# Endogenous 1-H-Pyrrole-2,3,5-tricarboxylic Acid (PTCA) in Hair and its Forensic Applications: A Pilot Study on a Wide Multi-Ethnic Population

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**Abstract**

Over the years, several studies have shown that many factors are likely to affect the results of forensic hair analyses and complicate their interpretation. Among these factors, one of the major drawbacks in hair analysis is the affectability of deposited xenobiotics by cosmetic treatments which could be eventually used to adulterate the sample. It is well known that some cosmetic treatments containing hydrogen peroxide, such as permanent dyeing or bleaching, lead to the formation of 1-H- pyrrole- 2,3,5- tricarboxylic acid (PTCA), a melanin degradation product. Considering that PTCA is also an endogenous compound,

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spontaneously formed by natural oxidation of melanin, its only detection in hair is not enough to confirm a cosmetic oxidative treatment. For this reason, the aim of the present work was to develop and validate a reliable liquid-liquid extraction method in ultra-high-performance liquid chromatographic–tandem mass spectrometry for the determination of endogenous PTCA in hair from a wide multi-ethnic population (African, Arab, Asian-Pacific, Caucasian, Hispanic, Indian). According to previous studies, untreated hair samples showed a PTCA content of 8.54 ± 5.72 ng/mg (mean ± SD), ranging between 0.44 and 23.7 ng/mg; after *in vitro* cosmetic bleaching, PTCA increased to 16.8 ± 6.95 ng/mg (range 4.16–32.3 ng/mg). Comparing baseline PTCA levels of each subgroup with the others, we could not observe any statistically significant difference, except for Caucasians ($p < 0.05$), wherein the concentrations were lower. Further studies and a wider sampling are necessary to elucidate the role of PTCA as diagnostic marker of cosmetic hair treatment in forensic field.

**Keywords:** hair analysis, cosmetic hair treatment, melanin degradation, Pyrrole- 2,3,5-tricarboxylic acid (PTCA)

## Introduction

In terms of hair testing for forensic purposes, it is important to consider the several factors that could affect the results of the analyses and complicate their interpretation. Factors including physiochemical and structural properties of drugs, such as melanin affinity and membrane permeability (1), external contamination, hair melanin content (2) and cosmetic treatment are just some of the many parameters that could hinder the interpretation of the analytical results. Moreover, there is a growing tendency of attempting to avoid detection of

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the investigated drugs into the hair matrix by undergoing physical and mechanical alteration and/or cosmetic treatment, such as bleaching or dyeing, using chemical products. The latter not only affect the hair fiber and its proteins, but they also degrade other components (i.e., melanin) (3). The hair shaft is composed by three layers: cuticle, cortex, and inner (but not always present) layer, medulla. Melanin is present in the cortex and medulla, not in the cuticle. Recent studies have focused on the action of different commercial hair products and the correlation between their use and the effects on incorporated xenobiotics (4, 5). Treating hair with high temperatures and aggressive chemical products leads to a damage of the cuticle, the outermost protective layer of the hair shaft (6), an increment of the porosity and a significant loss of xenobiotics included in hair (7). In addition, strong bleaching with hydrogen peroxide ($H_2O_2$) can be an irreversible destructive treatment for melanin and it leads to a loss of xenobiotic binding capacity (8). Briefly, agents usually containing ammonium hydroxide open the scales of the cuticle to facilitate the entry of other compounds, such as powerful oxidants like $H_2O_2$, that attack the pigment residing within the hair cortex and decolorizes the hair mass. After oxidative treatment, decreases in xenobiotics concentration were observed (7). Indeed, the effects of $H_2O_2$ could compromise the analytical results (5), by giving not only false negatives but also false positives (4). Hair color is determined by its melanin content maintained by the melanocytes functionality in the hair follicle (9); more precisely, hair pigmentation is given by the quantity and ratio between two main melanin subgroups coexisting within the same melanocyte: eumelanin and pheomelanin (10). Eumelanin is a heterogeneous macromolecule composed by 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomers derived by the oxidative metabolism of tyrosine, while pheomelanin is also a heterogeneous macromolecule but made

