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Pheomelanin markers in melanoma
with reference to their excretion into urine

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“Nobody can go back and start a new beginning,
but anyone can start today and make a new ending”

ABSTRACT

Skin pigmentation is an important issue in most cultures. Until recently we have not understood the most important elements of pigmentation regarding detailed chemical structure. The synthesis of melanin is very complex, and although core enzymes, other important proteins, and parts of the melanin structure have been identified much information in this context awaits disclosure.

The function of the melanocyte and the deposition of melanin pigments into the keratinocytes are very important in the protection against UV light. Melanin pigments consist of high-molecular structures often described as brown to black eumelanin and yellow to red pheomelanin. Eumelanin is photoprotective, whereas pheomelanin is believed to be carcinogenic after UV radiation. There is strong evidence that people of fair complexion with freckles who tan poorly are at higher risk of developing melanoma. These people have a higher pheomelanin to eumelanin ratio in their skin.

Melanoma, one of the most widely spread cancers, is derived from melanocytes. There is accumulating evidence that pigment constitution is highly involved in the development of melanoma. We found that patients with advanced melanoma secrete substantial amounts of pigment structures into the urine, in particular those with diffuse melanosis. In subsequently performed experiments we purified these pigments and subjected the product to chemical degradation by either hydrogen peroxide oxidation or hydriodic hydrolysis. Several new chromatographic methods were developed for the structural analysis of these products. Structural analysis of new chromatographic peaks was performed. In conclusion, complex pheomelanin structures as well as low molecular weight pigments and free benzothiazoles have been identified in the urine of patients with melanoma and diffuse melanosis.

The present thesis provides new insight into melanogenesis and melanoma progression. This opens the doorway to further approaches to the investigation of melanins and can help to understand fundamental problems about the structure and biosynthesis of natural melanins.

SAMMANFATTNING

Hudens pigmentbildning spelar en viktig roll för människan i de flesta kulturer. Förståelsen för pigmentbildning har stor betydelse i medicinska och biokemiska sammanhang, särskilt för uppkomsten av melanom och bedömning av dess utveckling. Melanom är en aggressiv typ av hudcancer som har ökat i omfattning under de senaste 20 åren. Avsikten med denna avhandling var att utveckla nya metoder för att bättre förstå sambandet mellan pigmentbildning och utveckling av melanom.

Melaninpigment är en heterogen biopolymer som bildas i speciella celler, melanocyter. I dessa celler finns specialiserade cellorganeller, melanosomer, där pigmentbildningen sker. Den epidermala melaninenheten i människans hud består av melanocyter och keratinocyter. Genom sina dendritiska utskott distribuerar melanocyterna sina pigmenterade melanosomer till näraliggande keratinocyter där deras närvaro ger huden dess karakteristiska färg och fotoprotektiva egenskaper. Den viktigaste yttre orsaken till melanom är den ultravioletta strålningen i solljuset och melanocyternas funktion är mycket viktig för hudens skydd mot UV-strålning.

Melanin utgörs av högmolekylära ringstrukturer och beskrivs oftast som brunt till svart eumelanin respektive gult till rött feomelanin. Eumelanin betraktas som fotoprotektivt medan feomelanin verkar gynna fototoxicitet och har samband med uppkomsten av melanom. En individ med högre andel av feomelanin än eumelanin i sin hud har större risk att utveckla melanom. Tidigare studier har visat att personer med rött hår och hudtyp med övervägande feomelanin och som lätt blir brända i solen har ökad benägenhet att få melanom. I vissa fall av melanom lagras melaninet i huden och andra vävnader i kroppen och patienten kan få en kraftig grå-blå missfärgning. Detta kallas för diffus melanos och är ett allvarligt tillstånd både för sjukdomen men även ur psykosocial synpunkt.

Melaninernas exakta struktur är inte känd. Den skiljer sig från individ till individ så att varierande hud- och hårfärg uppkommer. Ett av huvudproblemen i melanom-studier är brist på adekvata metoder för isolering av rena melaninpigment dvs tillgång till biologiskt pigment. Vid melanos utsöndras stora mängder av trikokromer – feomelaninliknande lågmolekylära föreningen i urinen. Den kemiska strukturen av dessa är känd och återfinns i melaninet som en delstruktur. Man har även hittat granula, större pigmentformationer, med okänd kemisk struktur inlagrade i bl.a. njurarna hos patienter med melanos. Urin från dessa patienter har varit en stor tillgång i mina strukturstudier av feomelanin.

Vid pigmentanalysen sönderdelades melaninet med två olika metoder; hydrolys med jodvätesyra respektive genom oxidation med väteperoxid i alkalisk miljö. Därefter utvecklade vi flera kromatografiska tekniker för bestämning av degra-dationsprodukterna. Det har tidigare visats att cysteinyldopa, en mellanprodukt vid

bildning av melanin, utsöndras i urinen hos patienter med melanom. Jag har i denna avhandlig visat att även andra lågmolekylära ämnen såsom benzotiazoler och också själva pigmentet eller större delar av pigmentet kan utsöndras i urinen. Jag har kunnat påvisa detta genom att jämföra det biologiska pigmentet med *in vitro* syntetiserat feomelanin. Detta stämmer väl med att man vid melanos får inlagring av pigmentet i makrofager i huden och i andra organ. Urinutsöndringen av pigment torde därför vara extra hög vid melanos.

Denna avhandling belyser melaninproduktionen vid melanom och ger en ny grund för att bättre förstå biosyntes och uppbyggnad av naturliga melaninpigment. Den öppnar möjligheten till att vidare studera prognosen vid melanom.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, which will be referred to by their Roman numerals:

I. Takasaki A, **Nezirević D**, Årstrand K, Wakamatsu K, Ito S, Kågedal B. HPLC analysis of pheomelanin degradation products in human urine. Pigment Cell Res 2003;16:480-486.

II. **Nezirević D**, Årstrand K, Kågedal B. Hydrophilic interaction liquid chromatographic analysis of aminohydroxyphenylalanines from melanin pigments. J Chromatogr A 2007;1163:70-79.

III. **Nezirević Dernroth D**, Rundström A, Kågedal B. Gas chromatography-mass spectrometry analysis of pheomelanin degradation products. J Chromatogr A 2009; 1216:5730-5739

IV. **Nezirević Dernroth D**, Årstrand K, Greco G, Panzella L, Napolitano A, and Kågedal B. Pheomelanin-related benzothiazole isomers in the urine of patients with diffuse melanosis of melanoma. Pigment Cell and Melanoma Research, Submitted August 2009

ABBREVIATIONS

4-AHP	4-amino-3-hydroxyphenylalanine
3-AHP	3-amino-4-hydroxyphenylalanine
ASR	Age Standardized Rate
BTCA-2	7-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole-2-carboxylic acid (BTCA-2)
BTCA-5	6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole-2-carboxylic acid (BTCA-5)
DHI	5,6-Dihydroxyindole
DHICA	5,6-Dihydroxyindole-2-carboxylic acid
ECD	Electrochemical Detector
ECF	Ethyl chloroformate
ESI	Electro spray ionization
eV	Electron volt
GC/MS	Gas chromatograph/mass spectrometry
HBTA-2	7-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole
HBTA-5	6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole
HI	Hydriodic acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatography
H_3PO_2	Phosphinic acid
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
PDA	Photo diode array
PDCA	Pyrrole-3,5-dicarboxylic acid
PTCA	Pyrrole-2,3,5-tricarboxylic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase HPLC
SPE	Solid phase extraction
SCX	Strong cation exchanger
2-S-CD	2-S-Cysteinyldopa
5-S-CD	5-S-Cysteinyldopa
TDCA	Thiazole-4,5-dicarboxylic acid
TTCA	Thiazole-2,4,5-tricarboxylic acid
UV	Ultraviolet

CONTENTS

INTRODUCTION

The color of hair, skin and eyes in animals depends mainly on the structure, quantity, and distribution of melanins, the most common light-absorbing pigments found in the animal kingdom. Melanins have very diverse roles and functions in different organisms. They can protect microorganisms, such as bacteria and fungi, against stress that involves cell damage by solar UV radiation or reactive oxygen species (ROS) (Meyskens et al. 2001). This includes high temperature as well as chemical (e.g. heavy metals and oxidizing agents) and biochemical (e.g. host defenses against invading microbes) stresses (Hamilton and Gomez 2002). In many species of fish, amphibians, and reptiles, melanin can be highly mobile within the cell in response to hormonal (or sometimes neural) control leading to visible changes in color that are used for behavioral signaling. In humans, in addition to the beneficial effects of melanin, disorders in melanin production can lead to serious consequences and unfortunately often to melanoma—the most aggressive form of skin cancer. There is strong evidence that UV-light exposure from the sun (Menzies 2008; Oliveria et al. 2006; Veierød et al. 2003) and from artificial UV radiation (Abdulla et al. 2005; Levine et al. 2005; Veierød et al. 2003) plays a major role in the development of cutaneous melanoma. People with sun-sensitive skin, red hair and freckles who never tan are at higher risk of getting melanoma. This group of people appears to have a higher amount of red pigment (pheomelanin) in their skin. The present thesis is based on studies of pheomelanin pigment.

The skin

The skin consists of three distinct layers: the epidermis, the dermis and the subcutaneous layer (Fig. 1). The top layer, the epidermis, is translucent and allows light to pass partially through it. It functions as a barrier, preventing dehydration and harmful irradiation from the sun, and blocks penetration of microbes and destructive chemicals. The epidermis consists mainly of epithelial keratinocytes (90-95 %) but also of pigment-producing melanocytes, antigen-presenting Langerhans cells, and touch-transducer Merkel cells. The main function of the epidermal melanin unit is to provide photoprotection, obtained through melanin synthesis in the melanocytes. The basement membrane attaches the epidermis firmly to the dermis—the layer below.

The dermis contains blood vessels, nerves, hair roots and sweat glands. It lies deeper and consists of fibroblasts, which synthesize collagen, elastic and reticular fibers that give the skin support and flexibility.

Below the dermis there is a layer of subcutaneous fat that covers muscles and bones, to which the whole skin structure is attached by connective tissues. It contains blood vessels and nerves, and is made up of clumps of adipose cells.

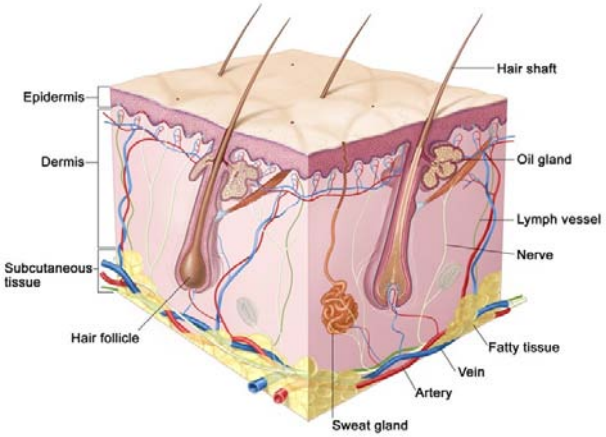


Fig. 1. *Schematic section of skin. ©2008 Terese Winslow, U.S. Govt. has certain rights*

The Melanocytes

Melanocytes are neural crest-derived cells located in the basal layer of the epidermis (Fig. 2).

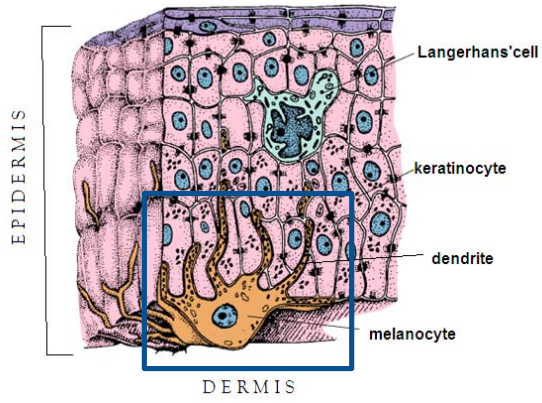


Fig. 2. *Schematic section of epidermis, modified from (Weiss 1988)*

These cells are also found in the hair follicles, in the choroid and retina of the eye, and in the leptomeninges. Melanogenesis – melanin production – occurs in the melanocytes containing specialized cell organelles. The melanin pigment is stored in these unique membrane-bound organelles that are thought to be specialized lysosomes (Van Den Bossche et al. 2006). The epidermal melanocyte unit consists

of melanocytes and keratinocytes. When early melanosomes mature into fully developed melanosomes, they are transferred to the tip of dendrites (Passeron et al. 2004) carrying melanin granules, which they drop off, via synapses, into the surrounding keratinocytes (approximately 36) to give the skin its characteristic color and photoprotective properties (Sturm et al. 1998) (Fig. 2b). The number, morphology and size of melanosomes are often genetically determined and influence the color of the skin of various races. The dark skin melanosomes are ellipsoidal, whereas the melanosomes of fair-skinned people are less regularly shaped and are generally smaller (Liu et al. 2005).

Melanin

Melanin is a biopolymer, a macromolecule with undefined structure and molecular weight. Because melanin is an aggregate of smaller component molecules, there are a number of different types of melanins of various proportions and bondings (Wenczl et al. 1998). For melanin synthesis two amino acids, L-tyrosine and L-cysteine, are required (Smit et al. 1997). Their availability and mutual ratio influence the chemical composition of the pigment.

Melanocytes produce two main groups of melanin: brown to black eumelanin and yellow to reddish pheomelanin (Ito et al. 2000). Dimeric pheomelanic pigment components called trichochromes are also produced in melanocytes in small quantities (Prota 1992b). In fact most melanins present in pigmented tissues appear to be mixtures or copolymers of eumelanins and pheomelanins (Ito 1993; Prota 1980). Their structures and ratios vary between individuals and give different colors of the hair and skin. Melanin pigments and their distribution in human skin are generally believed to be the most important factors in protecting human skin from the biochemical devastation caused by chronic exposure to solar irradiation. Melanin itself can serve both as an anti-oxidant and as a cellular pro-oxidant depending on its redox state, the presence of metal ions, and its state of polymerization (Meyskens et al. 2001). Pheomelanin and eumelanin not only differ in color but also in chemical structure and function. Eumelanin is photoprotective whereas pheomelanin is believed to be carcinogenic after UV radiation (Hill and Hill 2000; Meredith and Sarna 2006; Meyskens et al. 2001; Vincensi et al. 1998). It is believed that melanoma that develops from skin melanocytes results from the effect of UV radiation on pheomelanin. The higher incidence of UV-induced skin cancer among red-haired and fair-skinned individuals compared with individuals with dark skin and/or dark hair is hypothesized to result from photosensitization of pheomelanin (Chedekel et al. 1978; Ye et al. 2008).

The black or brown eumelanins afford protection against the damaging effects of the UV component of sunlight. UV radiation reaching the Earth is mainly in the UVA range (wavelength 320-400 nm) and to a minor extent in the UVB range

(wavelength 280-320 nm). UVB irradiation is much more potent in generating sunburns, tanning and DNA damage than UVA (Abdulla et al. 2005; Wolber et al. 2008). UVB irradiation also increases the number of melanocytes of the skin (Stierner et al. 1989). UVB light is considered to be non-destructive to eumelanin and produces reversible changes such as immediate pigment darkening and an increase in the number and nature of the unpaired electrons in the pigment (Chedekel et al. 1978; Riley 1997). The inflammatory skin response to UV light generates a massive production of cytokines and reactive oxygen, hydrogen peroxide, and/or superoxide (Meyskens et al. 2001). Fair-skinned humans exhibit a number of abnormal reactions to sunlight including freckling and high susceptibility to skin cancer. The skin of these people contains red-brown or yellow pigment, pheomelanin. It has a poor tanning capacity, contains little pigment and sunburns readily. Chedekel et al. (Chedekel et al. 1978) studied the effect of superoxide as a primary photoproduct of the irradiation of the pheomelanin which is formed by oxygen reaction with photochemical excited pheomelanin. The electron transfer enables the formation of the superoxide anion, which is biologically toxic and may contribute to the pathogenesis of many diseases (e.g. melanoma). The studies of Vincensi et al. on the pheomelanin to eumelanin ratio in hair melanin on one hand and minimal erythemal dose values on the other suggested a higher UV sensitivity with higher pheomelanin to eumelanin level (Vincensi et al. 1998). To study the regulation of melanogenesis and the biological roles of melanin, it is thus essential to analyze the contents of eumelanin and pheomelanin in biological samples. Researchers analyzed the melanin content in the epidermis and studied its relation to skin type and sun radiation (Thody et al. 1991; Vincensi et al. 1998). Their results showed that the melanin content itself is not a reliable indicator of UV susceptibility and that the efficiency of the skin photoprotection is rather a function of the balance of the pheomelanin to eumelanin pathways.

