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Abstract

Determination of eumelanin and pheomelanin in melanomas that exhibit different pigmentation was carried using a solid-phase extraction (SPE) preparation method based on weak anion exchange chemistry. This extraction significantly enhanced the chromatographic profile obtained by reverse phase high performance liquid chromatography - diode array detection (RP-HPLC-DAD). The SPE method was developed using aqueous standards of melanin markers: thiazole-2,4,5-tricarboxylic acid (TTCA), thiazole-4,5-dicarboxylic acid (TDCA), pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA) and non-pigmented cell lines spiked with those markers. An excellent average recovery, above 90%, was obtained for the four markers with a relative standard deviation below 7%. We have also optimized the stationary phase and the mobile phase (phosphate concentration and pH) to improve sensitivity and to reduce the analysis time. Elution of the four markers is achieved in 5 minutes and total analysis of biological samples is completed in 15 minutes. The quantification limits for TDCA, TTCA, PDCA and PTCA are 60, 50, 47 and 48 ng/mL respectively. Furthermore, DAD detection improves the marker identification in complex matrices through the analysis of UV spectra. We have successfully applied this method to melanoma tumors and cells. Murine B16BL6 tumor are highly pigmented with mostly eumelanin (98.1 % of eumelanin) while human SK-MEL-3 tumor contain about 30 % pheomelanin. B16BL6 and B16F10 are eumelanic cells lines and NHEM melanocytes contain about 24 % of pheomelanin.

Keywords: eumelanin, pheomelanin, melanoma tumors, solid-phase extraction (SPE), High performance liquid chromatography – diode array detection (HPLC-DAD)

1. Introduction

Skin, hair and eye color is determined by the quantity and ratio of eumelanin (EM) to pheomelanin (PM), the two classes of melanin [1–3]. Eumelanin is a black to brown insoluble pigment, whereas yellow to reddish-brown color and solubility in alkaline solvents characterize PM. These pigments also differ in their capacity of protection to ultraviolet (UV) exposure. While EM is known for its photoprotection action due to free-radical scavenging and broadband adsorption properties, PM is a pro-oxydant [4,5] with poor photoprotective potential [6]. Melanogenesis by definition is the production of the melanin pigments by melanocytes and starts with the production of dopaquinone from tyrosine, catalyzed by tyrosinase [7]. Pheomelanin is first synthetized in the presence of thiol compounds such as cysteine [8,9]. The reaction between cysteine and dopaquinone generate 5-S-cysteinyldopa (5SCD) and 2-S-cysteinyldopa (2SCD), which are subsequently oxidized by dopaquinone and yield the pheomelanin monomer entity, benzothiazine, that is progressively converted to benzothiazol [10]. The reduction of cysteine concentration contributes to the spontaneous cyclization of the highly reactive dopaquinone to give rise to dopachrome. Dopachrome rearrangement produces 5,6-dihydroxyindole (DHI) and 5,6- dihydroxyindole-2-carboxylic acid (DHICA). Tyrosinase-related protein 2 (Tyrp2) catalyzes the production of DHICA. Afterwards, both dihydroxyindoles are oxidized to form the eumelanin polymer. In mice, the tyrosinase-related protein (Tyrp1) oxidizes DHICA [7] and thus influences the degree of polymerization and the ratio DHI to DHICA in EM.

Quantification of EM and PM is achieved by indirect methods. Specific melanin markers are obtained after fragmentation of melanin polymers under controlled conditions. The most common degradation method uses alkaline peroxide oxidation [12–14] or reductive hydroiodic acid hydrolysis [15]. The markers are then separated by HPLC. Eumelanin breakup with the alkaline hydrogen peroxide oxidation produces PTCA and PDCA molecules that are specific markers of DHICA and DHI moieties respectively (Figure 1A) [12]. Additionally, under this treatment the pyrrole acids, pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA) and pyrrole-2,3,4-tricarboxylic acid (isoPTCA) can be detected. PTeCA reflects the cross-linking at the C2 and C3 positions of the DHI moiety of eumelanin and isoPTCA the cross-linking at the C3 position of DHI unit [16]. Pheomelanin is quantified through TTCA marker obtained from the benzothiazole monomer degradation, which also produces TDCA but in a low yield in comparison to TTCA (Figure 1B). If the treatment consists in a reductive hydroiodic acid hydrolysis, the specific marker 4-amino-3-hydroxyphenilalanine (4-AHP), originating from benzothiazine monomer, is used to measure the pheomelanin content. This reaction also generates the 3-amino-4-hydroxyphenilalanine (3-AHP) but this one is less pheomelanin-specific [17].

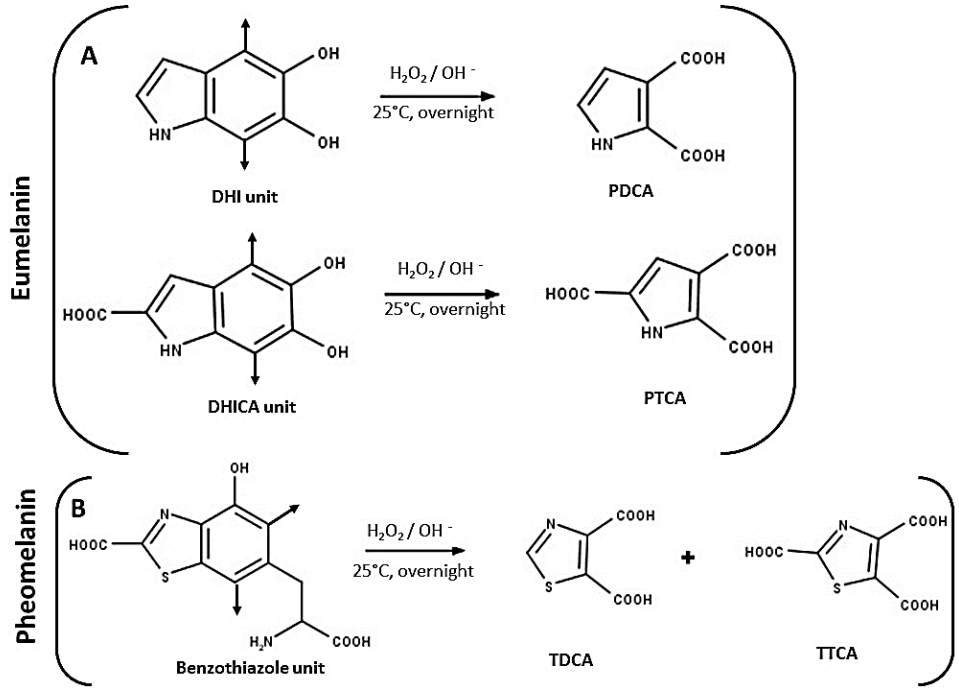

Figure 1. Eumelanin and pheomelanin markers obtained by alkaline peroxide oxidation. A) Peroxide oxidation of DHI and DHICA moieties of EM produces PDCA and PTCA markers respectively. B) Oxidation of the benzothiazole unit of PM yields TTCA and TDCA markers.

