ANALYSIS OF BLACK TATTOO INKS:

Ingredients, Interaction with Light, and Effects on Cellular Systems

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

an der Fakultät für Chemie und Pharmazie

der Universität Regensburg

vorgelegt von

Karin Lehner

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Für Alexis
&
meinen Vater Georg

„ Das Schönste, was wir entdecken können, ist das Geheimnisvolle “

(Albert Einstein)

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1. Black tattoo inks are a source of risky substances like dibutyl phthalate[1*]

Abstract

Tattooing has recently become increasingly popular. Using tiny needles, tattooists place the tattoo ink in the dermis along with numerous unknown ingredients. Most of tattoos consist only of black inks, which predominantly are composed of soot products like carbon black or polycyclic aromatic hydrocarbons (PAH).

Black tattoos cause different skin problems such as allergic reactions, whereas the responsible substance frequently remains unknown. We applied GC-MS analysis to search for potential hazardous compounds in 14 different commercially available black tattoo ink samples.

The analysis revealed that all inks contained the softener substance dibutyl phthalate (0.12 – 691.2µg/g). Some of the inks contained hexachloro-1,3-butadiene (0.08 to 4.52 µg/g), metheneamine (0.08 to 21.64 µg/g), dibenzofuran (0.02 – 1.62 µg/g), benzophenone (0.26 – 556.66 µg/g), and 9-fluorenone (0.04 – 3.04 µg/g). The sensitizing agent dibutyl phthalate acts directly on keratinocytes and can drive Th2 responses following skin exposure via induction of thymic stromal lymphopoietin gene expression. Hexachloro-1,3-butadiene is genotoxic *in vitro* and 9- fluorenone is cytotoxic generating reactive oxygen species under light exposure. The substances found in the inks might be partially responsible for adverse skin reactions to tattoos.

[1*]Results of this chapter has been published: Lehner K, Santarelli F, Vasold R, König B, Landthaler M, Bäumler W, "Black tattoo inks are a source of problematic substances such as dibutyl phthalate", Contact Dermatitis. **2011** Oct

1.1 Introduction

In recent years, tattoos have become very popular worldwide and millions of people mainly have black coloured tattoos. Despite the increasing number of tattooed individuals, presently there are few requirements, legislation and criteria for the safety of tattoos and permanent make-up (PMU). The list of ingredients on black tattoo inks is usually missing or incomplete. Frequently, there is no information on packaging such as expiration date, conditions of use, warnings or the guarantee of sterility of the contents. Risk assessment should be an essential part for protecting human health, and this applies also with tattoos,

Tattooing is a practice whereby a pigment suspension is deposited in the dermis by intradermal injection of the inks with tiny solid needles. Since black inks are produced by imperfect combustion, they consist mainly of carbon black. It is, therefore, unsurprising that such black inks contain high amounts of polycyclic aromatic hydrocarbons (PAH) and phenol. As previously reported, we established an extraction procedure for determination and quantification of 20 different PAH in various commercial available black tattoo suspensions using liquid chromatography detection (1).

Tattoo inks are not distributed by pharmaceutical companies and in many countries regulations of such inks are still missing. In Germany, a first tattoo regulation appeared in 2009; it interdicts the use of azo pigments that can be cleaved to hazardous aromatic amines. With black tattoo inks, PAHs and other substances can be introduced into skin, which might be responsible for health problems associated with tattoos (2). Therefore, we investigated black tattoo inks again by using GC-MS analysis and searched for other substances in 14 commercially available tattoo inks, which might have the potential to be harmful for humans.

1.2 Materials and Methods

1.2.1 Materials

14 commercially available black tattoo inks were purchased from different tattoo suppliers in Europe, US and Asia (trade name, supplier): Tribal black, Body Cult Tattoo Supply; Schwarz, Rotring; Black Magic, Faber; Liner-Black, Infernal Colour; Diabolo genesis, Deep Colours!; Pitch Black, Scream Ink; Tattoo Outlining Ink, Kuro Sumi; True Black, Intenze Prod; National Pelikan, Pelikan; Sailor Jerry, Deep Colours!; Ink Black, Nova Ink; New Intense Black, Lynx; Black Liner Ink, Spaulding; Calcutta Black, Spaulding;

Standard substances including hexachloro-1,3-butadiene (HCBD), dibenzofuran (DBF), hexamethylenetetramine (HET), 9-fluorenone (9F), benzophenone (BP) and dibutyl phthalate (DBP) were detected by mass spectroscopy (GC – MSD) and obtained from Sigma Aldrich (Steinheim, Germany) as analytical pure standards. The references with a 1.0 mg/mL stock solution in acetonitrile were combined to obtain a 0.88 mg/ml calibration solution. The purity of each standard was approximately 98 % as reported by the manufacturer.

Solvents used for extraction of PAH, including benzene and acetone were of reagent grade, obtained from Merck (Darmstadt, Germany). Acetonitrile as solvent for GC analysis was of gradient grade quality for liquid chromatography (LiChroSolv, Darmstadt, Germany). Millipore water as solvent for GC analysis was freshly produced by a Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim Cédex). All substances were dissolved in 1 mL Acetonitrile and treated by ultrasonic (Bandelin Sonorex Super RK 103 H). For internal standard (ISTD) hexamethylbenzene was obtained from Sigma Aldrich and prepared as a 0.2 mg/mL stock solution in acetonitrile.

1.2.2 Extraction procedure and sample preparation

As previously reported with PAH extraction, we used ultrasonic treatment of a defined amount of black ink suspension with benzene/acetone and centrifugation (1). The procedure was done in triplicate. The residual compounds were resolved in 1 mL acetonitrile, filtered using PTFE-filter (CHROMAFIL®, O-20/15, organic, pore-size 0.2 µm, Machery-Nagel, Düren, Germany) and analyzed with internal standard method using GC – MS analysis. The selected compounds were previously detected in a qualitative GC – MS run of the Ink extract samples.

1.2.3 Chromatography analysis

Analysis was done using Agilent Technologies GC/MS-System, consisting of 7890 A GC and 5975C Inert XL EI/CI MSD with a CTC Pal Autosampler. Method settings: Injection Volume 1.00 µL, He-Flow 1 mL/min. Oven Program: 40 °C for 3 min, then 15 °C/min to 280 °C for 5 min, then 25 °C/min to 300 °C for 5 min with Splitless mode (1 min); heater 250 °C. Transferline temp 300°C. The used column was an Agilent HP-5MS (30 m x 250 µm x 0.25 µm).

Qualification and Quantification was done using the Agilent MSD ChemStation E.02.00.493 and NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0f, July 23 2008. For Matchfactor NIST >900, a substance was regarded as clearly identified.

The concentration of investigated substances was quantified using the method of Internal Standard (ISTD). For each compound (i), the calibration factor (CFi) was determined in a calibration run (single level calibration). The respective concentration of the internal standard was chosen to be in the range of the concentration of the substances.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K the mass of compound i in the solution k and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

1.3 Results

1.3.1 Quantitatively determined ingredients

A stock solution (0.88 mg/mL) of each substance (HCBD, HET, DF, DBP, 9F, BP), which were qualitatively detected in a first GC run before, was measured using the Method of Internal Standard to calibrate the system.

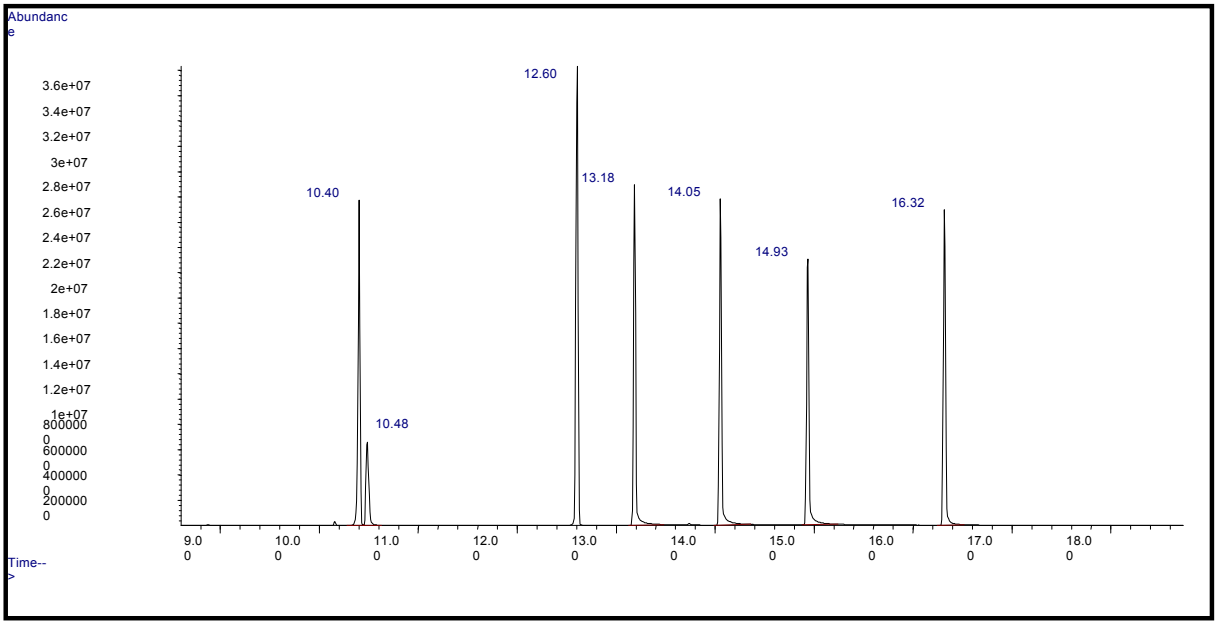


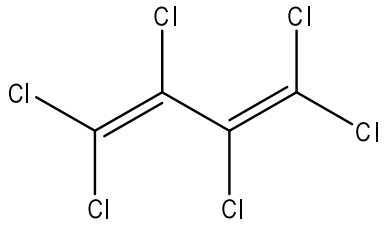
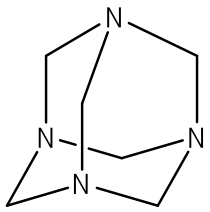
Fig. 1. Chromatography of the quantified substances. The retention times of the substances were 10.444 min (hexachloro-1,3-butadiene), 10.519 min (metheneamine), 13.229 (dibenzofuran), 14.101 min (benzophenone), 14.982 (9-fluorenone), and 16.349 min (dibutyl phthalate), and the retention time of the internal standard (hexamethylbenzene) was 12.642 min.

Figure 1 shows a clear separation of the investigated ingredients hexachlorobutadiene, benzophenone, hexamethylenetetramine, 9-fluorenone, dibenzofuran and dibutyl phthalate.

retention time [min]	reference substance
10.405	Hexachlorobutadiene
10.486	Hexamethylenetetramine
13.189	Dibenzofurane
14.075	Benzophenone
14.939	9 Fluorenone
16.321	Dibutylphthalate
12.608	**ISTD: Hexamethylbenzene**

Table 1. Retention time of the substances; ISTD, internal standard

The corresponding retention time of the substance peaks are listed in Table 1, whereby hexamethylbenzene (RT 12.608 min; 0.2 mg/mL) was used as Internal Standard. The chemical structures of these substances are displayed in Table 2.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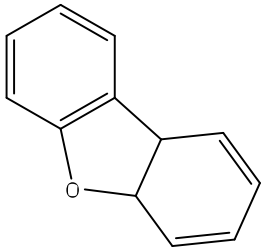
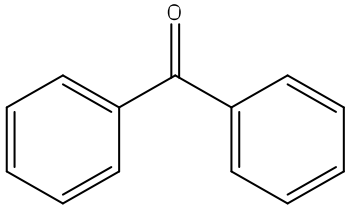
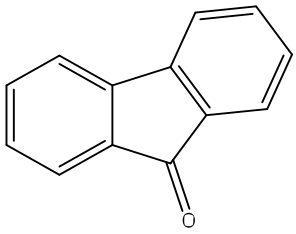
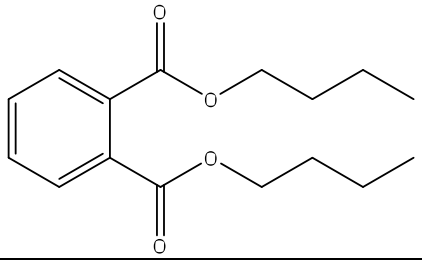
	
Dibenzofurane MW: 168.19 g/mol CAS: 132-64-9	Benzophenone MW: 182.22 g/mol CAS: 119-61-9
	
9-Fluorenone MW: 180.19 g/mol CAS: 486-25-9	Dibutyl phthalate DBP MW: 278.34 g/mol CAS: 84-74-2

Table 2. Chemical structures of identified and quantified ingredients in black tattoo inks

The substances exhibit some extend UV-active ring structures, an urotropine skeletal structure or a six-fold chlorinated butadiene. The total amounts of all clearly identified ingredients in selected black tattoo Inks are shown in Table 3.

Ink	**total amount*** [µg/*g*]
1: Tribalblack	96.08
2: Kapillarpatrone	42.38
3: Black Magic	21.28
4: Liner-Black	25.76
5: Diabolo genesis	0.48
6: Pitch Black	13.04
7: Tattoo Outlining Ink	0.24
8: True Black	164.88
9: National Pelikan	14.86
10: Sailor Jerry	0.18
11: Ink Black	556.78
12: New Intense Black	14.92
13: Black Liner Ink	716.94
14: Calcutta Black	6.12

Table 3. The total amounts of quantified ingredients found in black tattoo inks

After three fold repetition of the extraction procedure, the values range from 0.18 to 716.94 µg/g. A detailed list of the quantified ink ingredients is given in Table 4.