Melanoma and melanosis

Cutaneous melanoma

At various location of the melanocytes, neoplastic transformation generates different types of melanoma. Cutaneous melanoma is the most common type of melanoma, but other sites for melanoma are also known, e.g. the choroid of the eye, and mucous membranes (Ragnarsson-Olding et al. 2009). Historically, the first mention of melanoma was by Hippocrates in the fifth century, B.C. Approximately 2,400-years-old Inca mummies show evidence of melanoma both in the skin and bone tissue (Urteaga and Pack 1966). Robert Carswell, in 1838, first employed the term “melanoma” to designate these pigmented malignant tumors (Urteaga and Pack 1966).

According to a WHO report, about 48,000 melanoma-related deaths occur worldwide per year. The incidence of melanoma varies about 40 fold around the world, with the highest rates in Australia (34/100,000 ASR World Population) and New Zealand (31.5/100,000 ASR World Population), and the lowest rate in Eastern Asia (0.3/100,000 ASR World Population) (The Cancer Council 2008).

Melanoma is one of the cancers with the fastest rate of increase among white people in Europe. Currently, in almost all European countries the incidence is higher in women than in men. Melanoma is the most fatal of skin cancers accounting for 79 % of all skin cancer deaths. The number of new melanomas diagnosed in Sweden was 2,333 in 2007 (Socialstyrelsen 2009). According to Euro Melanoma (www.euromelanoma.org), 24 new melanoma cases were diagnosed in Sweden on Melanoma Monday, May 2008. The incidence of melanoma is increasing with 2.4 % per year in Sweden. This is also the case in the southeast region of Sweden (www.lio.se/us/onkologisktcentrum).

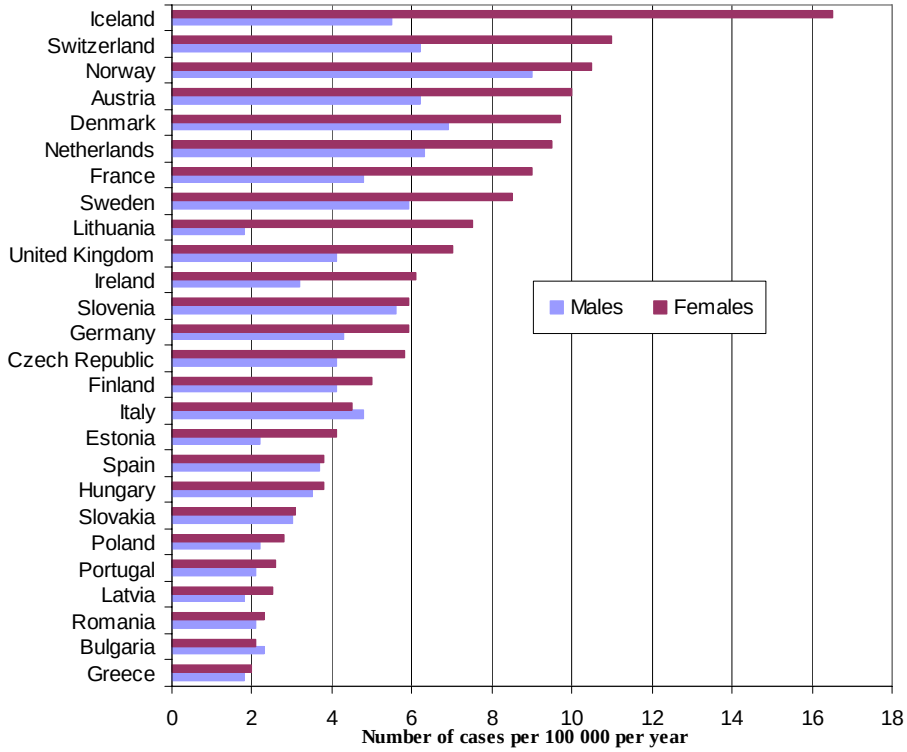


Fig. 3. *Age-standardized rates of melanoma incidence in people aged less than 55 years, selected European countries, 2002. Redrawn from Pirard et al. (Pirard and de Vries 2007)*

There is a strong correlation between the latitude of European regions and the incidence of melanoma in people aged less than 55 years (Fig. 3). The highest

incidences are found in northern (Denmark, Norway and Sweden) and western European countries (France, the Netherlands and the United Kingdom). The lowest incidence rates are found in southern Europe (Greece, Italy, Portugal and Spain) (Pirard and de Vries 2007). These variations are likely to be linked to specific behavior (winter holidays, sun-seeking behavior) as well as to improved detection of melanoma. The incidence of melanoma in white populations generally increases with decreasing latitude, with the highest recorded incidence occurring in Australia, where the annual rates are between 10 and over 20 times the rates in Europe. A large number of studies (Bliss et al. 1995; Elwood et al. 1990; Lock-Andersen et al. 1999; Veierød et al. 2003) indicate that the risk of melanoma correlates with genetic and personal characteristics, and a person's UV exposure behavior. The following is a summary of the main human risk factors:

1. A large number of atypical nevi (moles) is the strongest risk factor for malignant melanoma in fair-skinned populations (Pavel et al. 2004).
2. Melanoma is more common among people with a pale complexion, blue eyes, and red or fair hair (Veierød et al. 2003). Experimental studies have demonstrated a lower minimum erythema dose and more prolonged erythema in melanoma patients than in controls (Lock-Andersen et al. 1999).
3. High, intermittent exposure to solar UV radiation appears to be a significant risk factor for the development of cutaneous melanoma. Several epidemiological studies support a positive association with a history of sunburn, particularly sunburn at an early age (Elwood et al. 1990; Oliveria et al. 2006).

Diffuse melanosis

Diffuse melanosis is a rare condition which may occur in metastasizing melanoma and is associated with a very poor prognosis. The median survival after the appearance of diffuse melanosis is only about 6 months, with a range of a few days to 1 year (Gambichler et al. 2008). The process of diffuse melanosis may involve the total skin, mucous membranes and internal organs, resulting in a characteristic slate-gray discoloration. The color of the patients' urine is dark brown when freshly voided. The French physician Laënnec discussed the subject of melanoma, which he called "la mélanose," in 1806 (Urteaga and Pack 1966). In 1864 the German pathologist, Ernest Wagner, was the first to describe a 30-year-old patient with a melanoma arising in a congenital nevus who subsequently acquired a generalized bluish-gray discoloration (Böhm et al. 2001). Despite various explanations for the diffuse melanosis (Agrup et al. 1979; Konrad and Wolff 1974; Silberberg et al. 1968; Steiner et al. 1991; Tsukamoto et al. 1998), the process resulting in diffuse melanosis from metastatic melanoma is still unknown.

Melanin production—melanogenesis

Melanogenesis is usually described as two distinct pathways: pheomelanogenesis and eumelanogenesis. Both pheomelanin and eumelanin derive from dopaquinone, which is formed by tyrosinase hydroxylation and oxidation of L-tyrosine (Fig. 4). A number of reviews, books, and book chapters report the extensive research work on this topic (Nordlund et al. 2006; Prota 1992a; Simon et al. 2009).

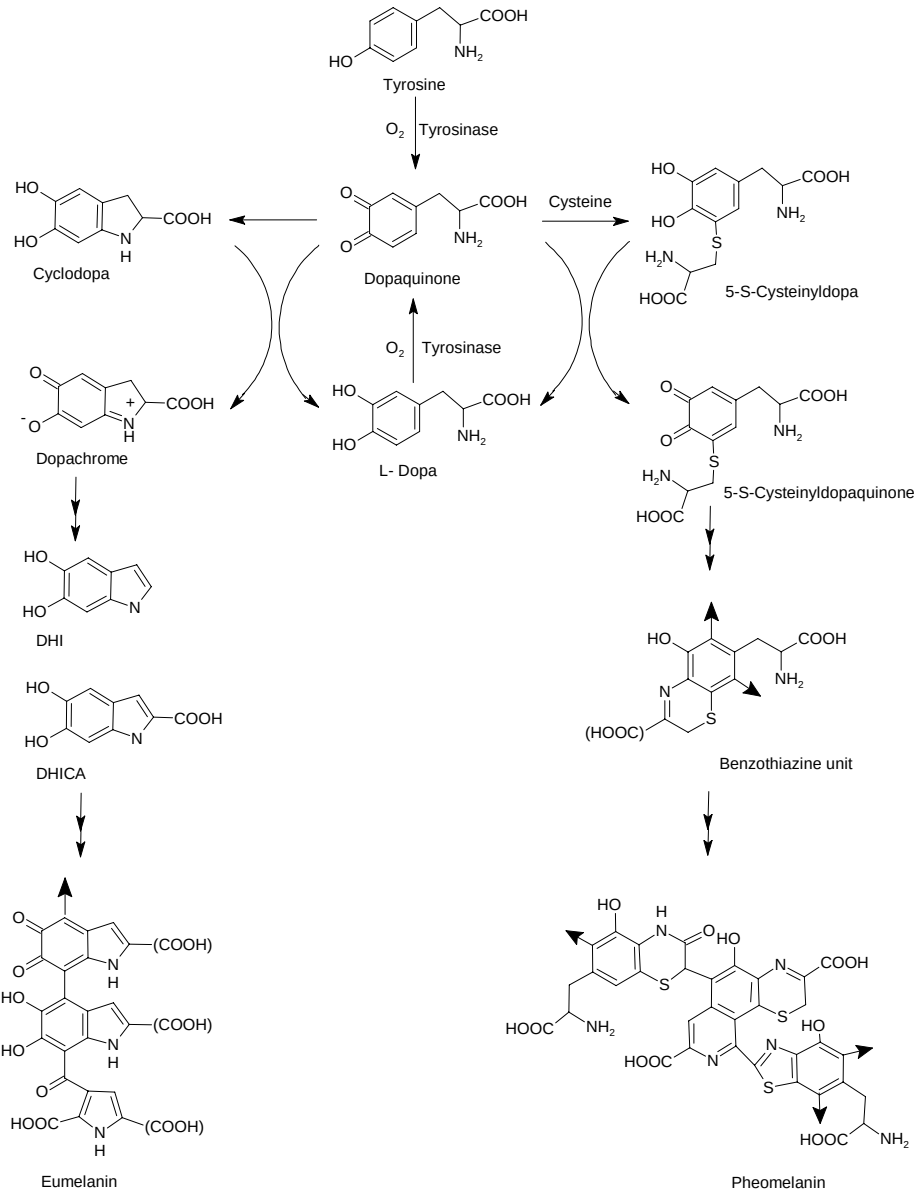


Fig. 4. *General outline of melanin formation (melanogenesis)*

Eumelanogenesis

Tyrosine metabolism in melanocytes can give rise to a broad range of products, which are continuously produced and excreted into body fluids in addition to their building up of melanins. Despite their different structures and properties, these ill-defined pigment structures can all be embraced by a biogenetic pathway in which dopaquinone is the crucial intermediate. Dopaquinone is a highly reactive intermediate, and in the absence of sulfhydryl compounds (thiols) it undergoes intramolecular cyclization to producing cyclodopa (Fig. 4). The cyclization reaction is a first-order reaction where in a nucleophilic addition the unpaired electrons of the nitrogen atom in the side-chain amino group complete the formation of a heterocyclic secondary ring. The redox exchange between cyclodopa and dopaquinone is a second-order reaction which gives dopachrome and L-dopa (Land et al. 2003). Rearrangement (decarboxylation and tautomerization) of dopachrome gives the dihydroxyindoles 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). These processes are supported by enzymatic activity of dopachrome tautomerase, also called Tyrp2 (Solano and Garcia-Borrón 2006). Both DHI and DHICA can be further oxidized to their respective quinones. The function of Tryp1 is still controversial, but it seems that Tyrp1 and tyrosinase are involved to various extend in different species in the next steps in eumelanogenesis (Solano and Garcia-Borrón 2006). Oxidations and polymerization of DHI and DHICA in various ratios lead to the formation of eumelanin (Ito 1986).

Pheomelanogenesis

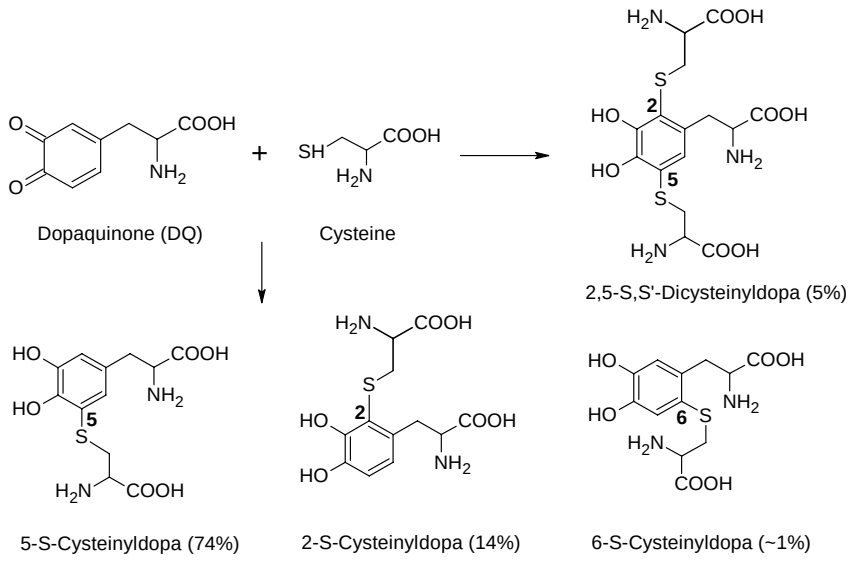
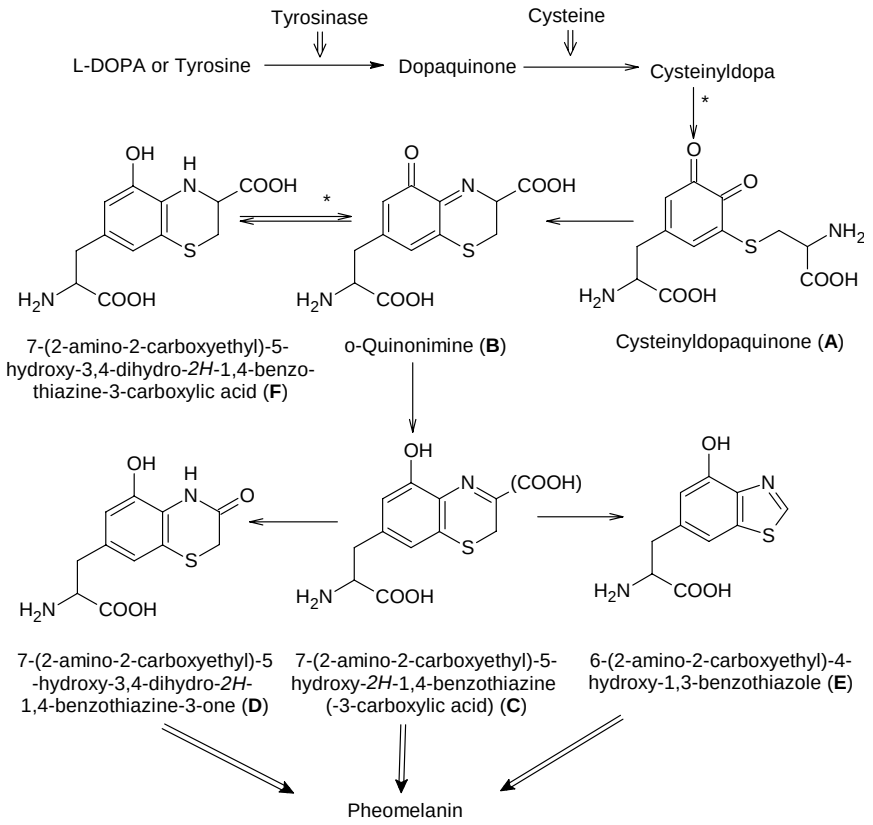


Fig. 5. *Formation of cysteinyldopa isomers*

The pheomelanogenesis pathway is quite different from eumelanogenesis and occurs when cysteine is present in the melanosomes (Potterf et al. 1999) (Fig. 4). Thus dopaquinone reacts with cysteine in a nucleophilic attack by the thiol group of the cysteine on the quinone (Huang et al. 1998). This reaction results in the formation of mainly 5-S-cysteinyldopa (5-S-CD) (74 %) with small amounts of the isomers 2-S-cysteinyldopa (2-S-CD) (14 %), 6-S-cysteinyldopa (6-S-CD) (~1 %) and 2,5-S,S'-dicysteinyldopa (5 %) (Ito and Prota 1977) (Fig. 5). Further oxidation of cysteinyldopa leads to the formation of cysteinyldopaquinone (**A**) which undergoes intramolecular cyclization via an attack by the cysteinyl side chain amino group on the carbonyl group to form a cyclic ortho-quinonimine (**B**) (Napolitano et al. 1994) (Fig. 4). Generation of the quinonimine represents a most critical event in the oxidation chemistry of cysteinyldopa and it is likely to control the further course of the reaction and the nature of the monomers that participate in the building up of the pheomelanin polymer (Napolitano et al. 1999).