The melanin markers can be synthetized through the methods reviewed by d'Ischia et al. [18]. The main advantage of alkaline H_2O_2 oxidation is the simplicity of sample treatment in comparison with reductive HI hydrolysis. It furthermore permits quantification of the four melanin markers (PTCA, PDCA, TTCA and TDCA) in a single reverse phase HPLC analysis using a classical UV detector, while the determination of 4-AHP employs electrochemical detection and two separated HPLC sample injections in order to quantify eumelanin and pheomelanin. Although the alkaline hydrogen peroxide oxidation method can be easily implemented, separation and quantification of markers by HPLC can be complicated, especially in complex biological samples. For example in melanoma cells or tumors, interferences from matrices lead to co-elution problems or production of unresolved chromatographic peaks, making the interpretation of chromatograms difficult. Recently, Ito et al. [19] have proposed an acidic hydrolysis with 6M HCl at high temperature before alkaline oxidation, allowing protein removal from hair samples and simplification of the chromatograms. However, this procedure induces the decarboxylation of the DHICA [20] and benzothiazole moieties and therefore modifies melanin markers concentrations. For example, PTCA, the marker used to quantify EM is approximately 50% reduced after strong acidic hydrolysis of natural hair melanins and fully reduced in synthetic DHICA melanins. In the case of pheomelanin markers, TDCA concentration increases significantly and TTCA drops to 37% in hair melanin [19].

Here we propose a SPE preparation method based on weak anion exchange chemistry prior to RP-HPLC, which significantly improves the chromatographic profile. Using two different C18 columns, we also evaluated analytical conditions, pH and phosphate buffer concentration in the mobile phase in order to decrease analysis time and improve sensitivity. We analyzed the UV response of each marker and optimized the detection wavelength by the use of the diode-array detection HPLC. Marker identification in complex matrices is significantly enhanced through the evaluation of UV spectra and the purity of the chromatographic peaks. We also show that the method can be successfully applied to different pigmented human and murine melanoma tumors and cell lines.

2. Material and methods

2.1 Reagents and standards

Phosphoric acid (49-51%), hydrogen peroxide (≥ 30%) and chlorhydric acid were provided from Fluka, France. Methanol (HPLC-PLUS-Gradient) was provided from Carlo Erba, Peypin, France. Monobasic sodium phosphate, sodium sulfite, sodium acetate, potassium hydroxide and synthetic melanin standard were purchased from Sigma-Aldrich, France. 25% ammonia solution for LC-MS was from Merck, Germany. Melanin standard markers: PTCA, PDCA, TDCA, TTCA were kindly provided by Dr. Ito and Dr. Wakamatsu (Fujita Health University School of Health Sciences, Toyoake, Aichi Japan). An aqueous stock solution containing 100 µg/mL of four melanin markers was prepared and stored at −20 °C. Other working standard solutions were prepared by dilution of stock solutions.

2.2 Samples

Murine melanoma B16BL6 cells were sourced from Professor Fidler's lab (Texas University, Houston, USA). Murine melanoma B16F10 cells and human melanoma SK-MEL-3 cells were purchased from ATCC (Manassas, USA). Human melanoma A375 cells were purchased from ECACC (Salisbury, UK). Normal Human Epidermal Melanocytes (NHEM) from juvenile foreskin of a Caucasian male were purchased from PromoCell (Heidelberg, Germany). B16BL6 and B16F10 cells were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM-Glutamax, Life Technologies, Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS, Eurobio, Courtaboeuf, France) and 4 µg/mL gentamycin (Life Technologies, Courtaboeuf, France). A375 cells were cultured in DMEM supplemented with 15% FCS and 4 µg/mL gentamycin. SK-MEL-3 cells were cultivated in McCoy's 5A Medium (Invitrogen) with 15% FCS and 4 µg/mL gentamycin. Normal Human Epidermal Melanocytes were maintained in Melanocyte Growth Medium M2 (Promocell) with Supplement Mix (Promocell) and 10 000 U/mL penicillin - 10 000 µg/mL streptomycin. Cells were grown at 37°C in a humidified incubator with 5% CO_2. Syngenic B16BL6 tumors were obtained after subcutaneous injection of

melanoma cells into the flank of C57BL/6J mice [21]. SK-MEL-3 human tumor xenografts were obtained after subcutaneous injection into the flank of male Swiss *nu/nu* mice as previously described [12].

2.3 Sample preparation

Samples were submitted to an alkaline H_2O_2 oxidation to obtain melanin markers as described by Ito et al. [12] with some modifications.

2.3.1 Tumour samples

About 10 mg of tumors were weighed into a gentleMACS M tube (Gentle MACS Dissociator, Miltenyi Biotec, Paris, France) and 500 µL of 1 mol/L KOH were added. Tumors were then homogenized using the pre-set gentleMACS™ Dissociator program m_heart_01.01. Homogenized samples were transferred into a 15 mL screw-capped conical tube. A volume of 100 µL was reserved for total melanin quantification at 405 nm using a standard curve generated from known concentrations of synthetic melanin. 25 µL of H_2O_2 were added (final concentration 1.5% v/v) to the remaining 400 µL and closed tubes were shacked overnight at 25°C. 50 µL of 10% (w/v) Na_2SO_3 were added to decompose residual H_2O_2 and samples were acidified by addition of 200 µL of 1 mol/L HCl. Samples were filtered with regenerated cellulose syringe filters (0.22 µm , Phenomenex).

2.3.2 Cell samples

The cell pellets of B16BL6 and B16F10 cell lines consisted of approx. 4.10^6 cells and the NHEM melanocyte pellets of 1.5 to 2.10^6 cells. A volume of 100 µL of 1 mol/L KOH was added for every 1.10^6 cells. After homogenization with vortexing, 50 µL was reserved for total melanin quantification at 405 nm using a standard curve generated from known concentrations of synthetic melanin. Next, the oxidation reaction was performed by addition of H_2O_2 to a final concentration of 1.5% v/v, followed by an overnight incubation at 25°C. Remaining peroxide was quenched by addition of a volume of 10% (w/v) Na_2SO_3 corresponding to twofold the H_2O_2 volume. The pH of the samples were adjusted to 3.3 by addition of 1 mol/L HCl. Samples were filtered with regenerated cellulose syringe filters (0.22 µm, Phenomenex).

2.4 Solid phase extraction

Prior to the sample extraction, SPE cartridges (Strata-X-AW 33µm polymeric weak anion 30mg/1mL, Phenomenex) were conditioned with 500 µL of acetonitrile and 500 µL of 100 mmol/L sodium acetate pH 5.2. Extracts of tumor samples were loaded onto the column prior to dilution 1:1 with 100 mmol/L sodium acetate pH 5.2. Samples were eluted from the column at a reduced flow rate followed by a column wash step with 500 µL of 100 mmol/L sodium acetate pH 5.2 and 500 µL of acetonitrile. The

cartridge was then dried under vacuum for 5 minutes. The SPE column was eluted twice with 500 µL of 5% (v/v) aqueous NH_4OH. Extracts were lyophilized overnight (Freeze Dryer/Lyophilisator Alpha 2-4 LD Plus, Christ, Germany) and reconstituted with 200 µL of 20 mmol/L aqueous potassium phosphate pH 3.3. Recoveries were calculated from at least three replicate measurements of each concentration in aqueous solutions of a standard mix containing PTCA, PDCA, TDCA and TTCA and spiked A375 cells using independent cartridges.