Ink	HCBD	Methen amine	DBF	Benzo phenone	9 Fluo renone	DBP
	[µg/*g*]					
1: Tribalblack	--*	0.32	0.04	95.34	0.04	0.34
2: Kapillarpatrone	0.08	0.14	1.62	34.74	3.04	2.76
3: Black Magic	0.30	--	0.02	19.94	0.62	0.4
4: Liner-Black	0.50	--	0.06	19.38	1.24	4.58
5: Diabolo genesis	--	--	--	--	--	0.48
6: Pitch Black	--	0.44	0.12	12.0	0.2	0.24
7: Tattoo Outlining Ink	--	0.08	--	--	0.02	0.14
8: True Black	--	--	--	164.7	--	0.18
9: National Pelikan	0.82	--	0.04	6.2	0.9	6.9
10: Sailor Jerry	--	--	--	--	--	0.18
11: Ink Black	--	--	--	556.66	--	0.12
12: New Intense Black	4.52	--	--	7.92	0.24	2.24
13: Black Liner Ink	1.0	21.64	0.02	2.96	0.16	691.2
14: Calcutta Black	--	--	--	0.26	1.8	5.68

Table 4. The amounts of the quantified ingredients found in black tattoo inks (µg/g)

Dibutyl phthalate could be definitely quantified in all ink samples. Total amounts ranged from 0.12 µg/g to 691.2 µg/g. The six fold chlorinated butadiene (HCBD) could be distinctively proven in six ink samples with a total amount of 0.08 up to 4.52 µg/g. High amounts for benzophenone could be extracted, ranging from 0.26 µg/g for black ink sample 14 up to 556.66 µg/g for Ink 11. Benzophenone could be detected in all ink extract samples except for sample 5, 7 and 10. Ink samples 2 and 13 contained all specified ingredients.

1.3.2 Qualitatively determined ingredients

Besides the 6 quantified ingredients, GC showed further peaks in several ink chromatograms.

Ink	Other ingredients
1: Tribalblack	3,6-dimethyl 1 heptyn-3-ol
2: Kapillarpatrone	1,6-hexandiole
3: Black Magic	1,6-hexandiole
4: Liner-Black	Oleamide*; 7-hexyl-2-oxepanone
5: Diabolo genesis	propyleneglycol; 7-hexyl-2-oxepanone
6: Pitch Black	propyleneglycol
7: Tattoo Outlining Ink	--+
8: True Black	1,1`oxybis-2-propanol; 2,2`oxybis-1-propanol
9: National Pelikan	7-hexyl-2-oxepanone
10: Sailor Jerry	Oleamide*; 7-hexyl-2-oxepanone
11: Ink Black	7-hexyl-2-oxepanone
12: New Intense Black	Oleamide*; 7-hexyl-2-oxepanone
13: Black Liner Ink	Carbitol cellosolve‡; 1,2,3,4-tetrahydro-1-phenyl-naphthalene
14: Calcutta Black	7-hexyl-2-oxepanone

Table 5. Qualitatively detected ingredients in black tattoo inks; *Oleaminde:IUPAC name (Z)-9-octadecenamide. +Value below detection limit. ‡Carbitol cellosolve: IUPAC name 2-(2-ethoxyethoxy) ethanol

Using NIST database for MSD analysis, additional compounds could be identified e.g. alcohol containing substances like 3,6-dimethyl 1 heptyn-3-ol, 1,6-hexandiole, propylene glycol or carbitol cellosolve as well as substances like oleamide, 7-hexyl-2-oxepanone and urea (Table 5). Figure 2 shows a GC chromatogram of black ink #13 as illustrative.

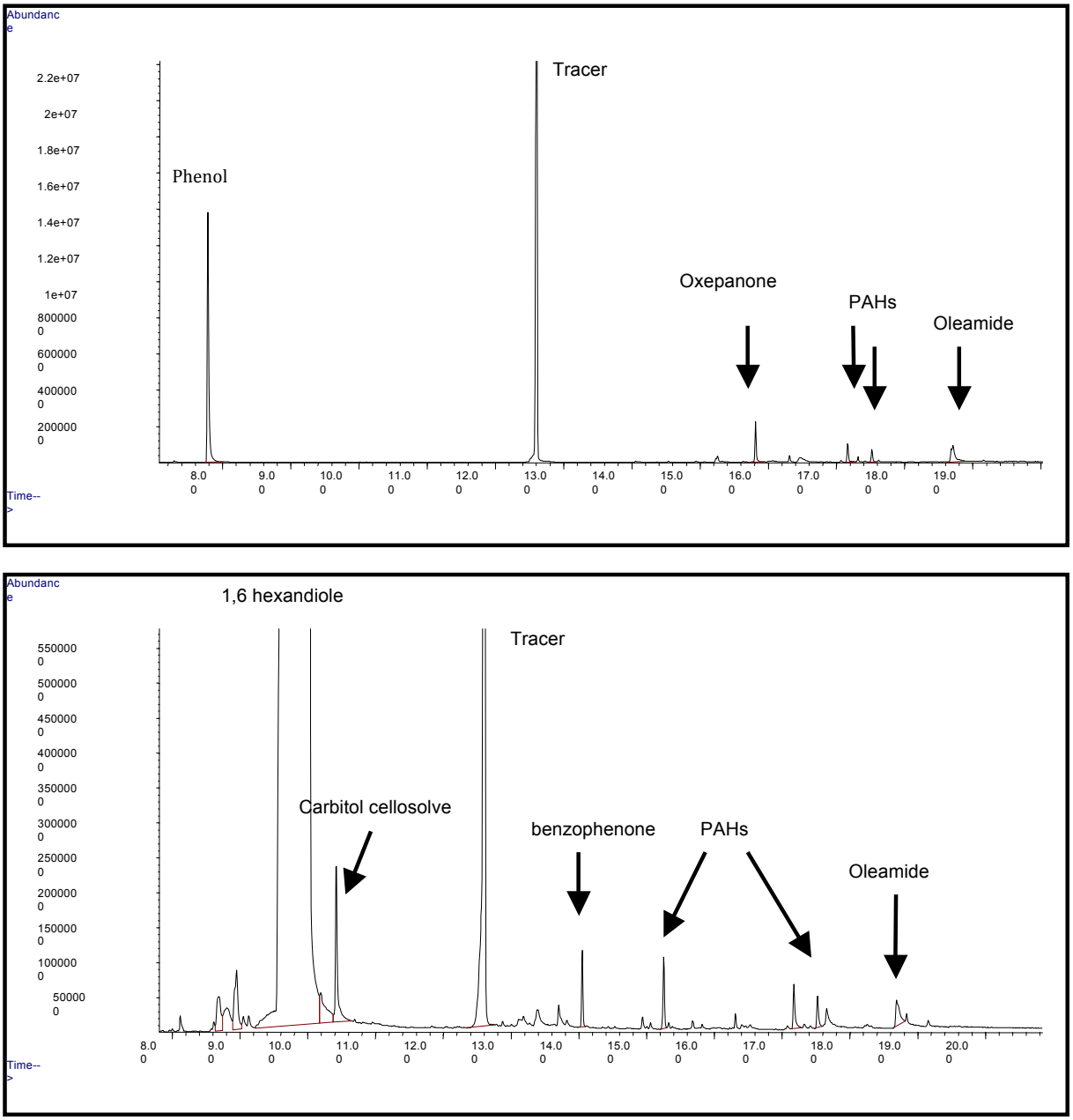


Fig. 2. Chromatograms of ink sample 13 (top) and ink sample 2 (bottom) showing additional substances that were either qualitatively determined or remained unknown. PAH, polycyclic aromatic hydrocarbon

Among quantitative and qualitative detected ingredients, the chromatogram still shows peaks which could not clearly be indentified using the NIST database.

1.4 Discussion

Recently, we investigated commercially available black inks regarding polycyclic aromatic hydrocarbons (PAHs) using HPLC technology (1). Using the established extraction procedure (3), we detected total concentrations of PAHs in the different inks ranging from 0.14 to 201µg/g. Even benz(a)pyrene, a known carcinogen, was found in four ink samples at a mean concentration of 0.3±0.2µg/g.

At the same time, we performed a nation wide survey in German speaking countries to reveal the incidence of health problems associated with tattooed skin (2). First of all, the survey showed that tattooed people have many (28%; ≥ 4) and large tattoos (36%; ≥ 900 cm^2) requiring the injection of several grams of tattoo inks into skin, which partly spread in the human body and probably stay lifelong in human tissue (2). However, it is possible that people who like tattoos might have been more willing to participate in such a survey.

The participants described skin problems (67.5%) or systemic reactions (6.6%) directly after tattooing and related to the tattooing process. 6 % of participants complained about persisting skin problems at the site of tattoo such as itching, burning, skin papules, small nodules, eczema, or redness of skin (erythema). In addition, 1.3 % reported burning and itching of tattooed skin when exposed to solar radiation (‘light sensitivity’).

In light of these skin reactions, we analyzed the extract of such black tattoo inks in more detail using GC – MS analysis to identify potential irritants or allergens. We found the substances HCBD, HET, DF, DBP, 9F, and BP, which were definitely quantified (Table 4). GC analysis and NIST database revealed the presence of other substances in the black inks, which could be not clearly identified and quantified (Table 5).

Below, we describe the usual sources of particular substances, their effects *in vitro* and in the body where known, with special regard to the skin. Any other health concerns of the substances are listed in Table 6.

Dibutyl phthalate	Genotoxic (4), teratogenic (5,6)
Hexachloro-1,3-butadiene	Genotoxic (7), carcinogenic (8), classified by the US EPA as a possible human carcinogen (Group C) (9)
Dibenzofuran	Not classifiable as to human carcinogenicity (Group D) (10)
Hexamethylenetetramine	Possibly genotoxic (11)
Benzophenone	Some evidence of carcinogenic activity (12)
9-Fluorenone	None

Table 6. Assessment of other health risks

1.4.1 Dibutyl phthalate (DBP)

In principle, synthetic materials, e.g. polyvinylchloride (PVC) contain several additives to obtain special physicochemical handling properties like softness, elasticity and plasticity (4). These synthetic materials are used for wallpapers, cloths, toys, plastic films and artificial leather. Such phthalates are also present in dispersions, and lacquers.

Thymic stromal lymphopoietin (TSLP) is an epithelial-derived cytokine expressed primarily in the lung, skin, and intestine, in response to inflammation, tissue damage, or Toll-like receptor ligation (5). In recent studies it was shown that DBP is capable of inducing expression of TSLP in the skin (6, 7). Allergic contact dermatitis from DBP is described (8).

In immunological experiments, DBP has been empirically included in the solvent system for fluorescein isothiocyanate (FITC) (9). A study showed that stimulation of sensory neurons via TRPA1 and TRPV1 is involved in the adjuvant effect during skin sensitization (9). This concept may reflect the connection between skin irritation and skin allergies. TRPA1 and TRPV1 may play a central role by transmitting noxious stimuli to the brain and immune cells such as APCs by sensing noxious compounds.

Immunohistochemical analysis of skin after epicutaneous application of dibutyl phthalate revealed a transient decrease in the number of macrophage C-type lectin positive cells in the dermis (10). Our results clearly reveal that DBP is present in all black inks under investigation showing concentrations of DBP of up to 691 µg/g. When skin is tattooed, the black inks together with DBP are punctured into skin. In this way, DBP contacts nearly all skin cells that may trigger TSLP. An animal model demonstrated for coloured tattoo pigments that such pigments can be partially transported to lymph nodes (11). Therefore, other ingredients of tattoo inks such as DBP might be transported away from skin.

1.4.2 Hexachlorobutadiene (HCBD)

The chlorinated alkene HCBD can be found predominantly as a by-product from the manufacture of chlorinated solvents and related products. The substance was used as a fumigant for treating *Phylloxera* in the former Soviet Union and to a lesser extent in Southern Europe (12).

Rabbits have been epicutaneously exposed to pure hexachlorobutadiene (0.25 to 1.00 mg/kg) during 8 hrs. HCBD has been found to be a skin penetrant and highly acutely toxic substance. HCBD led to epidermal and dermal necrosis, whereas the cutaneous changes increased with time for up to 5 weeks. The rabbits showed

damage of skin, liver and kidneys (13). Out of the 14 black inks, we found HCBD in 6 samples with concentrations up to 4.5 µg/g. So far, there are no case reports showing toxicity of HCBD in humans.

1.4.3 Dibenzofuran (DBF)

The substance DBF is the backbone of polychlorinated dibenzofurans, which belong to the group of dioxin-like chemicals (14) and is listed in the EPA´s Toxic Substances Control Act (TSCA) (15). DBF is found as combustion product in various percentages from the incomplete combustion of coal biomass, refuse, diesel fuel and residual oil, as well as tobacco smoke (16). DBF is frequently used as wood preservative. Out of the 14 black inks, we found DBF in 7 samples with concentrations up to 1.62 µg/g. We only detected the backbone of polychlorinated dibenzofurans and we can not decide whether the chlorine atoms were present or cleaved prior to our investigations. The frequently described dermal, hepatic, and gastrointestinal health problems in humans are related to the brominated or chlorinated dibenzofurans (17, 18).

In particular, little to no information is available on the effects of dibenzofuran exposure to skin. Information that does exist shows that short-term exposure to dibenzofuran can cause skin, eye, nose, and throat irritation (19). Clear data in the medical literature about DBF are missing and the concentration in tattoo inks is small. Thus, it remains unclear whether this substance may cause health problems when tattooed into skin.

1.4.4 Hexamethylenetetramine (MET)

The substance MET is used as preservatives in citrus washing solutions and in the manufacture of rubber, resins and coatings as well as in pharmaceuticals and

cosmetics. It is well known, that preservatives used in cosmetics constitute an important source of allergic contact dermatitis (20). Some of the most problematic preservatives are formaldehyde and formaldehyde-releasing agents (21). The formaldehyde-releaser MET as preservative in cosmetics must not exceed a maximum authorized concentration of 0.15% (22). In addition, MET is known to cause respiratory allergies (21). In case of tattooing, MET is placed in skin with concentrations of up to 21.6 µg/g.

1.4.5 Benzophenone (BP)

Benzophenone, an aromatic ketone (diphenyl ketone), is an important compound in organic photochemistry and perfumery as well as in organic synthesis. In the field of food packaging, benzophenone is used as initiator compound for hardening of printing inks by UV irradiation. Because of the volatile behaviour BP can be found in correspondent food.

According to EFSA, Benzophenone is characterized as irritant but not genotoxic (23). A similar molecule is benzophenone-3 (2-hydroxy-4-methoxy- benzophenone), which is frequently used in sunscreens. It can cause photocontact allergy and other types of hypersensitivity reactions to it such as contact allergy, photocontact urticaria, contact urticaria and even cases of contact anaphylaxis (24). BP showed phototoxic reactions and caused photosensitization in skin of guinea pigs under UV radiation (25).