* the place where redox exchange occurs

Fig. 6. *Benzothiazine pathway from 5-S-CD*

This quinonimine then tautomerizes, with or without decarboxylation, to the phenolic benzothiazine intermediates 7-(2-amino-2-carboxyethyl)-5-hydroxy-*2H*-1,4-benzothizine (**C**) and its 3-carboxy derivative 7-(2-amino-2-carboxyethyl)-5-hydroxy-*2H*-1,4-benzothizine-3-carboxylic acid (**C**), characteristic of pheomelanin formation (Fig. 6). Their ratio depends on many factors, including pH and metal ions (Di Donato et al. 2002; Napolitano et al. 2000a). Zn^{2+} promotes the retention of the carboxylic group (Greco et al. 2009a; Napolitano et al. 2001), whereas Fe^{3+} accelerates the ring contraction of benzothiazine to benzothiazole (Di Donato et al. 2002). Benzothiazine products (**C**) are unstable and decay over a few seconds and may lead to the formation of the 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-*2H*-1,4-benzothiazine-3-one (**D**) and 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole (**E**) (Fig. 6). By the redox exchange with cysteinyldopa, 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-*2H*-1,4-benzothiazine-3-carboxylic acid (**F**) can be formed.

Oxidation, cyclization, and dimerization of 5-S-CD and 2-S-CD lead to the formation of trichochromes (Napolitano et al. 2001; Prota 1992b), a class of molecules which are suggestive of pheomelanin. In the literature they first appeared under the name trichosiderin as early as 1879 (Prota 1980). The basic structural unit of trichochromes is a 1,4-benzothiazine ring system which can exist in 2H and 4H forms as well as in *cis* or *trans* forms (Fig. 7). Hence they could unambiguously be formulated as $\Delta^{2,2'}$-bi(*2H*-1,4-benzothiazines) (Di Donato and Napolitano 2003).

Early studies on trichochromes from red hair were reviewed by Thomson (Thomson 1974). Napolitano et al. (Napolitano et al. 2001) showed that trichochrome B and especially trichochrome C are the most abundant ones in red human hair. The trichochromes found in the urine of melanoma patients were trichochromes B and C (Agrup et al. 1978b). The origin of trichochrome C is benzothiazines derived from 5-S-CD and it is the main trichochrome excreted. Trichochromes E and F have been extracted from chicken feathers and hairs of several mammals (Simon et al. 2006), but have not been found in the urine of melanoma patients (Agrup et al. 1978b). In contrast to the latter, trichochromes B and C contain a carboxylic group at a benzothiazine nucleus which may account for their solubility and renal excretion. They are unusual molecules, insoluble in water (at neutral pH) and difficult to crystallize.

The studies performed by Simon et al. suggested that trichochromes may be photoprotective as no detectable or very low levels of ROS are produced by UV irradiation *in vitro* (Simon et al. 2006).

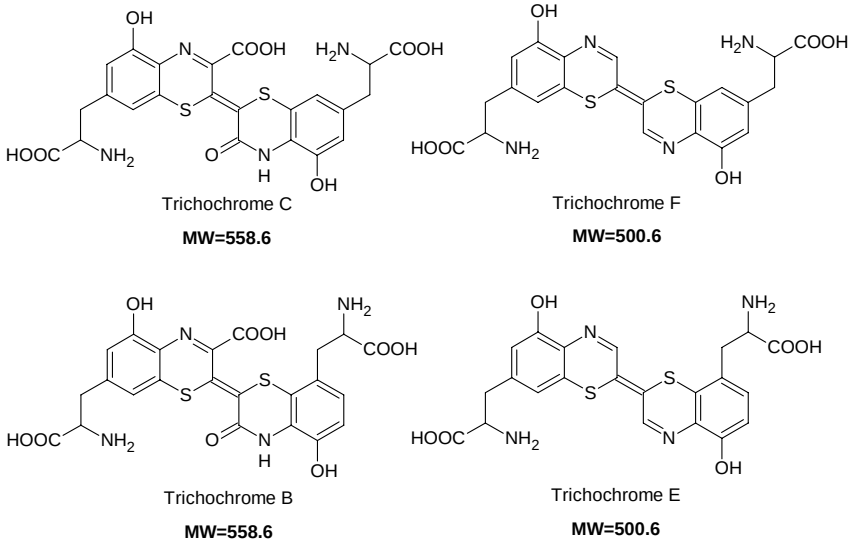


Fig. 7. *Trichochromes*

Tyrosinase activity seems to be the major factor controlling the course of melanogenesis. Ozeki et al. showed that pheomelanogenesis proceeds preferentially during the early phase of melanogenesis under condition of low tyrosinase activity and high cysteine concentration. Thus in mixed-mode melanogenesis, the switch from eumelanogenesis to pheomelanogenesis can bee achieved by lowering the tyrosinase activity (Ozeki et al. 1997). A wealth of information has been collected in recent years regarding genetic factors that influence the switch between eumelanogenesis and pheomelanogenesis. The connection between melanocortin 1 receptor (MC1R) and melanogenesis has recently been reviewed (Meyle and Guldberg 2009).

Land and Riley et al. studied melanogenesis using pulse radiolysis (Land et al. 2003; Land et al. 2001; Land and Riley 2000). Their studies indicate that the pheomelanin pathway predominates in the presence of cysteine and that early melanogenesis proceeds in three distinctive steps (Ito 2003; Wakamatsu et al. 2009):

1. Production of cysteinyldopa caused by a reaction between dopaquinone and cysteine, which continues as long as cysteine is present.
2. Oxidation of cysteinyldopa to cysteinyldopaquinone and benzothiazine moieties to give pheomelanin (continues as long as cysteinyldopa is present).
3. Production of eumelanins, which increases after most of the cysteinyldopa (and cysteine) is consumed.

Kinetic data show that the second step in pheomelanogenesis, the production of cysteinyldopaquinone, is slower than the production of cysteinyldopa. Therefore

cysteinyldopa isomers accumulate during the early phase of pheomelanogenesis (Wakamatsu et al. 2009). Thus it appears that the pheomelanin to eumelanin ratio can be a useful tool to study the melanin structure, the characters of the melanin defects and the genesis of diseases such as melanoma. Melanocytes producing high levels of eumelanin pigments appear to be able to afford protection against the damaging effects of UV radiation, whereas those producing high levels of pheomelanin, but little eumelanin, are usually found in skins with high UV light sensitivity. The susceptibility of fair-skinned individuals to developing melanoma indicates that this cancer results from the effect of UV radiation on pheomelanin substituents or precursors (Meyskens et al. 2001; Wolber et al. 2008). As a result we have concentrated our studies on this type of melanin.

Pigment analysis

Melanins are unique biopolymers with a high heterogeneity in their structural features. Under the microscope, melanin is brown, non-refractive and finely granular. The exact structures of the melanins are not known. Melanins from different sources (human hair, *Sepia officinalis*, commercially available eumelanin, melanin obtained by auto-oxidation of dopa, tyrosinase-enzymatic produced melanin, melanin from feathers of Rhode Island chicken and bacterial melanin) have been studied and significantly different composition of amino acids, C/N ratios and empirical formulas were found (Chedekel et al. 1992).

Lack of suitable methods for isolation and purification of natural melanin pigments is a real problem in structure studies. Major problems in the study of natural melanins are the lack of adequate methods to isolate pure melanins from biological material, the insolubility of the melanins over a broad pH range, and the effects of protein matrix. Many isolation methods affect the chemical structure of the pigment to be studied (Liu et al. 2003). Natural melanins are composed of two distinct portions, a protein fraction and a chromophoric backbone. The bonding between these two parts is still unknown and standard protocols used in protein purification fail to separate them. Many researchers (for review see (Ito 1998)) have used harsh isolation and purification agents which degrade and damage the chromophoric (melanin) part.

Liu et al. studied the structural and chemical properties of eumelanosomes and pheomelanosomes from human hair (Liu et al. 2005). Morphologic analysis of the surface of melanosomes showed that eumelanosomes are ellipsoid whereas pheomelanosomes are smaller and both spherical and ellipsoid. Furthermore, eumelanosomes maintain structural integrity during isolation from hair but pheomelanosomes tend to fall apart. However, synthetic models of eumelanin and pheomelanin are so far the best source for structural studies of these biopolymers.

Synthetic eumelanins can be prepared by oxidation of L-tyrosine or L-dopa at neutral pH in the presence of mushroom tyrosinase (Ito 1986). The most frequently used source of natural eumelanins is ink sacs of the cuttlefish, *Sepia officinalis*. Biosynthetic and degradative studies of natural and synthetic eumelanins indicate that eumelanins are highly heterogeneous polymers consisting of various monomer units, including DHI, DHICA and pyrrole units (Ito and Wakamatsu 2006; Prota et al. 1998; Wakamatsu and Ito 2001). Eumelanin is insoluble in both acidic and alkaline solutions, and contains nitrogen but not sulfur (Ito and Jimbow 1983; Ito and Wakamatsu 2006).

Synthetic pheomelanins are prepared by tyrosinase oxidation of either a mixture of L-dopa and L-cysteine or by oxidation of 5-S-CD in the presence of a catalytic amount of L-dopa (Ito 1989). Gallopheomelanin-1, the major protein-free pheomelanin pigment isolated from the red feathers of New Hampshire chickens, has been commonly used as a source of natural pheomelanin. The representative structure components of pheomelanin are benzothiazine, benzothiazole and isoquinoline units (Prota et al. 1998). Pheomelanins are soluble in alkaline solution and contain both nitrogen and sulfur (Ito and Jimbow 1983; Ito and Wakamatsu 2006). Under electron microscope the pheomelanin pigment exhibits a largely amorphous structure with deposits of various size and shape (Ye et al. 2008).

Pyrrole-2,3,5-tricarboxylic acid (PTCA) was identified as the main marker of eumelanin by Panizzi and Nicolaus in 1952 (Prota et al. 1998). Chemical degradation of eumelanin by permanganate or peroxide oxidation of eumelanin gives pyrrole-2,3,5-tricarboxylic acid (PTCA) and trace amounts of pyrrole-2,3-dicarboxylic acid (PDCA). PTCA, and PDCA are products arising from DHICA- and DHI-derived units respectively (Ito and Wakamatsu 1998) (Fig. 8).

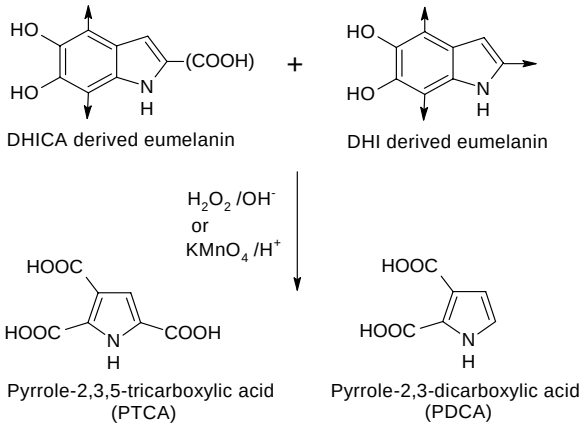


Fig. 8. *Chemical degradation of eumelanin*

The sulfur-carbon bonds in benzothiazine units of pheomelanins derived from 5-S-CD and 2-S-CD can be hydrolyzed by hydriodic acid. This results in the production of two isomers, 4-amino-3-hydroxyphenylalanine (4-AHP) and 3-amino-4-hydroxyphenylalanine (3-AHP), respectively, as the final products (Fig. 9). These compounds were first identified by Minale 1967 (Prota et al. 1998).

Besides PTCA and 4-AHP, several other degradation products are useful in characterizing various types of melanin (Napolitano et al. 2000b; Wakamatsu et al. 2003a; Wakamatsu and Ito 2002). These products include thiazole-2,4,5-tricarboxylic acid (TTCA) (Napolitano et al. 2000b), thiazole-2,4-dicarboxylic acid (TDCA) (Kongshoj et al. 2006; Wakamatsu et al. 2003a), and 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole (HBTA) (Chedekel et al. 1987; Ismail et al. 1980) as well as its carboxylic acid (BTCA) (Napolitano et al. 2008; Napolitano et al. 1996; Napolitano et al. 2000b). These markers of pheomelanin pigments are obtained by alkaline hydrogen peroxide oxidation of pheomelanin (Fig. 9).

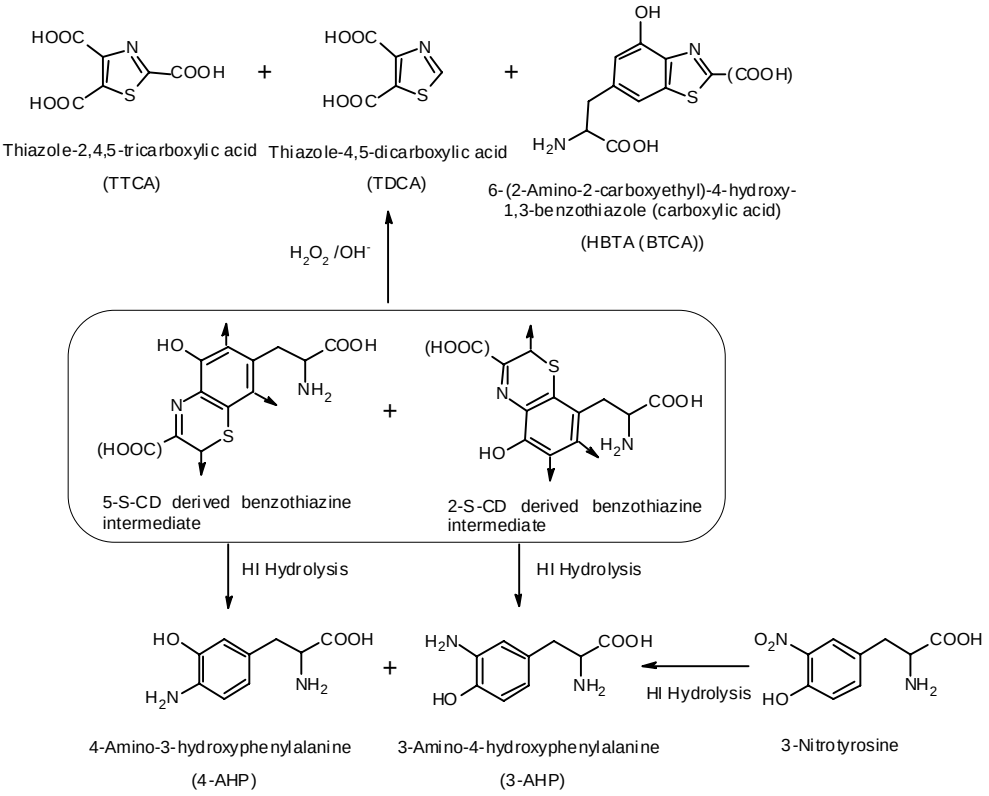


Fig. 9. *Chemical degradation of pheomelanin*

ANALYTICAL TECHNIQUES

Degradation and intermediate products of melanins have often been determined by high-performance liquid chromatography (HPLC). One of the earliest methods for analysis of cysteinyldopa as an intermediate pigment product was reported by Hanson et al. (Hansson et al. 1978). Ito and Jimbow were the first to report a chromatographic method for analysis of chemically degraded pigment (Ito and Jimbow 1983).