2.5 High performance liquid chromatography conditions

HPLC-DAD analysis conditions were determined by comparing two different C18 columns. Column 1 consisted of a Kinetex Evo C18, 150 x 3 mm, 2.6 µm particle size, 100 Å pore size from Phenomenex fitted with a SecurityGuard™ ultra LC filter 0.2µm (Phenomenex). Column 2 was a Gemini C18, 150 x 3 mm, 3 µm particle size, 100 Å pore size (Phenomenex) with a SecurityGuard™ cartridge for Gemini C18. The HPLC system was an Agilent 1200 HPLC separation module equipped with an online degasser, a quaternary pump, an automatic sampler, a thermostatic controlled column chamber and a DAD detector. The system was controlled by OpenLab CDS Agilent software version A.01.05. Separation of eumelanin (PDCA and PTCA) and pheomelanin (TDCA and TTCA) markers was obtained using 20 mM aqueous potassium phosphate pH 3.3 (A) and 100% methanol (B) as mobile phase, 0.6 mL/min flow rate and 36°C column temperature. The elution program differed for column 1 and 2. For column 1 the program starts from 0 to 6 min at 0% B isocratic, followed by 6-7 min linear gradient from 0% to 10% B for column cleaning and finally a re-equilibration period for 7-15 min with 0% B. The elution program use for column 2 was: 0 to 12 min 0% B isocratic, followed by 12-15 min linear gradient from 0% to 10% B for column cleaning and finally a re-equilibration period 15-25 min with 0% B. For both columns, the injection volume was 20 µL, and detection wavelengths were set a 225 and 275 nm for PTCA and TTCA, 254 nm for TDCA and 280 nm for PDCA. The UV spectra of reference compounds were recorded and integrated into an UV library. Spectra were used to confirm the identity of markers in biological samples. Total eumelanin was obtained by multiplying total PTCA by a factor of 60 for murine and 80 for human tumors. To convert TTCA to pheomelanin, TTCA was multiplied by a factor of 34 [12].

2.6 Validation of the HPLC method

Calibration curves were constructed in concentrations ranging from 0.25 to 10 µg/mL. The limits of detection (LOD) and quantification (LOQ) were calculated using LOD = 3.3(SD/S) and LOQ = 10(SD/S) respectively, where S is the slope and the SD is the residual standard deviation of calibration curves. To evaluate reproductibility, we used SD from three separated injections of markers that were realized on the same day.

3. Results and Discussion

3.1 Chromatographic condition optimization

To select the best stationary phase for melanin marker analysis, we chose to evaluate two octadecyl columns, which differ in the particle nature. Column 1 has core-shell particles (i.e a nonporous core inside a porous shell with a total diameter of 2.6 µm. Kinetex EVO C18, Phenomenex) and column 2 has fully porous particles of 3 µm diameter (Gemini C18, Phenomenex). Both columns tolerate 100% water. The mobile phase phosphate at 100 mM and pH 2.1 was used based on previously published results [12]. One of the goals was to optimize pH and concentration of the phosphate buffer in the mobile phase. A low concentration of phosphate allowed column-cleaning step with organic solvent after elution of melanin markers to avoid a contaminant carry-over from a previous injection. In addition, the salt precipitation risk is reduced at the column level, and also in the pump and HPLC valves. Peak shape analysis and retention time of melanin standard markers (10 µg/mL) obtained with 100, 70, 50, 30 and 20 mM phosphate buffer are illustrated in Table 1.

Table 1. Chromatographic peak performances of TTCA, PTCA, TDCA and PDCA standards using 10 µg/mL solutions.

Marker	Wavelength (nm)	Phosphate pH	Phosphate concentration (mmol/L)	Retention Time (min)	Area (mAU*s)	Area enrichment (%)	Symmetry (USP at 10% height)
TTCA	275	2.1	100	2.30 (0.13)	139 (12)		1.84 (0.04)
			70	2.22 (0.01)	128.2 (0.1)		2.20 (0.01)
			50	2.28 (0.01)	104 (16)		2.04 (0.04)
			30	2.38 (0.01)	121.4 (0.3)		2.12 (0.02)
			20	2.52 (0.02)	121.0 (0.4)		2.22 (0.02)
	275	3.3	20	2.14 (0.01)	116 (1)		2.02 (0.04)
	225		20	2.14 (0.01)	203 (1)	75	2.03 (0.03)
PDCA	275	2.1	100	2.89 (0.01)	243 (3)		1.47 (0.01)
			70	2.88 (0.01)	240.7 (0.2)		1.44 (0.03)
			50	2.90 (0.01)	242 (3)		1.42 (0.03)
			30	2.95 (0.01)	246 (1)		1.43 (0.01)
			20	3.04 (0.01)	250.3 (0.4)		1.43 (0.02)
	275	3.3	20	2.36 (0.01)	254.8 (0.5)		1.30 (0.02)
	280		20	2.77 (0.01)	278.1 (0.8)	9	1.30 (0.02)
TDCA	275	2.1	100	3.3 (0.2)	90 (6)		1.30 (0.04)
			70	3.23 (0.01)	86.3 (0.3)		1.28 (0.02)
			50	3.28 (0.01)	84 (7)		1.35 (0.03)
			30	3.37 (0.01)	89.9 (0.1)		1.33 (0.06)
			20	3.51 (0.01)	91.0 (0.7)		1.30 (0.03)
	275	3.3	20	3.22(0.01)	73.6 (0.2)		1.19 (0.03)
	254		20	3.22 (0.01)	228.8 (0.5)	210	1.76 (0.02)
PTCA	275	2.1	100	5.02 (0.24)	186 (10)		1.07 (0.01)
			70	4.86 (0.01)	176.4 (0.1)		1.08 (0.01)
			50	4.95 (0.01)	174 (3)		1.05 (0.01)
			30	5.03 (0.01)	178 (1)		1.08 (0.01)
			20	5.22 (0.04)	177 (1)		1.07 (0.01)
	275	3.3	20	4.22 (0.01)	163.9 (0.1)		1.17 (0.02)
	225		20	4.22 (0.01)	288 (2)	76	1.18 (0.02)

Values were calculated as means (standard deviation) of n=3. Area enrichment was calculated as the percentage of the difference between peak area at the maximum wavelength and peak area at 275 nm (obtained with 20 mM phosphate pH 3.3)

The data indicates that peak shape (symmetry) for PDCA, TDCA and PTCA detected at 275nm remains the same independently of phosphate concentration at pH 2.1. Diminution of phosphate concentration slightly increased retention times. The pheomelanin marker, TTCA, was the most affected by the reduction of phosphate concentration with a symmetry value of 1.84 at 100 mM and 2.22 at 20 mM phosphate pH 2.1. When a 20 mM pH 3.3 phosphate mobile phase is used instead of pH 2.1, the retention times of the four markers are reduced and the symmetry of TTCA peak reached 2.02, considered as an acceptable limit [22,23]. Chromatographic data were recorded in the range of 200 to 400 nm. Finally, PTCA and TTCA peaks were integrated at 225 and 275 nm respectively, PDCA at 280 nm and TDCA at 254 nm, which correspond to the maximum of absorbance wavelengths of each melanin marker (Figure 2C to 2F). Measurement at the specific wavelengths improves the detection as shown by the peak area enrichment of 75, 9, 210 and 76 % obtained for TTCA, PDCA, TDCA and PTCA respectively in comparison to the signal recorded at 275nm. A good separation is obtained with 20 mM pH 3.3 phosphate mobile phase delivered at 0.6 mL/min and column 1 maintained at 36°C (Figure 2A). The use of the fully porous column with the same mobile phase and elution conditions produced an excellent separation of the four markers (Figure 2B).