Fig. 3. Example of simple flasks containing black tattoo ink with no listing of ingredients.

Previous studies in humans have indicated that the dermal absorption of organic UV filters during the application of sunscreens is very limited; in fact, only very low concentrations of these UV filters were detected in the blood after repeated, intensive application (26). However, dermal penetration is maximal for such substances during tattooing and many of the black ink samples contained high concentrations of BP of up to 557 µg/g.

1.4.6 9-Fluorenone (9F)

The substance 9F is not commercially synthesized but is obtained from middle oil fraction of coal tar. It is used in manufacturing antimalaria drugs and other pharmaceuticals. 9F may cause phototoxic reactions *in vitro* and in *vivo*, in particular in oral mucosa (27, 28). Beyond that only little is known about any adverse reactions to 9F, in particular regarding skin. 9F was found in 10 samples of the black inks at low concentrations (up to 3.04 µg/g) and it remains unclear to which extent 9F can harm skin after tattooing.

1.5 Conclusion

The medical literature offers numerous case reports on dermatological diseases caused by tattoos, which includes pseudolymphoma, allergic or granulomatous skin reactions (29-31). Considering the results of the survey in German speaking countries, about 7.3 % of tattooed people describe persistent skin reactions at the site of tattooing including light sensitivity (2).

About 10 % of population are tattooed in Germany. If we extrapolate that percentage to all tattooed individuals, we tentatively assume that about 0.5 million of people might have persistent skin problems with tattoos. The investigated black inks are from different countries including US, where about 80 million people are tattooed (32). If the frequency of skin problems is the same in US, we tentatively calculate a number of about 5 million people with persistent skin problems at the tattooed site. Carbon black in the inks is not suspected of causing allergic or irritant skin reactions. Thus, the described health problems are more likely to be caused by some of the ingredients listed in this investigation, whereas the listing might be still incomplete. Many of the 14 inks contain more than one ingredient at the same time that might lead to complex skin reactions. However, reports on adverse reactions in tattoos to the substances detected in this investigation are missing so far. This first but probable incomplete list of tattoo ink ingredients may help physicians to search for the chemical trigger of adverse skin reactions.

In light of these results, we urgently recommend regulation of tattoo inks so that only those inks without hazardous substances may be used. This could be started with a first step: substances that are not permitted to be used in cosmetics should be prohibited from being punctured into skin. Moreover, lack of knowledge (see figure 3) should be removed by requiring complete listing of the ingredients as for cosmetics.

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2. Tattoo inks and hence tattooed skin contain polycyclic aromatic hydrocarbons: quantitative extraction method from human skin*

Abstract

Tattooing is a worldwide popular body adornment in which various inks are injected into the human skin. Black tattoo inks consist of Carbon Black that may act as a strong sorptive phase for hazard polycyclic aromatic hydrocarbons (PAH). PAH may additionally generate singlet oxygen during exposure of tattooed skin to solar radiation. To assess the potential health risk of tattooing using black inks, the concentration of PAH in tattooed skin should be determined.

We established an extraction method to recover PAH quantitatively from digested human skin. The extraction of phenol as well as 20 PAH was accomplished using recovery experiments and HPLC-DAD technology. PAH and phenol could be almost completely recovered from digested human skin with recovery rates of 96 to 99 %. Even the very volatile smaller aromatic two- and three-ring structures like naphthalene, acenaphthene, and acenaphthylene were successfully extracted using a combined vortex and ultrasonic procedure and a keeper compound. The presence of Carbon Black in the digested skin did not affect the recovery rate.

The use of the established procedure should allow a quantitative extraction of PAH from tattooed skin samples. The procedure should be also applicable for other tissue samples such as from lymph nodes.

* Results of this chapter are submitted: Lehner K, Santarelli F, Sidoroff A, Vasold R, König B, Landthaler M, Bäumler W, "Tattoo inks and hence tattooed skin contain polycyclic aromatic hydrocarbons: quantitative extraction method from human skin", Anal Bioanal Chem, 2012, under review

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous chemical pollutants and originate from incomplete combustion of fossil fuels, pyrolysis of organic material, vehicular emission, petroleum catalytic cracking and residential wood burning.[1] PAH eagerly adsorb to organic materials like soot and soil. It is well known that many of the PAH are carcinogenic, mutagenic or teratogenic.[2, 3] The presence of PAH in food, environment[4] and cigarette smoke[5] contribute to public health concerns. Recently, some of the PAH are suspected to be also endocrine disrupters.[6] Human exposure to PAH mainly occurs through inhalation of contaminated particles and intake of contaminated dietary products.

A new pathway of human PAH uptake was recently discovered along with the chemical analysis of black tattoo inks. The concentration of 20 different PAH and phenol was quantified in various commercially available Black inks by using HPLC.[7] The concentration of PAH in the inks ranged from 0.14 to 201 µg per gram dry ink. Tattooing is a worldwide phenomenon with increasing popularity,[8] e.g. about 25 % of people in US have one tattoo at least.[9] Thus, millions of people have many tattoos, which are predominantly black.[10, 11] Black tattoo inks mainly consist of Carbon Black, a mixture of different solvents and other ingredients, whereas the actual composition may vary for different inks. Carbon Black is listed as possible carcinogenic to humans (group 2 B).[12, 13]

Cancer formation in the skin and scrotum were found in groups of workers, which were mainly exposed to PAH via dermal absorption.[14] In contrast to that, tattooists damage the barrier of intact skin by using vibrating tiny needles (tattoo machines) and puncture PAH directly into tissue. [15, 16] In addition, the adsorbed PAH may stay in the dermis for long time or may be transported inside the human body, probably

together with PAH, that was observed for the lymph nodes.[17, 18] Besides the toxic or mutagenic risk, PAH are able to generate deleterious singlet oxygen upon exposure to ultraviolet radiation (UV) [7], to which skin is frequently exposed.

So far, the concentration of PAH in tattooed skin is unknown. To estimate the health risk, the concentration of PAH in tattooed skin should be known. From the analytical point of view, isolation of PAH from biological matrices most often involves complex extraction and clean up procedures to provide extracts ready for the accurate analytical determination and quantification. A large number of studies have been reported on the extraction of PAH from food and soil samples or water samples using Soxleth based procedures, pressurized liquid extraction and supercritical fluid extraction.[19-24]

Human skin consists of different structures such as cells containing lipids and proteins, adnexa (e.g. hair follicle, sweat glands) and extracellular matrix with collagen. After tattooing, most of the Carbon Black, together with adsorbed PAH, is taken up by phagocytes or can be found in secondary lysosomes, an example is shown in Figure 1.

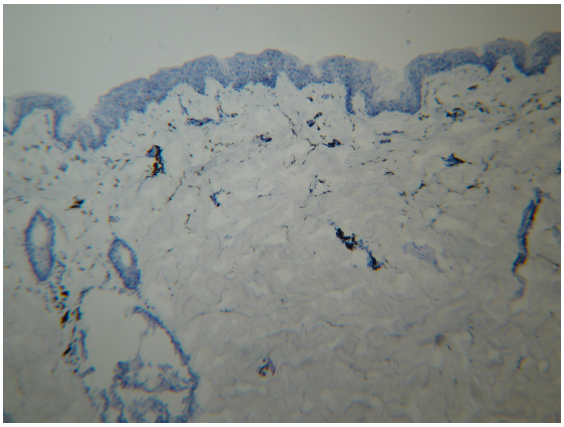

Figure 1 Punch biopsy and histology of tattooed skin. The ink can be seen as black spots with different sizes that are randomly distributed in the dermis of the skin. The ink particles (Carbon Black possibly loaded with PAH) can be usually found in macrophages or secondary lysosomes.

Any unbound PAH molecule could be either taken up in lipophilic structures of the skin or can be transported away from skin via lymphatic system. To determine the total PAH concentration in tattooed skin, a two-fold procedure is necessary. Firstly, tattooed skin samples must be disintegrated to extract Carbon Black nanoparticles including adsorbed PAH from skin matrix. Secondly, the extraction procedure must detach PAH from Carbon Black.

In case of commercial tattoo inks, an ultrasonic-assisted and heat dependent extraction procedure proved to be a convenient method for the extraction of PAH and phenol from Carbon Black.[7] Ramalhosa et al. used ultrasonic-assisted and vortex-assisted extraction procedure of PAH from fish.[25] The goal of the present investigations was to identify an optimal procedure to extract all 20 PAH from human skin, which were previously detected in commercial available black tattoo inks.

2.2 Experimental

Preliminary experiments were performed in duplicate. Optimized extraction experiments were done in triplicate.

2.2.1 Chemicals

Phenol and PAH were numbered (in brackets). As references for HPLC, phenol (1) (purity > 99%) was obtained from Riedel-de Haen and 20 well known PAH (purity ~ 99 %) were obtained from Sigma Aldrich (Steinheim, Germany): (2) naphthalene, (3, #5) acenaphthylene, (4) acenaphthene, (5) fluorene, (6) phenanthrene, (7) anthracene, (8) fluoranthene, (9) pyrene, (10) benzo[*a*]anthracene, (11) chrysene, (12) 5-methylchrysene, (13) benzo[*j*]fluoranthene, (14) benzo[*b*]fluoranthene, (15) benzo[*k*]fluoranthene, (16) benzo[*a*]pyrene, (17) dibenzo[*a,l*]pyrene, (18) dibenzo[*a,h*]anthracene, (19) benzo[*g,h,i*]perylene, (20) indeno[*1,2,3-c,d*]pyrene and (21) dibenzo[*a,e*]pyrene. Phenol (1) (purity > 99%) was obtained from Riedel-de Haen. For the internal standard (ISTD), 9,10-diphenylanthracene (purity > 99 %) was obtained from Riedel-de Haen.

2.2.2 Stock solutions

One milligram of each of the 20 US-EPA PAH and phenol were dissolved in one milliliter of acetonitrile and treated by ultrasonic (Bandelin Sonorex Super RK 103 H) for 10 min, respectively. 500 µL of each compound solution was combined in a 25 mL flask and filled with acetonitrile to obtain a concentration of 0.02 mg/mL of each investigated substance. For internal standard (ISTD), 9,10-diphenylanthracene (9,10 DPA) was prepared as an 0.4 mg／mL stock solution in acetonitrile. Benzene and acetone as solvents for the extraction were of reagent grade quality for liquid

chromatography (LiChroSolv, Merck, Darmstadt, Germany). Acetonitrile as solvent B for LC–MS analysis was of gradient grade quality for liquid chromatography (LiChro-Solv Darmstadt, Germany). Millipore water as solvent A for LC–MS analysis was freshly produced by a Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim, Cedex).

2.2.3 Enzymes

ATL buffer and proteinase K (>600 mAU/mL) were purchased from Qiagen (Hilden, Germany)

2.2.4 Skin Preparation

Human skin was obtained from surgical excisions (Department of Dermatology, University of Regensburg, Germany) and stored at -80 °C. For further treatment adipose tissue was removed by a scalpel, the skin was chopped up to slices with size of 1 cm^2 placed into Eppendorf cups (Eppendorf, Wesseling-Berzdorf, Germany), and 400 µL of PBS (PAA, Pasching, Austria) was added. Proteins were denatured by heating at 95 °C for 5 min according to Gaber et al. After cooling to room temperature, a total of 180 µL of buffer ATL and 20 µL of proteinase K were added to the skin, mixed by vortexing and incubated at 55 °C until the tissue was completely lysed.

2.2.5 Extraction Procedure

For recovery studies, one milliliter of the stock solution containing the respective 21 reference substances was added to 0.6 mL of digested human skin in a glass test tube (8 mm 10 mm, NS 14; Neubert-Glas, Ilmenau, Germany). The extraction solvents benzene/acetone (2 mL /1 mL) and acetonitrile (3 mL) were added,

respectively. Ultrasonic bath (Bandelin Sonorex Super RK 103 H) was used for 3, 10, 20 or 60 min and vortex 1 min using an appropriate mixer (MS 1 Minishaker, IKA, Brazil). After centrifugation at 4°C with 2500 ***g*** r.c.f. (Eppendorf Centrifuge, 5702 RH), the supernatant was collected and the solvent was removed in nitrogen stream (2 bar, 20 min, rt). Prior injection into the HPLC – DAD system, the residual crystal were resolved in one milliliter of MeCN and filtered through a PTFE-filter (CHROMAFIL®, O-20/15, organic, pore size 0.2 µm, Machery-Nagel, Düren, Germany) (figure 2).