A large number of techniques and methods have been used for qualitative and quantitative studies of melanin pigment. The main goal of the quantitative studies is to prove with acceptable probability that for an unknown constituent in a sample there is only one compound to be considered. In quantitative analysis the analytes of interests are known. Their physical and chemical property can be measured, related to its concentration, and used for quantification. In qualitative studies, however, the interpretation of unique spectral data obtained by advanced techniques, such as mass-spectrometry, leads to the identification of unknown compounds.

The following is a brief overview of the chromatographic techniques used in analytical methodologies in the present thesis.

Solid phase extraction

In connection with the present thesis, several approaches to sample preparation have been tested and evaluated. Sample isolation from the matrix is a critical point of the analysis. Matrix macromolecules and impurities can interfere with the analytical separation or cause a loss of capacity due to reduced mass transfer between the mobile phase and the stationary phase. Therefore, they have to be removed from the sample prior to analysis. Solid phase extraction (SPE) has been of particular interest and is described in more detail. The chemical properties of the analytes determine which extraction procedure will be the most efficient. General mechanisms for extraction are polar/non-polar partition interactions and ion exchange. The key element to any SPE is the sorbent, which is packed into a syringe barrel (Fig 10). The most popular SPE sorbents are chemically modified silica particles with functional groups covalently attached to the surface or polymeric sorbents. Retention mechanism on sorbents can be divided into four main categories:

1. Reversed-phase SPE, which involves non-polar sorbents and polar liquid phases. Here, the hydrophilic silanol groups at the surface of the raw silica packing have been chemically modified with hydrophilic alkyl or aryl functional groups (**Paper IV**).
2. Normal-phase SPE, which involves polar modified sorbents and non-polar liquid phases. Polar-functionalized bonded silicas are used.

3. Ion exchanger. Anionic (negatively charged) compounds can be isolated on strong anion exchanger (SAX) sorbents where aliphatic quaternary amine groups or aliphatic aminopropyl groups (weak anion exchanger, WAX) are bound to the silica surface. Likewise cationic (positively charged) compounds can be isolated by using strong cation exchanger (SCX) sorbents where strongly acidic aliphatic sulfonic groups are bound to the surface. Weak cation exchanger (WCX) contains an aliphatic carboxylic group that is bound to silica surface. In **Paper II** we used SCX sorbents.
4. Adsorption on unmodified materials such as alumina.

An SPE procedure normally consists of five basic steps: conditioning, equilibration, sample loading, washing and elution (Fig. 10).

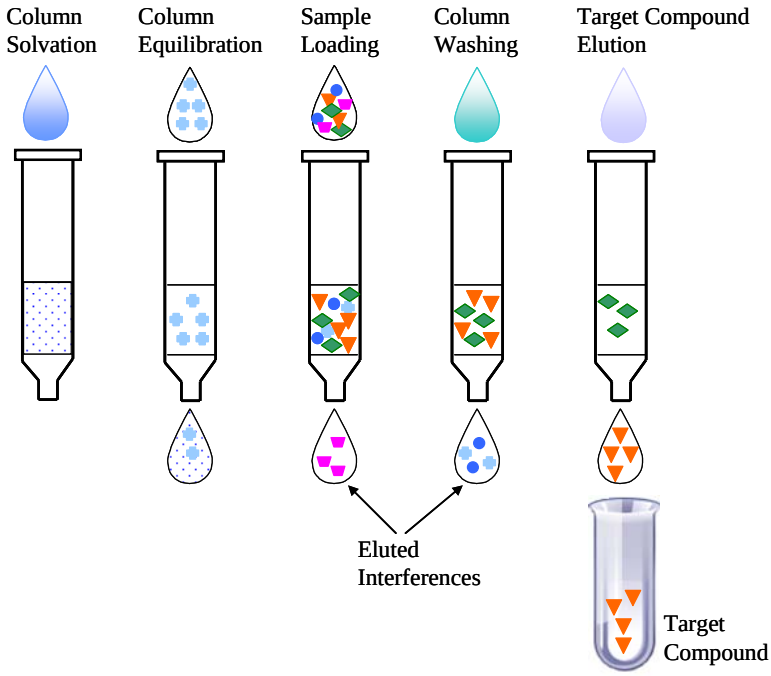


Fig. 10. *Schematic view of solid phase extraction procedure*

The solvent bed is rinsed with organic solvent to remove trapped air and prepare the chromatographic ligands for interaction with the sample. This activating solvent is then replaced by an equilibration solvent with composition similar to the diluted sample matrix. Sample preparation includes sample dilution with a “weak” solvent and pH adjustment to enhance the interaction between the analyte and the sorbent, and makes analytes or matrix components suitable for extraction. After applying the sample, retained contaminants are removed with a relatively “weak” solvent that is

not strong enough to disrupt the sorbent-analyte interactions. Before elution the sorbent can be dried to insure that all traces of wash solvent and contaminants are removed. The analytes are then eluted by adding a "strong" solvent designed to disrupt the interaction between the sorbent and the analyte.

Derivatization

To prevent unwanted interaction of functional groups with polar sites in the chromatography system, these groups have to be derivatized i.e. transformed into more neutral groups. Derivatization means that a non-polar chemical group is covalently attached to the acid. This also greatly affects the volatility and other properties of the compound. Various derivatization reagents are available to make active groups amenable for GC or sometimes for HPLC. Classically, silylation is a widely used derivatization approach, but the procedure requires strictly anhydrous conditions, sample heating and pre-treatment. In the 1990s, alkyl chloroformates were discovered as potential reagents (Hušek 1991). These do not require the exclusion of water; analytes can be directly treated in an aqueous matrix. Isolation of analytes from a matrix is not necessary. Hušek introduced alkyl chloroformates as general derivatizing reagents in GC (Hušek 1998; Hušek 2006). Single-step derivatization of amino acids is based on an aqueous amino acid solution treated in alkyl chloroformate and alcohol with pyridine (Guo et al. 2007; Hušek 2005; Namera et al. 2002; Wang et al. 1994; Zampolli et al. 2007). The reaction at room temperature is fast, the derivatives show good resolution with a few minutes retention time, and the method involves low reagent and instrument costs. **Paper III** reports a novel method for the study of N,O-alkoxycarbonyl-alkyl esters of a family of degradation products of pheomelanin.

Chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) whereas the other (mobile phase) moves in a definite direction.

High performance liquid chromatography

In high performance liquid chromatography (HPLC), the sample solved in a suitable solvent is forced through a column that is packed with irregularly or spherically shaped particles with very small diameters of 1-10 µm, or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. The column has to be able to withstand the high pressures that are applied, and must also be resistant to chemical degradation by the applied mobile phases. Smaller particles increase the stationary phase/mobile phase ratio, which increases the time spent by the analyte in

the stationary phase, which in turn enhances the separation of the peaks. The mobile phase can be water, buffers or organic solvents used separately or mixed.

HPLC is divided into sub-classes based on the polarity of the mobile and stationary phases. The main categories of HPLC are:

1. Partition chromatography. The first chromatography technique was developed by Archer John Porter Martin and Richard Laurence Millington Synge, who were awarded the Nobel Prize in chemistry in 1952 for their development of this technique which they used to separate amino acids. The separation of analytes is based mainly on differences between the solubilities of the components in the mobile and stationary phases. Molecules equilibrate (partition) between the stationary phase and the mobile phase, and separation is based on polar differences. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase, and liquid-liquid applications. It is also known as hydrophilic interaction liquid chromatography (HILIC).
2. Normal-phase chromatography (NP-HPLC) is also known as adsorption chromatography. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents.
3. Reverse-Phase Chromatography (RP-HPLC) was developed in the early 1970s. Because of better reproducibility of retention time and stability of the stationary phases it has replaced partition chromatography, HILIC as well as NP-HPLC. RP-HPLC uses non-polar stationary phase and an aqueous, moderately polar mobile phase. With these stationary phases, retention time is longer for molecules which are more non-polar, whereas polar molecules elute more readily. RP stationary phase operates on the principle of hydrophobic forces, which originate from the high symmetry in the dipolar water structure. Mobile phase condition, buffer concentration, choice of buffers, pH, amounts of organic phases, ion-pairing reagents, temperature, flow rate etc. affect separation and resolution as well. Traditionally RP-HPLC has been used for chromatography of the degradation products of melanin. We used this technique in **Paper I.**
4. Size exclusion chromatography, also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of molecular size.
5. Ion exchange chromatography retains analyte molecules based on ionic interactions. The stationary phase surface contains ionic functional groups that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. Cation exchange chromatography retains positively charged

cations because the stationary phase contains a negatively charged functional group whereas anion exchange chromatography retains anions using a positively charged functional group.

After passing through the column, the separated analytes are perceived by an in-line detector, and the resulting output in the form of electrical signals can be visualized in a chromatogram. A typical HPLC system is shown in Fig. 11. Most of the detectors are selective, which means that they respond to only certain compounds in the sample due to their unique characteristic. Detectors that have been used in the present thesis are UV-detector, electro chemical detector (ECD), photodiode array detector (PDA) and mass-spectrometric detector (MSD).

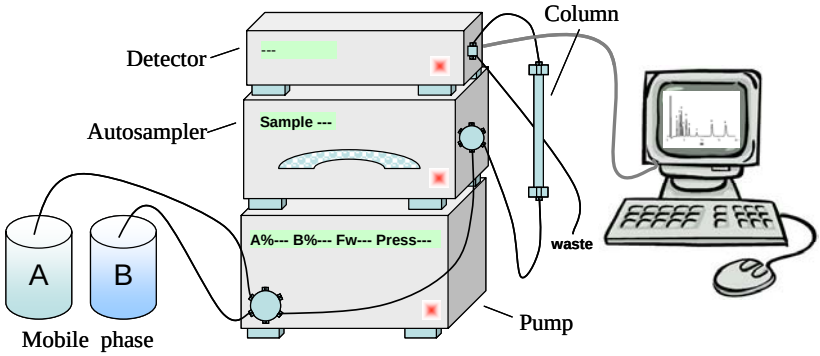


Fig. 11. *Typical scheme of an HPLC system*

Gas chromatography

In gas chromatography (GC), separations are achieved by partitioning of the solutes between an immobile solid or liquid stationary phase and a gas phase that percolates over the stationary phase. A suitable choice of stationary phase is a basic condition for obtaining separation of analytes, but several other parameters are important to the successful development of chromatographic methods. The temperature dependence of molecular adsorption and of the rate of progression along the column influences the level of separation. This is referred to as a temperature program. Electronic pressure control can also be used to modify flow rate during the analysis, aiding in faster run times while retaining acceptable levels of separation. The choice of carrier gas (mobile phase) is important. Although hydrogen is most efficient and provides the best separation, helium is the most common carrier gas used because it has the advantage to be non-flammable and works with a greater number of detectors. We used this technique coupled to a mass selective detector (Fig. 12) when we studied reduction products of pheomelanin in **Paper III**.

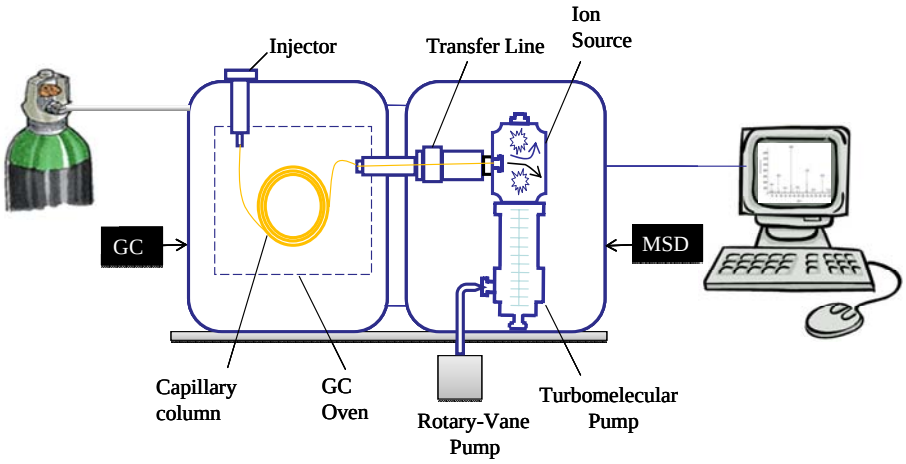


Fig. 12. *Typical scheme of a GC/MS system*

Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a well-adapted technique for separation of polar and hydrophilic compounds (Guo and Gaiki 2005). HILIC has similarities with partition and normal-phase chromatography with regard to the nature of the stationary phase. Recently it has become useful again with the development of HILIC bonded phases, which improve reproducibility (Appelblad et al. 2008; Hemström and Irgum 2006). Typical stationary phases are silica or polymer particles carrying polar functional groups (e.g. amino, amide, or zwitterionic groups).

The eluents, on the other hand, are similar to those known from reversed-phase chromatography, but solvent proportions likewise are the opposite. The weakest solvent for HILIC chromatography, acetonitrile, provides a higher increase in retention compared with methanol or water. Typical mobile-phase eluents consist of acetonitrile with low water or non-volatile buffer content and would therefore be appropriate as a liquid chromatography-mass spectrometry friendly technique.

The HILIC technique separates compounds by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity. The analyte is distributed between the water-rich stationary layer and the mobile phase with low water content (Fig. 13). More polar compounds will have a higher affinity to the stationary aqueous layer than less polar compounds. Retention is also influenced by electrostatic (ionic) interaction between the stationary phase and the analytes. The non-volatile salts such as formiate and acetate can be required in the mobile phase to disrupt these interactions for efficient analyte elution. HILIC was the key tool in the present thesis. We applied this chromatographic separation technique in **Paper I** and **Paper IV.**

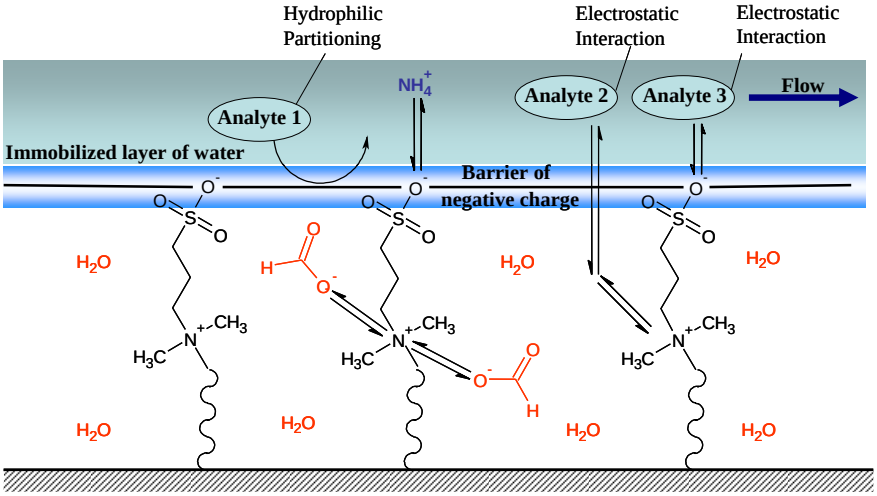


Fig. 13. *The retention processes in ZIC-HILIC illustrated by hydrophilic partitioning and electrostatic interactions with either positive or negative charges*

Detection

Electrochemical Detection

An electrochemical detector (ECD) is sensitive only to electroactive compounds and this distinguishes ECD from most other detection techniques where detection is based on the physical properties of an analyte (i.e. molecular mass and molar absorbance). The electrochemical detector responds to substances that are either oxidizable or reducible, and the electrical output is an electron flow generated by a reaction that takes place on the surface of the electrodes. If the reaction proceeds to completion (exhausting all the reactants) the current becomes zero and the total charge generated will be proportional to the total mass of material that has been reacted. The potential difference supplies the energy level needed to initiate or enhance the electrochemical reaction. Different analytes may have different oxidation or reduction potentials, which determines the specificity of ECD. Analyses of aminohydroxyphenylalanines with ECD are described in **Paper I** and **Paper II.**

UV, UV/vis and PDA Detection

UV detector functions on the capacity of compounds to absorb light in the wavelength range 180 to 350 nm (180-820 nm in the case of UV-vis lamp). Light from a UV light source passes through the sensor onto a flow cell (Fig. 14a). By interposing a monochromator between the light source and the cell, light of a specific wavelength can be selected for detection and thus improve the detector

selectivity. A UV detector is only selective in the sense that all solutes that absorb UV (or visible) light can be detected. The UV detector has wide applicability and can be used for general detection but it does not give any structural information about unknown compounds.

