631	1625	νC=O; νC=C aromatic (conjugated quinoid structure)
1440; 1370	1419; 1371	δCOH (phenolic)
1278	1247	νC-OH; OH deformation of alcoholic and carboxylic groups

Absorption peaks from IR spectrum obtained from *A. niger*'s purified melanin are nearly coincidental with the one from its extracted melanin (Fig. 3.7). Its extraction was easier than for *Ch. globosum* and *Cl. cladosporioides*, showing that without a purification process, the extracted melanin was already quite pure when compared with the extractions carried out for the other species. This could be associated with cellular location of the biologically synthesized melanin. *A. niger*'s melanin is located in its black conidiospores which are rather abundant [44], whereas *Cl. cladosporioides*' is located in the mycelia and conidia [51] and *Ch. globosum* in the perithecia, with conidia not generally produced [46]. *Ch. globosum* was in fact the hardest fungal melanin to extract, requiring larger amounts of mycelia in order to obtain a sufficient amount of sample for the analyses.

Comparing *A. niger*'s to Sigma's and other fungal melanins, characteristic peaks are also observed as previously described. However, differences are noticed particularly in the carbonyl and indolic regions, which is in agreement with reports from literature regarding the biosynthetic pathway for this fungal melanin's production in *A. niger* (L-DOPA). Another

absorption regions are observed with defined peaks: 1521 cm^{-1} attributed to N-H stretching and bending [51,56]; and 1092 cm^{-1} attributed to C-O stretching, to the aromatic ring C-H stretching and characteristic of C-N elongation, which may derive from the pyrrole units that make up the L-DOPA melanin structure [48,51], reported to be produced by this fungal specie. Moreover, the weak absorption peaks at 800-600 cm^{-1} could indicate some positions of aromatic rings were substituted and the conjugated system with low amount of aromatic hydrogen content was formed [57].

A proposed assignment for the specific peaks *A. niger*'s purified melanin is given in Table 3.2, regarding the proposed chemical structure of the L-DOPA-melanin polymer (Fig. 3.1. B).

Table 3.2. FTIR band wavenumbers (cm^{-1}) and assignments for A. niger; ν=streching mode.

FTIR band wavenumber (cm^{-1})	Proposed vibrational assignments
3362	$\nu N\text{-}H_2$; $\nu O\text{-}H$
2942	Aliphatic νCH_2; νCH_3
	νNH
1708	Oscillations of C-O groups
1604	$\nu C{=}O$; $\nu C{=}C$ aromatic (carbonyl groups conjugated benzene ring – typical structure of DOPA melanin); νCOO
1396	νCN (strong suggestive of a substantial amount of indole groups in the structure of the colourant – typical structure of DOPA melanin)
	νNH (indolic) νCOH (phenolic)
1271; 1222	$\nu C\text{-}OH$; OH deformation of alcoholic and carboxylic groups
1092	$\nu C\text{-}O$; aromatic ring C-H;
850	Some positions of aromatic rings were substituted and the conjugated system with low amount of aromatic hydrogen content was formed.

μ-Raman analyses

Raman Microscopy was carried out using a Labram 300 Jobin Yvon spectrometer, equipped 17 mW HeNe laser operating at 632.8 nm. Melanin samples were analyzed with a solid state laser operating at 532 nm. Spectra were recorded as an extended scan. The laser beam was focused either with a 50x or a 100x Olympus objective lens. The laser power at the samples' surface was varied with the aid of a set of neutral density filters (optical densities 0.6, 1 and 2), with laser power intensities varying between 2.5 and 0.1 mW.

The obtained spectra were dominated by two intense and broad peaks: a higher intensity band at circa 1580 cm^{-1} and a second band located at around 1370 cm^{-1} (Fig. 3.9).

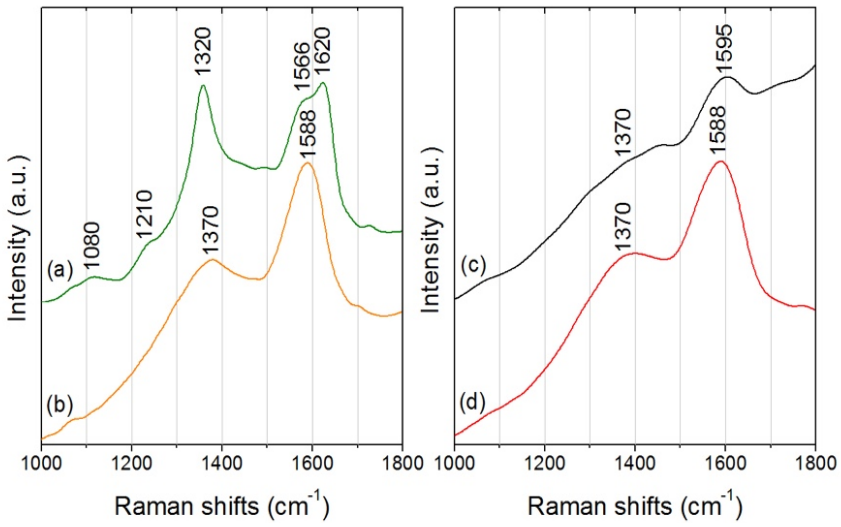

Figure 3.9. μ-Raman spectra from extracted melanins: Aspergillus niger (a), Chaetomium globosum (b), Cladosporium cladosporioides (c) and synthetic melanin (d) from Sigma Aldrich.

A broadband background is noticeable due to auto fluorescence emission properties of melanin. It has been proved [58], using multiple wavelengths and a variety of melanin sources, that the two prominent peaks observed in melanins are indeed due to inelastic Raman scattering rather than fluorescence or other nonlinear processes. These two peaks can be interpreted as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C-C bonds within the rings, along with some contributions from the C-H vibrations in the methyl and methylene groups.

Other bands or shoulders of lower intensity that appear in the spectrum of *A. niger* can be attributed to the non-pigment residues of fungi. For instance, lipid bands at 1210 and 1080 cm^{-1} that can be both assigned to C–C skeletal stretching.

However, the actual assignment of the two main melanin bands is not entirely agreed upon between authors, but it is most likely that the broad features of melanin are composed of many overlapping Raman bands because of the vibrations of the constituent monomers [59].

Table 3.3. Raman band wavenumbers (cm^{-1}) and assignments for A. niger, Ch. globosum, Cl. cladosporioides and synthetic melanins; s=strong, m=medium, w=weak, ν=streching mode.

Raman band wavenumber (cm^{-1})				**Proposed vibrational assignments**
A. niger	*Ch. globosum*	*Cl. cladosporioides*	Synthetic (Sigma)	
1566 (s) 1620 (s)	1588 (s)	1595 (s)	1588 (s)	νCCH aromatic
1320 (m)	1370 (m)	1370 (m)	1370 (m)	CH deformation

Solid-State NMR analyses

Samples were packed into 4 mm zirconium rotors and sealed with Kel-F caps. On selected experiments an insert was packed with the sample and placed inside the rotor.

^{13}C CP/MAS spectra was obtained on an Avance III WB spectrometer operating at 7.2 T, corresponding to a 300 MHz Larmor frequency for ^{1}H and 75 MHz for ^{13}C.

Magic angle spinning rate was 5-10 KHz. The cross-polarization was performed applying a variable spin-lock sequence RAMP-CP/MAS with a contact time 1.2 s and a recycle delay of 2 s. 5000 points were sampled and accumulated during 4000-15000 transients.

The ^{13}C CP/MAS spectra of the melanin samples revealed differences in chemical shift regions, maximum of those bands and in linewidth of the peaks (Fig. 3.10-3.12).