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up of benzothiazine units derived from cysteinyldopa (11, 12). Eumelanin gives human hair a brownish to black color (13), whereas pheomelanin is responsible for yellow to reddish shades of color and it is more resistant to oxidative degradation than eumelanin (14). According to Joseph et al., xenobiotics bind more into darker hair than lighter hair because of its higher eumelanin content (15). Moreover, melanin is known to exhibit autofluorescence; however, it is difficult to detect because it is a weak emitter with a very low quantum yield of fluorescence (16), but under oxidative conditions fluorescence increases considerably (17). Multiple studies on the oxidation of melanin demonstrated as strong treatment with $H_2O_2$ leads to the formation of different degradation products, such as pyrrole-2,3-dicarboxylic acid (PDCA), 1-H-pyrrole-2,3,5-tricarboxylic acid (PTCA) and aminohydroxyphenylalanine isomers (AHPs) (18). Specifically, PTCA and PDCA derive from the oxidative degradation of DHICA-derived units and DHI-derived units, respectively (19, 20), whereas AHPs are degradation products of pheomelanin (21). PTCA is regarded as a basis for eumelanin quantitative analysis since it is considered its major oxidation product (22). In a study published in 2018, Petzet-Witt et al. investigated how PTCA levels might vary after oxidative hair treatment in order to correlate its behavior with the one proper of other xenobiotics under the same stressful conditions. PTCA content showed an ascending trend in parallel with both incubation time and increasing $H_2O_2$ concentrations. For this reason, PTCA was proposed as a potential biomarker for the oxidative hair treatment (23). However, considering that PTCA is also an endogenous compound spontaneously formed by natural degradation of melanin, its detection alone cannot be considered enough for the confirmation of a cosmetic hair treatment. Therefore, determining a range for PTCA baseline using natural/untreated hair could be a useful tool for the discrimination between oxidatively

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treated and untreated hair. Moreover, it could be interesting to observe how PTCA baseline levels may vary between different ethnic subgroups (African, Arab, Asian-Pacific, Caucasian, Hispanic, Indian), considering that ethnicities usually characterized by darker hair might present higher eumelanin content; despite that, we cannot find in literature any reference that classifies ethnic hair in terms of melanin content. Different studies conducted on human hair showed that classification can be obtained by considering parameters such as texture, defined by diameter and section shape, mechanical properties and moisture (24, 25). The main structure of keratin fibers is the same for all the subgroups (25).

The aim of this work was to develop and validate a liquid-liquid extraction (LLE) method in ultra-high-performance liquid chromatographic–tandem mass spectrometry (UHPLC–MS-MS) for the determination of PTCA baseline levels in hair from six ethnic subgroups (n = 156). In addition, hair samples were analyzed before and after *in vitro* oxidation by both MS technique and fluorescence microscopy in order to investigated whether this treatment could be detected and confirmed also by the increment of fluorescence emission, as previously studied by Witt et al. in 2016 (8).

## Materials and Methods

### Chemicals

1H-Pyrrole-2,3,5-tricarboxylic acid (PTCA, 97%) and diazepam-$d_5$ used as internal standard (IS) were supplied by Fluorochem (Hadfield, UK) and Sigma Aldrich (Milan, Italy), respectively. Ultrapure water, ethyl acetate, methanol, dichloromethane, acetonitrile, hydrochloride acid (HCl, 37%), formic acid (98–100%) were provided by Carlo Erba (Milan, Italy). Ultrapure water and all other reagents were of UHPLC–MS grade. Wella Professional

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Welloxon Perfect 12% $H_2O_2$ 40 vol oxidation cream and Wella Professionals Blondor bleach powder were obtained from Wella (Darmstadt, Germany).