PDA detectors provide specific spectral data that describe properties of unknown compounds. The PDA detector is utilized with a deuterium or xenon lamp that emits UV light over the UV and part of the visible spectral range. The array consists of hundreds or thousands of photodiodes arranged as a one-dimensional array. Each photodiode acts as a capacitor by holding a fixed amount of charge. The detector measures the amount of current required to recharge each photodiode. Fig. 14b shows a schematic diagram of a diode array detector.

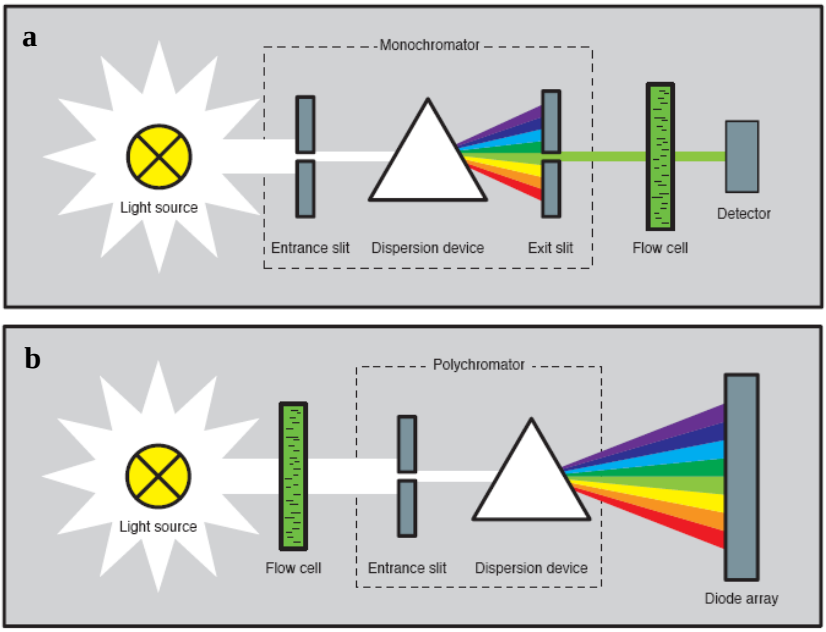


Fig. 14. *Schematic view of UV and PDA Detection principles*

Polychromatic light from a source is passed through the sample area and focused on the entrance slit of the polychromator. The polychromator disperses the light onto a diode-array, and each diode measures a narrow band of the spectrum. Each diode performs the same function as the exit slit of a monochromator (Fig. 14a). The detector computes absorbance by subtracting the dark current (loss of charge when the photodiodes are not exposed to light) and reference spectrum (measure of lamp intensity and mobile phase absorbance before any components are eluted) from the acquired spectrum.

Absorbance is based on the principles of Beer-Lambert Low:

$$A = \varepsilon c d$$

Where:
A= absorbance
ε= molar extinction coefficient, L/mol cm
c= molar concentration, mol/L
d= length of the flow cell, cm

The great advantage of the PDA detector over the UV detector is that it takes the UV spectrum of the eluent continuously. This can be used when the separation of two peaks is not successful. The ratio of absorptions at two wavelengths can show whether studied peaks contain impurities. A chromatogram can be reconstructed by monitoring at a specific wavelength, depicting only those substances that absorb UV light at the chosen wavelength. We used this technique in **Paper IV**.

Mass Spectrometry

Mass Spectrometry (MS) is an analytical tool for measuring the molecular mass of a sample. In MS, ions are monitored according to their mass over charge ratio (m/z). A mass spectrometer works with electrically charged compounds. Before a mass spectrum can be obtained, the substances to be analyzed must be ionized if they are not already ionic. In all MS techniques, the mass separator works at very low pressure, close to vacuum.

MS detectors can be divided into three fundamental parts: the ionization source, the analyzer and the detector.

The sample can be inserted directly into the ionization source, or can undergo some type of chromatography before it reaches the detector. In the latter case the sample introduction usually involves the mass spectrometer being coupled directly to a high pressure liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) separation column.

The commonest liquid-chromatography mass spectrometry technique used for most biochemical analysis is Electrospray Ionization (ESI) (Fig. 15). The mobile phase matrix must be eliminated and analytes must be presented as ions in a gas phase before entering the mass separator device. During standard ESI the sample is dissolved in a polar, volatile solvent and pumped through a narrow stainless steel capillary at low flow rate. A high voltage is applied to the tip of the capillary. As a consequence of this strong electric field, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets. This process is aided by co-axially introduced nebulizing gas flowing around the outside of the capillary. This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip toward the mass spectrometer. The charged droplets diminish in size by solvent

evaporation, assisted by a warm flow of nitrogen known as the drying gas. The charged sample ions free from solvent are released from the droplets. Some of them pass through a sampling cone into an intermediate vacuum region and from there through a small aperture into the analyzer of the MS, which is held under high vacuum. All these parameters were studied during method development and experiments done to study the structures of degradation products of pheomelanin (**Paper II**).

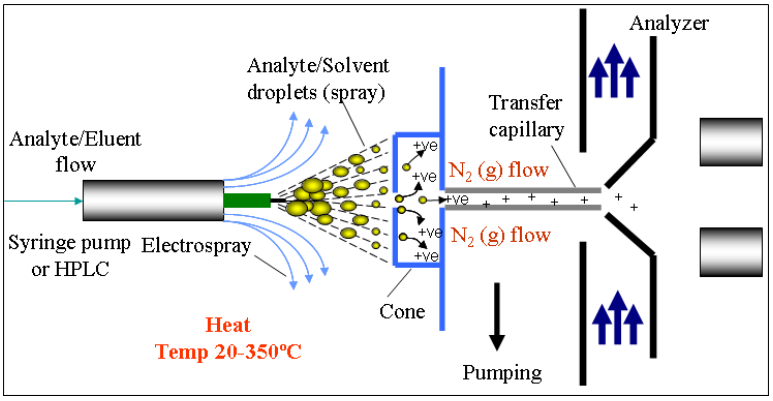


Fig. 15 *Schematic view of Electrospray Ionization MS*

The most common type of mass spectrometer associated with a gas chromatograph (GC) is the quadrupole mass spectrometer, sometimes referred to as a Mass Selective Detector (MSD) (Fig. 16). After passing through the GC, the chemical pulses continue to the MS. The molecules are blasted with electrons, which cause them to break into pieces and turn into positively charged particles, ions. A detector counts the number of ions with a specific mass. This information is sent to a computer and a mass spectrum is created. We used this technique in **Paper III** (Fig. 12).

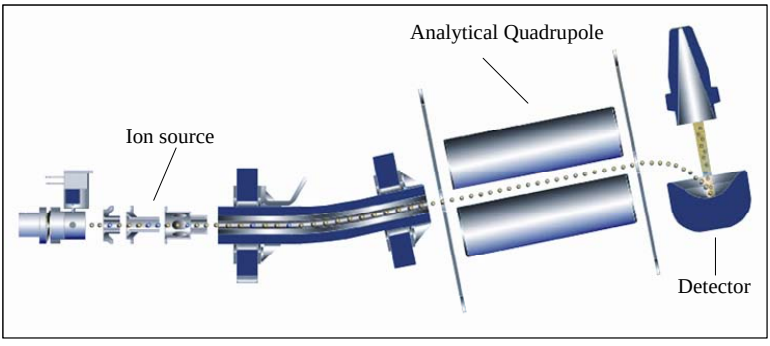


Fig. 16. *Schematic view of a quadrupole MS*

HYPOTHESIS

The main focus of the present thesis was to develop methods for studying the degradation product of melanin pigments. Pheomelanin is widely thought to be related to the susceptibility of melanocytes to the harmful effects of UV radiation. Human subjects with pheomelanin type of skin pigment are more prone to develop melanoma than subjects with eumelanin skin type (Kadekaro et al. 2003; Thody et al. 1991). Whether this is the result of changes in the synthesis or break-down in melanosomes or a combination of both is not clear yet. Whatever the case, quantitation and structure analysis of pheomelanin are therefore of importance to understanding the development of melanoma. It is also reasonable to speculate that specific molecules contained in pheomelanin, but not presented in eumelanin, may contribute to the increased likelihood of developing melanoma (Meyskens et al. 2001).

Earlier findings showed that metastatic melanoma can be diagnosed and followed up by measuring 5-S-CD in urine (Kågedal and Pettersson 1983; Kärnell et al. 2000; Wakamatsu et al. 2002b). The concentration of 5-S-CD varies over the year and is dependent on sun exposure (Stierner et al. 1988). Hence, 5-S-CD is not an ideal marker for following melanoma progression.

Some other markers such as AHP or pyrrole, thiazole and benzothiazole carboxylic acids had not been analyzed in urine before our studies started.

The main purpose of this thesis was to determine, either by analyzing known markers or finding new markers, whether there are melanin pigment structures in the urine and whether such structures increase in the urine of patients with metastatic melanoma.

This hypothesis also includes the notion that there are other degradation products of melanin that have not yet been identified with the classical chromatographic techniques. To test this hypothesis we synthesized pheomelanin and compared the degradation products of pheomelanin with those from urine, hair, melanocytes etc. The degradation products have been analyzed with HPLC and GC coupled to different detections techniques such as electro-chemical, photo-diode array and mass spectrometry.

AIMS

The general aim of the present work was to elucidate the chemical properties and biological relevance of melanin markers in different natural samples. We also proposed to develop and validate bioanalytical methods to shed light on the structure of pigments released into the urine of patients with disseminated melanoma.

Throughout the present thesis, my intention was to answer the following questions:

1. Can we identify pigment structures in the urine of patients with melanoma?
2. Can we identify new markers of melanin with the help of chromatographic techniques?
3. What is the molecular and macromolecular structure of melanin and how is it related to melanoma progression?
4. Can we determine melanoma type by using those new analyses?

STUDY DESIGN

In order to address the paradigm that people who have a high ratio of pheomelanin to eumelanin are at higher risk of developing melanoma, we designed a non-randomized study, which implies analytical quasi-experimental design and method development. Because no clinical trial was planned to be performed in the present study, the samples for analysis of melanin content in urine and hair were obtained without randomization or blinding when the volunteers or patients had been chosen.

The excretion of 5-S-CD in the urine of the patients was abnormally high, which means that pigment was probably excreted in high concentration too. This was the incentive for measuring pheomelanin degradation products in **Paper I** and **Paper IV.** Patients in **Paper II** and **Paper III** were chosen according to the condition of their disease, e.g. high pigment exertion, which provides good biological material for method development.

The urine and hair of healthy subjects with different hair colors and skin types was also collected without randomization or blinding. To avoid measurement bias, the influence of pigment leakage in urine was investigated by analysis of the urine of people with different hair color and skin type. The samples were categorized to three groups as blond, red and black hair.

The study design was basic biochemical research on melanin pigment in various biological situations. This design was chosen as no research on degradation of melanin in urine had been conducted earlier.

Specimens and pretreatments

Urine

Urine was obtained from healthy subjects with different hair colors and from patients with melanoma. Most of the urine samples from melanoma patients were obtained from the routine analysis for clinical diagnosis. In selected cases we obtained urine samples from patients specified as having melanosis from melanoma. These patients had earlier been operated for melanoma and had developed melanoma metastases and melanosis. Specimens, collected for 24 h in plastic bottles containing 35 ml concentrated acetic acid, were aliquoted and stored at -20°C. After thawing, the aliquots of urine required for further analysis were centrifuged at 3000 rpm for 10 min at 15 ºC or just mixed before analysis. The urinary sediment from 10 ml urine was washed twice with 5 ml deionized water, centrifuged, and taken to oxidation.

Hair samples

To evaluate different kinds of melanins in hair samples we proposed that 20 samples of human hair colored blond, red, or black be analyzed for pheomelanin (AHP) and eumelanin (PTCA).

Human samples were collected from 20-40-year-old volunteers of different ethnical backgrounds. The hair colors were classified according to Schwarzkopf's hair color card.

A hair sample was cut from the vortex of the skull. The hair was washed once with isopropanol and once with isopropanol:water 2:1 (v/v) to remove grease and hair treatment chemicals. Homogenization was performed in a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen Germany). The hair was cut into small pieces, and a weighted amount (10-15 mg) was added to a shaking flask containing a steel grinding ball. The sample was frozen at –70 °C for 30 min. Homogenization was performed at 3000 rpm for 1 min. The sample was refrozen and the process was repeated another three times. Then water was added to the vial to bring the hair concentration up to 10 mg/ml.

Melanoma tissue and melanoma cells

Melanoma tissue and melanoma cells were added to lysis buffer (100 mM Tris-HCl pH 7.6, 500 mM LiCl, 10 mM EDTA, 5 mM DTT, 1 % SDS) to give a concentration of 50 mg/ml. The tissue was homogenized using the Mikro-Dismembrator S at 3000 rpm for 1 min. Melanoma cells were homogenized in water in the similar way at a concentration of 10^7 cells/ml. We chose two types of melanoma cells: FM55.P and SK-Mel 28. The first showed marked pigmentation in earlier studies, whereas the second was weakly pigmented and contained lower amounts of pheomelanin (Johansson et al. 2002).

Synthetic pheomelanin

Synthetic pheomelanin was prepared by oxidation of L-dopa with cysteine, following the standard procedure (Ito 1989) by which 1.0 mmol of L-dopa and 1.5 mmol of L-cysteine were dissolved in 100 ml of 0.05 M sodium phosphate buffer, pH 6.8. Pheomelanin was synthesized by incubating with mushroom tyrosinase, and the resulting pheomelanin was centrifuged, dried and stored in a desiccator at ambient temperature.

Methods

The samples were hydrolyzed and oxidized as described earlier (Panzella et al. 2007; Takasaki et al. 2003) and by applying different chromatographic techniques such as: solid-phase extraction (with strong cation exchanger), gas chromatography,

preparative chromatography, RP-HPLC, and HILIC. They were then analyzed for the content of degradation products. In **Paper IV** we analyzed urine samples without degradation of the pigment and compared the result with that obtained after degradation. The contents were identified using the following detectors: electrochemical (**Paper I** and **Paper II**), MSD (**Paper II** and **Paper III**) and PDA (**Paper IV**). A short review of the analytical and technical details of methods applied in **Papers I-IV** is given below.

Hydriodic reduction

Urine (100 µl) or homogenate (**Paper I and Paper III**) or 200 µl urine (**Paper II**) was mixed with 30 µl 50 % H_3PO_2 and 500 µl of 57 % HI in a glass screw-capped tube and hydrolyzed at 130 ºC for 16 h. After cooling to room temperature 600 µl of 6 M hydrochloric acid (HCl) was added to the final volume, slightly modified from Ito et al. (Ito and Fujita 1985).

Oxidation procedure

We optimized the method described by Panzella et al. (Panzella et al. 2007) to match our conditions. NaOH was added to 5 ml urine to give a concentration of 1 M. Urinary sediment or synthetic pheomelanin was dissolved in 1 ml of 1 M NaOH. The samples were treated with H_2O_2 to give a concentration of 1.5 % and were vigorously stirred at room temperature. After 24 h the mixture was treated with 5 % $Na_2S_2O_5$ after which 85 % H_3PO_4 was added to pH 4.