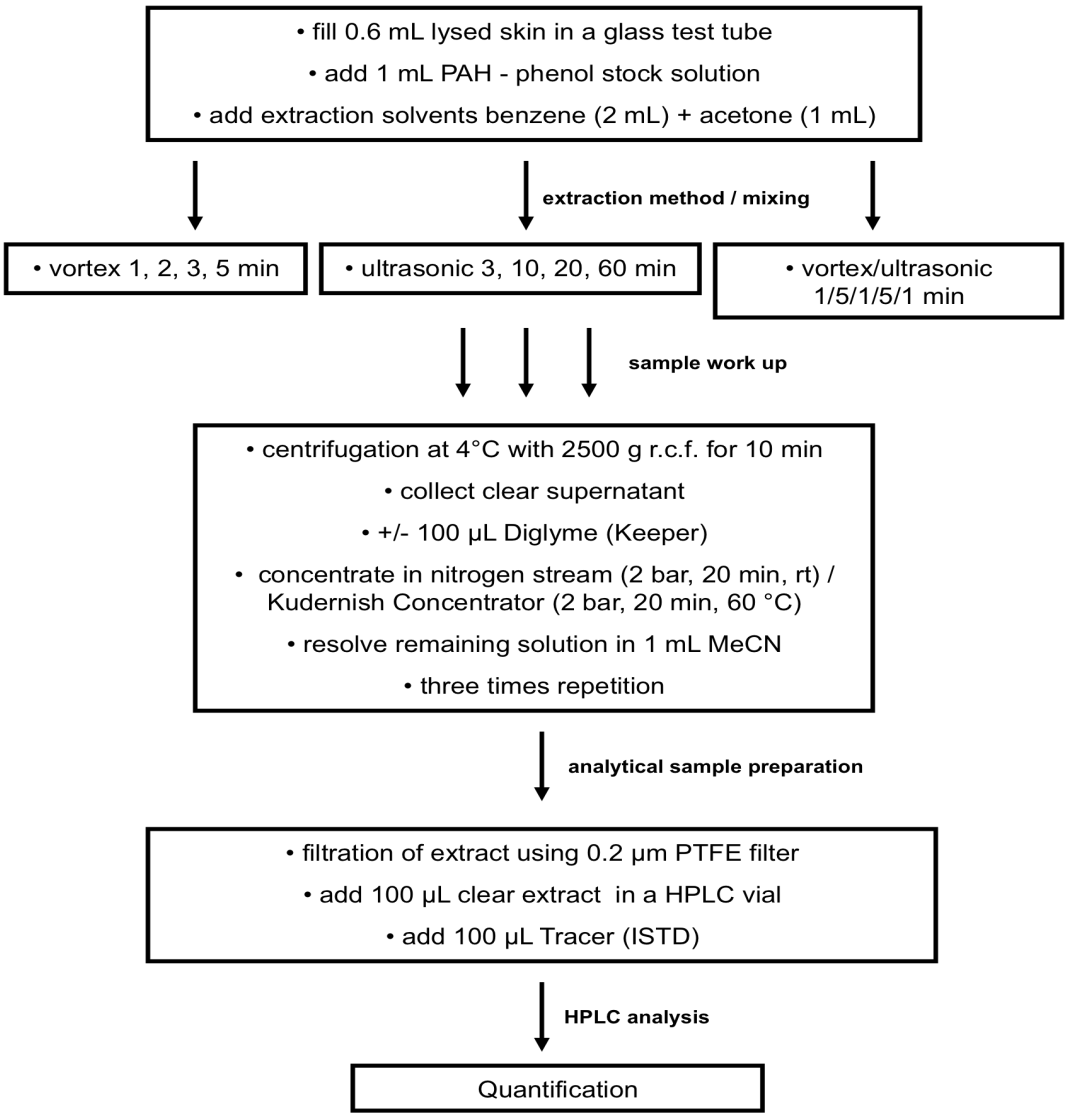

Figure 2: Extraction scheme for recovery of phenol (1) and PAH (2-20) from digested human skin: concerning the mixing step, three methods were tested: vortex, ultrasonic bath and a combination of both

2.2.6 Liquid chromatography analysis

The extracts were filtered using a PTFE filter (Chromafil, O-20/15, organic, pore size 0.2 µm; Machery-Nagel, Düren, Germany). A 100 µL sample was analyzed using a model 1100 HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column for nanoscalic environmental analysis (Phenomenex Environsep PP, particle size 3µm, 125 x 2 mm, Aschaffenburg, Germany) and diode array detector (DAD). The Injection volume was 10 µL. The data-files were analysed using a HPLC-3D-ChemStation Rev. B.03.02. The PAH could be separated by gradient elution with water [0.0059 w % trifluoroacetic acid] (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.3 mL/min. A gradient profile with the following proportions of solvent B was applied [t (min), % B]: (0, 40), (2, 40), (27, 98), (35, 98). The chromatograms were monitored at 220 nm.

The concentration of phenol and PAH in the solutions was determined by the method of internal standard. For each compound (i), the calibration factor (CF_i) was determined in a calibration run (single level calibration). The respective concentration of the internal standard was chosen to be in the range of the concentration of the PAH.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K the mass of compound i in the solution k and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

2.3 Results and Discussion

2.3.1 Limit of Quantification (LOQ), Limit of Detection (LOD)

To investigate precision and accuracy regarding PAH and phenol extraction, LOD and LOQ were defined as the minimum detectable amount of analyte with a signal to noise ratio of 3:1 and 10:1, respectively, from acetonitrile solution. The 21 reference substances were dissolved in acetonitrile and a dilution series followed. A separation of the 20 PAH and phenol could be achieved with an HPLC injection volume of 10 µL and the described gradient elution. Under the applied HPLC conditions the limits of detection and the limits of quantification could be calculated for diode array detection (DAD) monitoring at 220 nm: LOQs of 500 µg/L for dibenzo[a,e]pyrene, 250 µg/L for indeno[*1,2,3-cd*]pyrene, 100 µg/L for acenaphthylene, fluorene, phenathrene, anthracene, pyrene, benz[*a*]anthracene, chrysene, 5-methylchrysene, benzo[*j*]fluoranthene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,l*]pyrene, dibenzo[*a,h*]anthracene, benzo[*g,h,i*]perylene, phenol and 50 µg/L for naphthalene, acenaphthene and fluoranthene could be obtained. According to our extraction procedure, corresponding LODs were in the range 100 µg/L for dibenzo[a,e]pyrene, 50 µg/L for acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, 5-methylchrysene, benzo[*j*]fluoranthene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,l*]pyrene, dibenzo[*a,h*]anthracene, benzo[*g,h,i*]perylene, indeno[*1,2,3-c,d*]pyrene, phenol, 17 µg/L for naphthalene and 10 µg/L for acenaphthene. These values are highly reproducible for the applied extraction procedure and HPLC –DAD monitoring for all 21 investigated substances at 220 nm at the same time.

2.3.2 Extraction experiments – preliminary investigations

Concerning the extraction of 20 PAH and phenol in our previous study, a mixture of benzene (2 mL) and acetone (1 mL) proved to attain highest extraction yields.[7] In contrast to that, acetonitrile is frequently used[26, 27] that yielded high recovery rates for PAH extraction from tissue such as fish.[25] To find the most suitable solvent for human tissue extraction, we investigated the use of both solvents benzene/acetone and acetonitrile in a first step.

The digested organic material was loaded with PAH as described and extracted at four different time spans: 3 min, 10 min, 20 min and 60 min using a 2:1 mixture of benzene/acetone or acetonitrile. The results of the ultrasonic assisted procedure using the two different extraction solvents are listed in table 1.

Compound	Recovery +/- RSD (%)							
	ultrasonic bath (benzene/acetone)				ultrasonic bath (acetonitrile)			
	3 min	10 min	20 min	60 min	3 min	10 min	20 min	60 min
1 Phenol	3.9+/-0.4	1.7+/-2.4	1.6+/-2.3	3.7+/-0.4	2.85+/-4.0	3.0+/-1.2	6.0+/-3.6	4.1+/-0.2
2 Naphthalene	0.7+/-0.9	0.4+/-0.6	0.7+/-0.6	0.5+/-0.1	0	0	0	0
3 Acenaphthylene	11.1+/-1.6	21.8+/-2.5	24.2+/-5.9	2.2+/-1.8	0.5+/-0.7	12.8+/-3.9	5.2+/-5.0	1.8+/-0.2
4 Acenaphthene	11.4+/-0.9	24.4+/-5.5	31.8+/-1.4	4.0+/-2.8	2.5+/-0.4	13.0+/-1.6	5.3+/-3.0	3.2+/-0.1
5 Fluorene	33.4+/-5.9	62.2+/-4.7	59.9+/-9.1	18.5+/-3.8	20.4+/-0.1	18.8+/-3.3	18.5+/-3.7	22.3+/-2.6
6 Phenanthrene	79.1+/-12.4	54.8+/-3.9	64.8+/-0.5	79.4+/-12.7	78.2+/-0.2	84.5+/-11.1	69.8+/-0.2	72.9+/-5.8
7 Anthracene	87.1+/-6.9	88.6+/-12.1	86.6+/-8.7	86.0+/-7.3	86.2+/-0.8	96.2+/-1.9	91.0+/-4.2	74.2+/-13.3
8 Fluoranthene	102.8+/-1.6	102.4+/-4.4	99.7+/-0.9	103.0+/-5.9	103.0+/-2.5	102.0+/-0.5	81.9+/-11.8	94+/-9.0
9 Pyrene	98.0+/-2.8	99.8+/-0.5	95.0+/-5.9	97.8+/-0.2	94.1+/-1.4	96.6+/-4.8	84.7+/-12.8	90.0+/-0.5
10 Benz(a)Anth.	93.8+/-7.6	93.4+/-5.9	91.8+/-7.0	93.0+/-0.2	92.0+/-5.6	92.4+/-6.7	83.0+/-4.8	75.4+/-5.9
11 Chrysene	97.2+/-8.4	99.6+/-2.8	103.4+/-9.3	98.9+/-1.8	93.9+/-0.7	97.6+/-7.9	80.6+/-8.2	76.9+/-13.3
12 5Methylchry	104.4+/-3.3	103.8+/-2.5	106.0+/-2.8	103.6+/-3.3	106.8+/-3.9	107.4+/-1.9	69.5+/-13.0	88+/-5.0
13 Benzo(j)Fl	105.8+/-2.4	105.2+/-2.8	102.8+/-1.6	105.7+/-8.9	105.2+/-1.1	105.0+/-0.2	69.5+/-6.9	88.0+/-14.0
14 Benzo(b)Fl.	96.8+/-10.7	96.9+/-8.9	93.0+/-11.0	96.1+/-4.6	93.2+/-7.2	90.2+/-6.5	83.6+/-5.6	82.6+/-0.2
15 Benzo(k)Fl.	97.2+/-10.1	96.2+/-7.6	93.7+/-10.2	96.8+/-0.0	88.6+/-1.9	95.4+/-9.9	84.6+/-3.1	70.2+/-8.2
16 Benzo(a)Pyrene	91.9+/-7.4	92.1+/-7.2	89.9+/-10.2	97.4+/-0.8	89.4+/-3.0	92.5+/-5.5	79.2+/-3.9	73.8+/-7.0
17 Dibenzo(al)P	95.3+/-4.2	93.6+/-2.1	87.9+/-3.3	94.7+/-1.6	89.7+/-1.7	91.1+/-2.1	81.4+/-3.4	72.8+/-5.2
18 Dibenzo(ah)A.	107.4+/-1.4	106.2+/-4.2	101.9+/-0.4	109.4+/-0.2	104.2+/-3.1	104.6+/-0.8	60.2+/-0.2	82.4+/-9.1
19 Benzo(ghi)Peryl	51.0+/-6.5	51.2+/-5.0	49.2+/-6.2	53.0+/-5.9	54.2+/-0.8	58.8+/-7.9	53.8+/-0.2	45.2+/-5.6
20 Indeno(123-cd)P	93.2+/-3.9	92.4+/-2.2	89.8+/-3.1	93.0+/-3.1	95.7+/-3.5	90.0+/-3.9	83.2+/-9.0	75.7+/-8.8
21 Dibenzo(ae)Pyr	55.8+/-5.8	56.9+/-6.0	66.2+/-1.9	78.2+/-5.3	39.7+/-3.1	38.2+/-0.7	29.6+/-3.1	40.8+/-6.2

Table 1: Effect of extraction solvent benzene/acetone and acetonitrile is shown using ultrasonic extraction method. High recovery rates for PAH could be obtained using a 2:1 mixture of benzene and acetone. The smaller aromatic ring structures phenol, naphthalene, acenaphthylene, acenaphthene and fluorene could not be extracted in quantitative manner using benzene/acetone or acetonitrile.

The large PAH, ranging from phenanthrene to dibenzo[*a,e*]pyrene, predominantly yielded better recovery when using benzene/acetone instead of acetonitrile. In addition, the recovery of these PAH (benzene/acetone) was nearly independent of the extraction time (table 1).

It was not surprising that the small, volatile PAH molecules partially disappeared during an evaporation step, in which the extract was dried. However, the mean recovery for these substances was again clearly better for benzene/acetone than for acetonitrile (figure 3). Taking into account that the solvent mixture benzene/acetone yielded high PAH extraction from tattoo inks, this solvent mixture was considered superior as compared to acetonitrile.

At this experimental stage, the lost of the small PAH was minimized by using short but still efficient extraction times in the following experimental investigations. A time of about 10 min was considered to be optimal for small PAH extraction from benzene/acetone solution (figure 3).

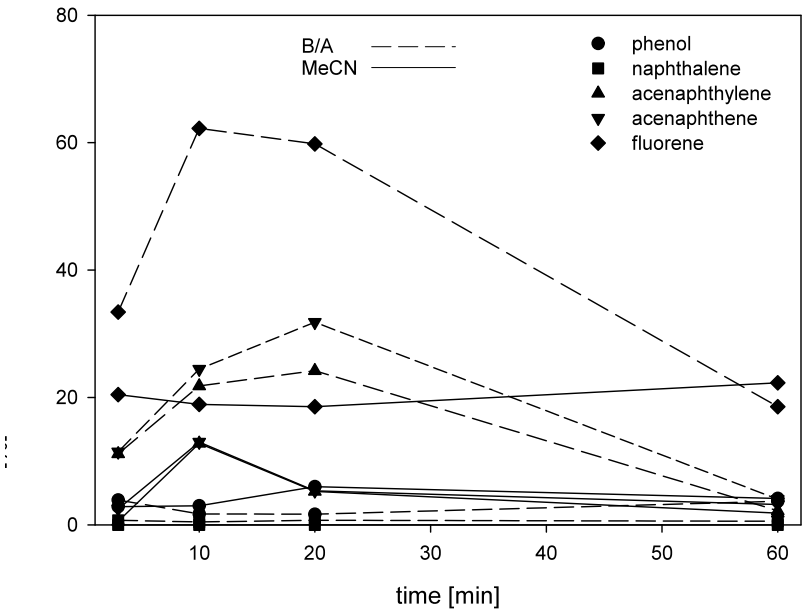

Figure 3: Mean recovery of extracted phenol, naphthalene, acenaphthylene, acenaphthene and fluorene for different times using ultrasonic bath and a mixture of benzene/acetone (B/A) or acetonitrile (MeCN) as extraction solvents. Standard deviation [%] was ± 18.9 for B/A extraction and ± 7.6 for extraction using acetonitrile.

To further optimize this extraction procedure, the ultrasonic treatment of the solution was spilt in two time intervals of 5 min that were combined with three short steps of using vortex.