**Instrumental**

Analyses were performed on a 1290 Infinity UHPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a Q Trap 5500 linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt, Germany) and equipped with an electrospray ionization (ESI) source. Chromatographic separation was carried out on a Luna Omega Polar C18 column (100 mm length x 2.1 mm i.d, 1.6 particle size) at 25°C using a linear gradient elution with two solvents: 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent A and B were 97.5% and 2.5% at 0.00 minutes, respectively. Solvent B was increased to 15% from 0.00 to 2.00 min, then increased to 17% from 2.00 to 3.00 minutes, to 20% from 3.00 to 3.50 min, to 40% from 3.50 to 6.50 min to 98% from 6.50 to 8.00, held at 98% from 8.00 to 10.50 minutes, and then decreased back to 2.5% from 10.50 at 12.50 for re- equilibration. The flow rate was kept constant at 0.45 mL/min during the analysis. The separated analytes were detected with a triple quadrupole MS operated in multiple reaction monitoring (MRM) mode via both positive and negative ESI using the precursor ion and product ions transition showed in Table I. The instrumental conditions were optimized by direct infusion (flow rate 7 μL/min) of PTCA and diazepam-$d_5$ solution (100 ng/mL) and were as follows: Entrance potential 10 eV, curtain gas 25 psi, ion spray voltage 5500 eV (+ESI) and -4500 (-ESI), ion source temperature 450°C, ion source gas 1 and 2 55 psi. Data acquisition and processing was performed using Analyst®1.6.2 and MultiQuant®2.1.1 software (Sciex, Darmstadt, Germany), respectively.

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**Hair samples collection and PTCA extraction**

Untreated hair samples (n = 156) were collected from the back region of the head and the 3–6 cm proximal segment was used for analysis. Sampling was performed among brown/dark male hair from six ethnic subgroups (African, Arab, Asian-Pacific, Caucasian, Hispanic, Indian) with self-reported no previous cosmetic treatments. Albino rabbit hair was provided by Prof. Giuseppe Rossoni of the Department of Medical Biotechnology and Translational Medicine (University of Milan) and was used as PTCA- free hair samples for calibration standards and for quality controls. All samples were washed with 3 mL dichloromethane, dried at room temperature and then cut into small pieces (2–4 mm). Afterwards, 50 mg were weighed and IS was added in the amount of 20 ng. PTCA and diazepam-$d_5$ extraction from matrix was adapted from the procedure used by Petzel-Witt et al. (23), introducing minimal modifications in acid concentration, solvent volume and hydrolysis features. The final procedure included samples incubation with 2 mL HCl (0.125 N) for 18 hours at 45°C. After centrifugation at 13,000 g for 10 min, 1-mL aliquot of supernatant was transferred into a glass tube and mixed with 3 mL of ethyl acetate. After mixing for 10 minutes, the samples were centrifuged for 10 minutes, the supernatant was evaporated and then reconstituted with 80 µL of methanol. A 0.5-µL aliquot was injected into UHPLC–MS-MS system for PTCA determination.

**Effect of *in vitro* bleaching treatment using professional products on PTCA**

Among the 156 collected samples, n = 60 (10 for each ethnic subgroup) hair samples were treated by professional bleaching products with 12% $H_2O_2$, described at paragraph 2.1, for 30

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minutes in the dark, according to the manufacturer's instructions. Hair samples were rinsed with water until the pH of the washing water was neutral. Hair samples were dried at room temperature, extracted as described above and analyzed for PTCA content in UHPLC–MS-MS. Statistical evaluation of group differences (untreated and treated) was performed using the non- parametrical Mann- Whitney- U test (GraphPad Prism 7.00, CA, USA).

**Validation**

Method validation was performed in accordance with international recommendations for the validation of new analytical methods in hair endorsed by the Society of Hair Testing (SoHT) (26). Selectivity was assessed using hair samples of PTCA- free albino rabbits with and without addition of internal standard (blank and zero samples). Linearity was assessed by the analysis of 8 calibration levels (0, 0.1, 0.2, 0.5, 1, 2, 5, 10 ng/mg) based on the peak area ratios of the analytes to the IS against nominal analyte concentration and using a weighted $1/x^2$ linear regression. Sensitivity was expressed in terms of limit of detection (LOD) and lower limit of quantification (LLOQ). The LLOQ was determined as the lowest concentration with values for precision and accuracy within ±20% and a signal-to-noise (S/N) ratio of the peak areas ≥10, the LOD as the lowest concentration with a signal- to-noise (S/N) ratio of the peak areas ≥3. For evaluation of intra- and inter-day precision and accuracy, low- , medium- , and highquality control (QC) samples (0.1, 1, 10 ng/mg) were used. Precision and accuracy of the method were determined through the analysis of three independent replicates of QC samples by calculating the coefficient of variation (CV%) and the Bias (BIAS%). The matrix effect (%) was analyzed by spiking PTCA-free hair extracts and HCl 0.125 N using three different concentrations and comparing the mean peaks areas.