Chromatography conditions

Paper I

After hydrolysis the samples were evaporated and the residues were dissolved in 990 µl 0.1 M HCl. To determine 4-AHP and 3-AHP, 10 µl of the samples were taken to reversed-phase HPLC. A Nova-Pack C18 column (3.9 x 150 mm, 4 µm particle size) was used with isocratic condition of the mobile phase containing 25 mM ammonium acetate buffer pH 4.5:methanol (99:1, v/v) and 0.5 mM sodium octanesulfonate as the ion-pairing reagent. The proportion of methanol and octanesulfonate was critical for good selectivity and optimal retention. The flow rate was 0.2 ml/min. Detection was performed with a digital electrochemical amperometric detector equipped with an analytical cell. The detector was set at +400mV (nA range) versus the Ag/AgCl reference electrode.

Paper II

As the evaporation procedure takes a lot of time, an SPE method was developed. Hydrolyzed samples were diluted 1:20 with 0.1 M HCl. A strong cation exchanger was used as the solid-phase extraction material (500 mg, 3 ml column). The

cartridge was activated with methanol and equilibrated with 0.1 M HCl before the sample (2 ml) was applied. Utilizing sorbent and analyte properties, ion exchange occurs between the cation bound to the sorbent and cation of the analytes (amino acid group). After washing off interferences, AHP isomers were eluted in a 2-ml fraction with acetonitrile:ammonium acetate 0.2 mM (60:40 v/v). In order to establish good stability of the AHPs, 20 µl glacial acetic acid was added to the vials before the eluate was collected.

The extraction method was developed to be compatible with HILIC chromatography. The mobile phase for chromatographic separation of AHP on a ZIC-HILIC column (2.1 x 150 mm, particle size 3.5 µm) with a preceding matched guard column was acetonitrile:0.1 M ammonium acetate buffer, pH 4.5 (82:18, v/v). The proportion of water phase in the sample matrix was a critical point for chromatographic performance. Thus, eluted samples could be almost directly injected onto the HPLC system. The flow rate was 0.2 ml/min, and injection volume was 2 µl. Detection was performed by an electrochemical detector at +400 mV and a mass spectrometer with electrospray ionization. The method was validated with respect to stability and selectivity, linearity, LOD, extraction recovery and reproducibility. The main aim of this new HPLC method was to study degradation products of HPLC with mass spectrometry.

Paper III

To identify degradation products obtained after hydriodic reduction of urinary pheomelanin a GC-MS method was developed and used for study of AHPs and benzothiazole and benzothiazine units. The method employed derivatization with ethyl chloroformate, and concurrent extraction with chloroform. As a suitable internal standard for this separation, deuterated L-dopa was used. For derivatization 300 µl of hydrolyzed sample spiked with internal standard (10 µl, 750 mg/ml) was mixed with 25 µl ethyl chloroformate and 200 µl ethanol:pyridine (4:1 v/v). At the same time 500 µl chloroform was added, and the mixture was gently shaken for 1 min. After phase separation for 5 min the organic phase was transferred to the injection vial, and 1 µl was injected onto the gas chromatograph supplied with a CP-Sil 8 Low Bleed/MS WCOT fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The analytes was separate under helium flow with constant pressure (45 kPa) and a temperature program as follows: initial temperature 50 ºC (1 minute) raised to final temperature of 300 ºC by 20 ºC/min and kept at the final temperature for 5 minutes. The injection port temperature was set at 250 ºC and the GC-transfer line at 280 ºC. Detection and analysis were performed by a mass spectrometer in scan mode from 50-550 Da. The selective ion monitoring (SIM) mode was established for quantification of AHPs with the intention of comparing this with our earlier methods (**Paper I** and **Paper II)**.

Paper IV

The high amounts of 5-S-CD in the urine of some patients with melanoma led to the notion that other small molecules originating primarily from pheomelanin may be excreted into the urine. Injection of urine onto a HILIC column (described in **Paper II**) confirmed the theory. A significant amount of benzothiazole carboxylic acid isomers was found in the urine of melanoma patients with diffuse melanosis. In addition to free pigment compounds, degraded products obtained after hydrogen peroxide oxidation (described above) were also found. As the urine of these patients contained a number of interfering substances an SPE method for purification of the benzothiazole isomers was developed. C18 cartridges were used (200 mg, 3 ml). The samples were adjusted to pH~1 in order to protonate acidic analytes. Protonating a molecule or ion alters many chemical properties such as change of charge and mass but also hydrophilicity and therefore makes them more readily retained by non-polar interactions. C18, non-polar sorbents previously conditioned with methanol (2 ml) and 0.1 M HCl (1 ml) were able to interact with protonated benzothiazole carboxylic acid isomers. The compounds were eluted with 1 ml of acidified acetonitrile, and after dilution with HPLC mobile phase, 5 µl was injected to a ZIC-HILIC column (2.1 x 150 mm, particle size 3.5 µm). The isomers were separated at a flow rate of 0.3 ml/min of a mobile phase containing acetonitrile:0.1 M ammonium acetate buffer pH 5 (82:18, v/v). The isomers were identified and quantified by a photo-diode array detector by scanning of the absorbance at wavelengths 190-400 nm. The HILIC-PDA method was validated with respect to the linearity, limit of detection, extraction recovery, and precision.

Biochemical markers

The biomedical interest in degradation products of pheomelanin is related to the possibility that pheomelanins can act as potent UV photosensitizers. The studies of components that are built into pheomelanin and to which pheomelanin can be degraded are of pivotal importance to understanding pheomelanin phototoxicity and UV susceptibility. Fig. 17 shows an assembled family of chromophores that originate from pheomelanin and were fundamental reasons for my research and the present thesis.

4-AHP (***1***) and 3-AHP (***2***) obtained after hydriodic reduction of urine of patients with melanoma were analyzed by reversed-phase chromatography (**Paper I**). They were also analyzed by HILIC (**Paper II**), and other degradation products (***5***) were recognized by mass detection. These products were identified by GC/MS in **Paper III** (***5***, ***9*** and ***11***). BTCA isomers (**7** and **8**) obtained from 5-S-CD and 2-SCD were studied by direct analysis of the urine of patients with melanoma (**Paper IV**). In additional experiments HBTA isomers (**5** and **6**) were identified together with AHP isomers (**1** and **2**) after HI hydrolysis of urinary pigment.

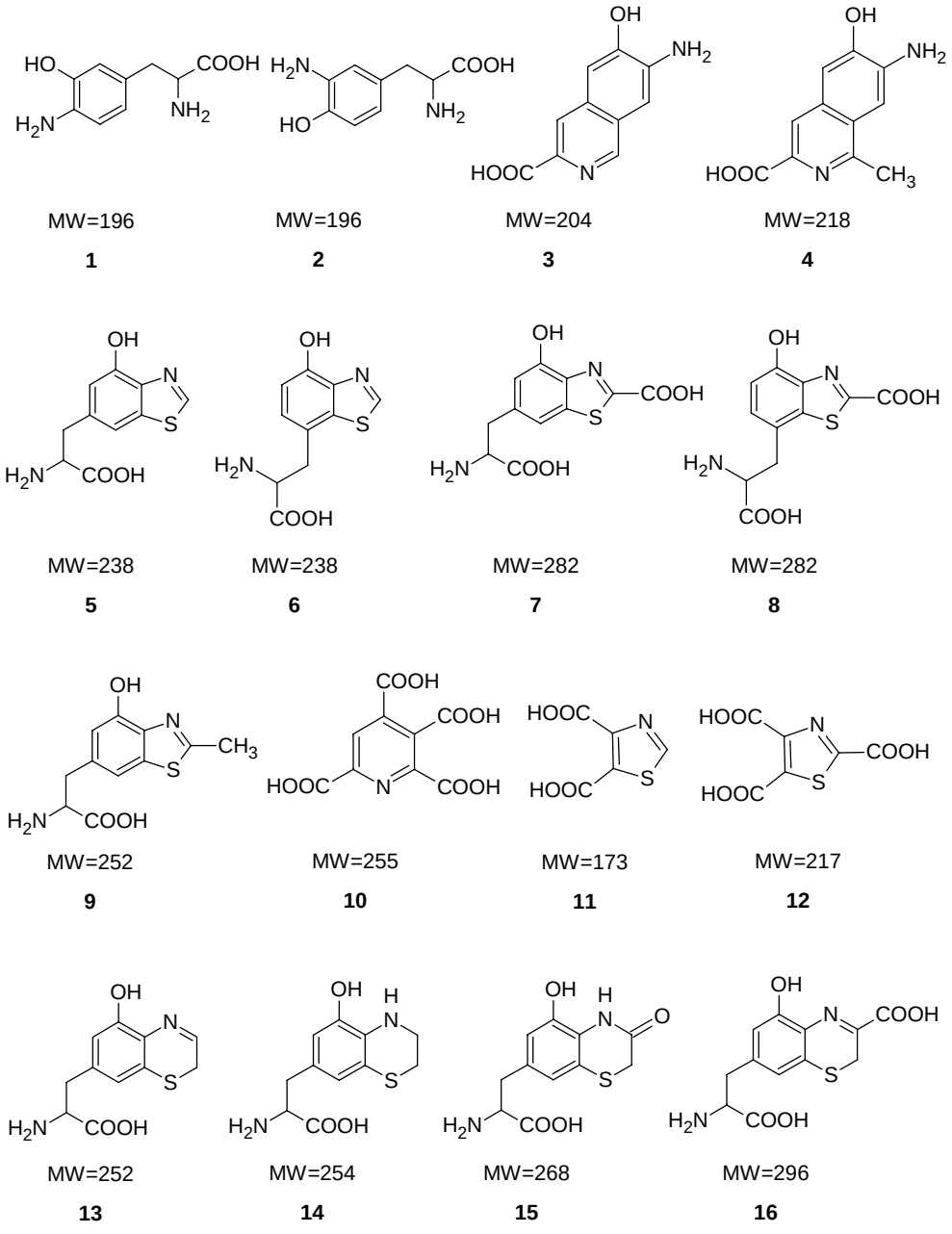


Fig. 17. *Structures and denotations of pheomelanin related substance*

1/ *4-amino-3-hydroxyphenylalanine (4-AHP)*
2/ *3-amino-4-hydroxyphenylalanine (3-AHP)*
3/ *7-amino-6-hydroxyisoquinoline-3-carboxilic acid*
4/ *7-amino-6-hydroxy-1-methylisoquinoline-3-carboxilic acid*
5/ *6-(2-amino-2-carboxyethyl)-4-hydroxybenzothiazole (HBTA-5)*
6/ *7-(2-amino-2-carboxyethyl)-4-hydroxybenzothiazole (HBTA-2)*
7/ *6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole-2-carboxylic acid (BTCA-5)*
8/ *7-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole-2-carboxylic acid (BTCA-2)*
9/ *6-(2-amino-2-carboxyethyl)-4-hydroxy-2-methyl-benzothiazole*
10/ *pyridine-2,3,4,6-tetracarboxylic acid*
11/ *thiazole-4,5-dicarboxylic acid (TDCA)*
12/ *thiazole-2,4,5-tricarboxylic acid (TTCA)*
13/ *7-(2-amino-2-carboxyethyl)-5-hydroxy-2H-1,4-benzothizine*
14/ *7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-2H-1,4-benzothizine*
15/ *7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-2H-1,4-benzothizine-3-one*
16/ *7-(2-amino-2-carboxyethyl)-5-hydroxy -2H-1,4-benzothizine-3-carboxylic acid*

RESULTS AND DISCUSSIONS

In degradation studies of melanins 4-AHP and 3-AHP are obtained from the benzothiazine units of pheomelanin (Ito 1989) and pheomelanin-related metabolites such as trichochromes (Ito and Fujita 1985). In early studies the total amount of AHP was used as a single marker of pheomelanin because these two isomers could not be separated in the chromatography (Ito and Fujita 1985). A drawback of this was that 3-AHP from other sources than pheomelanin could influence the results. The background level of 3-AHP can be derived from 3-nitrotyrosine residues in proteins or from free nitrotyrosine (Crowley et al. 1998) (Fig. 9). In cells that do not produce melanin, only the 3-AHP isomer can be found (Kolb et al. 1997). Hence, measuring total AHP can be misleading. Therefore, the separation of 4-AHP and 3-AHP is very important in determining the true quantity of pheomelanin. Later on, the 4-AHP isomer was characterized as a specific marker for pheomelanin (Wakamatsu and Ito 2002; Wakamatsu et al. 2002a).

Therefore we developed an HPLC method to analyze 4-AHP and 3-AHP in the urine of patients with melanoma **(Paper I).** The method is based on hydriodic acid hydrolysis of the melanin polymer followed by reversed-phase HPLC with electrochemical detection. The mobile phase consists of 25 mM ammonium acetate and sodium octanesulfonate as an ion-pairing reagent. The 4-AHP and 3-AHP peaks were well separated when prepared from biological samples (Fig. 18), and the detector response was linear within the range 0-2 ng injected for both compounds.

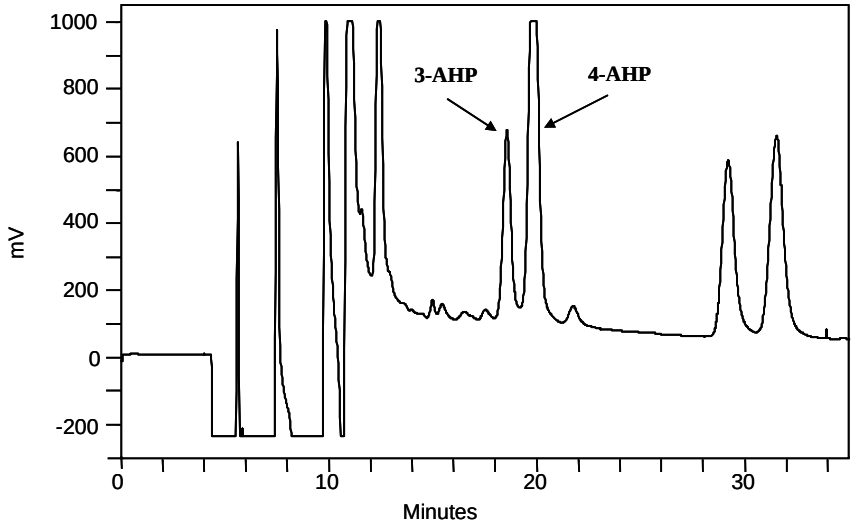


Fig. 18. *HPLC chromatogram of a urine sample from a patient with melanoma obtained after HI degradation*

We analyzed 4-AHP and 3-AHP in the urines of 50 patients with melanoma and studied the correlation between the two AHPs as well as their correlation with 5-S-CD. There was a strong correlation between 4-AHP and 3-AHP (R=0.977). Separately the two compounds were also strongly correlated with 5-S-cysteinyldopa in urine, the correlation coefficients being 0.862 and 0.907, respectively (Fig. 19).

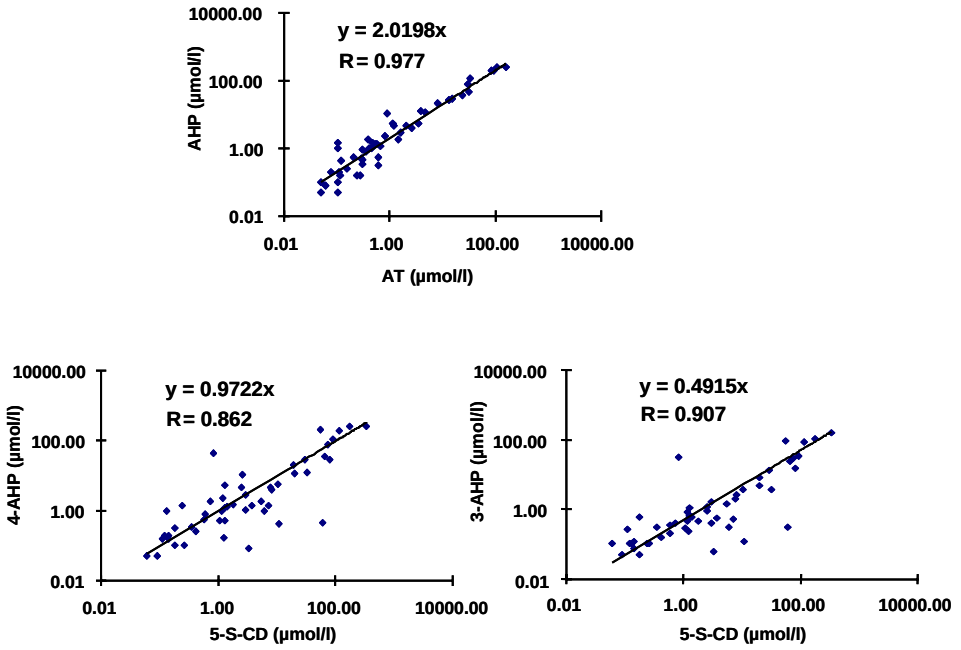


Fig. 19. *The concentrations of 4-AHP and 3-AHP in urine and their correlation with 5-S-CD*

We also analyzed synthetic pheomelanin and found that the mean ratio of 4-AHP to 3-AHP in urine samples was close to the ratio in synthetic pheomelanin. These findings suggest that 4-AHP and 3-AHP in urine are derived from pheomelanic pigment that was formed in melanoma cells, leaked to the blood and then excreted into the urine. In addition the biological and chemical influence of nitrotyrosine-derived 3-AHP in studies of pheomelanin in urine was investigated. The results indicated that 3-AHP in urine is not influenced by excreted 3-nitrotyrosine.