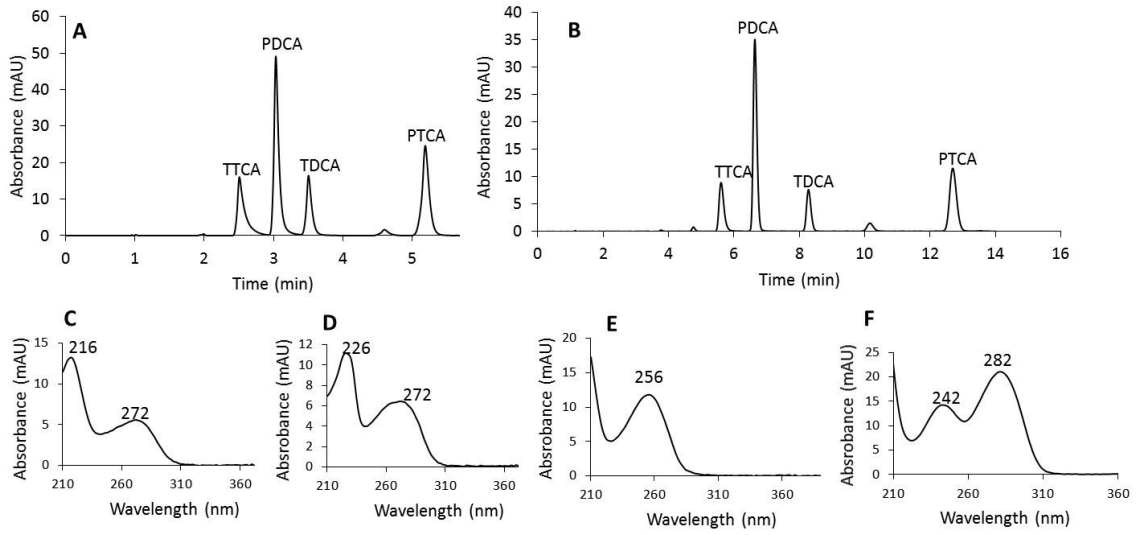

Figure 2. RP-HPLC-DAD chromatograms of a mixture of four melanin markers (10µg/mL) obtained with 20mM phosphate pH 3.3 as mobile phase at 0.6 mL/min, 36°C, signal detected at 275nm and 5µL injection volume. A) Column: Kinetex Evo C18, 150 x 3 mm, 2.6 µm particle size, 100 Å pore size from Phenomenex B) Column: Gemini C18, 150 x 3 mm, 3µm particle size, 100 Å pore size, Phenomenex C) TTCA UV- spectrum D) PTCA UV- spectrum E) TDCA UV- spectrum and F) PDCA UV- spectrum

However, the duration of analysis increased three-fold. The analysis time reduction with the core-shell particles is in accordance with the particle morphology favoring a quick mass transfer from the mobile phase through to the outer layer of stationary phase and back again. Validation of the method under the optimized conditions for the core-shell and fully porous column is presented in Table 2. Calibration curves were linear for all markers between 0.25 and 10 µg/mL with $r \geq 0.9999$. The calculated limit of

detection and quantification (LOD and LOQ) were similar for both column over the range from 0.015 to 0.020 µg/mL for the core-shell column and from 0.013 to 0.016 µg/mL for the fully porous column.

Table 2. Validation of the method with respect to linearity of calibration, retention time of the four melanin markers studied.

Markers (Wavelength, nm)	Linearity range (µg/mL)	Retention time (RT) * (min)	Regression equation	Correlation (r^2)	LOD♣ (µg/mL)	LOQ♣ (µg/mL)	%RSD (RT)	%RSD (peak area)
TTCA (225)	0.25-10	2.13 5.11	y = 663.119X + 1.347 y = 66.536x – 0.791	1.0000 1.0000	0.017 0.016	0.050 0.048	0.03 0.10	0.17 0.03
PDCA (280)	0.25-10	2.80 6.34	y = 119.861x -1.2203 y = 129.436x -1.595	1.0000 1.0000	0.015 0.014	0.047 0.041	0.04 0.06	0.27 0.06
TDCA (254)	0.25-10	3.26 7.85	y = 87.620x – 2.299 y = 88.175x – 0.713	1.0000 1.0000	0.020 0.013	0.060 0.039	0.02 0.06	0.06 0.04
PTCA (225)	0.25-10	4.34 12.23	y = 115.390x +1.063 y = 119.803x -1.269	1.0000 1.0000	0.016 0.013	0.048 0.040	0.05 0.06	0.39 0.03

Values were calculated as means of n = 3

*Retention times of standards at 10 µg/mL, injection volume of 20µL

♣LOD: limits of detection calculated as LOD = 3.3(SD/S); LOQ: limits of quantification; calculated as LOQ = 10(SD/S); RSD (%): percentage relative standard deviation.

The first line corresponds to results obtained with Kinetex EVO C18 column and the second with the Gemini C18 column

3.2 SPE extraction optimization of standards

In order to take into account the ionization capacity of melanin markers at basic pH, a weak anion exchange extraction was realized. Analytical conditions are critical for satisfactory elution and recovery of compounds in SPE. For that we first tested the extraction method using aqueous melanin standards. Strata-X-AW 33µm cartridges were selected to provide relatively high recoveries due to their capacity of interaction with acidic compounds (pKa<5) as the sorbent consists of a weak anion polymer. In addition, the polymer tolerates a wash step with 100% of organic solvent. For the first step, the sorbent was conditioned with 100% acetonitrile and 100 mmol/L sodium acetate pH 5.2. At this pH, at least one acidic function of melanin markers loses a proton and is retained by the sorbent. The elution step was performed comparing 5% (v/v) aqueous NH_4OH and 5% (v/v) NH_4OH in 95% methanol or acetonitrile. Direct HPLC injection of extracts after adjustment to pH 3.3 produces deformed chromatographic peaks (data not shown), principally due to composition differences between sample solvent and mobile phase. To overcome these problems, extracts were lyophilized overnight and reconstituted with the mobile phase before injection. Lyophilisation of aqueous NH_4OH is easier to handle in contrast to organic solvents since sample freezing is achieved at -20°C. Another advantage of reconstitution of lyophilized extracts is the possibility of pre-concentration of analytes, in case of samples with low melanin content. The recovery results of aqueous standard solution were calculated by comparison of analyte amounts obtained after extractions and those in the initial working solutions. Average recovery at 2 and 4 µg/mL (Table 3) exhibit very high recovery values between 91 and 96%, with an RSD between 5 to 7%.

Table 3. Recoveries obtained by SPE from aqueous solutions of standards containing PTCA, PDCA, TDA and TTCA at 2 and 4 µg/mL.