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Additionally, due to the different polarity of PTCA and IS, the matrix effect was assessed also by fortifying PTCA-free hair, drug-free human hair and HCl 0.125 with diazepam-$d_5$ (20 ng) in duplicate. Recovery (%) was determined by comparing the mean peak areas of PTCA-free hair samples fortified at three concentrations prior and after extraction. The stability of PTCA in QCs extracts was assessed by comparing the response factor after 24 h and 7 days at room temperature, and after one freeze and thaw cycle with the original vial at T0.

**Fluorescence microscopy**

Untreated and treated hair samples (n = 3 for each ethnic subgroup), prepared as described at paragraph 2.5, embedded in 100 µL glycerol based ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific, USA) were mounted on a microscopic slide and enclosed with a cover slip. Inspection of hair segments was performed using a fluorescence microscope (BX51 Olympus, Japan). For documentation, photographs were taken with an Olympus X10 camera (Olympus, Japan). For bright-field microscopy, transmitted light from a long-life halogen lamp (12 V, 50W) was used. For fluorescence microscopy, reflected light of a mercury lamp in combination with different excitation-emission filters (Olympus, Japan) was used: U-MNU2 (360-370nm - 420nm, DAPI), U-MNB2 high pass (470-490nm - 520nm, FITC), U-N41002 (510-560nm - 570-640nm, TRITC), U-N41001 band pass (460-500nm - 510-560nm, GFP) and U-N49006 (585-655nm - 660-740nm, CY5). Samples were inspected in the bright-field microscopic mode with 20x optical magnification first, then the fluorescence properties were analyzed using the 5 excitation/emission filters. Fluorescent images were acquired with the exposure time fixed at 100 msec, except for CY5 (1000 msec). Images were analyzed using Fiji ImageJ® software: for each image, three areas of the

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same dimensions were randomly selected and quantified using the measure tool. The resulting mean values of the two groups, untreated and treated, were compared.

## Results and discussion

### Analytical procedure

Figure 1 shows the representative extracted ion current (XIC) chromatograms obtained following the extraction of 50 mg PTCA-free hair sample spiked with 5 ng PTCA (0.1 ng/mg, A) and 20 ng IS (B). For linearity, 1/x2 weighted regression was evaluated by the correlation coefficient $r^2$, over eight replicates for each calibration level. Values >0.998 were achieved, thus the calibration model was considered acceptable. LOD (0.005 ng/mg) and LLOQ (0.02 ng/mg) values were adequate for the purpose of the present study. Intra- and inter-day precision (measured as coefficient of variation) and accuracy (measured as percentage error), at three concentrations (0.1, 1, 10 ng/mg), were below 15% (Table II). No additional peaks due to endogenous substances or drugs typically detected in forensic samples were observed in PTCA-free hair samples. The matrix effect ranged from 85% to 113%, recovery from 70% to 87%. Diazepam-$d_5$ proved to be adequate as internal standard since its chemical properties are similar to PTCA. Moreover, no considerable matrix effect was found on diazepam-$d_5$ both in PTCA-free hair and human drugs-free hair (107.8% and 107.4%, respectively). The stability of PTCA in QC extracts, kept in the autosampler at 25°C for 24 h and 7 days from the first analysis, showed a decrease of the nominal concentration lower than 15%.

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**PTCA endogenous levels in a multi-ethnic population**

Untreated hair samples (n = 156, color from dark brown to black) were analyzed by the present method exhibiting PTCA contents of 8.54 ± 5.72 ng/mg(mean ± SD, range 0.44 – 23.7 ng/mg).Despite the higher number of samples and the multi-ethnicity variability, our data appear similar to Petzel-Witt et al. (23)findings. In detail, results obtained for each ethnic sub group are showed in Table III. Comparing each group with the others, we could not observe any statistically significant difference, except for Caucasians ($p < 0.05$).From the literature, there is no evidence of any ethnic difference in human hair in terms of melanin content and, since it is a preliminary study, we could not exclude that the analysis might suffer of an omitted variable bias. The lower PTCA baseline mean level was found in Caucasians, while the higher one in Indians. Moreover, Indians present the broadest concentration range of PTCA, ranging from 0.44 to 23.7 ng/mg, which suggests a higher heterogeneity of melanin content.