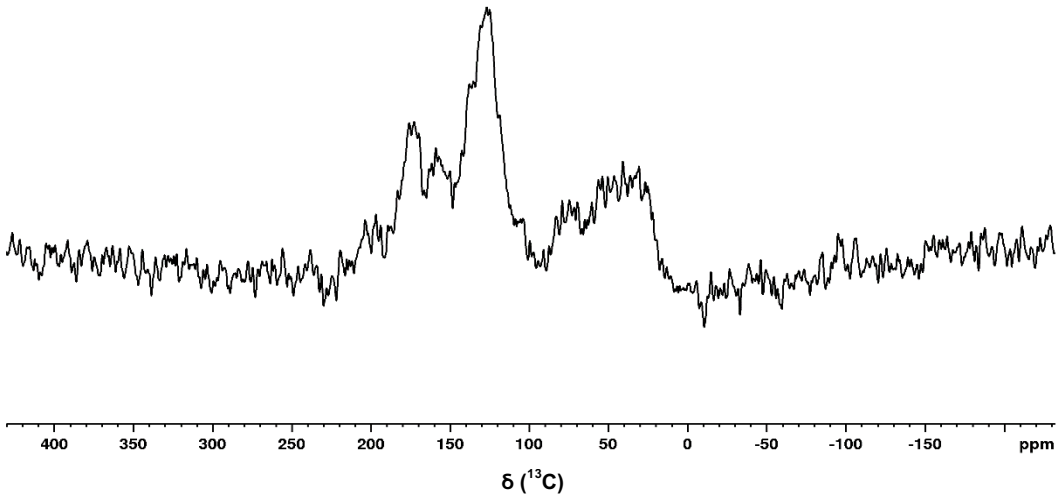

Figure 3.10. Cl. cladosporioides ^{13}C CP/MAS spectrum

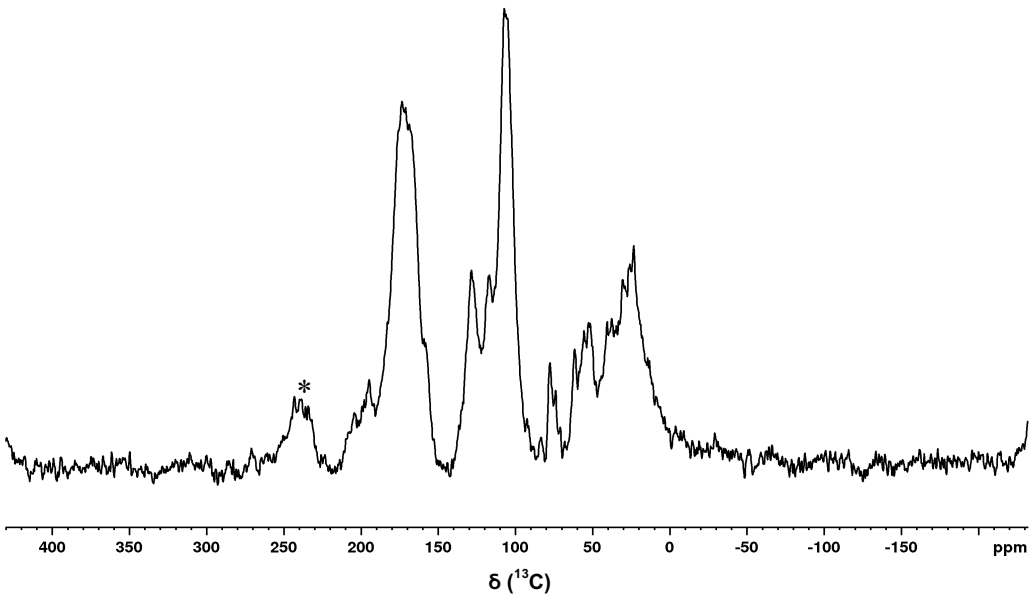

*Figure 3.11. A. niger ^{13}C CP/MAS spectrum. *Spinning Side Bands.*

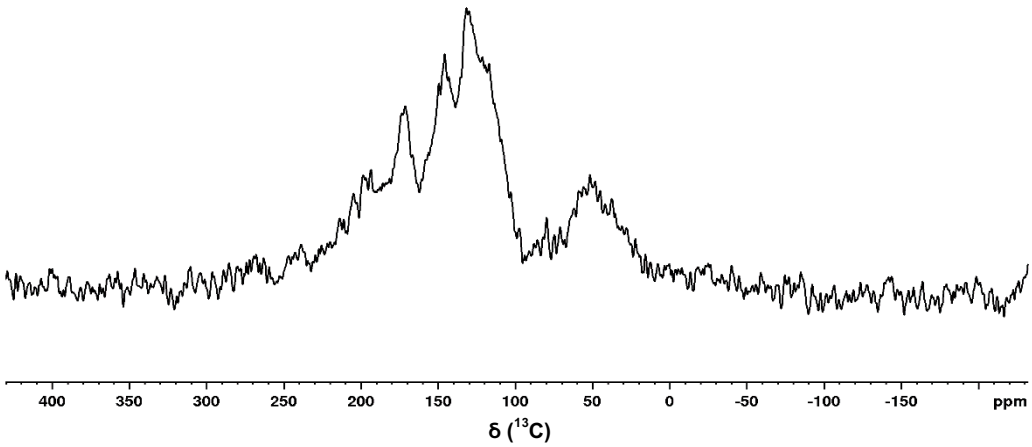

Figure 3.12. Sigma-Aldrich synthetic melanin ^{13}C CP/MAS spectrum

Unfortunately, it was only possible to obtain sufficient melanin sample for two of the selected species, namely *Cl. cladosporioides* (reported to produce DHN-type melanin) and *A. niger* (reported to produce L-DOPA-type melanin). This is a solid state analyse, so a major amount of sample must be sparred to do so. Characteristic chemical shift regions for the spectra indeed obtained are given in Table 3.4.

Table 3.4. Characteristic Chemical shift regions in ^{13}C CP/MAS Spectra.

Sample	Carbonyl	Aromatic		Aliphatic
		Cq	CH	
A. niger	215-150 (Carbonyl + Cq)		140-88	84-0
Cl. cladosporioides	190-165	165-150	144-100	81-9
Sigma-Aldrich	185-163	163-137	140-90	70-12

Note: Chemical shifts are reported in ppm; CH represents protonated carbons and Cq represents non-protonated carbons.

Analyzing the obtained spectra, the samples obtained are predominantly aromatic, with phenol groups, having some fragments oxidized to carbonyls, which is consistent with the type of structures expected from the consulted literature and with the previously carried out analyzes, also showing carbonyl, phenolic and aromatic groups.

The synthetic melanin sample from Sigma-Aldrich is known to be from L-Tyrosine origin, oxidized in DMSO with H_2O_2. Comparing that spectrum with the one from the *A. niger* sample and the reported ^{13}C chemical shifts for tyrosine derived compounds in Table VI.1 (Appendix VI), the melanin production pathway in *A. niger* can be assigned to a L-DOPA pathway (Appendix II), which is in agreement with the reports from literature [48] and the previously

carried out analyses - namely indolic vibrations in the IR spectrum, indicating Nitrogen content (correspondent to the typical structure of the L-DOPA-melanin).

Regarding characteristic regions, the aromatic and carbonyl regions are different between *A. niger*'s and synthetic melanin, which can be attributed to a lower oxidation of the monomers [60] in the synthetic melanin, resulting in fewer signals in the carbonyl region. The linewidth is also different, with *A. niger*'s melanin having much sharper peaks. This broadening effect is caused, in the synthetic melanin, by either a higher molecular weight that decreases the molecule correlation time and makes the NMR signal decay faster, or to a higher crosslinking in the polymer which has the same effect as the size of the polymer, by restricting the molecular motion inside the polymer structure.

The molecular weight (MW) difference between natural and synthetic melanin in literature is conflicting, with reports of similar MW regardless of the source, and of higher size in the natural produced melanins [61]. The natural polymer being of higher size than the synthetic polymer does not fit the experimental data, as it would lead to sharper peaks in the synthetic sample rather than in the *A. niger's* melanin. Therefore, the synthetic sample should have a more crosslinked structure than the produced by *A. niger*.

Cl. cladosporioides melanin sample has a similar peak width to the Sigma-Aldrich sample, also indicating a very crosslinked or a higher molecular weight polymer than *A. niger*'s.

Cl. cladosporioides melanin is reported in literature to be of 1,8-DHN pathway [62]. Comparing the observed ^{13}C chemical shift with model compounds present in Table VI.1 (in Appendix VI) from literature, it is likely to be of the DHN pathway as the observed signals are in better agreement with the naphthalene derived structures. At 160 ppm the naphthalene phenol groups fit the one of the observed shifts in the *Cl. cladosporioides* melanin, as the 140 ppm shift from the catechol ring found in L-DOPA fits the shifts in the synthetic Sigma melanin and the *A. niger* sample.

Without further analyses, it is only possible to ascertain that, regarding the observed differences between the carbonyl and aromatic regions of the obtained spectra, the melanins produced by the two species analyzed, namely *A. niger* and *Cl. cladosporioides*, are clearly from two different biosynthetic pathways. This is in agreement with the initially consulted literature and with the other analyses carried out. Also, melanin sample produced by *A. niger* is considerably more crystalline and organized than *Cl. cladosporioides'* melanin, which is evidenced by the difference in peak width between both spectra.

Conclusions

The successful cleaning of fungal stains from paper is considered a priority by paper conservators, but data from the literature review show that, despite the relevance of this subject, there is still a lack of information since most of the authors refer only the stain colour and patterns (e.g. black, purple, etc.) but they do not indicate the colourant or colourants (or chemical compound) responsible for the stain or the colorimetric parameters. Therefore, the present work was of the utmost importance in linking the knowledge between the conservation literature concerning fungal stains on paper and the remaining literature (from e.g. food, chemical and pharmaceutical industries) concerning fungal colourants production.

The paper conservation literature shows that the most common stains produced by fungi on paper substrates were brown or black, having no clear correspondence with the bright colours (e.g. red and yellow) reported by studies from other areas of knowledge. However, stains' colour produced by fungi on paper may be a result of the colourants' degradation in the given medium, causing its darkening, or a convergence of different colourants produced by the same, or different species, present in the fungal stains.

It was assessed that these fungal stains on paper are in no doubt a serious problem to conservators, particularly because the two most common fungal genera producing these colourants are the same as the ones identified on paper substrates: *Aspergillus* spp. and *Penicillium* spp.

Each colourant, with different properties, may require distinct removal methods. Thanks to the present work, innovative cleaning techniques targeting the most concerning type of stain for paper conservators, namely black stains caused by melanised fungi, will be addressed and tested specifically for these molecules.

The extraction and characterization of fungal melanins produced by fungi isolated from paper was a stepping stone in the current investigation concerning which colourants may be present in a given stain, in order to develop new reliable and safer methods for these stains' removal.

A successful melanin extraction of the three selected fungal species was obtained according to the UV-Vis, μ-FTIR and μ-Raman data, showing a predominantly aromatic structure with carbonyl and phenolic groups for all samples, with a special difference noticed in the IR spectrum of the sample obtained from *A. niger* – namely nitrogen content, indicating indolic groups, typical of the L-DOPA melanin structure.

For the two species where enough melanin sample was obtained to carry out SSNMR analyses, a proposed monomer type melanin was given, namely DHN type melanin for *Cl. cladosporioides* and L-DOPA melanin for *A. niger*. These analyses were consistent with the other analytical techniques that were possible to carry out to this point.