Concentration (µg/mL)	% Recovery			
	TTCA	PDCA	TDCA	PTCA
2	87.6	89.2	85.9	86.4
	98.4	100.2	99	98.9
	87.3	91.3	88.8	88.6
4	87.7	91.6	86.9	87.0
	99.0	101.8	92.6	94.2
	102.0	104.2	93.8	97.6
mean	93.7	96.4	91.2	92.1
STD	6.8	6.4	4.9	5.5
RSD (%)	7.3	6.6	5.4	6.0

3.3 SPE extraction in spiked biological samples

The SPE extraction was performed using fortified depigmented cell extracts (A375 cell lines) at 1, 2 and 5 µg/mL. HPLC-DAD analysis of all fractions from the different steps of SPE extraction was performed and compared to the untreated sample analysis. Satisfactory recovery values for TTCA, PTCA, PDCA and TDCA standards were obtained (97.8; 97.7; 99.1 and 103.4% respectively with RSD $\leq$ 5.6%) for the spiked depigmented cell line samples (Table 4), validating the use of weak anion exchange SPE method fo r the analysis of melanin markers in biological matrix.

Table 4. Recoveries obtained after SPE from spiked A375 cells at three concentrations.

	Expected mass (µg)	Measured mass (µg)	Recovery (%)
TTCA	0.877	0.881	100.4
	2.196	2.105	95.8
	4.393	4.266	97.1
mean			97.8
SD			2.4
RSD (%)			2.4
PTCA	1.003	1.041	103.9
	2.511	2.350	93.6
	5.022	4.804	95.7
mean			97.7
SD			5.4
RSD (%)			5.6
PDCA	0.989	1.041	102.9
	2.478	2.350	96.9
	4.955	4.804	97.5
mean			99.1
SD			3.3
RSD (%)			3.4
TDCA	0.980	0.980	100.0
	2.454	2.580	105.1
	4.908	5.160	105.1
mean			103.4
SD			3.0
RSD (%)			2.9

Chromatograms of untreated samples and the elution SPE fraction of non–spiked and spiked A375 samples are presented in Figure 3. Chromatograms of untreated samples (Figure 3A) present weak retained compounds with high response signals of about 2000 mAU. In Figure 3B, chromatogram overlay of a sample before and after SPE shows that SPE disminishes large quantities of highly polar products that are weakly retained by the stationary phase. A peak at 8.30 min that co-eluted with TDCA is totally removed after SPE extraction, as are small interference peaks at the retention time of TTCA (5.42 min). Figures 3C and 3D allow the comparison of a crude sample before SPE and the sample spiked with a mixture of four markers at a concentration of 1 µg/mL after SPE. This comparison shows that melanin standard peaks are well separated from minor matrix sample peaks that remain after SPE extraction. All together, these results demonstrate the improvement of the chromatographic profile using SPE extraction. The remaining peaks after SPE extraction, other than melanin standards, are probably ionic compounds that have very similar UV spectra with a maximum of absorption around 254nm.

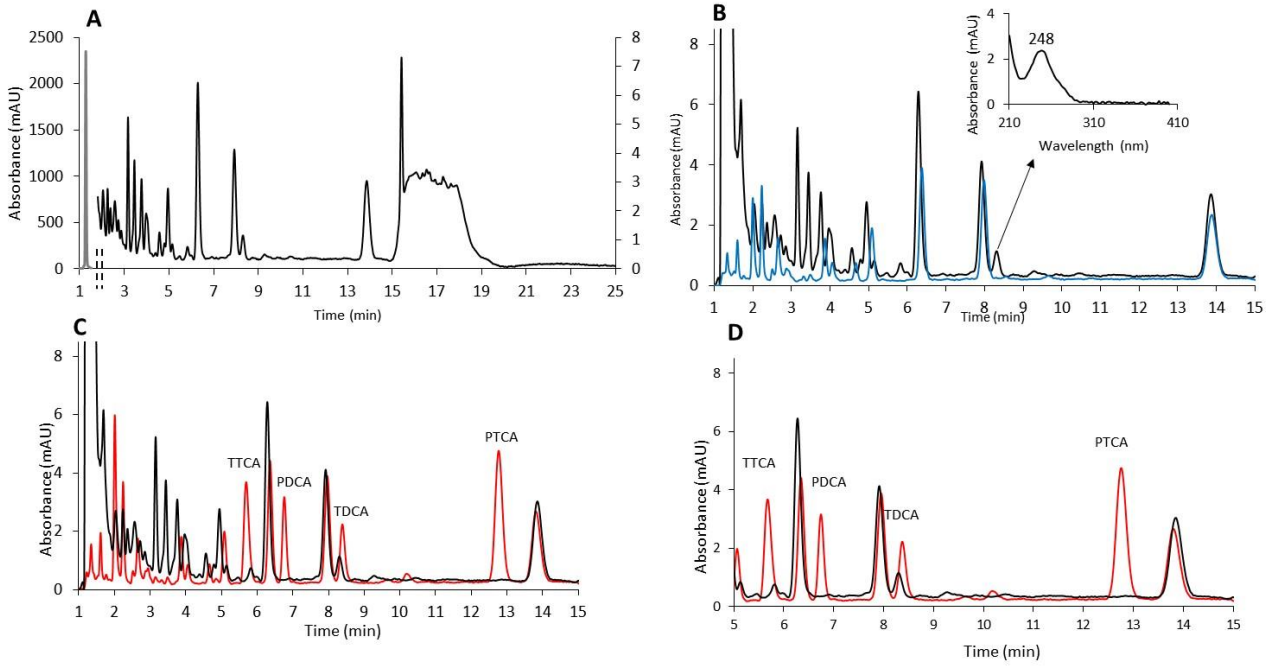

Figure 3. Chromatograms obtained by RP-HPLC-DAD (Column: Gemini C18, 150 x 3 mm, 3µm particle size, 100 Å pore size, Phenomenex) of A375 non-pigmented cells. A) crude sample before SPE method. A break was introduced in the x-axis and note the differences in y-axis scales. B) overlay of a crude sample before and after SPE. C) overlay of a crude sample before SPE and a spiked sample with a mixture of four markers (at a concentration of 1 µg/mL) after SPE. D) zoom of chromatogram C that shows the zone of interest between 5 and 15 min of analysis.

3.4 Application to melanoma samples

The analysis of eumelanin and pheomelanin in melanoma cells and tumors was then achieved with the validated method. In Figure 4, we present an example of a chromatogram of a SK-MEL-3 pigmented tumor. Figure 4A shows the reduction of matrix peaks after SPE. Focus in analysis time between 1.5 and 2 min (Figure 4B) highlights the TTCA markers present in SK-MEL-3 samples. Different detection wavelengths and UV spectrum library of melanin markers were used to confirm the identity of markers. The correlation of UV spectra between reference standards and markers in samples is expressed as a match factor (values from 0 to 1000). To confirm the peak identity we fixed the match factor upper limit to 970, corresponding to 97.0% peak purity. For Figure 4B, the match factor of peaks in the load and elution fraction that have the same retention time as TTCA are 905 and 996.5 respectively, confirming the presence of TTCA in the elution fraction and the removal of interference in the load fraction. The PTCA peak at 4.2 min is detected in the elution fraction.