To further minimize the loss of PAH, 100 µL of diethylene glycol dimethyl ether (Diglyme) (Fluka, Deisenhofen, Germany), a low volatile liquid, was added. This compound worked as keeper, which enabled the solvents to evaporate and momentarily prevents vaporization of the volatile compounds. The substance Diglyme was already successfully used in previous experiments, in which azo pigments were extracted from human tissue.[15]

2.3.3 Extraction experiments – final procedure

Taking all the results of preliminary experiments in consideration, extraction of PAH from digested human skin was done using a combination of the described methods:

vortex and ultrasonic alternating 1/5/1/5/1 minutes, the procedure is shown in figure 2. After adding Diglyme, the solution was concentrated to 100 µL under a gentle stream of nitrogen (20 min, 2 bar, rt). For the HPLC analysis, each sample, consisting of the extracted compounds concentrated in keeper, was reconstituted in 1 mL of acetonitrile, filtered and finally a 100 µL solution of internal standard was added. After the final step, the total volume of each sample was 1.2 mL. As seen in figure 4, the optimized extraction procedure yielded a high recovery rate for each single PAH of 96 to 99 %, rather independent of the volatility of the molecule.

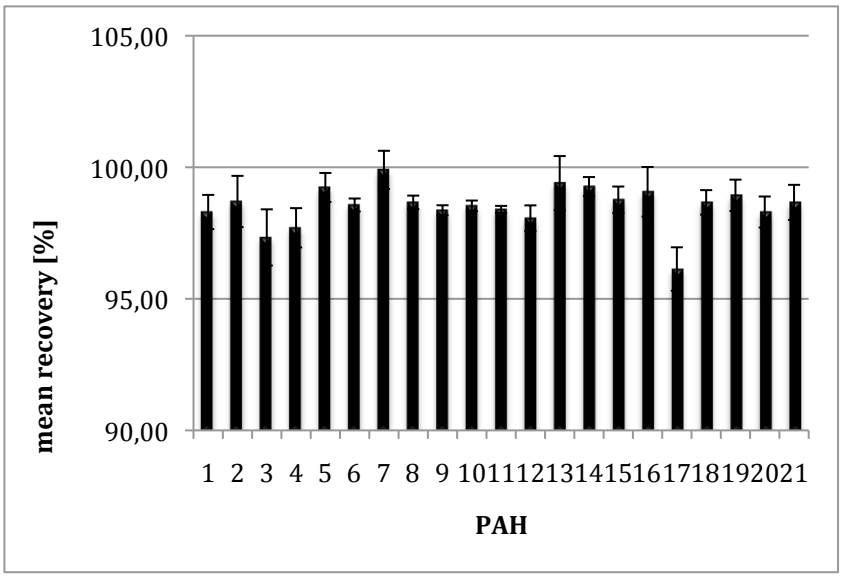

Figure 4: Recovery of phenol (1) and PAH (2-21) from digested human skin using a combination of vortex and ultrasonic extraction (1/5/1/5/1) min in a 2:1 benzene/acetone solution. Recovery yields from 96 to 99 % could be obtained for each single PAH and phenol, respectively, whereby the mean RSD (n = 3) did not exceed 3%.

In a next step, 20 mg of pure Carbon Black was added to the skin-PAH-suspension to approach the real skin tattoo conditions. The presence of carbon black as sorptive phase for PAH did not affect the recovery rates as shown in figure 5.

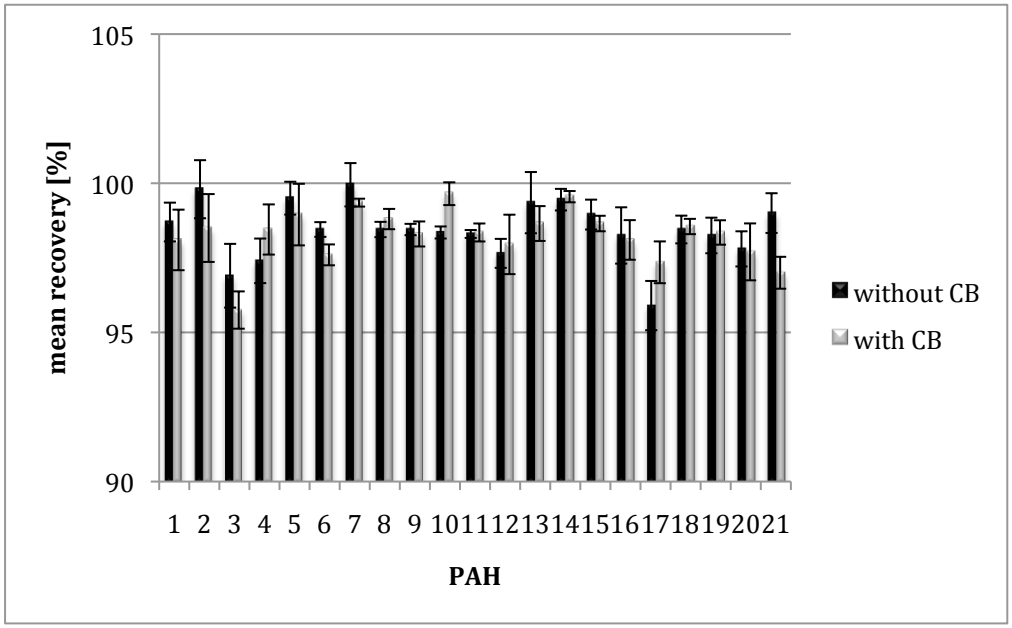

Figure 5: Recovery of phenol (1) and PAH (2-21) from digested human skin using a combination of vortex and ultrasonic extraction in a 2:1 benzene/acetone solution and keeper without (blue) and loaded with (red) Carbon Black particles. Recovery ranges from 96 – 99 % for both, without and with adding Carbon Black particles whereby the mean RSD (n = 3) did not exceed 3%.

The experiment showed that the extraction procedure is an effective and quantitative procedure to extract PAH even in the presence of carbon black, present in black inks in original tattoo suspensions.

2.4 Conclusion

Clinical observations, case reports and surveys show that tattoos are often associated with a variety of adverse skin reactions, [16, 28-34] whereas the causation of tattoo inks and the documented occurrence of cancer in tattooed skin is still controversially discussed.[35] Being frequently unregulated, the black tattoo inks may contain a variety of substances, which originate either from the production process or from intentional additives.[31, 36, 37]

The most deleterious substances, which were detected in tattoo inks so far, are PAH and phenol. To elucidate the role of such substances in skin, the concentration of PAH or phenol in tattooed skin should be determined. The concentration directly or months after tattooing may help to estimate the toxic or mutagenic risks of such black tattoo inks. The concentrations years after tattooing may elucidate the role of such substances regarding long-term effects that include bioavailability of PAH when adsorbed to Carbon Black. Thus, a quantitative procedure is required that allows quantitative extraction of PAH and phenol from tissue. This goal was achieved and the extraction procedure will be applied in the near future to investigate tattooed tissue samples.

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3. Detection of polycyclic aromatic hydrocarbons (PAH) in black tattooed human skin and related locoregional lymph nodes*

Abstract

Millions of people worldwide have black or colored tattoos. To create a tattoo, tattooists inject high amounts of inks or pigments in the dermis along with numerous ingredients. Most of the tattoos are black and the black ink frequently contains soot products like carbon black or polycyclic aromatic hydrocarbons (PAH). After tattooing of the skin, a substantial part of the ink and hence PAH are transported to the locoregional lymph nodes.

To estimate to the health risk of such tattoo inks, we extracted and quantified the amount of PAH in black tattooed skin and the locoregional lymph nodes.

Sixteen specimens of human tattooed skin and lymph nodes were digested and tested for 20 different PAH. The amount of these substances was quantified by using HPLC – DAD technology. PAH were found in twelve of the sixteen tattoos and in eleven locoregional lymph nodes. The PAH concentration ranged from 0.07 – 0.57 μg/cm^2 in the tattooed skin and 0.05 – 11.75 μg/g in the lymph nodes.

The present results provide evidence that PAH can be found in tattooed skin months or years after tattooing. In addition, carbon black obviously transported the PAH to the respective lymph nodes. Thus, PAH in tattoo inks may pose a health risk on both, skin and lymph nodes, because some of PAH are carcinogenic.

* Results of this chapter are part of a manuscript: Lehner K, Santarelli F, Vasold R, Penning R, Sidoroff A, König B, Landthaler M, Bäumler W, "Detection of polycyclic aromatic hydrocarbons (PAH) and Carbon Black in black tattooed human skin and related proximate lymph nodes", Br J Derm 2012, in preparation

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAH) such as benz[a]pyrene (B[a]P) belong to a large class of well studied chemical pollutants with ubiquitous occurrence in the environment. They consist of two or more fused benzene rings and are generated naturally or notably found as a result of incomplete combustion of organic materials, fossil fuels, vehicular emission or even tobacco smoke. Inhalation, ingestion and skin contact are the three main pathways humans are exposed to PAH. Some PAH are classified by the International Agency of Research in Cancer [1] as human carcinogens (e.g. B[a]P) and several others as probably or possibly carcinogenic to humans [1].

Epidemiologic studies have associated PAH exposure with multiple adverse health effects affecting lung, skin, larynx, kidney and bladder [2-4]. This has been linked to mutagenic properties of PAH metabolites. Benz[a]pyrene has been thoroughly studied and requires metabolic activation by cytochrome P450 enzymes through covalent binding to DNA (DNA adduct formation) [5, 6]. The active metabolite benz[a]pyrene-7,8-diol-9,10-epoxide (BPDE) represents probably the ultimate carcinogen [7]. In addition to carcinogenic properties, PAH exert a wide range of deleterious effects towards tissue and cells. PAH are thus potent immunotoxic agents that impair functional activation of lymphocytes [8, 9] and inhibit macrophage differentiation [10]. They reduce fertility, which may reflect altered survival of ovocytes [11], favour the development of cardiovascular diseases and trigger apoptosis in various cell lines [12-14]. However, PAH can also induce oxidative lesions resulting from the production of reactive oxygen species (ROS) [15].

In our previous studies, a new source of PAH could be discovered by chemical analysis of commercially available black tattoo inks. 20 different PAH and phenol

could be quantitatively detected in black tattoo suspensions using an established extraction procedure with HPLC – DAD technique and the method of internal standard. The amount of extracted PAH was in the range of 0.14 to 201 µg/g [16]. This is an alarm signal since millions of people have many and large tattoos, which are predominantly black [17, 18]. Regulation of ink composition is frequently missing. Black tattoo inks mainly consist of Carbon Black (CB), a mixture of different solvents and other ingredients, whereas the actual composition may vary for the different ink products. Carbon Black is already listed by IARC as possibly carcinogenic to humans (group 2 B) [19, 20]. In addition, the black inks, which are placed in the skin, are partially transported in the human body via lymphatic system and can be also found in the locoregional lymph nodes [21, 22].

Thus, the present study was designed to analyse the amount of PAH and phenol in real black tattooed human skin as well as in the corresponding locoregional lymph nodes by using our established extraction procedure and HPLC - DAD technology.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

As reference, 20 well known PAH (purity ~ 99 %) were obtained from Sigma Aldrich (Steinheim, Germany): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benz[b]fluoranthene,benz[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, benz[ghi]perylene, indeno[1,2,3-cd]pyrene, dibenz[a,e]pyrene, dibenz[a,l]pyrene, 5-methylchrysene and benz[j]fluoranthene. Phenol (purity > 99%) as analytical reference was obtained from Riedel-de Haen. For the internal standard (ISTD), 9,10-diphenylanthracene (purity > 99 %) was obtained from Riedel-de Haen.

3.2.2 Stock solution

One milligram of each of the 20 US-EPA PAH and phenol was dissolved in one milliliter of acetonitrile to obtain a 1.0 mg/mL PAH stock solution and treated by ultrasound (Bandelin Sonorex Super RK 103 H) for 10 min, respectively. 200 µL of each compound stock solution were combined and filled up with acetonitrile to obtain a concentration of 0.01 mg/mL of each investigated substance. As internal standard (ISTD), 9,10-diphenylanthracene (9,10 DPA) was prepared as a 0.08 mg／mL stock solution in acetonitrile. Benzene and acetone as solvents for the extraction were of reagent grade quality for liquid chromatography (LiChroSolv, Merck, Darmstadt, Germany). Acetonitrile as solvent B for LC–MS analysis was also of gradient grade quality for liquid chromatography (LiChro-Solv Darmstadt, Germany). Millipore water as solvent A for LC–MS analysis was freshly produced by a Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim, Ce´dex).

3.2.3 Enzymes

ATL buffer and proteinase K (> 600 mAU/mL) were purchased from Qiagen (Hilden, Germany).

3.2.4 Skin specimens and related locoregional lymph nodes

Sixteen skin specimens with black tattoos and related locoregional lymph nodes were provided by the Department of Forensic Medicine at the Ludwig Maximilians University Munich. No cosmetic impairment occurred during the skin harvest because the samples were taken from the periphery of large injuries of heavily traumatized bodies, for instance from people killed by train accidents. Skin sampling was performed as soon as possible after exitus and frozen to -80°C. For further analysis, adipose tissue was removed by a scalpel, the skin was chopped up to slices at a size of 1 cm^2 and placed into Eppendorf cups (Eppendorf, Wesseling-Berzdorf, Germany), and 400 µL of PBS (PAA, Pasching, Austria) was added. Proteins were denatured by heating at 95°C for 5 min. After cooling to room temperature, a total of 180 µL of buffer ATL and 20 µL of proteinase K were added to the skin, mixed by vortex and incubated at 55 °C until the tissue was completely lysed. The respective locoregional lymph nodes were weighted, cleaved and treated in an analogue work-up procedure. The experiments were performed according to the Helsinki Declaration of 1975.

3.2.5 Extraction procedure

0.6 mL of digested tattooed human skin and 0.6 mL of digested locoregional lymph node were placed in a glass test tube (8 mm · 10 mm, NS 14; Neubert-Glas, Ilmenau, Germany), respectively. Our previously reported method for the extraction of PAH from black tattoo ink suspensions [16] was just enlarged to obtain optimal conditions

for organic matrices like human tissue specimens with recovery rates ranging from 96 – 99 percent. The extraction solvents benzene/acetone (2 mL /1 mL) were added. The extraction of PAH and phenol from the tattooed digested human skin was done using an alternating combination of vortex mixer (MS 1 Minishaker, IKA, Brazil) and ultrasonic bath (Bandelin Sonorex Super RK 103 H) for 1/5/1/5/1 minutes. After centrifugation at 4°C with 2500 ***g*** (Eppendorf Centrifuge, 5702 RH), the supernatant was collected, 100 µL of keeper "Diglyme" was added to avoid the loss of volatile components during the extraction procedure. Finally, the solvent was removed under a gentle stream of nitrogen (2 bar, 20 min, rt) and concentrated to 100 µL. For the HPLC analysis, each sample, consisting of the extracted compounds concentrated in keeper, was reconstituted in 0.3 mL of acetonitrile, filtered through a PTFE-filter (CHROMAFIL®, O-20/15, organic, pore size 0.2 µm, Machery-Nagel, Düren, Germany) and finally 50 µL of internal standard stock solution were added. After the final step, the total volume of each sample was 0.35 mL.