**Effect of *in vitro* oxidative treatment on PTCA content**

Among all the collected hair samples, n = 60 (10 for each ethnic subgroup) were treated by professional bleaching products, as described at paragraph 2.4. After *in vitro* bleaching, the mean PTCA content increased to 16.8 ± 6.95 ng/mg (range 4.16 – 32.3 ng/mg). Figure 2 shows a comparison of the PTCA resulting peak areas for a Caucasian sample before (A) and after (B) treatment. Data obtained for each ethnic subgroup are showed in Table IV. The mean ratio between treated and untreated hair samples ranged from 1.77 to 3.70, whereas bleached hair showed a percentage increase in PTCA concentration ranging from 32.6 to 63.4. Figure 3 shows the comparison of untreated and treated hair samples for each ethnic

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subgroup. The bleaching treatment was significantly more effective in increasing the PTCA content in Caucasian ($p = 0.0005$) and Hispanic ($p = 0.0021$). However, the increase of the PTCA content in each subgroup in comparison to the untreated hair was statistically significant ($p < 0.05$), except for African.

**Effect of *in vitro* oxidative treatment using fluorescence microscopy**

Eighteen hair samples (3 for each ethnic subgroup) were analyzed before and after *in vitro* oxidative treatment by fluorescence microscopy, as described at paragraph 2.6. Figure 4 shows the fluoresce emission increase after oxidative treatment for each ethnic subgroup using five different fluorescence filters ranging from 360 up to 740 nm excitation wavelength. In detail, for each filter the fold increase was obtained comparing the mean florescence values obtained from the treated samples in comparison to the untreated ones. According to Witt et al. (8), an increase of fluorescence occurred after *in vitro* oxidative treatment, so it could be an indicator of oxidized melanins. Comparing to the previous work by Witt et al. (8), here the inspection of hair segments was performed using five different excitation-emission filters (from 360 up to 740 nm) expanding the excitation wavelength range. These results were in accordance with the information provided by the volunteers concerning previous hair treatments. The fluorescence observed was markedly different from untreated and oxidatively treated hair (Figure 5). Surprisingly, the CY5 filter did not show visible fluorescence emission increase after treatment. These data were slightly in accordance with PTCA variations obtained by UHPLC–MS-MS analysis, although a correlation between fluorescence emission and PTCA has not been demonstrated yet. Indeed, in African hair a lower fluorescence was found mainly when excited at 360-370 nm (DAPI filter), which

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provides the higher response in all subgroups. Corresponding PTCA mean ratio between treated and untreated for African subgroup was the lowest (Table IV).

## Conclusion

In the present study, a wide multi-ethnic population was screened for PTCA baseline level and after oxidative treatment *in vitro* for the first time. It was showed that PTCA is formed during oxidative treatment of hair melanin using a professional cosmetic treatment. By the analysis of our samples, the PTCA baseline level was found in the range of 8.54 ± 5.72 ng/mg, completely in accordance to Petzel-Witt (23), despite the higher number of samples and the multi-ethnicity variability. After *in vitro* bleaching the PTCA content increased to 16.8 ± 6.95 ng/mg. Moreover, all the analyzed samples confirmed the information provided by the volunteers concerning previous hair treatments and showed a fluorescence increment under controlled conditions of bleaching. Up to now, an indicative value of excessive oxidation remains controversial. For this reason, in order to obtain a clear evidence of an oxidative hair treatment, we aim to analyze in the next future a larger number of untreated hair samples to establish a PTCA cut-off level as diagnostic marker in forensic field.

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## Data availability

The data underlying this article are available in the article.

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figure captions

Figure 1. MRM chromatograms of PTCA-free hair sample spiked with PTCA (0.1 ng/mg) (A) and ISs (B).

Figure 2. MRM chromatograms of untreated (A) and *in vitro* treated (B) Caucasian hair sample showing PTCA (RT 2.51 min) and diazepam-$d_5$ (IS, RT 7.73 min).

Figure 3. PTCA concentrations in untreated and treated hair samples belonging to six different ethnic subgroups (in each subgroup, the PTCA content was significantly higher than in untreated hair except for African, $p < 0.05$).

Figure 4. Results of fluorescence microscopy of hair samples from six ethnic subgroup using five different filters ranging from 360 up to 740 nm excitation wavelength.