The data presented in **Paper I** showed that pheomelanin pigments are excreted into the urine of patients with metastatic melanoma. These structures are not formed from excreted metabolites, being intermediates in the biosynthesis of pigment. We believe that these pigments originate from degraded melanoma cells. Our studies also showed that the two isomers could be analyzed in other biological materials (hair, melanoma cell, tumor biopsies) as well by this method.

1 ***Table 1.*** *Literature overview of the different HPLC methods for determination of AHPs*

Author	Analyte	Source	Amount (%)	Method	Chromatography method
Ito and Jimbow (Ito and Jimbow 1983)	AHP tot	Hair Melanoma		RP-HPLC	Column: Yanaco ODS-T 4.0 x 250 mm; 7 µm; 40ºC Mobile phase: (0.1 M sodium citrate pH 4. 1 mM sodium octanesulphonate, 0.1 mM Na_2EDTA)/MeOH, (97:3) Detection ECD + 0.50 V
Ito and Fujita (Ito and Fujita 1985)	AHP tot	Synthetic pheomelanin 5-S-CD melanin Trichochrome F	18 21 50	Evaporation RP-HPLC	Column: Yanapack ODS-T 4.0 x 250 mm; 7 µm; 60ºC Mobile phase: (0.1 M sodium citrate pH 4. 0, 1 mM sodium octanesulphonate, 0.1 mM Na_2EDTA)/MeOH, (97:3) Detection ECD + 0.40 V
Ito (Ito 1989)	AHP tot AHP tot	Synthetic pheomelanin 5-S-CD melanin	20 23	Evaporation RP-HPLC	Column: Yanapack ODS-T 4.0 x 250 mm; 7 µm; 60ºC Mobile phase: (0.1 M sodium citrate pH 4. 0, 1 mM sodium octanesulphonate, 0.1 mM Na_2EDTA)/MeOH, (97:3) Detection ECD + 0.40 V
Kolb et al. (Kolb et al. 1997)		Melanoma cells and hair		Evaporation SPE and RP-HPLC	Column: Supelcosil LC-18-D 4.6 x 250 mm; 5 µm; 35ºC Mobile phase: (0.01 M potassium phosphate buffer pH 5.7. 1 mM sodium octanesulphonate, 30 mg Na_2EDTA)/MeOH, (99:1) Detection ECD + 0.35 V
Borges et al (Borges et al. 2001)	3-AHP 4-AHP	2-S-CD melanin 5-S-CD melanin	16 23	Evaporation SPE and RP-HPLC	Column: Luna C18 4.6 x 250 mm; 5 µm; 35ºC Mobile phase: (0.01 M potassium phosphate buffer pH 5.7, 1 mM sodium octanesulphonate, 0.1 mM Na_2EDTA)/MeOH, (99:1) Detection ECD + 0.40 V
Wakamatsu et al. (Wakamatsu et al. 2002a)	AHP tot 4-AHP	Synthetic pheomelanin	13 11	Evaporation RP-HPLC	Column: Catecholpack 4.6 x 150 mm; 7 µm; 35ºC Mobile phase: (0.1 M sodium citrate pH 4. 0, 1 mM sodium octanesulphonate, 0.1 mM Na_2EDTA)/MeOH, (98:2) Detection ECD + 0.50 V
Paper I (Takasaki et al. 2003)	3-AHP 4-AHP	Synthetic pheomelanin	3.7 10	Evaporation RP-HPLC	Column: Nova Pack C18 3.9 x 150 mm; 4 µm; 25ºC Mobile phase: (25 mM ammonium acteate buffer pH 4.5, 0.5 mM sodium octanesulphonate)/MeOH, (99:1) Detection ECD + 0.40 V

Results from **Paper I** showed that we can detect pigment compounds in urine and that some compounds might still be unidentified (Fig 18). Corresponding compounds could be identified by LC-MS/MS. However, the HPLC methodology reported in **Paper I** and by other researchers (Table 1) included high water and ion-pairing reagent concentrations in the mobile phases and these are not compatible with ESI-MS technique. We attempted, therefore, to develop a new method for analysis of degradation products of pheomelanin by using hydrophilic interaction liquid chromatography. The two isomers could be separated by optimizing several factors such as the choice of sample solvent and ammonium ion concentration in the mobile phase as well as the content of acetate or formiate ions. We developed and validated an HPLC method for separation of 4-AHP and 3-AHP with ZIC-HILIC stationary phase **(Paper II).**

As HI gives very high signals in the electrochemical detector, it was necessary to remove it for trouble-free performance of the HPLC system. The original method for sample preparation before HPLC (**Paper I**) employs a long and complicated evaporation procedure. In the new method (**Paper II**) pheomelanin samples were hydrolyzed and extracted with solid-phase extraction columns using strong cation-exchange (SCX) cartridges. Retention in the HPLC was affected by pH, ionic strength and counterions of the mobile phase as well as by the net charge of the analytes that contain at least three functional groups. 4-AHP and 3-AHP were separated on a ZIC-HILIC column with a mobile phase consisting of acetonitrile: ammonium acetate buffer. Detection was performed with an electrochemical detector at +400 mV. Run time was 30 min. The method was fully validated (Table 2) according to the criteria of the Washington report (Shah et al. 1991) and considered satisfactory.

Table 2. *Validation parameters of ZIC-HILIC method*

	4-AHP	**3-AHP**
Capacity factor k’	3.26	3.94
Linearity 0.05-2.5 µg/ml	R = 0,9994	R = 0,9998
LOD (pg)	73	51
Extraction Recovery (%)	~70	~70
Within-day (%) imprecision	2.5	2.3
Between-day (%) imprecision	7.5	6.5
Stability: - hydrolyzed samples - eluates at 4°C - autosampler at 10°C.	 1 year 4 days 2 days	

With the earlier method we were able to analyze a maximum of three to four samples per day. The new method allowed analysis of thirty samples in one set and is also suitable for routine work with human hair and melanoma cells. The method was a prerequisite for further LC-MS studies of degradation products of pheomelanin.

High contents of pigment structures were found in the urine of patients with advanced melanoma. These components were isolated and used in attempts at structure analysis. We therefore developed and adapted an easy-to-run solid-phase extraction procedure for purification of AHPs from reduction reagents prior to the chromatography.

The benefit of ZIC-HILIC as the stationary phase is that the separation mechanism is different from that of reversed-phase stationary phase. Thus 4-AHP was eluted before 3-AHP (Fig. 20), the opposite of the elution order found in our earlier ion-pair RP-HPLC (**Paper I**) (Fig. 18). When a potential at +700 mV was used, several interesting peaks could be detected in the chromatogram. 4-AHP, 3-AHP, and some of the new peaks were identified with both electrochemical and mass spectrometric detection (Fig 20). Ion-pairing reagents were also avoided, which made the method suitable for further analysis of degradation products from pheomelanins using mass spectrometric detection. With the mass spectrometer coupled to HILIC, we were able to characterize peaks appearing in samples both from a patient's urine and from synthetic pheomelanin.

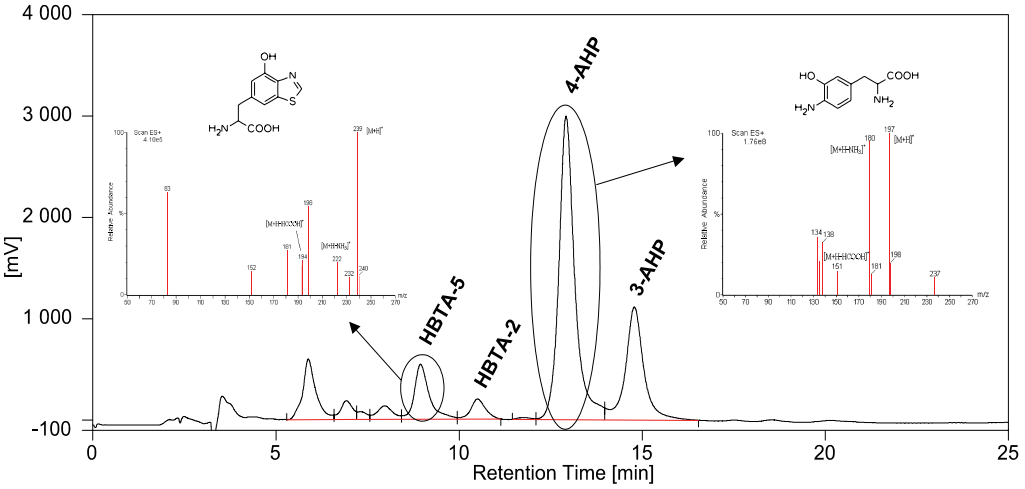


Fig. 20. *Chromatogram of 4-AHP and 3-AHP from 1 mg synthetic pheomelanin. Detection was performed by ECD at +700 mV. A hitherto unidentified compound was identified as hyroxybesothiazole by LC/MS/MS.*

The mass spectra indicated that these compounds can originate from benzothiazole units. The identities of these compounds were later proven by comparison with authentic standards, see below.

Because of the insufficiency of our LC-MS/MS instrumentation we continued our studies of urinary pheomelanin degradation products by GC/MS in order to relieve their structures **(Paper III**). The method was based on chemical degradation of pheomelanin with hydriodic acid and GC/MS analysis of specific degradation products. The derivatization process and the fragment pattern of AHPs were studied. The method involved the employment of a simple derivatization with ethyl chloroformate (ECF) and temperature gradient gas chromatographic separation. The derivatization requires a minimum of sample preparation. It offers rapid reaction, and the reaction conditions are ambient temperature and aqueous medium. Chromatographic baseline separation between the two isomers was complete using a CP-Sil 8 CB Low Bleed/MS WCOT fused-silica capillary column.

On the basis of the fragmentation pattern of N,O-ethoxycarbonyl ethyl esters of 4-AHP and 3-AHP, we characterized three other degradation products from both synthetic pheomelanin and from the urine of a patient with advanced melanoma. These products were identified from their mass spectra to be 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-2H-1,4-benzothizine-3-one (**A**), 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole (**B**), and 6-(2-amino-2-carboxyethyl)-4-hydroxy-2-methyl-1,3-benzothiazole (**C**) (Fig. 21).

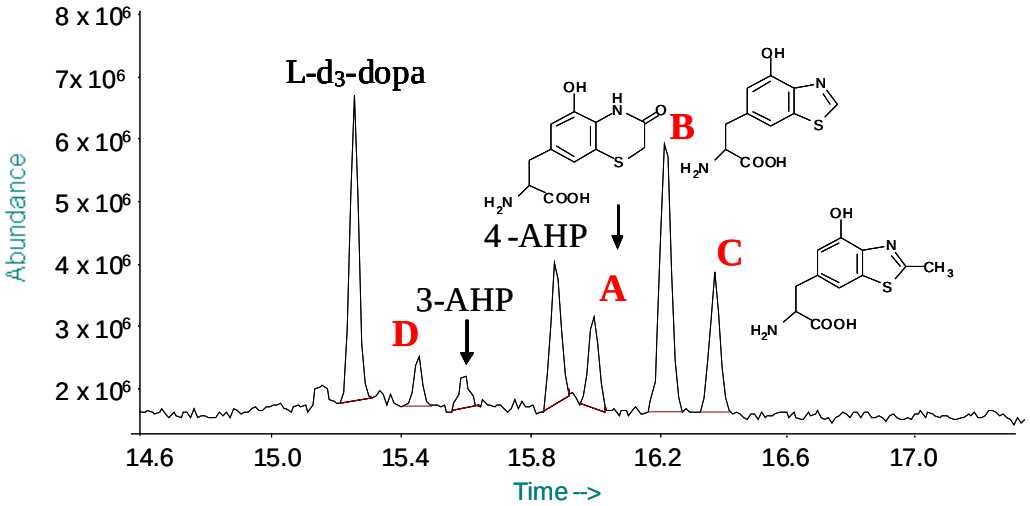


Fig. 21. *GC/MS chromatogram of degradation products of urinary pheomelanin. The peaks A, B, and C were identified as 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-2H-1,4-benzothizine-3-one, 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole, and 6-(2-amino-2-carboxyethyl)-4-hydroxy-2-methyl-1,3-benzothiazole*

The findings of benzothiazinone and benzothiazole derivatives among the degradation products of pheomelanin samples strongly suggest that heterocyclic pheomelanin-type units are incorporated into the pigment structure.

Previous chemical, biosynthetic, and spectroscopic studies indicated that pheomelanins may be composed of 4-hydroxybenzothiazole and 5-hydroxy-1,4-benzothiazine moieties and hydriodic acid reduction of pheomelanin yields 4-AHP as a major product. AHPs are easily formed from the 5-hydroxy-1,4-benzothiazine moiety with alanyl side chain, but only to a minor degree from the 4-hydroxybenzothiazole compound with the alanyl side chain (Ito and Fujita 1985). Therefore the presence of 4-hydroxybenzothiazole could remain in the sample after its formation.

Our findings of benzothiazoles as degradation products of urinary pigment of patients with melanoma as well as previous findings of large amounts of 5-S-CD and pigment in the urine of patients with diffuse melanosis (Agrup et al. 1979; Rorsman et al. 1986) prompted the study of free BTCA in urine from such patients (**Paper IV**). Biosynthetic studies of pheomelanin employ oxidation of 5-S-CD as a main precursor (Borges et al. 2001; Ito et al. 1980; Ito and Prota 1977), but findings of AHP isomers in urinary pigments from melanoma patients (**Paper I** and **Paper II**) indicate that 2-C-SD is also involved in pigment building. Oxidation, cyclization and dimerization of 5-S-CD and 2-S-CD also lead to the formation of mixed type melanin and trichochromes (Ito et al. 1980; Prota 1980).

As 5-S-CD (Agrup et al. 1978a; Kärnell et al. 2000; Tsukamoto et al. 1998; Wakamatsu et al. 2002b; Wakamatsu et al. 2003b) and trichochromes (Agrup et al. 1979; Agrup et al. 1978b; Prota et al. 1976; Rorsman et al. 1986) were found in large amounts in the urine of patients with melanosis, we applied the previously developed HILIC method (**Paper II**) to analyze urine samples without previous degradation. We also developed a solid-phase extraction method for purification of BTCA isomers from urine matrix. BTCA isomers were identified with a photo-diode array detector, which provides UV spectra and enables the isomers to be matched against known library spectra. Among patients with melanoma, three out of twenty-one had diffuse melanosis. We found free BTCA isomers in all of these three patients and in one other patient with advanced melanoma **(Paper IV)**.