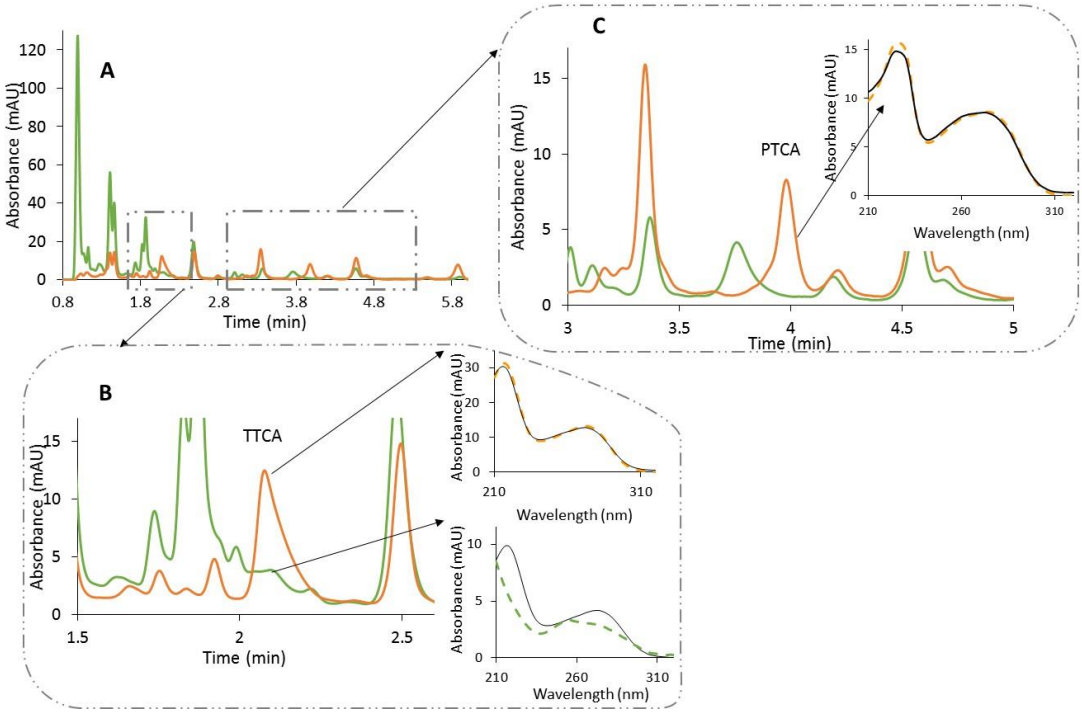

Figure 4. Chromatograms of SK-MEL-3 tumor obtained by RP-HPLC-DAD (column: Kinetex Evo C18, 150 x 3 mm, 2.6 µm particle size, 100 A pore size from Phenomenex) after SPE: ___ fraction obtained during sample loading, ___Elution-fraction, ___UV spectrum of standards, ___ UV spectrum of the sample. A) Chromatograms between 0 and 6 min of analysis corresponding to maximum elution time of melanin markers B) Zoom between 1.5 and 3 min corresponding to the TTCA elution zone C) Zoom between 3 and 5 min of analysis corresponding to the PTCA elution zone.

We use the previously reported factors of 34 to convert TTCA to pheomelanin and 80 for conversion of PTCA into eumelanin [12], for human melanoma samples. We find a good positive ratio (0.91 +/-

0.22; n=10) between EM+PM obtained by HPLC and total melanin measured by the standard spectrophotometric method at 405nm, indicating a good fit of the conversion factor employed. However, for murine tumors the best fit was obtained when eumelanin was calculated by multiplying PTCA by a factor of 60 (EM+PM/TM of 1.04 +/- 0.25) instead of 80 (EM+PM/TM of 1.35 +/- 0.35). The PTCA conversion factor is subject to several variables such as DHI/DHICA ratio in EM, which can be modified by the Tyrp activity. We hypothesize that the variation comes from a different ratio of DHI/DHICA in melanoma tumors between mice and humans.

The determination of EM and PM in melanoma tumors and cells of different pigmentation is presented in Figures 5A and 5B respectively. B16BL6 murine melanoma tumors are highly pigmented with 98.2 % of eumelanin. In parallel, TTCA was below of the detection limit for the B16BL6 cells, highlighting the eumelanic pigmentation of this cell line. Similarly, the murine B16F10 cell line is mainly eumelanic but it is 35% less pigmented, although this difference is not perceived visually. These results are in agreement with the literature on cells from C57BL/6J black mice which contain mostly the EM pigment [15,24,25]. Less pigmented SK-MEL-3 human melanoma tumors and NHEM cells contain about 30% and 24% of pheomelanin respectively. The obtained percentage of pheomelanin in SK-MEL-3 tumors and NHEM can be compared to recent published data of melanin content in human epidermis that show approximately 26 % of pheomelanin and 74% of eumelanin independently of the degree of pigmentation [26].

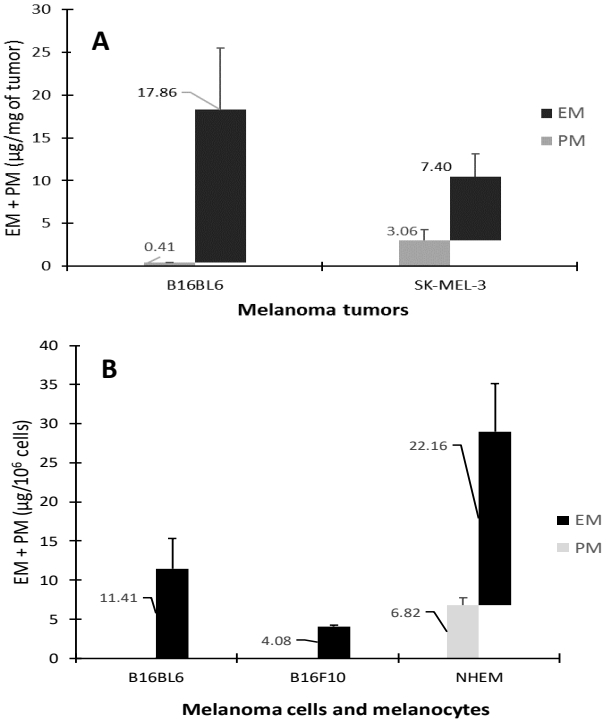

Figure 5. Content of eumelanin and pheomelanin in: A) murine melanoma tumors B16BL6 (n=10) and human melanoma tumors SK-MEL-3 (n=10); B) murine melanoma cells B16BL6 (n=2), B16F10 (n=2) and human melanocytes (n=4).

A good correlation (r^2 = 0.7849 and 0.9046; P <0.0001) is observed between total melanin obtained by chemical degradation method followed by SPE and HPLC analysis and total melanin measured by spectrophotometric method after potassium hydroxide solubilization for tumors and cells (Figure 6A and 6B), another indication of the good fit of the employed conversion factors.