Figure 5. Untreated and treated Caucasian hair sample inspected by bright-field microscopy with transmitted light (BF) followed by fluorescence microscopy with five different fluorescence filters ranging from 360 to 740 nm excitation wavelength.

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Table I. MRM parameters: Precursor and Product Ion Transitions for PTCA and Diazepam-$d_5$, Dwell time, De-clustering Potential (DP), Collision Energy (CE)

| Compound | Precursor ion (m/z) | Product ions (m/z) | Dwell time (msec) | DP | CE (eV) |
|---|---|---|---|---|---|
| PTCA | 198.1 | 153.9<br>110.0 | 90<br>90 | -60<br>-60 | -20<br>-12 |
| Diazepam-d5 | 290.1 | 198.1<br>262.1 | 90<br>90 | 120<br>120 | 25<br>25 |

Table II. Intra-day and Inter-day Precision (CV%) and Accuracy (BIAS%)

| Compound | Amount (ng/mg) | CV(%) | | BIAS(%) | |
|---|---|---|---|---|---|
| | | Intra-day | Inter-day | Intra-day | Inter-day |
| PTCA | 0.1 | 6.62 | 8.20 | 6.76 | 10.2 |
| | 1 | 2.96 | 3.04 | 5.71 | 9.11 |
| | 10 | 2.20 | 1.44 | 4.13 | 4.50 |

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Table III. Mean, Median, Standard Deviation (SD), 95% Confidence Intervals (CI) and Range Concentration of Baseline PTCA in Six Different Ethnic Subgroups

| Ethnicity | Mean (ng/mg) | Median (ng/mg) | SD (ng/mg) | 95% CI (ng/mg) | Range (ng/mg) |
|---|---|---|---|---|---|
| African (n = 20) | 9.60 | 7.85 | 6.00 | 6.83, 12.4 | 1.87–22.5 |
| Arab (n = 31) | 9.01 | 7.20 | 5.83 | 6.89, 11.1 | 1.14–23.1 |
| Asian-Pacific (n = 30) | 9.77 | 9.54 | 5.54 | 7.72, 11.8 | 1.97–20.5 |
| Caucasian (n = 24) | 4.78 | 4.67 | 2.25 | 3.83, 5.72 | 1.47–9.77 |
| Hispanic (n = 26) | 7.93 | 7.32 | 3.19 | 6.65, 9.20 | 3.02–15.1 |
| Indian (n = 25) | 10.7 | 7.13 | 7.95 | 7.25, 13.1 | 0.44–23.7 |

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Table IV. Mean ± SD, Range Concentration, Mean Ratio and Mean Increment (%) of PTCA after Oxidative Treatment in Six Different Ethnic Subgroups

| Ethnicity | Mean ± SD (ng/mg) | | Range (ng/mg) | | Mean ratio treated/untreated | Mean increment (%) |
|---|---|---|---|---|---|---|
| | untreated | treated | untreated | treated | | |
| African (n = 10) | 13.9 ± 9.49 | 18.3 ± 8.48 | 1.87–23.5 | 6.63–30.1 | 1.77 | 32.6 |
| Arab (n = 10) | 6.34 ± 4.20 | 13.8 ± 4.88 | 1.52–19.6 | 5.00–20.1 | 2.15 | 50.0 |
| Asian-Pacific (n = 10) | 11.1 ± 5.42 | 19.7 ± 7.18 | 1.97–18.2 | 9.93–30.8 | 3.13 | 56.4 |
| Caucasian (n = 10) | 4.66 ± 2.39 | 13.4 ± 4.85 | 1.46–9.76 | 4.46–19.1 | 3.29 | 63.4 |
| Hispanic (n = 10) | 8.64 ± 3.22 | 17.9 ± 7.04 | 3.01–15.1 | 6.90–29.0 | 2.11 | 50.0 |
| Indian (n = 10) | 8.79 ± 7.13 | 17.1 ± 7.76 | 0.44–22.3 | 4.16–32.3 | 3.70 | 46.2 |