Also, through SSNMR analyses, it was possible to asses that, besides two different biosynthetic pathways, the melanins from *A. niger* and *Cl. cladosporioides* have different structural organization. *Cl. cladosporioides'* melanin shows an amorphous structure, or a more crosslinked structure, with a higher MW, when compared to *A. niger*'s.

A SSNMR spectrum of *Ch. globosum*'s melanin should be carried after further extractions, in order to confirm if its similarity with *Cl. cladosporioides'* sample in the remaining analyses is indicative of the same biosynthetic pathway, as reported in the available literature (namely DHN melanin synthesis pathway), since their spectral profile in the IR spectra are extremely similar.

Also, MALDI-TOF analyses should be optimized and carried for the given samples, in order to assess their degree of polymerization and the monomers. This will allow to make a clear assessment of the already obtained data, considering the actual constituent monomers and respective molecular weight of the polymers.

Research is still needed regarding other types of colourants; the correlation between fungal stains on paper and its culprit; about how the colourants are altered by different types of paper substrates; how the colourants change with ageing; and finally, about cleaning methodologies for the removal of this aesthetic damage, without damaging the paper substrate, which is aimed for a final phase of the CleanART project.

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Appendices

Appendix I

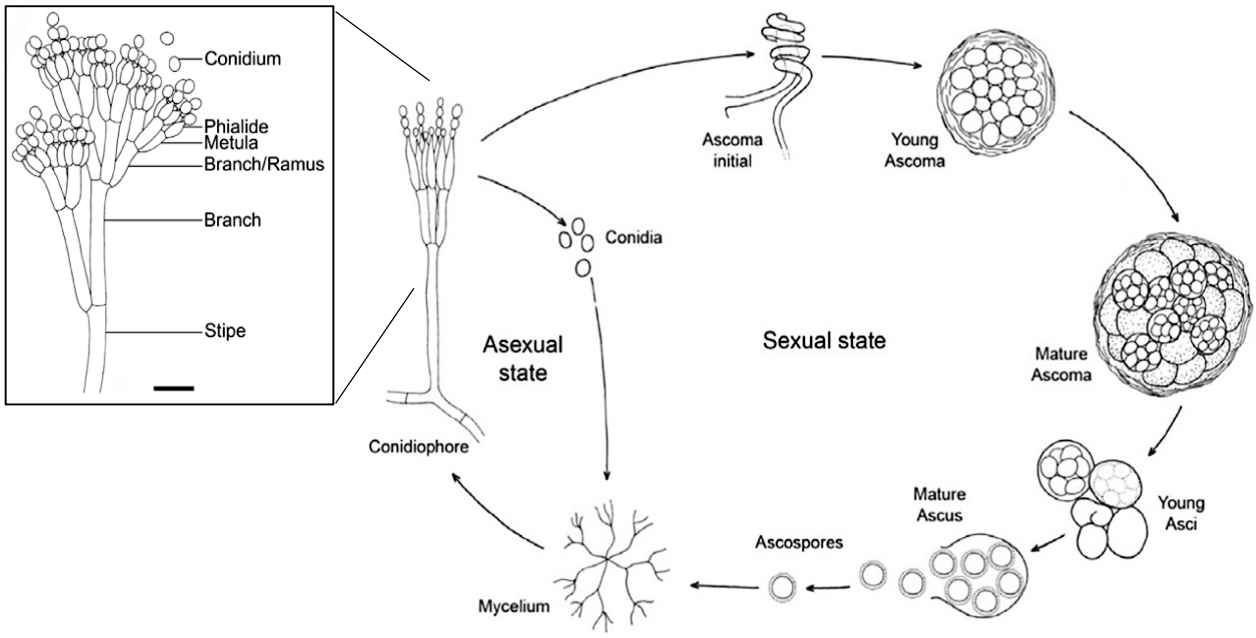


Figure I.1. Life cycle of fungi where both the teleomorphic and anamorphic states are observed and, thus, both ascospores and conidia are produced [12]. Terms used for describing parts of conidiophores are discriminated. Scale bar = 10 μm [63].

Appendix II

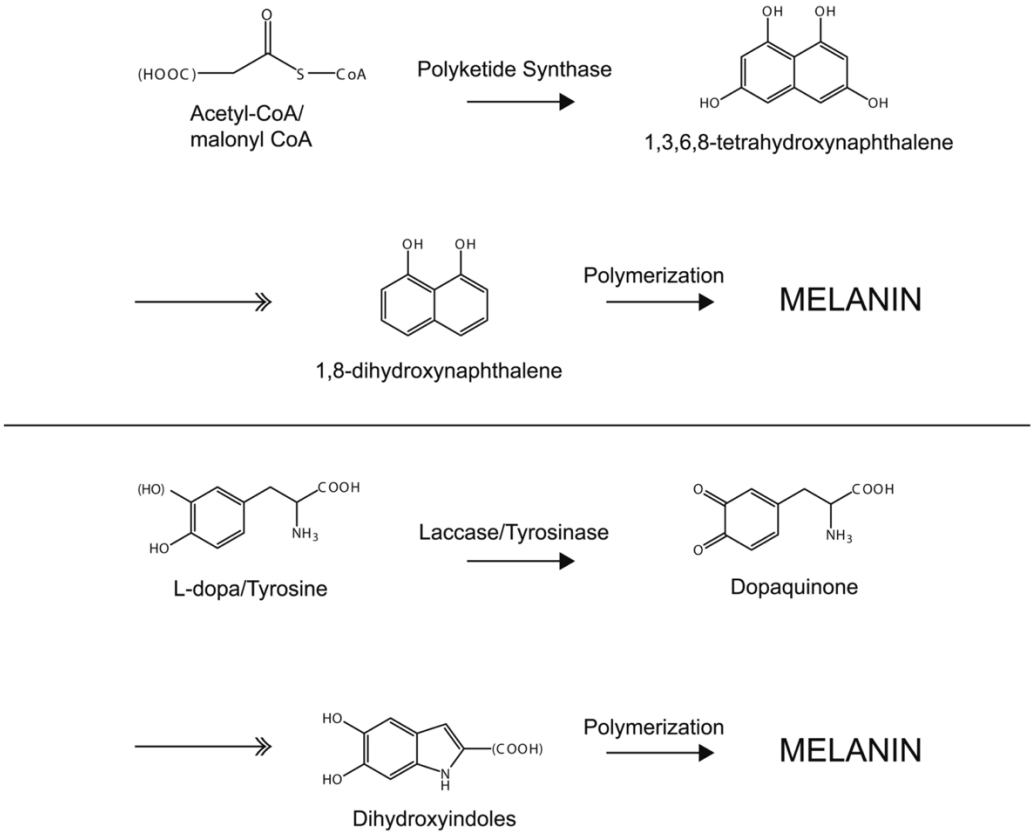


Figure II.1. Comparison of DNH (top) and L-DOPA (bottom) melanin synthesis pathways [23].

Appendix III

Table III.1. Case studies reviewed in this work together with the type of paper, type of study, location and respective reference are presented chronologically organized. Information regarding if the paper was already colonized or if it was inoculated under laboratorial conditions, is also given.

Work number		Type of paper (century)	Type of study	Paper already colonized by fungi	Local and/or country	Reference
1.		Weathered cardboard (20^{th} century)	Identification of fungi by culture.	Yes	New England, Coastal area (USA)	[33]
2.		Saunders Company medium-weight, pliable, rag paper – no heavy sizing (20^{th} century)	Rapid production of fungal stains on paper. Analyses of stains by SEM.	No	(USA)	[64]
3.	**a)**	Flemish etching (19^{th} century)	Effectiveness of stain laser removal from prints, drawings, and artworks on paper. Analyses of stains by SEM.	Yes	New York (USA)	[65]
	b)	Wove paper of medium weight, manufactured by Rives and Arche		No		
4.		Rag paper (16^{th}-20^{th} century)	Investigation about the role of fungi in foxing.	Yes	Libraries in England, South Africa, Brasil, Canada and the USA.	[66]
5.		Paper documents (18th-19th century)	Isolation/microscopic examination of fungi colonizing the documents.	Yes	Easton, Maryland (USA)	[67]
6.	**a)**	Untreated linen rag paper (19^{th} century)	Irregular fungal fox spots on the untreated rag paper in the book; alum-rosin-treated paper in the same book and another one were examined. Analyses of stains by EDX and SEM.	Yes	New York (USA)	[68]
	b)	Alum-rosin-treated paper (18-19^{th} century)				
7.		Hemp paper (20^{th} century)	Identification of "foxing-causing fungi"	Yes	Hoodo of Byodoin Temple (Japan)	[32]
8.		Paper (20^{th} century)	Isolation and culturing of fungi that can produce the foxing effect.	No		
9.		Israeli stamps (20^{th} century)	Investigation on foxing, to identify the fungi responsible. Analyses of stains by SEM.	Yes	(Israel)	[69]
10.	**a)**	Samples of model-paper (20^{th} century) inoculated with suspensions of spores	Culture-independent molecular methods applied to identify fungal communities colonizing paper samples of different composition and age. Molecular biology analysis	No	-	[70]
	b)	Paper samples with naturally occurring fungal infections (20^{th} century)		Yes		
	c)	Paper samples with 20-years-old inocula and naturally occurring infections (20^{th} century)				
11.		Book made of linen and hemp (19th century)	Identification of isolates from brownish areas by culture-independent approaches using MB techniques.	Yes	Paris (France)	[71]
12.	**a)**	Topographic map (18^{th} century)	Investigate the fungistatic properties of the commercial 4-HB spray and calcium propionate (in both aqueous and ethyl alcohol solutions) on pure cultured fungal strains isolated from foxing patches of some old prints. FTIR-ATR.	Yes	Museo di Sant'Agostino in Genoa (Italy)	[10]
	b)	Topographic map N° 17 (19^{th} century)				
	c)	Cardboard backing Lignin-containing (20^{th} century)				

Table III.1. (continuance).