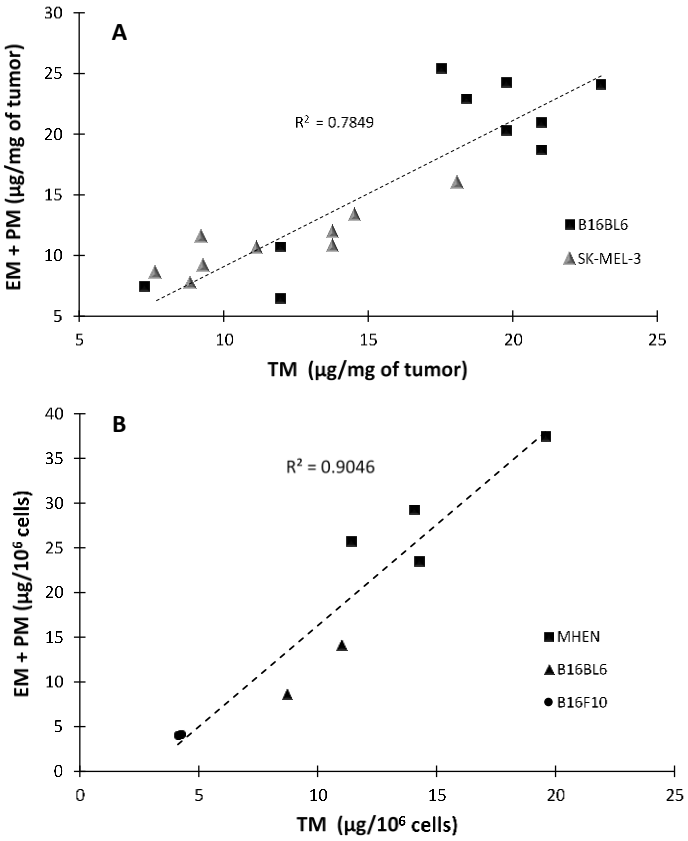

Figure 6. Correlation between total melanin obtained by chemical degradation method followed by SPE and HPLC analysis (eumelanin EM, and pheomelanin PM) versus total melanin (TM) content after potassium hydroxide solubilisation measured at 405nm. A) Melanoma tumors B16BL6 (n=10) and human melanoma tumors SK-MEL-3 (n=10); B) murine melanoma cells B16BL6 (n=2), B16F10 (n=2) and human melanocytes (n=4). Correlation are significant at P < 0.0001.

In order to confirm the advantages of the developed SPE-HPLC method, we present in Table 5 the comparison of results obtained after the analysis of melanoma tumors (B16BL6 and SK-Mel-3) and cell lines (B16BL6, B16F10 and human melanocytes) with and without SPE. The match factors between UV spectra of TTCA and PTCA markers of standards and samples are presented in heat map form. We fix minimum, middle and upper match factor limits at 960, 970 and 990 respectively. We show that match factors of chromatographic peaks corresponding to the retention time of TTCA obtained without SPE for SK-MEL-3 tumors are between 860 and 967, indicating the low purity produced by co-elution of matrix compounds. The TTCA marker was not detectable in the NHEM cells in this condition. After SPE

treatment of the same sample, we observe an excellent improvement of TTCA match factors to 968 to 999, reflecting the elimination of matrix interferences. In the case of NHEM cells, the TTCA becomes quantifiable and is unambiguously identified (match factor 974 to 999). The impact of SPE is less pronounced for the PTCA marker. Notwithstanding, improvements are evident in the analysis of B16BL6 and B16F10 cell lines, resulting in a best match between the melanin detected by HPLC after SPE and the total melanin measured by the spectrophotometric method.

Table 5. Comparison of melanin content in tumor and cells samples after HPLC analysis with and without the developed SPE method.

Sample	HPLC without SPE							HPLC with SPE							UV		
	TTCA		PM	PTCA		EM	PM+EM	TTCA		PM	PTCA		EM	PM+EM	Total melanin 405 nm	%PM	%EM
	match	Aire (mAU*s)		match	Aire (mAU*s)			match	Aire (mAU*s)		match	Aire (mAU*s)					
Tumor B16L6 #1	929.1			997.4	839	27.16	27.16	972.0	26.4	0.32	998.0	2024	23.84	24.16	23.05	1.3	98.7
Tumor B16BL6 #2	860.4			996.7	815	26.95	26.95	968.1	38.4	0.47	995.7	2075	24.96	25.43	17.53	1.8	98.2
Tumor B16BL6 #3	919.4			993.2	889	26.39	26.39	978.8	37.8	0.38	997.1	2280	22.57	22.95	18.39	1.7	98.3
Tumor SK-MEL-3 #1	966.8	37.4	1.13	991.8	99	3.94	5.07	998.9	209.9	2.12	996.9	427	5.64	7.76	8.81	27.3	72.7
Tumor SK-MEL-3 #2	962.0			993.1	189	8.56	8.56	998.5	186.5	2.34	997.1	419	6.89	9.23	9.27	25.4	74.6
Tumor SK-MEL-3 #3	960.5			991.9	232	10.99	10.99	999.1	292.5	3.85	997.3	556	9.58	13.42	14.51	28.7	71.3
Cell B16BL6 #1		<LOD		978.2	133	15.22	15.22		<LOD		994.5	719	14.16	14.16	11.00		100
Cell B16L6 #2		<LOD		982.8	112	11.86	11.86		<LOD		989.9	429	8.66	8.66	8.71		100
Cell B16F10 #1		<LOD		968.7	63	6.20	6.20		<LOD		997.4	210	4.16	4.16	4.26		100
Cell B16F10 #2		<LOD		965.3	62	6.03	6.03		<LOD		995.6	205	4.00	4.00	4.12		100
Cell MHEN #1		<LOD		996.3	181	33.4	33.38	998.5	245.9	7.82	995.3	711	29.6	37.46	19.58	20.9	79.1
Cell MHEN #2		<LOD		992.3	173	23.3	23.27	992.4	174.6	5.60	992.2	426	17.9	23.48	14.27	23.9	76.1
Cell MHEN #3		<LOD		993.0	199	28.1	28.08	992.3	208.4	6.61	993.1	545	22.6	29.25	14.06	22.6	77.4
Cell MHEN #4		<LOD		990.4	229	50.8	50.75	973.9	107.2	7.26	992.3	209	18.5	25.76	11.408	28.2	71.8

- Values of EM, PM, EM+PM and Total melanin at 405 nm are expressed in µg/mg tumor or µg/10^6 cells
- Match factor between UV spectra of melanin markers standards and markers in sample is presented in heat map form. Minimum, middle and upper limit were fixed at 960, 970 and 990, respectively.
- <LOD below the limit of detection

4. Conclusions

Weak anion exchange solid-phase extraction offers a consistent, inexpensive and suitable preparation method to improve eumelanin and pheomelanin determination in melanomas with high recoveries (97 to 103%) of melanin markers and good precision (RSD $\leq$ 5.6%). The optimized HPLC-DAD method increases the detection sensitivity of melanin markers, with detection limits ranging from 0.015 to 0.020 µg/mL. This method reduces the time of analysis and enables a decrease of solvent consumption, since complete marker elution is achieved in 5 min and the total analysis of complex samples is accomplished in 15 min.

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References

[1] S. Ito, K. Wakamatsu, Quantitative Analysis of Eumelanin and Pheomelanin in Humans, Mice, and Other Animals: a Comparative Review, Pigment Cell Res. 16 (2003) 523–531. doi:10.1034/j.1600-0749.2003.00072.x.

[2] J.D. Simon, D.N. Peles, The red and the black, Acc. Chem. Res. 43 (2010) 1452–1460. doi:10.1021/ar100079y.

[3] S. Ito, K. Wakamatsu, Diversity of human hair pigmentation as studied by chemical analysis of eumelanin and pheomelanin: Human hair pigmentation, J. Eur. Acad. Dermatol. Venereol. 25 (2011) 1369–1380. doi:10.1111/j.1468-3083.2011.04278.x.