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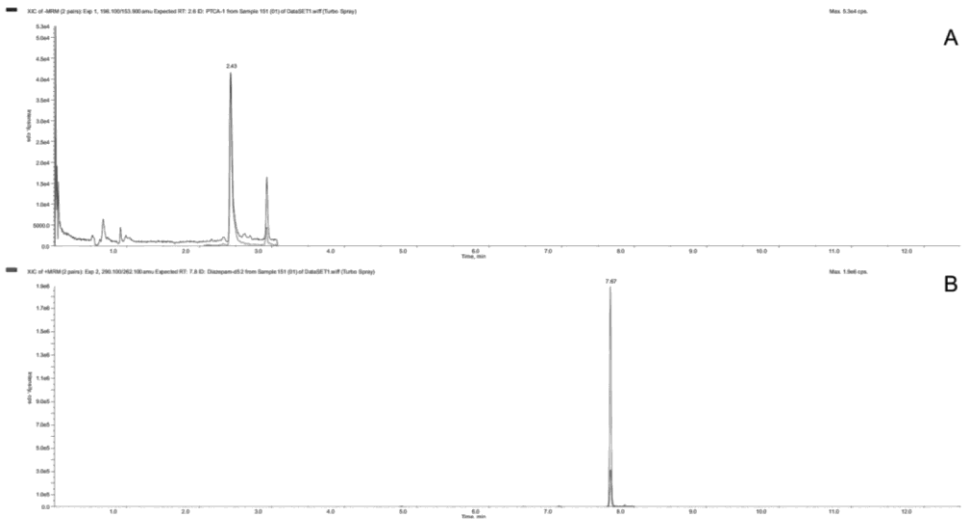