Work number		Type of paper (century)	Type of study	Paper already colonized by fungi	Local and/or country	Reference
13.	**a)**	Topographic map (18th century)	Verifying the presence of fungi in biodeteriorated 18th century etchings, and characterizing the paper surface by means of Fourier transform infrared (FTIR) spectroscopy and fluorescence under UV radiation.	Yes	Genoa (Italy)	[72]
	b)	Topographic map (19th century)				
	c)	Cardboard backing Lignin-containing. (20th century)				
	d)	Ancient print GE1 (19th century)				
	e)	Ancient print GE2 (18th century)				
	f)	Ancient print GE3 (19th century)				
14.		Book (cotton linters fibers) (16th century)	Study of microbiological damage.	Yes	Rome (Italy)	[73]
15.		Italian Manuscript (13th century)	MB analysis and microscopy.	Yes	Italy	[5]
16.	**a)**	Passepartout (20th century)	FTIR-ATR spectroscopy - utilized for a clearer understanding of the controversial nature of foxing.	Yes	Genoa (Italy)	[74]
	b)	Passepartout (21st century)				
	c)	Backing cardboard 21st century				
17.		Printed book and inoculation on sample paper.	Procedures required for a conservative approach to the evaluation and description of the damage and the organisms. SEM	Yes	Rome (Italy)	[75]
18.	**a)**	Atlantic Codex set of drawings by Leonardo da Vinci (15-16th century) - Codex folio	Molecular biology methods were used to assess the current microbiological risk to stained pages of the manuscript.	Yes	Milan (Italy).	[76]
	b)	Atlantic Codex set of drawings by Leonardo da Vinci (15-16th century) - Modern paper frames				
19.		Historic paper (18-20th century)	Evaluation, through a combination of cultural and molecular methods, of the microbial diversity of different kinds of stains.	Yes	Martin (Slovakia).	[77]
20.		One paper from the 17th century and two from the 20th centurys	Focused studies on black stains which are prevalent on art rendered on paper.	Yes	-	[4]
21.		Three paper photos (F1, F2 and F4), one book (L1) and two maps (M1 and M3), two paper notarial acts (P1a, P1b and P2) (19th Century)	Study microbial contamination of the environment and its influence on biodeterioration by the biofilm formation; analyze the relationship between environment microbiota and biofilm formation in materials stored at four archives.	Yes	(HAMLP), (AHCRD), (AN), (PL) and (ML) of the (NARC)*	[78]
22.		General information on the main fungi and bacteria that attack the paper	Highlight the role played by biocides in the destruction of microorganisms that attack irreversibly the paper, but also in the processes of preservation, restoration and conservation.	Yes	Iaşi (Romania)	[1]
23.		Japanese paper in the underlining of a painted folding screen and paper envelope	On-site investigations and a culture- based analysis were carried out to determine the extent of the microbial deterioration of paper-based objects.	Yes	Japan	[79]
24.		Ancient paper (19th century)	Identification and characterization of the microflora that damage historical manuscripts books from an old library of the Medina of Fez.	Yes	Medina of Fez (Morocco)	[80]
		Sterile Whatman paper (21th century)	The effect of fungal contamination in paper artificially attacked for 18 months at 25 °C was examined.	No		
25.		Handmade sheet – cotton (17th century)	Explores black stains on paper attributed to Dematiaceous, meristematic fungi and their interactions with the paper matrix.	Yes	Collection of the Maltese Archives (Malta)	[15]
		Engraving on machine-made paper – cotton (20th century)				
26.		Leonardo da Vinci's self-portrait (16th century)	Investigation on the possible causes of a very famous foxing through molecular and microscopic techniques.	Yes	Royal Library in Turin 'Biblioteca Reale'(Italy)	[81]

*Historical Archive of Museum of La Plata (HAMLP), Archive of Historical and Cartographic Research Department from the Geodesy Direction (AHCRD), Archive of Notaries of Buenos Aires Province (AN), Argentina; at two repositories: Photo Library (PL) and Map Library (ML) of the National Archive of the Republic of Cuba (NARC).

Table III.2. Fungal genera or specie found staining on paper together with their stain colour, colourants, fungal identification method and respective references. (C – Culture; MB – Molecular Biology)

Fungi	Stain Colour	Colourants	Fungal Identification Method	References
Alternaria sp.	Red, purple, yellow, brown, black, etc.	-	-	[1,78]
Altemaria solani	Black	-	C	[65]
	Black stain that adheres very strongly to the paper	Black pigments	C	[64]
Aureobasidium pullulans	Brownish-red (foxing)	-	C	[10,72]
Aspergillus	Brown spot (foxing)	-	C	[10,72]
Aspergillus carneus	Foxing (light yellow to reddish bown)	-	C	[69]
	Brown spot (foxing)	-	C	[72]
Aspergillus flavus	Foxing (light yellow to reddish bown)	-	C	[69]
	Brown spot (foxing)	-	C	[72]
Aspergillus fumigatus	Black-brown	-	C and MB	[77]
	Black which is diffused in the whole surface	-	C and MB	[77]
	Black and pink	-	C and MB	[77]
	Foxing (light yellow to reddish bown)	-	C	[69]
Aspergillus japonicus	Brown (foxing)	-	MB	[71]
Aspergillus melleus	Brown (foxing)	-	C	[74]
	Green yellow	-	C	[80]
Aspergillus nidulans (Emericella)	Purple	Purple-brown	C and MB	[73]
Aspergillus niger	Foxing (light yellow to reddish bown)	-	C	[69,78,82]
	Black	-	MB	[80]
Aspergillus oryzae	Brown (foxing)	-	MB	[71]
	Green yellow	-	MB	[80]
Aspergillus penicilloides	Brown (Foxing)	Melanoidines	C	[83]
Aspergillus sclerotiorum	Pale brown (foxing)	-	C	[74]
Aspergillus tamarii	Foxing (light yellow to reddish bown)	-	C	[69]
Aspergillus terreus var. aureus	Foxing; Brown pigmentation; Yellow spots	-	C	[69]

Table III.2. (continuance).

Fungi	Stain Colour	Colourants	Fungal Identification Method	References
Aspergillus ustus	Brown (Foxing)	-	MB	[71]
Aspergillus versicolor	Purple	Pink-orange (Versicolorine)	C and MB	[73]
	Foxing- light yellowish coating	Secreted yellowish pigments to the culture media	C	[84]
Bjerkandera adusta	Brown (foxing)	-	MB	[71]
Chaetomium (Dematiaceous fungi)	Dark pigmented individual fruiting bodies attached	Melanin	CLSM; LCA; SEM-VP; TLM	[15]
	Black	Melanized fruiting structures	SEM; CLSM	[34]
Chaetomium globusum	Brownish gray	-	C	[65]
	Black	-	C	[5] *
	Brown (foxing)	-	MB	[71]
	Yellow (3 days); becomes grayish-brown in 2 days	Tomichaedin	C	[64]
Cladosporium sp.	Brown	-	C	[85]
	Black	Melanized spores/conidia	SEM; CLSM	[34]
Cladosporium cladosporioides	Black	Melanoidines (melanized cell walls)	C	[33]
	Brown spot (foxing)	-	C	[72]
Cladosporium sphaerospermum	Pale brown (foxing)	-	C	[74]
Doratomyces stemonitis	Brown spot (foxing)	-	C	[10,72]
Engyodontium album	Green–black staining	-	MB	[76]
Eurotium spp. (repens or *rubrum*)	Foxing	-	SEM	[68]
Eurotium amstelodami	Foxing	-	SEM	[68]
Eurotium halophilicum	Foxing	-	MB, SEM	[81]
Eurotium herbariorum	Brown (Foxing)	Melanoidines	C	[83]
Eurotium repens	Foxing - light yellowish coating	Secreted yellowish pigments	C	[84]

Table III.2. (continuance).

Fungi	Stain Colour	Colourants	Fungal Identification Method	References
Eurotium rubrum	Foxing - light yellowish coating	Secreted yellowish pigments	C	[84]
Fusarium sp.	Red, purple, brown, black, etc	-	-	[1]
Fusarium oxysporum	Pinkish	-	C	[65]
	Purple-pink	Fusarubin	C	[64]
Geomyces pannorum	Brownish-red (foxing)	-	C	[10,72]
Geosmithia putterillii	Brownish-red (foxing)	-	C	[10,72]
Gliocladium roseum	Foxing (light yellow to reddish bown)	-	C	[69]
Gloeotinia temulenta	Brown (Foxing)	-	MB	[71]
Hypocrea lixii	Green	-	MB	[80]
Mucor racemosus	Dark grey	-	MB	[80]
Mucor spinosus	Black	-	C and MB	[77]
	Black and pink	-	C and MB	[77]
Myxotricum deflexum	Light-red	Red pigmentation	Light microscopy; LV-SEM; MB	[79]
Paecilomyces variotii	Brown spot (foxing)	-	C	[72]
Penicillium atrovenetum	Green–black staining	-	MB	[76]
Penicillium chrysogenum	Yellow-green	-	C	[86]
Penicillium citrinum	Brown (foxing)	-	MB	[71]
Penicillium commune	Black-brown	-	C and MB	[77,78]
Penicillium coralligerum	Green–black staining	-	MB	[76]
Penicillium funiculosum	Foxing (light yellow to reddish bown)	-	C	[69]
Penicillium minioluteum	Brown (foxing)	-	MB	[71]
Penicillium notatum	Light green	-	C	[65]
	Yellow-green stain	Yellow compounds secreted	C	[64]
Penicillium purpurogenum	Brown-yellowish (foxing)	-	C	[74]
Penicillium restrictum	Brownish-red (foxing)	-	C	[10,72]
Penicillium spinulosum	Brownish-red (foxing)	-	C	[10,72]
Phoma herbarum	Black-brown	-	C and MB	[77]
	Black	-	C and MB	[77]

Table III.2 (continuance).