[4] L. Panzella, L. Leone, G. Greco, G. Vitiello, G. D'Errico, A. Napolitano, M. d'Ischia, Red human hair pheomelanin is a potent pro-oxidant mediating UV-independent contributory mechanisms of melanomagenesis, Pigment Cell Melanoma Res. 27 (2014) 244–252. doi:10.1111/pcmr.12199.

[5] D. Mitra, X. Luo, A. Morgan, J. Wang, M.P. Hoang, J. Lo, C.R. Guerrero, J.K. Lennerz, M.C. Mihm, J.A. Wargo, K.C. Robinson, S.P. Devi, J.C. Vanover, J.A. D'Orazio, M. McMahon, M.W. Bosenberg, K.M. Haigis, D.A. Haber, Y. Wang, D.E. Fisher, An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background, Nature. 491 (2012) 449–453. doi:10.1038/nature11624.

[6] H.Z. Hill, G.J. Hill, UVA, Pheomelanin and the Carcinogenesis of Melanoma, Pigment Cell Res. 13 (2000) 140–144. doi:10.1034/j.1600-0749.13.s8.25.x.

[7] S. Ito, K. Wakamatsu, Chemistry of Mixed Melanogenesis—Pivotal Roles of Dopaquinone, Photochem. Photobiol. 84 (2008) 582–592. doi:10.1111/j.1751-1097.2007.00238.x.

[8] H. Ozeki, S. Ito, K. Wakamatsu, I. Ishiguro, Chemical characterization of pheomelanogenesis starting from dihydroxyphenylalanine or tyrosine and cysteine., Biochim. Biophys. Acta BBA - Gen. Subj. 1336 (1997) 539–548. doi:10.1016/S0304-4165(97)00068-8.

[9] V. del Marmol, S. Ito, B. Bouchard, A. Libert, K. Wakamatsu, G. Ghanem, F. Solano, Cysteine deprivation promotes eumelanogenesis in human melanoma cells, J. Invest. Dermatol. 107 (1996) 698–702.

[10] K. Wakamatsu, K. Ohtara, S. Ito, Chemical analysis of late stages of pheomelanogenesis: conversion of dihydrobenzothiazine to a benzothiazole structure, Pigment Cell Melanoma Res. 22 (2009) 474–486. doi:10.1111/j.1755-148X.2009.00580.x.

[11] R.E. Boissy, C. Sakai, H. Zhao, T. Kobayashi, V.J. Hearing, Human tyrosinase related protein-1 (TRP-1) does not function as a DHICA oxidase activity in contrast to murine TRP-1, Exp. Dermatol. 7 (1998) 198–204. doi:10.1111/j.1600-0625.1998.tb00324.x.

[12] S. Ito, Y. Nakanishi, R.K. Valenzuela, M.H. Brilliant, L. Kolbe, K. Wakamatsu, Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins: Alkaline hydrogen peroxide oxidation of melanin, Pigment Cell Melanoma Res. 24 (2011) 605–613. doi:10.1111/j.1755-148X.2011.00864.x.

[13] A. Napolitano, M.R. Vincensi, P. Di Donato, G. Prota, G. Monfrecola, Microanalysis of Melanins in Mammalian Hair by Alkaline Hydrogen Peroxide Degradation: Identification of a New Structural Marker of Pheomelanins, J. Invest. Dermatol. 114 (2000) 1141–1147. doi:10.1046/j.1523-1747.2000.00977.x.

[14] L. Panzella, P. Manini, G. Monfrecola, M. d'Ischia, A. Napolitano, An easy-to-run method for routine analysis of eumelanin and pheomelanin in pigmented tissues, Pigment Cell Res. 20 (2007) 128–133. doi:10.1111/j.1600-0749.2006.00359.x.

[15] S. Ito, K. Fujita, Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography, Anal. Biochem. 144 (1985) 527–536. doi:10.1016/0003-2697(85)90150-2.

[16] S. Ito, K. Wakamatsu, K. Glass, J.D. Simon, High-performance liquid chromatography estimation of cross-linking of dihydroxyindole moiety in eumelanin, Anal. Biochem. 434 (2013) 221–225. doi:10.1016/j.ab.2012.12.005.

[17] K. Wakamatsu, S. Ito, J.L. Rees, The Usefulness of 4-Amino-3-hydroxyphenylalanine as a Specific Marker of Pheomelanin, Pigment Cell Res. 15 (2002) 225–232. doi:10.1034/j.1600-0749.2002.02009.x.
[18] M. d'Ischia, K. Wakamatsu, A. Napolitano, S. Briganti, J.-C. Garcia-Borron, D. Kovacs, P. Meredith, A. Pezzella, M. Picardo, T. Sarna, J.D. Simon, S. Ito, Melanins and melanogenesis: methods, standards, protocols, Pigment Cell Melanoma Res. 26 (2013) 616–633. doi:10.1111/pcmr.12121.
[19] S. Ito, S. Miyake, S. Maruyama, I. Suzuki, S. Commo, Y. Nakanishi, K. Wakamatsu, Acid hydrolysis reveals a low but constant level of pheomelanin in human black to brown hair, Pigment Cell Melanoma Res. 31 (2018) 393–403. doi:10.1111/pcmr.12673.
[20] S. Ito, Reexamination of the structure of eumelanin, Biochim. Biophys. Acta BBA. (1986) 155–161. doi:doi.10.1016/0304-4165(86)90146-7.
[21] Z. Boudhraa, F. Rondepierre, L. Ouchchane, R. Kintossou, A. Trzeciakiewicz, F. Franck, J. Kanitakis, B. Labeille, J. Joubert-Zakeyh, B. Bouchon, J.L. Perrot, S. Mansard, J. Papon, P. Dechelotte, J.-M. Chezal, E. Miot-Noirault, M. Bonnet, M. D'Incan, F. Degoul, Annexin A1 in primary tumors promotes melanoma dissemination, Clin. Exp. Metastasis. 31 (2014) 749–760. doi:10.1007/s10585-014-9665-2.
[22] C FDA, Rockville., Guidance, Center for Drug Evaluation and Research, U.S. Food and Drug Administration. Reviewer Guidance, Validation of Chromatographic Methods, (1994).
[23] Bose, Anirbandee, HPLC Calibration Process Parameters in Terms of System Suitability Test., Austin Chromatogr. 1 (2014) 1–4.
[24] K. Jimbow, Y. Miyake, K. Homma, Characterization of Melanogenesis and Morphogenesis of Melanosomes by Physicochemical Properties of Melanin and Melanosomes in Malignant Melanoma, 44 (1984) 8.
[25] H.Z. Hill, G.J. Hill, K. Cieszka, P.M. Plonka, D.L. Mitchell, M.F. Meyenhofer, P. Xin, R.E. Boissy, Comparative action spectrum for ultraviolet light killing of mouse melanocytes from different genetic coat color backgrounds, Photochem. Photobiol. 65 (1997) 983–989.
[26] S. Del Bino, S. Ito, J. Sok, Y. Nakanishi, P. Bastien, K. Wakamatsu, F. Bernerd, Chemical analysis of constitutive pigmentation of human epidermis reveals constant eumelanin to pheomelanin ratio, Pigment Cell Melanoma Res. 28 (2015) 707–717. doi:10.1111/pcmr.12410.