3.2.6 HPLC analysis

The calibration solution consisted of 0.3 mL of PAH-phenol stock solution and 50 µL tracer stock solution. All samples were analyzed using a model 1100 HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column for nanoscalic environmental analysis (Phenomenex Environsep PP, particle size 3 µm, 125 x 2.00 mm, Aschaffenburg, Germany) and diode array detector (DAD). The Injection volume was 10 µL. The data-files were analysed using a HPLC-3D-ChemStation Rev. B.04.02. The PAH and phenol could be separated by gradient elution with water [0.0059 w % trifluoroacetic acid] (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.3 mL/min. A gradient profile with the following

proportions of solvent B was applied [t (min), % B]: (0, 40), (2, 40), (27, 98), (40, 98). The chromatograms were monitored at 220 nm.

The concentration of PAH in the solutions was determined by the method of internal standard. For each compound (i), the calibration factor (CF_i) was determined in a calibration run (single level calibration). The respective concentration of the internal standard was chosen to be in the range of the concentration of the PAH.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K the mass of compound i in the solution k, and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

3.3 Results and Discussion

Sixty percent of tattooed individuals have predominately black coloured tattoos [18]. Therefore, during the last years, we focused on the chemical analysis of the ingredients of commercially available black ready-to-use suspensions [16]. Among others, we recently detected substances like dibutyl-phthalate or dibenzofuran in such black tattoo inks [23]. These impurities of the inks have the potential to cause adverse skin reactions that is frequently described in the medical literature [24-28]. The extent of health problems was investigated by a nation-wide survey [18].

Beside such adverse skin reactions, also case reports about skin malignancies in tattoos are published. It is still under discussion whether these malignancies such as basal cell carcinoma [29-32] or melanoma [24, 26, 27, 33, 34] coincidentally occurred only. Frequently, melanomas in tattoos were found on pre-existent melanocytic nevi [34]. It has been suggested that ultraviolet radiation, a photoallergic effect, a persistent inflammatory reaction, or even trauma may promote malignant transformation [34].

Tattooists usually do not spare melanocytic nevi when puncturing the black inks into the skin. It is a fact that many black tattoo inks contain high amounts of PAH of up to 201.1 ± 19.5 µg/g [16], which may harm the skin in different ways. Firstly, it is well documented that many of PAH such as benz[a]pyrene are mutagenic or carcinogenic. Secondly, PAH generate reactive oxygen species (ROS) such as singlet oxygen when exposed to solar ultraviolet radiation during years after tattooing.

Due to hydrophobic nature, Carbon Black as fundamental matrix of the ready-to-use suspensions acts as strong sorptive phase for large conjugated aromatic systems such as PAH [35]. Nevertheless, PAH in tattoo inks can either be bound to CB or be

dissolved in the solvents of the inks suspension. After tattooing, unbound PAH should be immediately transported away from skin via lymphatic system. Adsorbed PAH can leave Carbon Black in skin after tattooing to an unknown extent, whereas the time kinetics might range from months to years. It is known that concentration of tattoo inks may change over years after tattooing [36].

It is well documented that tattoo inks are transported to locoregional lymph nodes [22, 37-44]. Along with CB nanoparticles also PAH compounds are transported to the lymph nodes. Therefore, we had established an extraction scheme for the quantification of 20 PAH and phenol from disintegrated human tissue with a recovery rate of 96 – 99 % for each substance (*chapter 2*).

Then, the concentrations of PAH in sixteen samples of tattooed skin and respective lymph nodes were determined, an example is shown in figure 1. These tattoos existed for months or years, the exact time spans could be not evaluated.

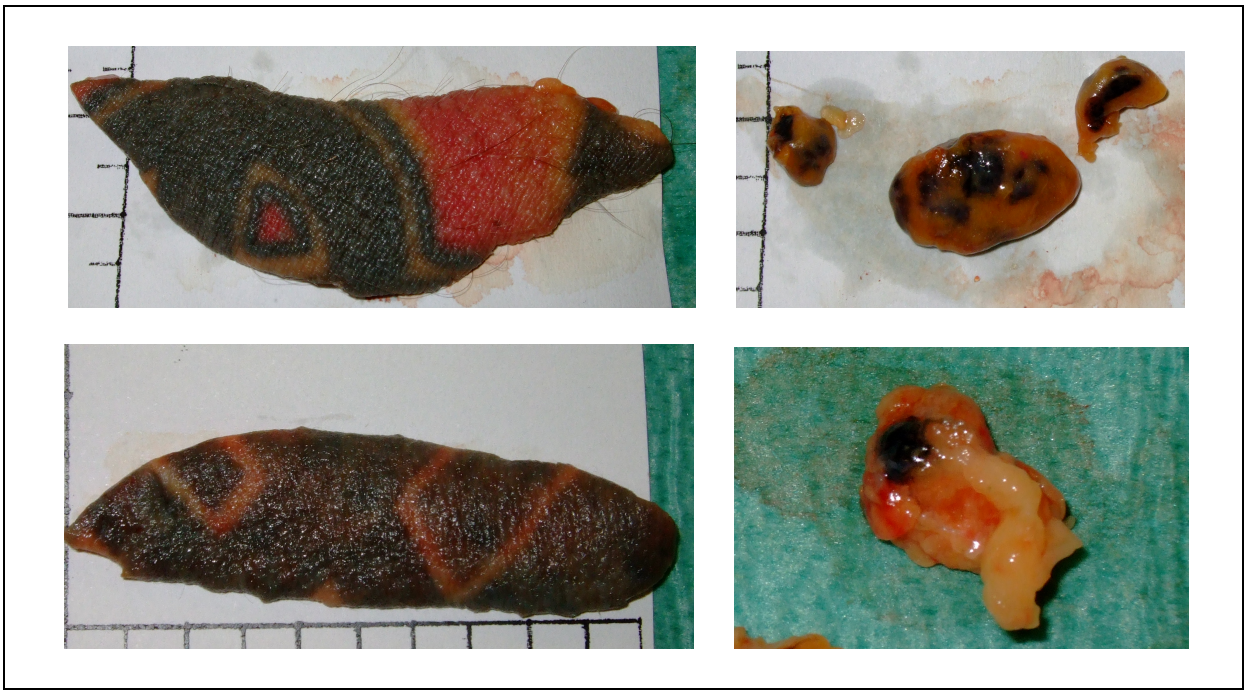

Figure 1: Black tattooed human skin specimen #4 (top, left) and #5 (bottom, left) and the corresponding locoregional lymph nodes. The most frequent tattoo colorant was black and the size of the tattoo was around 10 cm^2, each. Every lymph node contained black color, which could be seen macroscopically.

3.3.1 PAH in tattooed skin specimen

In the present study, we selected tattooed skin samples showing predominantly black areas. The size of the tattoo specimens was in the range of 1.75 – 12 cm^2. Skin for extraction was excised from black areas only and the calculated PAH concentration was referred to the size of this area. The extracted and lysed skin samples were screened using HPLC – DAD technology. This was based on the retention time and UV spectra of the 20 PAH naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benz[b]fluoranthene, benz[k]fluoranthene, benz[a]pyrene, dibenz[a,h]anthracene, benz[g,h,i]perylene, inden[1,2,3-c,d]pyrene, dibenz[a,e]pyrene, dibenz[a,l]pyrene, 5-Methylchrysene, and benz[j]fluoranthene. These PAH are substances, listed by the

United States Environmental Protection Agency (US EPA) due to their toxicity and carcinogenicity (USEPA, 1982; National Toxicology Program, 1998; Warshawsky, 1999) or Scientific Committee on Food (SCF) of health and consumer protection directorate-general in Europe.

Tattoo	amount PAH µg/cm^2
# 1	0.08
# 2	0.57
# 3	n.d.
# 4	0.41
# 5	0.19
# 6	0.40
# 7	0.18
# 8	0.07
# 9	n.d.
#10	0.12
#11	0.08
#12	0.22
#13	n.d.
#14	n.d.
#15	0.14
#16	0.16

Table 1: Total amount of 20 investigated PAH [µg/cm^2] in sixteen black tattooed human skin specimens. The values range from 0.07 µg/cm^2 to 0.57 µg/cm^2. PAH was below the detection limit and could not be quantified within experimental accuracy according to the applied procedure (n.d.: not detected) for tattoo #3, #9, #13 and #14

Using the method of internal standard, the total amount of PAH in twelve of sixteen tattooed skin samples ranged from 0.07 to 0.57 µg/cm^2 (table 1). Black tattoo sample #2 contained the highest amount of investigated pollutants. As our extraction procedure shows a rather high experimental accuracy, this broad range of values is probably caused by different tattooing procedures using different pigment

concentrations together with various admixtures. In fact, differences might also be explained by the tattooed skin site.

Phenanthrene and acenaphthene were present in seven of sixteen skin samples, fluorene, anthracene, benz[j]fluoranthene and naphthalene were found in two tattoos, respectively. Pyrene, benz[k]fluoranthene, benz[b]fluoranthene and fluoranthene could be quantified in almost one specimen. PAH dibenz[a,e]pyrene, indeno[1,2,3-c,d]pyrene, benz[g,h,i]perylene, dibenz[a,h]anthracene, dibenz[a,l]pyrene, benz[a]pyrene, 5-methylchrysene, chrysene, benz[a]anthracene, and acenaphthylene were below the detection limit and could not be detected in any tattoo sample within the experimental accuracy. There are some indications for the presence of phenol in few tattoo samples but not quantitatively detectable. PAH investigated are listed in table 2.

Phenanthrene was detected with highest amount in both, commercially available black tattoo ink suspensions as published and black tattooed human skin. There might be some correlation between PAH present in black tattoo inks and PAH punctured into human skin because the same substances found in human matrices were recently detected in tattoo ink suspensions.

PAH	mean amount in ink suspensions* [µg/g]	mean amount in skin [µg/cm^2]
Phenanthrene	24.5 ± 6.0 (12)[#]	0.1 ± 0.4 (7)
Acenaphthylene	14.5 ± 5.5 (8)	n.d.
Benz[b]Fluoranthene	4.5 ± 4.3 (2)	0.1 ± 0.1 (1)
Pyrene	4.4 ± 0.8 (12)	0.1 ± 0.1 (1)
Anthracene	3.3 ± 0.8 (8)	0.2 ± 0.1 (2)
Fluoranthene	2.8 ± 1.0 (14)	0.1 ± 0.1 (1)
Chrysene	1.7 ± 0.8 (4)	n.d.
Benz[a]Anthracene	1.6 ± 0.2 (6)	n.d.
Benz[g,h,i]Perylene	1.2 ± 1.5 (3)	n.d.
Indeno[1,2,3-cd]Pyrene	1.1 ± 1.0 (2)	n.d.
Acenaphthene	0.9 ± 0.3 (8)	0.1 ± 0.04 (6)
Fluorene	0.9 ± 0.2 (6)	0.2 ± 0.2 (2)
Benz[k]fluoranthene	0.4 ± 0.2 (2)	0.03 ± 0.03 (1)
Benz[a]Pyrene	0.3 ± 0.2 (4)	n.d.
Naphthalene	0.3 ± 0.1 (7)	0.05 ± 0.04 (2)
Dibenz[a,h]Anthracene	0.1 ± 0.1 (1)	n.d.
Benz[j]fluoranthene	n.d.[§]	0.1 ± 0.1 (2)
Dibenz[a,l]Pyrene	n.d.	n.d.
5-Methylchrysene	n.d.	n.d.
Dibenz[a,e]Pyrene	n.d.	n.d.

Table 2: Mean value of PAH found in investigated tattooed skin samples [µg/cm^2]. *Mean PAH value [µg/g] as previously detected in commercially available black tattoo ink suspensions [16]. [#]The number in brackets indicates the total number of inks or human tissue samples in which the respective PAH was found. [§]PAH was below the spectral detection limit and could not be quantified within experimental accuracy according to the applied procedure (n.d.: not detected)

3.3.2 PAH in locoregional lymph nodes

Due to lymphatic transportation of Carbon Black, PAH may appear also in the locoregional lymph nodes. Figure 2 shows an example of histology of a tattooed skin sample and lymph node.

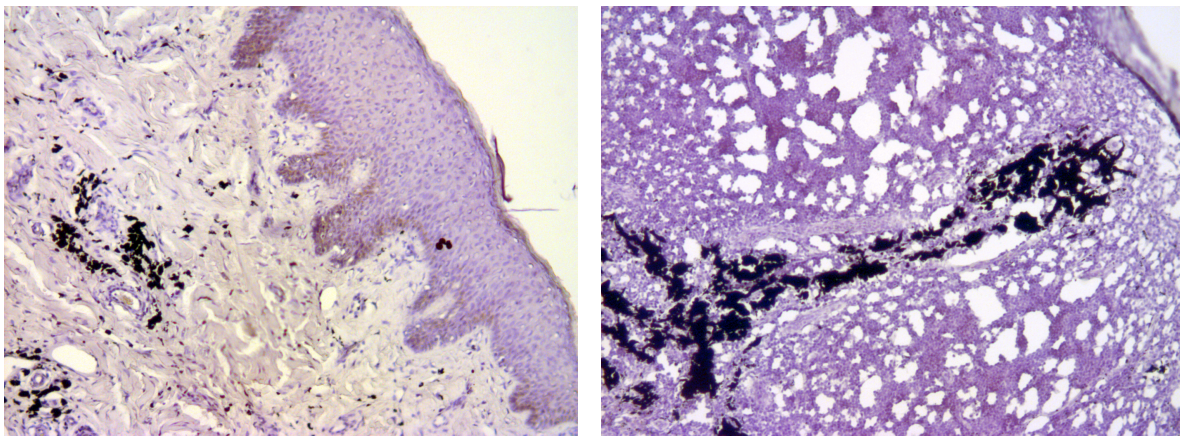

Figure 2: Histological cross section of black tattooed human skin (left) and related locoregional lymph node (right), embedded in paraffin and after hematoxylin-eosine (H&E) staining, recorded at a Zeiss Axiostar Plus microscope (10 fold optical magnification). The black tattoo particles are deposited inside the dermis together with adsorbed hazardous PAH (left). Lymph node specimen (right) containing black tattoo pigment and adsorbed PAH which may be transported from the site of the black tattoo during month or years after tattooing.