Fungi	Stain Colour	Colourants	Fungal Identification Method	References
Polyporus brumalis	Brown (foxing)	-	MB	[71]
Ramichoridium apiculatum	Green–black staining	-	MB	[76]
Saccharicola bicolor	Brown (Foxing)	-	MB	[71]
Stachybotrys chartarum	Black	-	Microscopy;SEM;MB	[79]
Taeniolella sp.	Black	Melanins	SEM;CLSM	[34]
Trichoderma citrinoviride	Brown (Foxing)	-	MB	[71]
Trichoderma koningii	Brown (Foxing)	-	MB	[71]
Trichoderma pseudokoningii	Brownish-red (foxing)	-	C	[10,72]
Ulocladium chartarum	Brown (foxing)	-	MB	[71]
Ulocladium cucurbitae	Brown (foxing)	-	MB	[71]

Table III.3. Fungi known to produce melanins and to colonize paper.

Fungi	Reference
Alternaria alternata	[87]
Aspergillus fumigatus	[22,23,26,87–89]
Aspergillus nidulans	[22,23,26,87,90,91]
Aspergillus niger	[11,22,23,30,44]
Aspergillus penicilloides	[32]
Aureobasidium pullulans	[5]
Chaetomium sp.	[15]
Chaetomium globosum	[50]
Cladosporium cladosporioides	[33]
Eurotium herbariorum	[32]

Table III.4. Azaphilone colourants chemical structure (base structure in bold), colour and the fungi responsible for its production. Note that only the genera that are also found on paper are reported here.

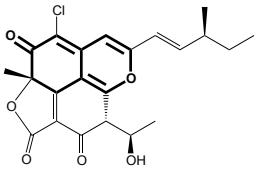
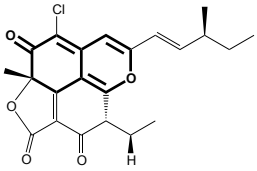
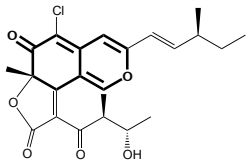
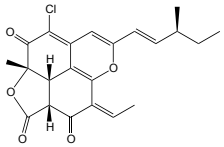
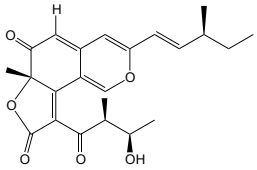
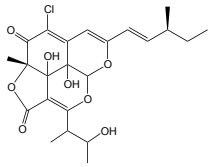
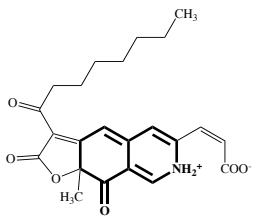
Colourant	Colour	Fungi	References
4'-Epichaetoviridin A 	Orange	*Chaetomium globosum*	[92]
4'-Epichaetoviridin F 	Orange	*Chaetomium globosum*	[92]
5'-Epichaetoviridin A 	Orange	*Chaetomium globosum*	[92]
Chaetoviridin G 	Orange	*Chaetomium globosum*	[92]
Chaetoviridin H 	Yellow	*Chaetomium globosum*	[92]
Chaetoviridin I 	Yellow	*Chaetomium globosum*	[92]
Monascus-like pigment 	-	*Penicillium*	[31]

Table III.4. (continuance).

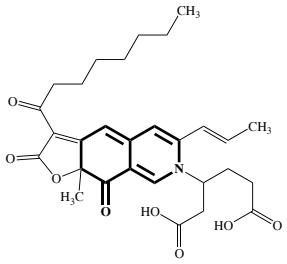
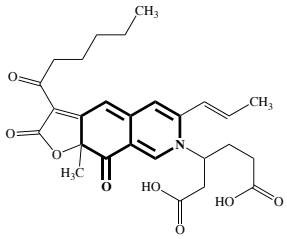
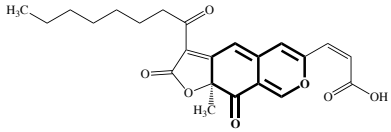
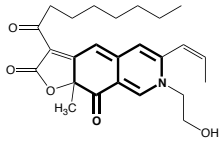
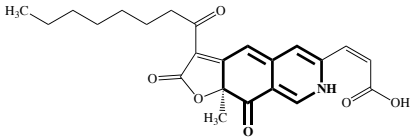
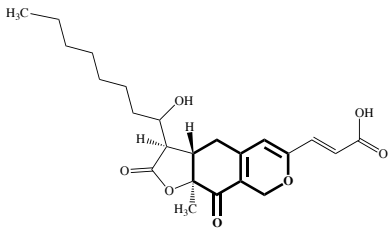
Colourant	Colour	Fungi	References
N-glutarylmonascorubramine 	Red	*Penicillium purpurogenum*	[93]
N-glutarylrubropunctamine 	Red	*Penicillium purpurogenum*	[93]
PP-O 	Orange	*Penicillium purpurogenum*	[94]
PP-R or 7-(2-hydroxyethyl)-monascorubramine 	Purple-red	*Penicillium purpurogenum*	[95,96]
PP-V or 12-Carboxyl-monascorubramine 	Purple-red	*Penicillium sp.* *Penicillium purpurogenum*	[20,94,97–100]
Sequoiamonascin C 	Yellow	*Penicillium sp.*	[20]

Table III.5. Quinone, HAQN and Naphthoquinone colorants chemical structure (base structure in bold), colour and the fungi responsible for its production. Note that only the genera that are also found on paper are reported here.

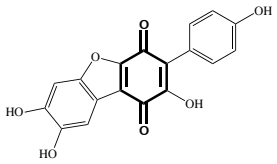
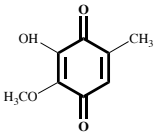
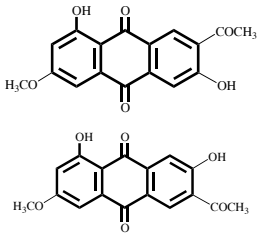
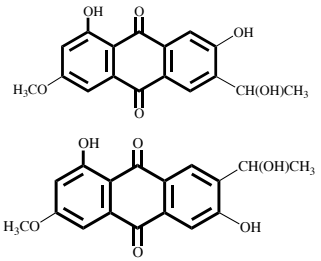
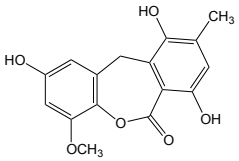
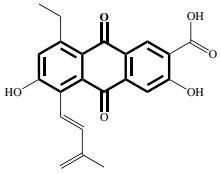
	Colourant	Colour	Fungi	Reference
	Cycloleucomelone 	Green	*Aspergillus niger*	[31,101,102]
	Fumigatin° 	Yellowish-brown	*Aspergillus fumigatus*	[29]
HAQN (Hydroxyanthraquinoid)	**2-Acetyl-3,8-dihydroxy-Gmethoxy or 3-acetyl-2,8-dihydroxy-6-methoxy-anthraquinone** 	Yellow	*Fusarium oxysporum*	[103]
	2-(1-Hydroxyethyl)-3,8-dihydroxy-6-methoxy or 3-(Z-hydroxyethyl)-2,8-dihydroxy-6-methoxy-anthraquinone 	Orange	*Fusarium oxysporum*	[103]
	2-O-methyleurotinone 	Brown	*Eurotium rubrum*	[104]
	Arpink red™ 	Red	*Penicillium oxalicum*	[31,36,105]

Table III.5. (continuance).

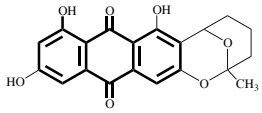
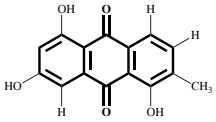
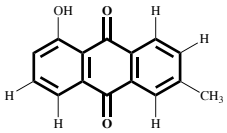
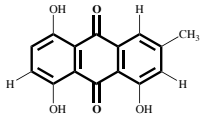
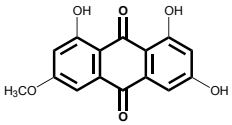
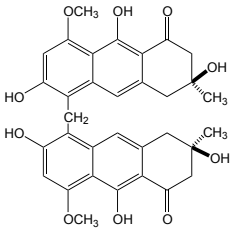
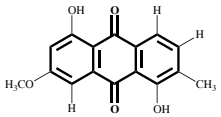
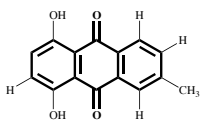
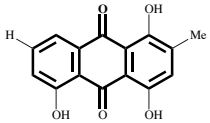
	Colourant	Colour	Fungi	Reference
HAQN (Hydroxyanthraquinoid)	**Averufin** 	Red	*Aspergillus versicolor*	[38,106–108]
	Catenarin 	Red	*Aspergillus glaucus, A. cristatus and A. repens*	[31]
			Eurotium spp., Fusarium spp.	[36]
	Chrysophanol 	Red	*Eurotium spp., Fusarium spp.*	[36]
	Cynodontin 	Bronze	*Eurotium spp., Fusarium spp.*	[36]
	Emodin 	Yellow	*Penicillium citrinum*	[31,109]
			Penicillium islandicum	
			Aspergillus glaucus, A. cristatus and A. repens	
	Eurorubrin 	Brown	*Eurotium rubrum*	[104]
	Erythroglaucin 	Red	*Aspergillus glaucus*	[30]
			A. glaucus, A. cristatus and A. repens	[31]
			Eurotium/Fusarium spp.	[36]
	Helminthosporin 	Maroon	*Eurotium spp., Fusarium spp.*	[36]
	Islandicin 	Green/orange-red	*Penicillium islandicum*	[29]

Table III.5. (continuance).