During the systematic studies of BTCA isomers in different urinary fractions of patients with melanoma and diffuse melanosis (mixed urine, supernatant after centrifugation of urine, and urine sediment) we found strong indications that BTCA isomers are excreted in urine, either as free components or as constitutive parts of low molecular or pheomelanin structures. We compared the results of free BTCA isomers with that obtained after hydrogen peroxide oxidation of urine and found that most of the pigment remained in solution probably as low molecular weight components such as trichochromes and/or dimers formed from 5-S-CD and 2-S-CD (Table 3). It is not clear what the original source of BTCA is. This compound may

possibly be formed in the melanoma cells of the metastatic tumors. In the studies by Gambichler et al. (Gambichler et al. 2008) the histopathologic appearance of the kidney tissue was consistent with acute kidney injury caused by melanin accumulation within the tubules, tubular cells, and endothelial cell of the vessels and glomeruli. An interesting theory is that tumor-derived melanin granules are liberated to lymph and blood, and are circulated to various organs where they are phagocytosed by endothelial cells and then the pigment is deposited in macrophages and other cells (Gambichler et al. 2008). It is not clear at present how such excretion can occur although even if melanosomes are found circulating to cell-free material in tissues (Böhm et al. 2001) these particles measure up to 1.5 µm in diameter and such subcellular granules as well as melanin granules of comparable size are unlikely to cross an intact glomerular membrane. However, our findings of free BTCA isomers in urine from patients with melanosis strongly suggest that sulfur containing pigment structures are secreted during melanoma progression.

In previous work (**Paper II** and **Paper III)** we found that pheomelanin is degraded to several products other than AHPs (Fig. 20 and Fig. 21). In additional experiments we applied HI hydrolysis to different fractions of urine from patients with diffuse melanosis and identified significant amounts of AHPs and HBTA isomers (Table 3). As AHPs are mostly degradation products of benzothiazines (Ito 1989; Ito and Fujita 1985), the molar concentrations of AHPs presented in Table 3 indicate that urinary pigments consist mostly of benzothiazine structures, which is in agreement with earlier findings (Ito 1998). The occurrence of 1,4-benzothiazine units in the pheomelanin polymer is also supported by isolation of 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-2H-1,4-benzothizine-3-one by degradation with HI (**Paper III**).

Wakamatsu et al. (Wakamatsu et al. 2009) recently showed that the benzothiazole unit is incorporated into pheomelanin during melanogenesis as a result of 5-S-CD conversion to 7-(2-amino-2-carboxyethyl)-5-hydroxy-3,4-dihydro-*2H*-1,4-benzothiazine-3-carboxylic acid, which then gradually degrades to form a benzothiazole moiety in a later stage of oxidation. The study by Ismail et al. showed that the benzothiazole moiety is a dominant monomeric unit in pheomelanin (Ismail et al. 1980). HBTA isomers have not been studied in urine before. It was shown that natural pheomelanins have a higher benzothiazole to benzothiazine ratio (Wakamatsu et al. 2009). Our preliminary data (Table 3) indicate that HBTA isomers can originate from BTCA isomers as a result of decarboxylation of BTCAs and/or from pigment structures, either as a result of ring contraction of benzothiazine and/or as cleavage of benzothiazole parts. The control study based on HI hydrolysis of BTCAs shows that 90 % of BTCAs transforms to HBTAs and 5 % to AHPs. In a parallel experiment, it was shown that HBTAs convert to 5 % AHP and 90 % remains as HBTAs. Hence the relative yield for conversion of BTCA to HBTA was estimated to be 100 %.

Table 3. *Relation between the isomers of pheomelanin identified in different fractions of urine of patients with melanosis; mixed urine (A), supernatant (B) and sediment (C)*

Patient	Sample fraction	[a] BTCA-5/BTCA-2		[b] HBTA-5/HBTA-2		[b] 4-AHP/3-AHP	
		µmol/l	ratio	µmol/l	ratio	µmol/l	ratio
Patient N – 730 (1)	Free	342.7/81.4	4.2				
	A	856.9/262.0	3.6	440.4/79.9	5.5	624.3/251.1	2.5
	B	681.6/245.9	2.8	265.4/49.2	5.4	481.8/142.5	3.4
	C	105.7/44.6	2.4	56.7/19.78	2.9	128.9/46.3	2.8
Patient N-550 (2)	Free	94.4/80.5	1.2				
	A	632.8/290.0	2.2	573.9/150.3	3.8	631.1/217.1	2.9
	B	457.1/227.0	2.0	349.8/176.0	4.6	359.6/115.4	3.1
	C	257.2/75.7	3.4	218.3/60.8	3.6	157.6/42.8	3.7
Patient B	Free	118.7/37.4	3.2				
	A	436.7/92.4	4.7	92.2/22.3	4.1	257.9/108.6	2.4
	B	362.1/74.4	4.9	84.9/23.5	3.6	223.9/101.8	2.2
	C	60.9/13.3	4.6	2.7/0.7	3.9	6.5/1.9	3.4
Patient R	Free	28.9/6.8	2.3				
	A	101.8/38.2	2.7	13.4/7.3	1.9	95.0/20.4	4.7
	B	75.3/29.8	2.5	7.3/2.2	3.3	47.5/33.9	1.4
	C	34.7/10.4	3.3	4.5/1.3	3.5	22.5/7.5	3.0
Pheomelanin		14.9/2.7[c]	5.5	2.0/0.4[c]	4.7	11.2/4.0[c]	3.1

[a] Obtained either as free or after H_2O_2 oxidation
[b] Obtained after HI hydrolysis
[c] % on weight basis

Whereas AHP isomers are derived mostly from benzothiazine structures in the pigment and only to a low degree from benzothiazole units, BTCAs and HBTAs seem to be not just degradation products of pheomelanin but also constitutive parts of natural pheomelanin. The benzothiazole monomer units seem to be present in the pheomelanin pigment in such form that they may be readily hydrolyzed off without requiring an oxidative step. This is in accordance with the study by Napolitano et al. (Napolitano et al. 1996) (Fig 22). The ability of the skin to produce pheomelanin rather than eumelanin has negative implications for photoprotection. Our results indicate that conversion of benzothiazine to benzothiazole in melanogenesis may also have an implication for pheomelanin phototoxicity and melanoma progression.

The yield of HBTA obtained after HI hydrolysis of synthetic pheomelanin is lower than the yield of BTCA obtained after H_2O_2 oxidation. Anyhow, when using the molar concentrations of BTCA, HBTA and AHP and conversion factors based on the percents showed in Table 3 the calculated amount of pheomelanin are in the same range or slightly higher than the amount we calculated earlier (900 µg/ml) using 4-AHP as the marker of pheomelanin (**Paper II**). This might be because of the influence of free BTCA.

In the early study of pheomelanin markers both 5-S-CD and 2-S-CD were studied (Fattorusso et al. 1969; Morishima et al. 1983), and their excretion ratio in

serum and urine was found to be 5:1 or higher. 5-S-CD and degradation products from 5-S-CD-dervived pheomelanin played a central role in studies performed afterwards. Greco et al. recently showed that not only 5-S-CD-derived components but also 2-S-CD-derived components are important building blocks in pheomelanin of red human hair (Greco et al. 2009b). In the present thesis we show that isomers derived from 2-S-CD, *viz.* 3-AT, BTCA-2, and HBTA-2, are also excreted in the urine, either as free or as a part of larger pigment structures. This is a new finding and may have implications on further pheomelanin research since according to Greco et al. (Greco et al. 2009b) these components may be more stabile than the 5-S-CD-derived components.

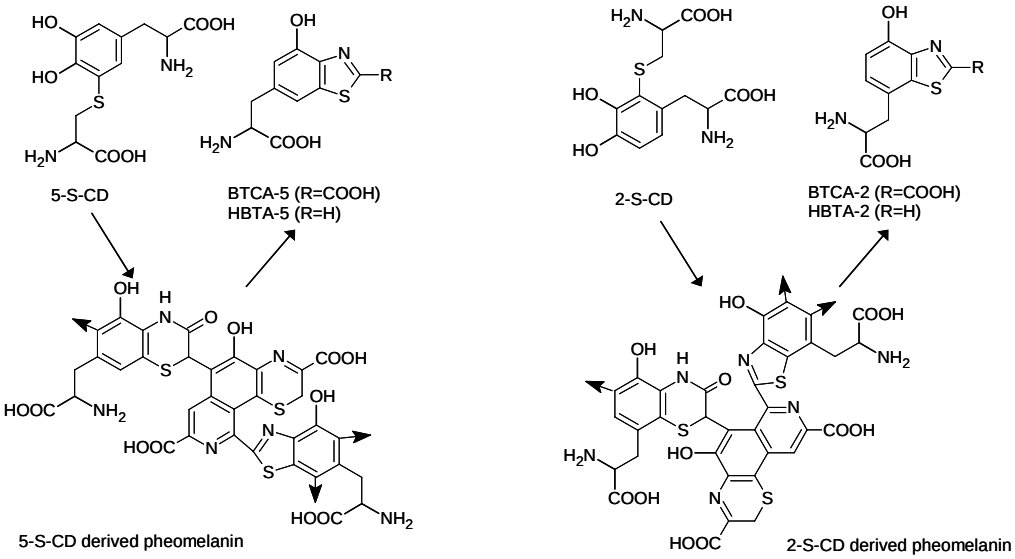


Fig. 22 *Structures of pheomelanin compounds. The figure illustrates pheomelanic structures formed from 5-S-CD and 2-S-CD and the benzothiazole compounds obtained by chemical degradation of these pheomelanins.*

CONCLUSION

A key step in my studies of pheomelanin structures was the development of tailored analytical protocols for identification of degradation products of pheomelanin. From the result obtained in **Papers I-IV** the following conclusions can be drawn.

- ✓ Pheomelanin pigment structures are excreted into the urine of patients with melanoma and diffuse melanosis. The ratio of 4-AHP and 3-AHP, obtained after hydriodic degradation of urine of patients with melanoma by a conventional RP-HPLC, was very close to that obtained after the same degradation procedure of synthetically prepared pheomelanin. There was a strong correlation between AHPs and 5-S-CD in the urine of the patients. This suggests that 4-AHP and 3-AHP are derived from pheomelanin pigment formed in melanoma cells, leaked into the blood and then excreted into the urine.
- ✓ Identification of other markers of pheomelanin has been demonstrated with a significant improvement of the analytical protocol for analysis of AHPs. The main strength of the ZIC-HILIC stationary phase is that the separation mechanism is different from that of RP stationary phase. An effect of this was that 4-AHP was eluted before 3-AHP, which is the opposite of the elution order found in our earlier ion-pair RP-HPLC. Ion-pairing reagents were avoided, which made the method suitable for further analysis of degradation products from pheomelanins using mass spectrometric detection. With the mass spectrometer coupled to HILIC, we were able to characterize at least another three peaks in samples from patient urine and from synthetic pheomelanin. The spectra obtained indicated that these compounds might originate from benzothiazine or benzothiazole units. We also developed and adapted a solid-phase extraction procedure for these compounds for the following chromatography. With SPE, harmful effects of iodic alcohol fumes are avoided. The method developed allowed for analysis of at least 30 samples a day, which is much better that the method we used before, which allowed just 1-2 samples a day.
- ✓ GC/MS studies of N,O-ethoxycarbonyl ethyl esters strongly indicate that benzothiazole and benzothiazine structures are constitutive parts of pheomelanin pigment. These structures have been found both in synthetic pheomelanin and urine sediment after hydriodic degradation. The results suggest that they do not specifically originate from trichochromes, the soluble low molecular pigment structures.

- ✓ Studies on patients with melanoma and melanosis showed that BTCA isomers are excreted into the urine as free components and as constitutive parts of low molecular or pheomelanin structures. Identification of free benzothiazoles provides important information in the field of urinary melanogenesis, which offers considerable insight into the biosynthetic activity of normal and pathologic melanocytes. This opens the doorway to further approaches in the investigation of pheomelanins and can help to understand fundamental problems about the structure and biosynthesis of natural melanins.

The reported papers prove the possibility of identifying several different pigment compounds in the urine by the use of chromatography and several detection techniques.

IMPLICATIONS AND FUTURE RESEARCH

The results obtained in the present study show that urinary pigments are a suitable material for structure analysis of melanin from biological sources. The relationship between bioactivity in melanoma cells and the excretion of the pigment structures in the urine remains unclear and represent a major focus for further biochemical and clinical studies. In the future, it would be of interest to study pigment excretion in the urine of patients with melanoma in different stages. These categories would be primary melanoma, primary melanoma with positive sentinel nodes, patients with palpable lymph nodes and patient with disseminated diseases. It would be interesting to follow up melanoma progression or the results of treatment using the methodology described.

We intend to apply this analytical approach to the analysis of synthetic eumelanin, sepia melanin, synthetic pheomelanin, urine from healthy persons, urine from patients with melanoma, the urinary sediment from the urine of patients with melanoma and diffuse melanosis of melanoma, and even other biological samples such as dog coat, tumor tissues etc.

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♥ ♥ ♥

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APPENDIX

Bioanalytical method validation is the process used to establish that quantitative analytical method is suitable for biomedical applications

Selectivity and specificity

The term specificity refers to a method which produces response for only a single analyte whereas selectivity refers to a method which provides response for a number of chemical entities which may or may not be separated. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. There is a variety of ways to test selectivity which can provide validation. The simplest test in chromatographic analysis is to demonstrate a lack of response in the blank biologic matrix. Another approach is to test whether the intercept of the calibration curve is significantly different from zero (Karnes et al. 1991).

Capacity factor k'

The capacity factor is used to describe the retention of a compound in a chromatogram. The compounds that have no interaction with the stationary phase pass by the column at the speed of the mobile phase and this elution is defined as "dead time", t_0. If compounds have interaction with both stationary phase and mobile phase, their time in the chromatographic system is defined as retention time t_R. The magnitude of k´ can be estimated from the equation $(t_R-t_0)/t_0$. To achieve a reasonable compromise between the retention time and the required analysis time, k´ should be a value between 1 and 5. The nature of the analyte, mobile phase composition, stationary phase properties, as well as temperature has an effect on k´ value.

Calibration curve and linearity

The concentration range (calibration curve) over which the analytes will be determined is based on the evaluation of actual standard samples including their statistical variation. The number of standards to be used is a function of the dynamic range and nature of the concentration-response relationship. In many cases, five to eight concentrations (including blank values) define the standard curve (Shah et al. 1991). The calibration curves are plotted using peak response vs. concentration. This is referred to as external calibration. Another very common technique in bioanalytical methodology is internal calibration. The internal calibration requires an internal standard (a functional or isotopic analogue of the analyte) which is added to standards and samples prior the sample pretreatments. The calibration curves are then plotted using ratios of the signal of the analytes to that of the

internal standard vs. concentration. Although some analytical procedures may require nonlinear calibration it is conventional to use a liner model. Linear regressions, equations and regression coefficients (R) in the present thesis were calculated by the software Chromeleon.

Limit of detection (LOD)

Limits of detection can be estimated using equations from the linear regressions obtained by plotting peak height vs. the concentration of the calibrators. The noise was measured as the maximum distance between the highest and the lowest data points of the background signal at the retention times of the references in a blank sample. LOD is based on signal-to-noise ratio 3, corresponding to 99.9 % level of confidence (Karnes et al. 1991).

Extraction recovery

Recovery is usually defined as the percentage of reference material which is measured to that which has been added to a blank (Karnes et al. 1991). The absolute recovery of a bioanalytical method constitutes the response of a standard spiked to the matrix and is calculated as a percentage of the response of a pure standard which has not been subjected to the sample pretreatment process (Causon 1997). Result of experiments that compare responses of the compound in the matrix and in the pure solvent is referred to as relative recovery. Although it is desirable to achieve absolute recovery as close to 100 % as possible, recoveries of 50 % or more are acceptable if good precision and accuracy can be obtained.

Precision and accuracy

The precision of a bioanalytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variation (% CV) or relative standard deviation (R.S.D). Within-day imprecision (intra-assay precision) defines the ability to repeat the same methodology with the same analyst, using the same equipment and the same reagents in a short interval of time. The ability to repeat the same methodology under different conditions across longer time intervals is covered by the between-day imprecision (inter-assay precision).

As the true value for the real sample is not known, an approximation is obtained based on spiking free matrix with a nominal concentration. The accuracy of the bioanalytical method is determined as the agreement between the measured and nominal concentration.

Precision and accuracy are considered together because they are interdependent in assessing the acceptability of a method. They should be accomplished by analysis of replicate sets of samples and the mean value should be within ± 15 % (Shah et al. 1991).

Stability

Stability data is required to show that the concentration of the analyte in the sample at time of analysis corresponds to the concentration of analyte at the time of sampling. The stability of the analytes in biological matrix should be studied at the intended storage temperature and the influence of a minimum of two freeze/thaw cycles should be established. The Washington report set 15 % as the upper limit of acceptance (Shah et al. 1991).