Extraction and analysis procedure was also applied for the lymph nodes. In eleven of the sixteen lymph nodes, identification and quantification of PAH was possible (table 3). The weight of the respective lymph node specimens ranged from 0.06 to 0.59 g and the concentration of PAH ranged from 0.05 – 11.75 µg/g. Phenanthrene could be detected in seven samples, acenaphthene in four. Benz[j]fluoranthene was detected two times and anthracene, indeno[1,2,3-c,d]pyrene, fluorene, benz[k]fluoranthene and naphthalene were quantified in one specimen, each (table 4).

Lymph node sample	amount PAH [µg/LN]	Weight of lymph node [g]	amount PAH [µg/g]
# 1	0.02	0.36	0.05
# 2	1.88	0.16	11.75
# 3	n.d.[§]	0.04	n.d.
# 4	0.20	0.23	0.87
# 5	0.39	0.17	2.29
# 6	0.44	0.15	2.93
# 7	0.52	0.17	3.06
# 8	n.d.	0.21	n.d.
# 9	n.d.	0.12	n.d.
#10	0.10	0.59	0.17
#11	0.74	0.07	10.57
#12	0.61	0.06	10.16
#13	n.d.	0.13	n.d.
#14	n.d.	0.08	n.d.
#15	0.10	0.23	0.43
#16	0.46	0.21	2.19

Table 3: Amount of 20 investigated PAH [µg/g] in the sixteen proximate lymph nodes #1 - #16. The value for hazardous polycyclic aromatic compounds ranged from 0.05 µg/g for lymph node #1 up to 10.50 µg/g for lymph node #11 and 11,75 µg/g for lymph node #2. [§]PAH was below the spectral detection limit and could not be quantified within experimental accuracy according to the applied procedure (n.d.: not detected) for lymph node #3, #8, #9, #13 and #14

PAH	mean amount in ink suspensions* [µg/g]	mean amount in lymph nodes [µg/g]
Phenanthrene	24.5 ± 6.0 (12)[#]	2.2 ± 1.8 (7)
Acenaphthylene	14.5 ± 5.5 (8)	n.d.
Benz[b]Fluoranthene	4.5 ± 4.3 (2)	n.d.
Pyrene	4.4 ± 0.8 (12)	n.d.
Anthracene	3.3 ± 0.8 (8)	9.7 ± 9.7 (1)
Fluoranthene	2.8 ± 1.0 (14)	n.d.
Chrysene	1.7 ± 0.8 (4)	n.d.
Benz[a]Anthracene	1.6 ± 0.2 (6)	n.d.
Benz[g,h,i]Perylene	1.2 ± 1.5 (3)	n.d.
Indeno[1,2,3-cd]Pyrene	1.1 ± 1.0 (2)	2.1 ± 2.1 (1)
Acenaphthene	0.9 ± 0.3 (8)	0.2 ± 0.3 (4)
Fluorene	0.9 ± 0.2 (6)	2.5 ± 2.5 (1)
Benz[k]fluoranthene	0.4 ± 0.2 (2)	4.2 ± 4.2 (1)
Benz[a]Pyrene	0.3 ± 0.2 (4)	n.d.
Naphthalene	0.3 ± 0.1 (7)	0.3 ± 0.3 (1)
Dibenz[a,h]Anthracene	0.1 ± 0.1 (1)	n.d.
Benz[j]fluoranthene	n.d.[§]	2.0 ± 0.2 (2)
Dibenz[a,l]Pyrene	n.d.	n.d.
5-Methylchrysene	n.d.	n.d.
Dibenz[a,e]Pyrene	n.d.	n.d.

Table 4: Mean value of PAH found in investigated related locoregional lymph nodes [µg/g]. *Mean PAH value [µg/g] as previously detected in commercially available black tattoo ink suspensions [16]. [#]The number in brackets indicates the total number of inks or human tissue samples in which the respective PAH was found. [§]PAH was below the spectral detection limit and could not be quantified within experimental accuracy according to the applied procedure (n.d.: not detected)

The polycyclic compounds dibenz[a,e]pyrene, benz[g,h,i]perylene, dibenz[a,h]anthracene, dibenz[a,l]pyrene, benz[a]pyrene, benz[b]fluoranthene, 5-methylchrysene, chrysene, benz[a]anthracene, pyrene, fluoranthene and acenaphthylene were below the spectral detection limit and could not be sufficiently identified within experimental accuracy according to the applied procedure (table 3).

The origin of PAH in the lymph nodes and the resulting concentration may not necessarily correlate to the results of skin extraction. For the present study, single lymph node was excised from the adjacency of the respective tattooed skin. It remains unclear which skin area was drained to which extent by which lymph node. Also other locoregional lymph nodes could contain black tattoo inks and PAH.

3.4 Conclusion

Millions of people worldwide have tattoos. In a recent survey in German speaking countries revealed that 28% of tattooed individuals have more than four tattoos and 36% tattoos have tattoos, which are larger than 900 cm^2. This implies that several grams of tattoo inks are injected into skin. For the sixteen tattooed specimens investigated, a mean total PAH concentration of 0.24 ± 0.17 $\mu g/cm^2$ was found. The tattooed pigment mainly resides in the dermis. Due to their hydrophobic character and insolubility, carbon black is resistant to enzymatic degradation in the skin. This feature provides the permanence of a tattoo for decades. Frequently, pigments in a tattoo are aggregated into crystals with a size ranging from about 0.1 to 10 µm [45]. Some of the deposited pigment may be recognized by macrophages as foreign bodies and carried from the site of the tattoo via the lymphatic system. At the same time, PAH molecules, which are adsorbed to Carbon Black nanoparticles, are also transported to lymph nodes. Thus, it was not surprising that we found a mean total value of 4.04 ± 4.49 µg/g in such locoregional lymph nodes. Benz[j]fluoranthene was found in one black tattoo specimen and also in the related locoregional lymph node. High amounts of phenanthrene were extracted from both, the ink suspensions and digested human skin samples. In addition, we suggest further correlation caused by lymphatic transportation of the pigments because phenanthrene was also detected in the corresponding locoregional lymph node (table 5).

phenanthrene	tattoo specimen [µg/cm^2]	lymph node specimen [µg/g]
#1	n.d.[§]	n.d.
#2	**0.17**	**5.63**
#3	n.d.	n.d.
#4	**0.20**	**0.87**
#5	**0.14**	**2.29**
#6	**0.14**	**0.67**
#7	**0.12**	**3.06**
#8	n.d.	n.d.
#9	n.d.	n.d.
#10	n.d.	n.d.
#11	n.d.	n.d.
#12	n.d.	n.d.
#13	n.d.	n.d.
#14	n.d.	n.d.
#15	**0.08**	**0.43**
#16	**0.16**	**2.19**

Table 5: PAH phenanthrene detected in both, tattoo specimen and corresponding locoregional lymph node; Phenanthrene was also quantified in black tattoo ink suspension with highest amounts [16]. Other investigated PAH behave similar. [§]PAH phenanthrene was below the spectral detection limit and could not be quantified within experimental accuracy according to the applied procedure (n.d.: not detected)

Finally, our results indicate a relation between PAH actually present in black inks before and in human tissue and the related locoregional lymph nodes after tattooing. The medical literature contains various case reports on adverse skin reactions after tattooing [48] including cutaneous pseudolymphoma [49], granulomatous tattoo

reactions [50, 51], allergic reactions [52] or pseudoepitheliomatous epidermal hyperplasia. These adverse reactions could be caused by the ink particles, by additives, impurities or microbial contamination. To analyze the actual causes of these unwanted reactions, at least, detailed information and declaration of the composition of tattoo inks must be provided. Only with the knowledge of the ingredients, individual cases as well as epidemiological studies can clarify the impact on public health for this decorative body art.

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4. Carbon Black as fundamental matrix in black tattoo inks: quantitative determination in tattooed human skin and related locoregional lymph nodes*

Abstract

Millions of people worldwide have tattoos that are predominantly black colored. Black tattoo inks mainly consist of Carbon Black (CB), which is known to be a strong sorptive phase for polycyclic aromatic hydrocarbons (PAH). The concentration of CB in skin is unknown so far. In addition, these substances are partly transported away to locoregional lymph nodes.

To estimate the potential risk of such tattooing procedure, we aimed to determine the concentration of CB in skin of preexisting tattoos as well as in lymph nodes. Based on previous studies, the initial concentration was determined in freshly tattooed pig skin samples that yielded a mean value of 517.2 ± 198.3 µg/cm^2. Then, CB concentration was quantified in sixteen tattooed human skin samples yielding a mean value of 110.8 ± 48.3 µg/cm^2. This indicates a remarkably decrease of black inks during the years after tattooing. The assumption of lymphatic transportation is substantiated by CB concentrations that were found in locoregional lymph nodes (0.11 mg/g - 17.0 mg/g).

*Results of this chapter are part of a manuscript: Lehner K, Santarelli F, Vasold R, Penning R, Sidoroff A, König B, Landthaler M, Bäumler W, "Detection of polycyclic aromatic hydrocarbons (PAH) and Carbon Black in black tattooed human skin and related proximate lymph nodes", Br J Derm 2012, in preparation;

*Sample preparation was done by F. Sanatrelli

The results show that substantial amounts of Carbon Black are placed in black tattooed skin. The results also allow an estimation of the amount of other substances in black inks such as PAH, which should be found in tattooed skin and in locoregional lymph nodes.

4.1 Introduction

Carbon Black (CB) is defined as black fine powdered pure elemental carbon in the form of colloidal particles, which are produced by incomplete combustion or thermal decomposition of hydrocarbons [1]. In form of amorphous carbon, CB contains high surface area to volume ratio [2], contrary to graphite or diamond, which belong to the class of crystalline carbon. It is used in tires, rubber and plastic products as well as for printing inks, coatings and consumer goods.

The International Carbon Black Association (ICBA) clearly distinguishes between Carbon Black, manufactured or engineered by furnace or thermal processes in a closed reactor by atomizing feedstock oil, and on contrary soot or black carbon, which results due to incomplete combustion containing a variety of unwanted carbonaceous by-products [3]. Manufactured Carbon Black contains more than 97 % elemental carbon arranged in graphitic-like cluster particles.

In contrast, combustion-derived soot nanoparticles are chemically and physically distinct and less than 60 % of the total particle mass is composed of carbon. Commercially carbon black contains hydrophobic contaminants, such as polycyclic aromatic hydrocarbons (PAH), which are adsorbed onto the large conjugated aromatic π – system of the carbon skeleton. Being usually not regulated and frequently not controlled, the source of black tattoo inks, which consists of Carbon Black, is unclear in the majority of cases.

Tattooing is a very popular body adornment for thousands of years. About sixty percent of tattoos predominantly contain black color [4]. Using tiny solid needles, the process of tattooing involves the simply injection of Carbon Black and several ingredients, whereas the exact chemical composition may vary for different ink suspensions. By means of phagocytosis, part of the pigment, beneath CB

nanoparticles, punctured into the dermis is recognized by macrophages as foreign bodies and removed from the site of the tattoo via lymphatic system. As a consequence, lymph nodes located next to the tattoo frequently showed black pigmentation mimicking positive sentinental lymph node in melanoma [5] or metastatic malignant melanoma [6].

We aimed to determine the concentration of CB in tattooed skin and in locoregional lymph nodes. This should allow a first estimation of the health risk regarding black tattoos. Since Carbon Black acts as carrier for other hazard substances, this estimation could be tentatively extended to e.g. PAH.

4.2 Materials and Methods

4.2.1 Chemicals, Enzymes and Reagents

Pure powdered Carbon Black was obtained from Sigma Aldrich. Proteinase K (> 600 mAU/mL) and ATL Buffer were provided by Quiagen (Hilden, Germany). PBS was obtained from PAA (Pasching, Austria). Tattoo ink dispersant (ingredients: aqua, glycerin, isopropanol, povidone, crospovidone) to dilute and disperse tattoo ink, was obtained from “The Ink Factory”. A tattoo device including 15 Magnum shading needles was provided from Polttechnology, Cologne, Germany.

4.2.2 Standard curve for CB concentration

To determine the amount of carbon black in tattooed skin samples, a standard curve was established. Therefore, a 1.0 mg/mL stock solution of pure Carbon Black in tattoo ink dispersant was diluted in a series of 100, 50, 25, 10, 1, 0 µg/mL. After calibration of the spectrophotometer (DU 640, Beckman Coulter GmbH, Krefeld, Germany) at 950 nm, using a blank sample (pure tattoo ink dispersant, consisting of aqua, glycerin, isopropanol, povidone, crospovidone and used to dilute and disperse tattoo ink), the optical density (OD) was measured.