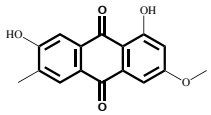
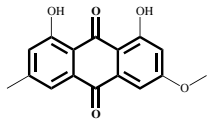
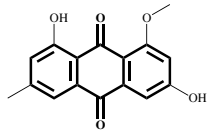
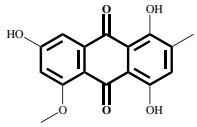
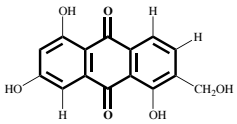
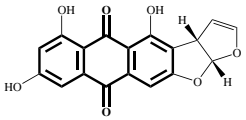
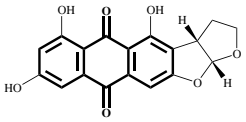
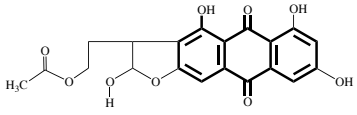
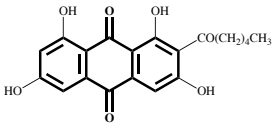
	Colourant	Colour	Fungi	Reference
HAQN (Hydroxyanthraquinoid)	**Macrosporin** 	Yellow	*Alternaria sp.*	[31]
	Physcion 	Yellow	*Aspergillus glaucus, A. cristatus and A. repens*	[31,96]
			Alternaria sp. *Eurotium herbariorum*	
	Questin 	Yellow to orange-brown	*Aspergillus glaucus, A. cristatus and A. repens* *Eurotium rubrum*	[31,104]
	Rubrocristin 	Red	*Aspergillus glaucus, A. cristatus and A. repens*	[31]
	Rubroglaucin (mixture of Physcion and Erythroglaucin)	Red	*Aspergillus glaucus*	[29,110,111]
	Tritisporin 	Red-brown	*Eurotium spp., Fusarium spp.*	[36]
	Versicolorin A 	Pink-orange	*Aspergillus versicolor*	[38,73,112–114]
	Versicolorin B, C 		*Aspergillus versicolor*	[73,112–114]
	Versiconal hemiacetal acetate 	Orange	*Aspergillus versicollor*	[38]
	Unidentified 	Red	*Aspergillus versicolor*	[112]

Table III.5. (continuance).

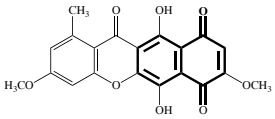
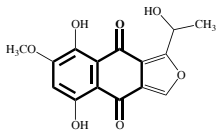
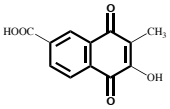
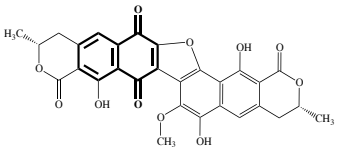
	Colourant	Colour	Fungi	Reference
Naphthoquinones	**Bikaverin** 	Red	*Fusarium sp.*	[20,115]
	Nectriafurone 	Yellow-brown	*Fusarium sp.*	[20]
	Tomichaedin 	Yellow-brown	*Chaetomium globosum*	[64,116]
	Viopurpurin 	Purple-black	*Aspergillus sp.* *Aspergillus melleus*	[20,37]

Table III.6. Other colorants chemical structure, colour and the fungi responsible for its production. Note that only the genera that are also found on paper are reported here.

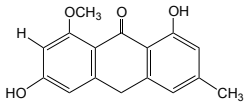
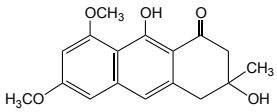
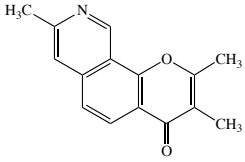
Colourant	Colour	Fungi	Reference
Anhydroasperflavin 	Yellow	*Aspergillus flavus*	[117]
Asperflavin 	Yellow	*Aspergillus flavus* *Eurotium rubrum*	[104,117]
Aspergillin –hexahydroxyl pentacyclic quinoid (HPQ) and a melanin pigment	Brown	*Aspergillus niger*	Frey in [118] [47]
Aspergillitine 	Yellow	*Aspergillus versicolor*	[119]

Table III.6. (continuance).

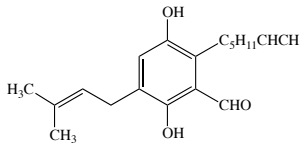
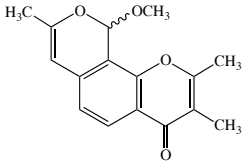
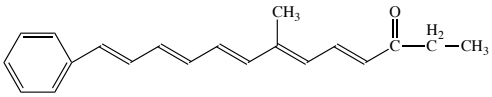
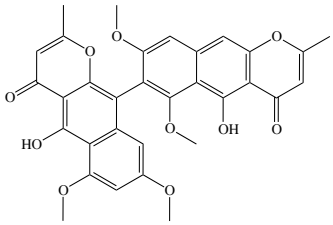
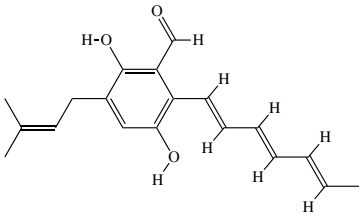
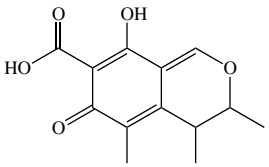
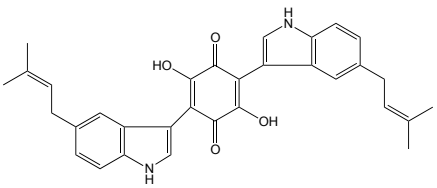
Colourant	Colour	Fungi	Reference
Aspergin 	Yellow	*Eurotium herbariorum*	[96,117]
Aspergione C 	Yellow	*Aspergillus versicolor*	[119]
Asperyellone 	Yellow	*Aspergillus niger*	[120,121]
Aurasperone 	Yellow	*Aspergillus niger*	[44,120,121]
Auroglaucin 	Orange-red	*Aspergillus glaucus Aspergillus repens Eurotium amstelodami, E. chevalieri and E. herbariorum*	[29,30,110,111]
Citrinin 	Yellow	*P. purpurogenum*	[122,123]
Cochliodinol 	Purple	*Chaetomium globosum*	[29,124,125]

Table III.6. (continuance).

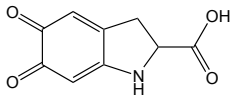
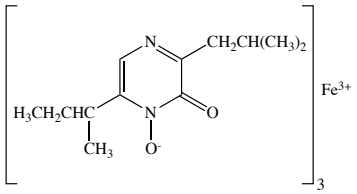
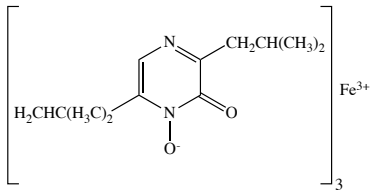
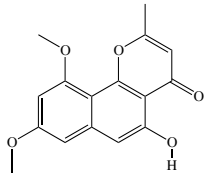
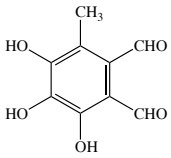
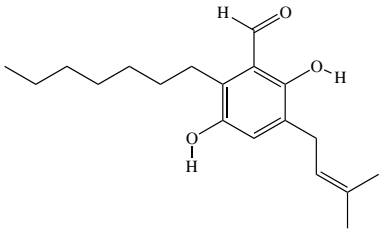
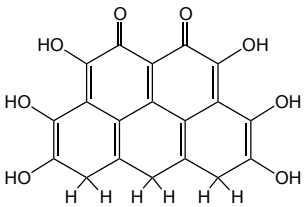
Colourant	Colour	Fungi	Reference
Dopachrome 	Pink	*Aspergillus nidulans*	[26]
Ferriaspergillin 	Red	*Aspergillus flavus*	[126]
Ferrineoaspergillin 	Red	*Aspergillus melleus* *A. sclerotiorum Huber*	[126]
Flavasperone 	Yellow	*Aspergillus niger*	[44,120,121]
Flavipin 	Yellow	*Epicoccum purpurascens*	[36,127]
	Orange-Yellow	*Aspergillus flavipes* *Aspergillus terreus*	[128]
Flavoglaucin 	Yellow	*Aspergillus glaucus* *Aspergillus flavus* *Eurotium amstelodami, E. chevalieri and E. herbariorum*	[29,30,96,110, 117]
Hexahydroxyl pentacyclic quinoid (HPQ) 	Green	*Aspergillus niger*	[44,47]

Table III.6. (continuance).