Fig 1

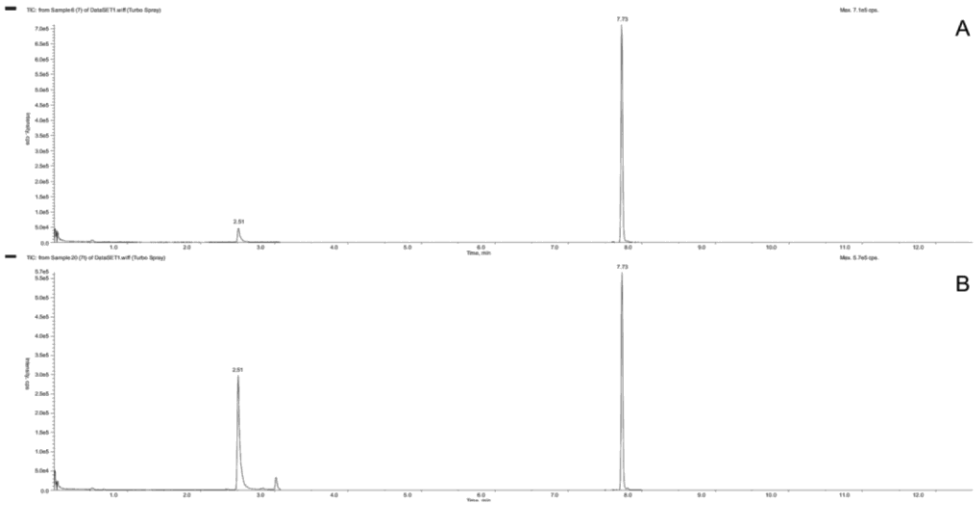


Fig 2

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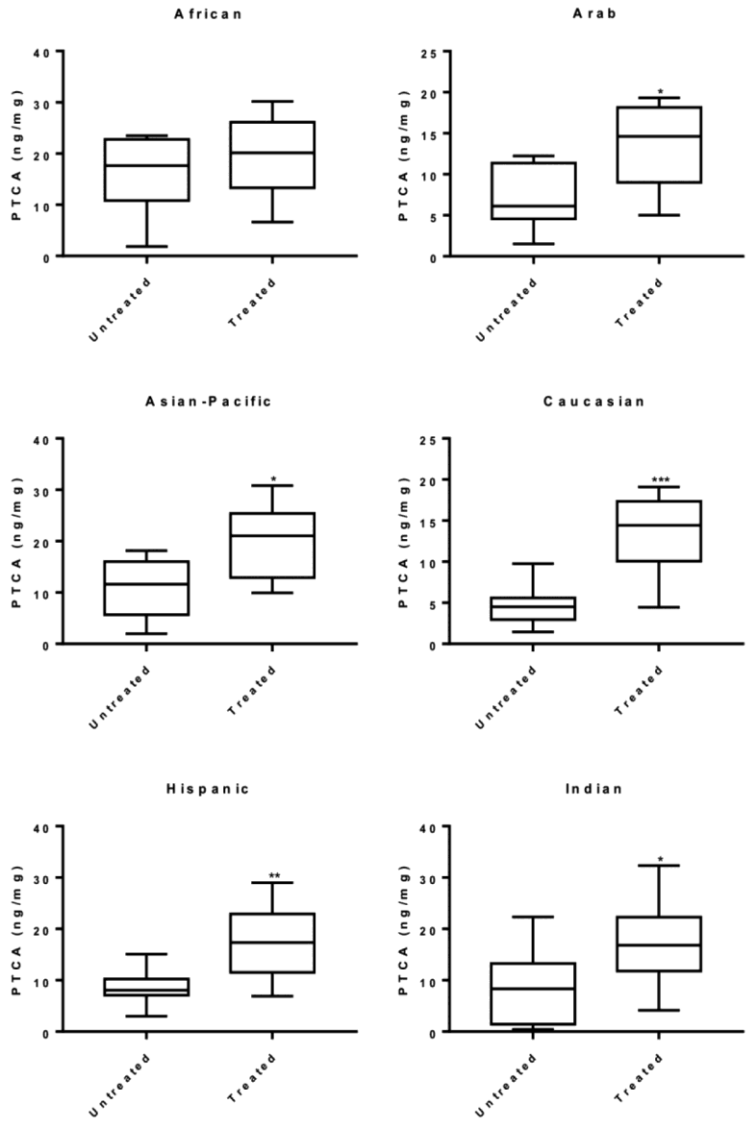


Fig 3

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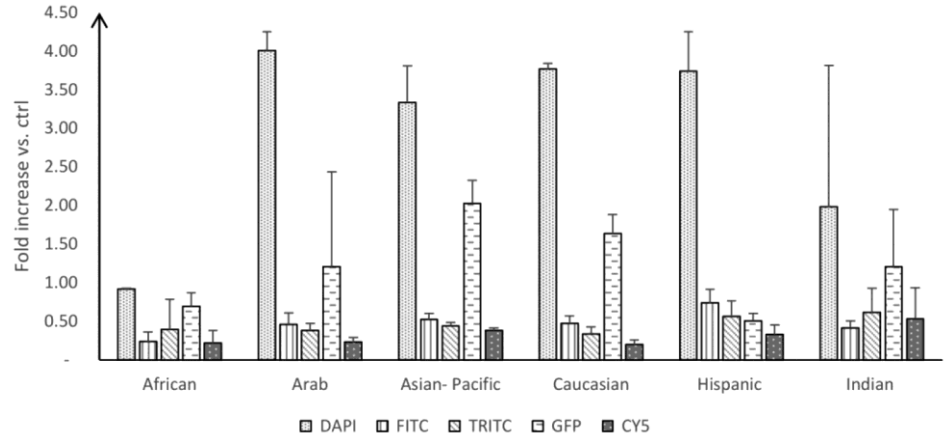


Fig 4

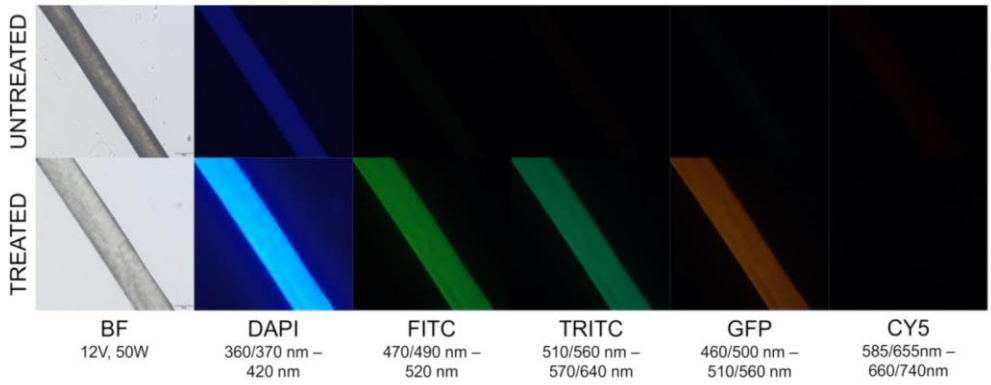


Fig 5

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