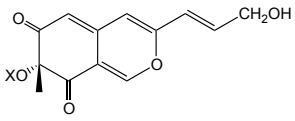
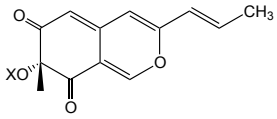
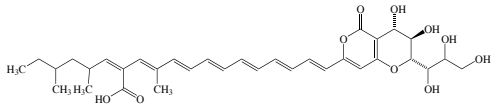
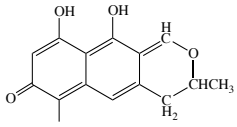
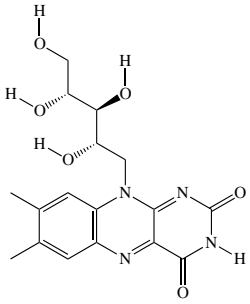
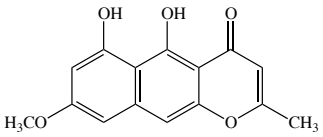
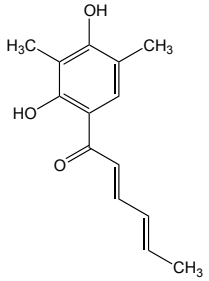
Colourant	Colour	Fungi	Reference
Melanochrome (intermediary in the formation of DHN-melanin)	Purple	*Aspergillus nidulans*	[26]
Mitorubrinol 	Orange-red	*Penicillium purpurogenum*	[21,96]
Mitorubrin 	Yellow	*Penicillium purpurogenum*	[96]
Orevactaene 	Yellow	*Epicoccum purpurascens* Ehrenb (formerly known as *Epicoccum nigrum* Link)	[20,31,129,130]
	Orange		[36], [126]
Purpurogenone 	Yellow-orange	*Penicillium purpurogenum*	[96,97,122]
Riboflavin 	Yellow	*Aspergillus niger*	[118,121]
Rubrofusarin 	Red	*Fusarium sp.*	[29]
Sorbicillin 	Yellow	*Penicillium chrysogenum*	[131]

Table III.6. (continuance).

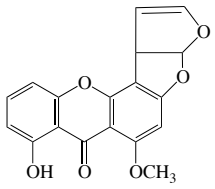
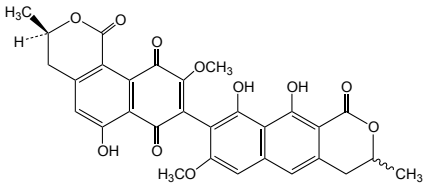
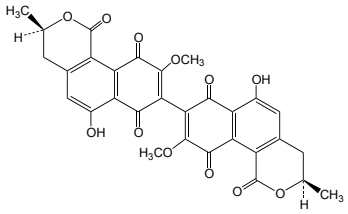
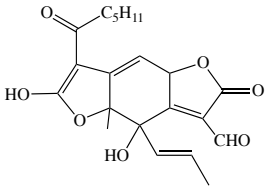
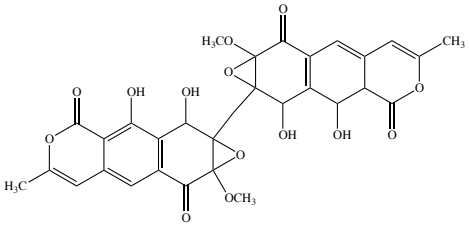
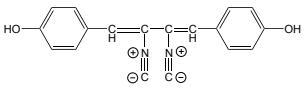
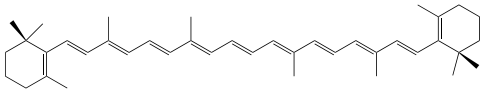
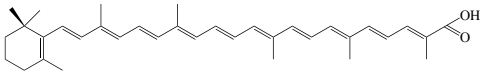
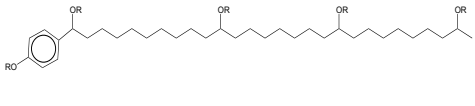
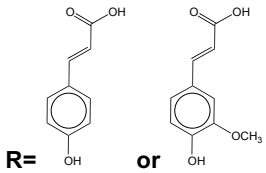
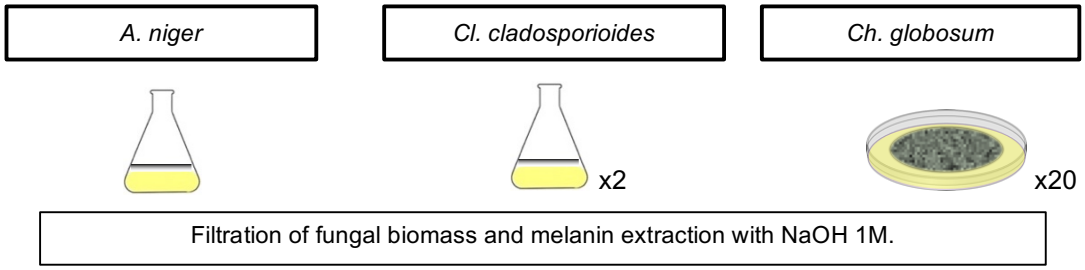
Colourant	Colour	Fungi	Reference
Sterigmatocystin 	Yellow	*Aspergillus versicolor*	[106,132]
Viomellein 	Reddish-brown	*Aspergillus sp.* *Aspergillus melleus* *Aspergillus ochraceus* *Penicillium sp.*	[30,37,96]
Xanthomegnin 	Orange	*Aspergillus sp.* *Aspergillus melleus* *Aspergillus ochraceus* *Penicillium sp.*	[30,37,96]
Xanthomonascin A 	Yellow	*Monascus sp.*	[133]
Xanthoepocin 	Yellow	*Penicillium brevicompactum* *Penicillium simplicissimum*	[96,134]
Xanthocillins 	Yellow	*P. chrysogenum*	[135] 25

Table III.7. Carotenoid colorants chemical structure, colour and the fungi responsible for its production. Note that only the genera that are also found on paper are reported here.

Colourant	Colour	Fungi	References
β-carotene 	Yellow	*Aspergillus giganteus*	[136,137]
		Penicillium sp.	[137,138]
Neurosporaxanthin 	Orange	*Neurospora crassa*	[18,29,30,136, 139–141]
		Fusarium sp.	[139–143]
Sporopollenin  	Brown	*Neurospora crassa*	[35,144,145]

Appendix IV



Filtration of fungal biomass and melanin extraction with NaOH 1M.

In this part of the process, liquid media was separated from the fungal structure. For Ch. globosum, solid media was necessary in order to favour its sporulation. This complicated the method of harvesting the fungal structure, since the media had to be dissolved and reheated in order to filter the fungal structure. Only the filtered fungal structures were independently crushed with NaOH, in order to dissolve melanins, since these colourants are not soluble in any common solvents, requiring harsh basic solutions to do so.

↓

Sterilization of the obtained solutions in the autoclave (120ºC, 20 min).

↓

Filtration to remove fungal structures followed by precipitation of fungal melanins with 2M HCl.

↓

Centrifugation of the resulting precipitates, in order to obtain the melanin pellets.

↓

Removal of carbohydrates and proteins by acid hydrolysis (6M HCl at 100ºC for 2h).

This is a very important stage of the purification, since it's responsible for the removal of the cell wall component present in the IR analyses of the extracted melanins - chitin. After this purification by acid hydrolysis, which is catalysed by temperature (100ºC, 2h), chitin's characteristic bands in the IR spectra of the extracted colourants are no longer present in the purified melanin's spectra.

↓

Removal of lipids with organic solvents (chloroform, ethyl acetate and ethanol)

Different solvents, with growing polarity, are used to remove any fungal debris of lipid nature.

↓

Drying and dissolution of melanin in 2M NaOH followed by filtration.

Melanin samples were dried in vacuum, dissolved again in NaOH and filtered to leave out any traces of fungal debris.

↓

Acidification of supernatants collected (6M HCl) – precipitates washed with H_2O and dried.

The collected supernatants, containing the melanin colourants dissolved in NaOH, were acidified with 6M HCl, in order to precipitate and obtain the purified melanin pellets. These were centrifuged and washed three times with H_2O to elevate its pH to near 5.

↓

Melanins' characterization by UV-Vis, μ-FTIR, μ-Raman and SSRMN.

Figure IV.1. Diagram of the experimental procedure for the extraction of melanin from fungal biomass from Aspergillus niger, Cladosporium cladosporioides and Chaetomium globosum.

Appendix V

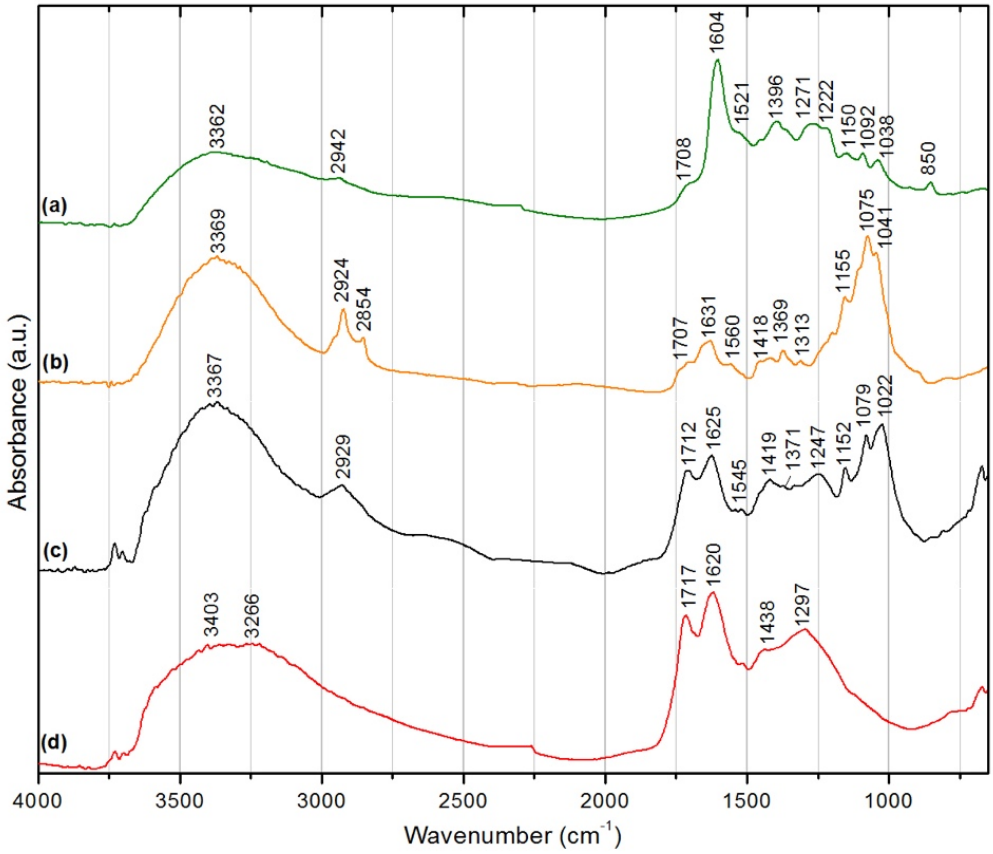


Figure V.1. Infrared spectra from extracted melanins: Aspergillus niger (a), Chaetomium globosum (b), Cladosporium cladosporioides (c) and synthetic melanin (d) from Sigma Aldrich.

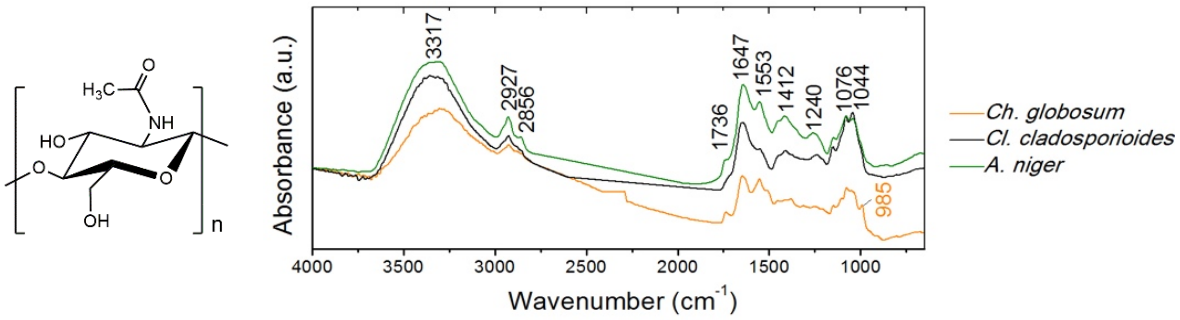


Figure V.2. Chemical structure of chitin (left) [146]. ***μ*-FTIR spectra of A. niger, Ch. globosum and Cl. cladosporioides mycelia (right).**

Table V.1. FTIR band wavenumbers (cm^{-1}) and proposed assignments for chitin in melanin extractions for A. niger, Ch. globosum and Cl. cladosporioides; ν=streching mode; δ=bending mode [146,147].

FTIR band wavenumber (cm^{-1})			**Proposed vibrational assignments**
A. niger	*Ch. globosum*	*Cl. cladosporioides*	
3362	3369	3367	νN-H; νO-H
2942	2924; 2854	2929	νCH_2; νCH_3
1521	1560	1545	νC-N (C-N-H); δN-H (Amide II)
1150	1155	1152	(νC-O-C) ring
1038	1075; 1041	1079; 1022	(νC-O) skeletal

Appendix VI

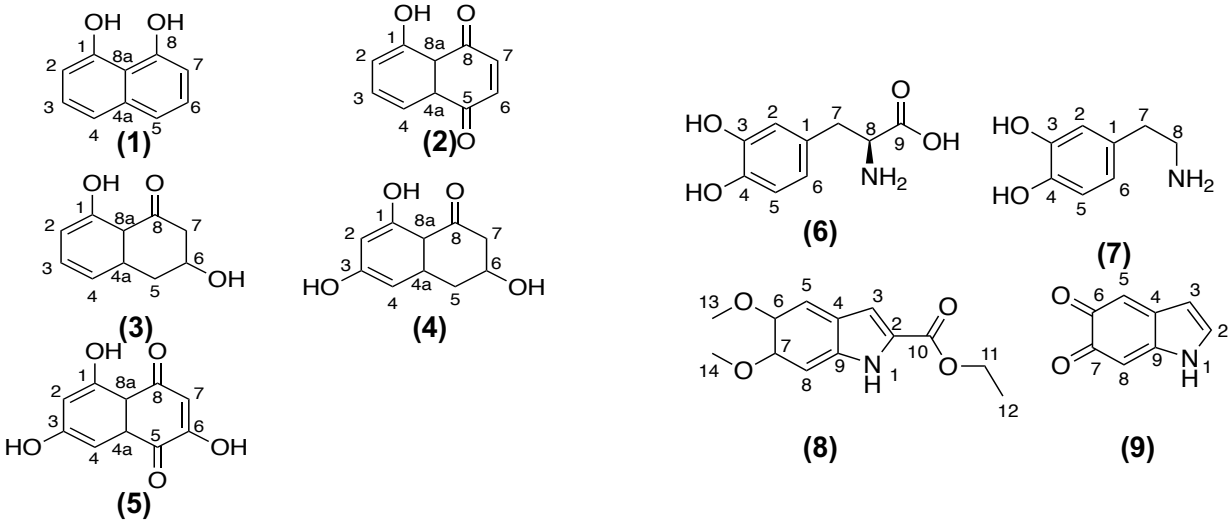


Table VI.1 – ^{13}C Chemical shifts of L-DOPA and 1, 8-DHN melanin model compounds

1,8-DHN	(1) [148]	(2) [149]	(3) [150]	(4) [151]	(5) [152]	L-DOPA	(6) [153]	(7) [153]	(8) [153]	(9) [154]
1	152.5	161.4	162.7	165.9	163.6	1	123.8	128.4	-	-
2	114.5	124.5	116.2	101.3	109.0	2	117.8	113.6	121.6	60
3	126.7	136.5	137.0	165.4	164.5	3	141.4	138.6	102.5	40
4	109.4	119.1	120.0	108.9	108.2	4	139.9	142.2	116.6	125
4a	137.0	131.7	141.4	145.7	133.3	5	117.2	113.3	97.0	118
5	*	184.2	38.3	38.8	181.6	6	112.7	119.4	131.1	170
6	*	138.6	66.3	66.3	160.7	7	35.2	30.2	146.6	170
7	*	139.6	47.2	47.1	111.3	8	55.7	38.7	90.0	120
8	*	190.2	203.1	202.0	191.0	9	174.2	-	130.7	150
8a	120.6	114.9	116.8	111.4	108.0	10	-	-	160.0	-
						11	-	-	58.6	-
						12	-	-	12.5	-
						13	-	-	51.4	-
						14	-	-	52.0	-

Note: * denote magnetic equivalent carbons

Glossary

Anamorph. The asexual or conidial form of a fungus.

Ascocarp. A fruiting body in Ascomycetes—containing **asci** and **ascospores**.

Ascomycetes A sub-division of the fungi in which the spores (the ascospores) are contained within a small structure like a bag, which is known as an **ascus**.

Ascus (pl. asci). A thin-walled sac containing ascospores.

Asexual reproduction. Reproduction that does not involve fusion of nuclei and meiosis.

Cellulolytic. Metabolic capacity to catabolize cellulose.

Colony. A colony is defined as a visible cluster of cells, often resulting from an initial cell, on solid medium.

Conidium (pl. conidia). An asexually produced spore.

Conidiophore. A specialized **hypha** on which the **conidia** (spores) are borne.

Family. A group of related objects or organisms. In the taxonomic hierarchy, a family is a subdivision of an order which encompasses different **genera**.

Fungi. Heterotrophic organisms that obtain their nutrients by the absorption of compounds from their surroundings.

Genus (plural: genera). Low-level taxonomic rank below the **family** and above the **species**.

Heterotrophs. Organisms using organic compounds as carbon source to synthesize their cellular constituents. The heterotrophy opposes the notion of autotrophy.

Hyphae. Thread-like filaments that form the basic structural units in fungi. The vegetative apparatus, composed of a mass of filaments (hyphae), is named **mycelium**.

Metabolism. Whole biochemical processes occurring within a living cell or organism.

Metabolite. Any substance produced by metabolism within an organism.

Metula. (pl. metulae) apical branch of a stipe-bearing phialides, especially in *Penicillium* and *Aspergillus*

Mitospore. A nucleated spore formed by mitosis.

Mycelium. Vegetative part of the fungi, consisting of a set of filaments; these filaments are traditionally referred to as **hyphae**.

Natural colourants. Pigments made by living organisms (i.e. they exist in nature).

Perithecium. A rounded ascocarp from which the spores are discharged via a small hole.

Phialide. a conidiogenous cell which produces conidia in basipetal succession, without an increase in the length of the phialide itself

Sclerotium. (pl. sclerotia) a resting body, usually globose, consisting of a compacted mass of mycelium, often very hard.

Sexual reproduction. Reproduction that includes fusion of nuclei and meiosis.

Species. Set of organisms which are present in different populations and can exchange genetic material.

Spore. A microscopic structure that functions in the reproduction and dispersal of fungi rather like a seed.

Strain. Pure cultures of microorganisms obtained from a single isolate.

Teleomorph. The forms representing sexual reproduction.