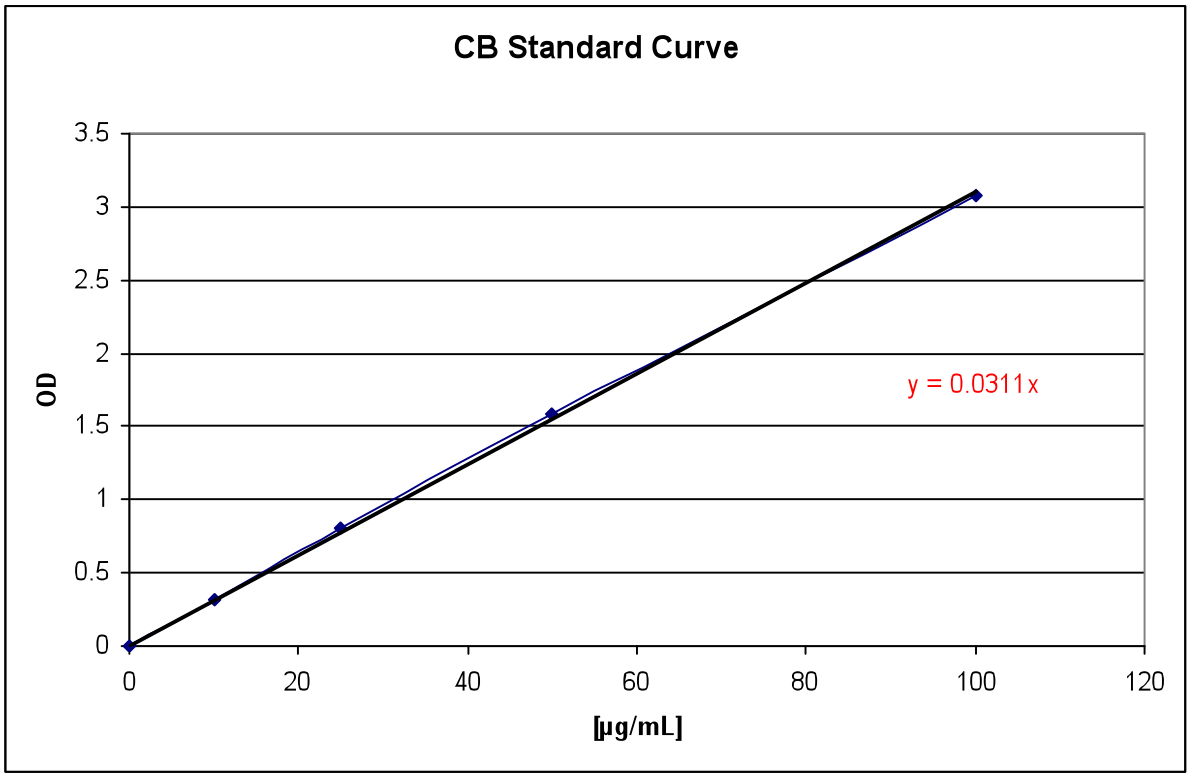

Figure 1: Optical Density (950 nm) of Carbon Black at different concentrations.

The figure clearly shows a linear dependence of OD and Carbon Black concentration. The slope was y = 0.0311 x (figure 1).

4.3 Preliminary experiments

To obtain optimal conditions for the spectro-photometrical detection of Carbon Black in tattooed human skin and related locoregional lymph node specimens, two different preliminary experiments were done using pig skin, because its morphology is almost equal to human skin. A local butcher provided freshly excised pig skin that was shaved, whereas the fatty layer of tissue was removed by a scalpel.

Firstly pure Carbon Black was suspended in tattoo ink dispersant (consisting of aqua, glycerin, isopropanol, povidone, crospovidone and used to dilute and disperse tattoo ink) to obtain tattoo ink like conditions ("special" black ink). Secondly, original black tattoo ink was used for tattooing.

4.3.1 Tattooing of pig skin with a "special" tattoo ink

A weighted amount of pure powdered Carbon Black was suspended in tattoo ink dispersant (consisting of aqua, glycerin, isopropanol, povidone, crospovidone and used to dilute and disperse tattoo ink) to obtain a finally CB concentration of 30 mg/mL. This "special" black ink was tattooed into pig skin with a commercially available tattoo machine. The experiment was done in triplicate.

4.3.2 Skin lysis

The tattooed skin was chopped up to slices at a size of 1 cm^2 and placed into Eppendorf cups (Eppendorf, Wesseling-Berzdorf, Germany), and 400 µL of PBS was added. Proteins were denatured by heating at 95°C for 5 min. After cooling to room temperature, a total of 180 µL of buffer ATL and 20 µL of proteinase K were added to the skin, mixed by vortex and incubated at 55 °C by 900rpm in a Thermomixer (Eppendorf, Wesseling-Berzdorf, Germany) until the tissue was completely lysed.

Quantification of CB was done by a spectrophotometer. For each tattooed sample, a reference sample (control; disintegrated pig skin without ink) was kept.

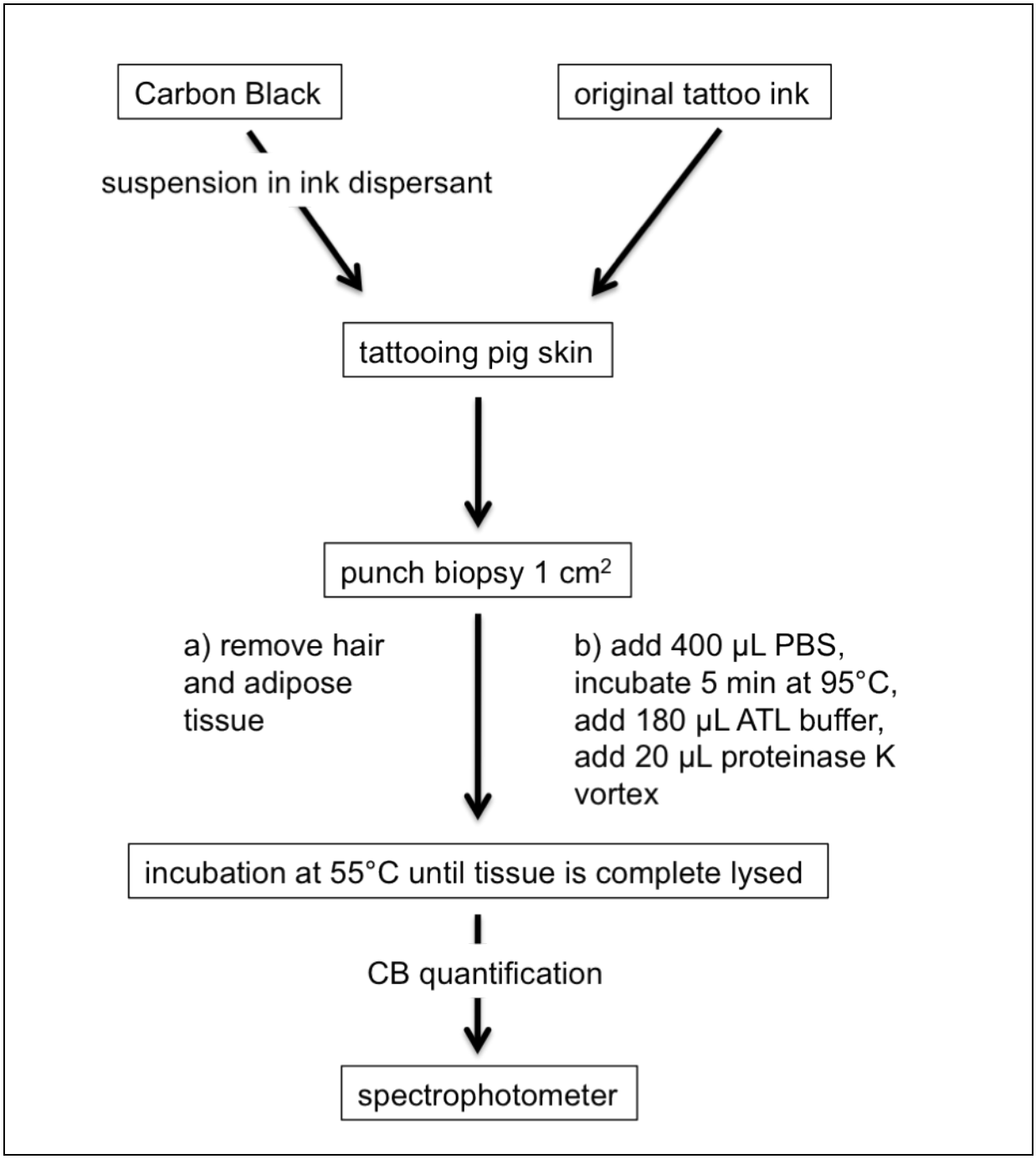

Figure 2: Flow – scheme: preliminary experiments: tattooing of pig skin with either “special” fabricated or originally black tattoo ink

The experiment was performed in triplicate.

4.3.3 Tattooing of pig skin with commercially available black tattoo ink

In parallel, tattooing of pig skin with commercially available black tattoo ink and skin lysis were done in analogues work – up procedure. The residual digested pig skin samples containing Carbon Black were analyzed by spectrophotometer detection.

4.3.4 Human skin specimens and related locoregional lymph nodes

Sixteen skin specimens with black tattoos and related locoregional lymph nodes were provided by the Department of Forensic Medicine at the Ludwig Maximilians University Munich. No cosmetic impairment occurred during the skin harvest because the samples were taken from the periphery of large injuries of heavily traumatized bodies, for instance from people killed by train accidents. Skin sampling was performed as soon as possible after exitus and frozen to -80°C. Further digestion procedure followed as described above for the lysis of pig skin. The respective locoregional lymph nodes were weighted, cleaved and treated in an analogue work – up procedure.

4.4 Results and Discussion

Literature attempt to investigate the relationship between exposure to CB nanoparticles and inhalation mainly associated with respiratory morbidity (respiratory symptoms, lung function and chest radiographs) [7-10]. Maternal exposure to carbon nanoparticles has been described leading to an increase of collagen expression in tubular cells of the renal cortex in the offspring [11], which can lead to the development of fibrosis [12]. Toxicogenomic effects in dams and offspring are suggested caused by intratracheal instillation of carbon black to pregnant mice [13-15]. Later stages of lung cancer carcinogenesis in oil refinery and carbon black production workers have been monitored [16-19]. In addition, several recent subchronic studies in rats and mice have shown that particle size and surface area dose of CB nanoparticles influence the pulmonary inflammation response [20-23]. Recently, the IARC has classified CB as a possible human carcinogen (group 2B) [24, 25].

For quantitative analysis of CB nanoparticles in the sixteen tattooed human skin specimens and the corresponding locoregional lymph nodes, the present report presents the establishment of a spectro-photometrical method for the quantitative detection of Carbon Black in human tissue. Since pig skin is morphological similar to human skin, preliminary experiments were done by tattooing pig skin using a “special” fabricated black ink as well as originally black tattoo suspension.

4.4.1 Preliminary investigations – tattooing of pig skin

Pig skin was tattooed with a commercial available tattoo machine. In a first step, a "special" fabricated black tattoo ink was used, consisting of pure powdered Carbon Black suspended in tattoo ink dispersant. After skin lysis work – up procedure, recovery of Carbon Black was done spectro – photometrical. A mean Carbon Black concentration of 270.2 ± 87.4 µg/cm^2 was detected, analyzed from 1 cm^2 of tattooed pig skin. In case of original black tattoo ink, a Carbon Black concentration of 517.2 ± 198.3 µg/cm^2 was quantified after tattooing pig skin. Due to various additional solvents and softening agents being present in the original tattoo ink a better miscibility of CB particles and density is available. In case of "special" fabricated ink, a rapid sedimentation of CB enables only moderate pigmentation during the tattooing procedure.

4.4.2 Carbon Black in tattooed human skin specimens

In the present study, sixteen tattooed human skin specimens were investigated. The most dominant color of the tattooed area was black and the size of the tattoo specimens was in the range of 1.75 – 12 cm^2. Skin lysis and extraction was performed according to our established procedure. The digested samples were analyzed and the amount of Carbon Black was quantified by spectrophotometer detection. The concentration of Carbon Black was in range of 21.1 ± 14.9 µg/cm^2 for tattoo # 3 up to 194.9 ± 21.1 µg/cm^2 for tattoo # 6. The results are shown in table 1.

Tattoo	amount CB µg/cm^2
#1	103.2 ± 51.4
#2	88.4 ± 61.1
#3	21.1 ± 14.9
#4	61.9 ± 29.0
#5	152.0 ± 49.7
#6	194.9 ± 21.1
#7	157.0 ± 67.8
#8	89.4 ± 38.1
#9	82.8 ± 22.1
#10	105.3 ± 52.5
#11	94.3 ± 31.5
#12	42.1 ± 32.4
#13	157.3 ± 20.6
#14	167.4 ± 72.1
#15	167.5 ± 29.3
#16	87.6 ± 20.2

Table 1: Amount of CB [µg/cm^2] extracted from real existing human tattoo specimens #1 - #16 were in the range of 21.1 ± 14.9 µg/cm^2 for tattoo # 3 up to 194.9 ± 21.1 µg/cm^2 for tattoo # 6

The overall mean CB concentration was 110.8 ± 48.3 µg/cm^2. The preliminary experiments clearly reveal a decrease of pigment concentration in tattooed skin of about 80 % (table 2).

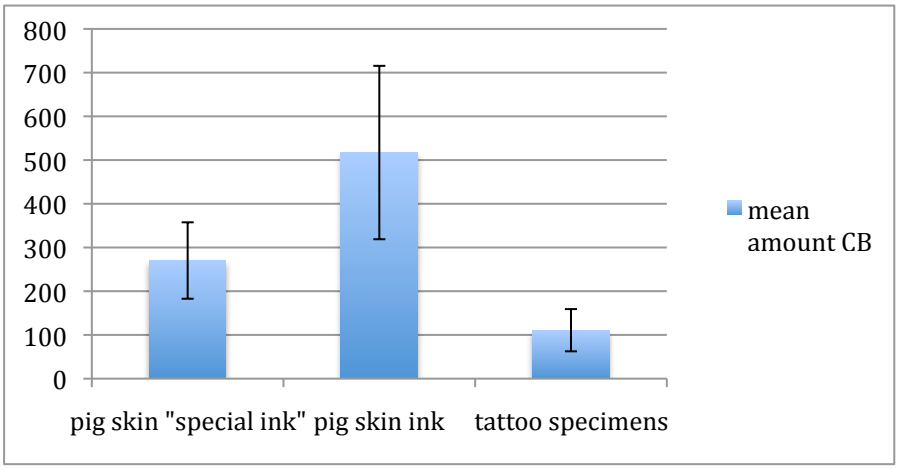

Table 2: Mean amount of CB [µg/cm^2] extracted from pig skin, tattooed with either “special” ink (left) or commercial available black ink (middle) as well as mean amount of CB [µg/cm^2] extracted from human skin specimens, month or years after tattooing (right)

This perception could be already demonstrated in our previously studies for red azo pigments years after tattooing [26]. The pigment either decomposes or migrates into the human body. Due to lymphatic transportation of tattoo pigments, we expect remaining Carbon Black being transported to related locoregional lymph nodes.

4.4.3 Carbon Black in related locoregional lymph node specimens

Sixteen related locoregional lymph nodes were investigated. All lymph node specimens contained black color (figure 3).

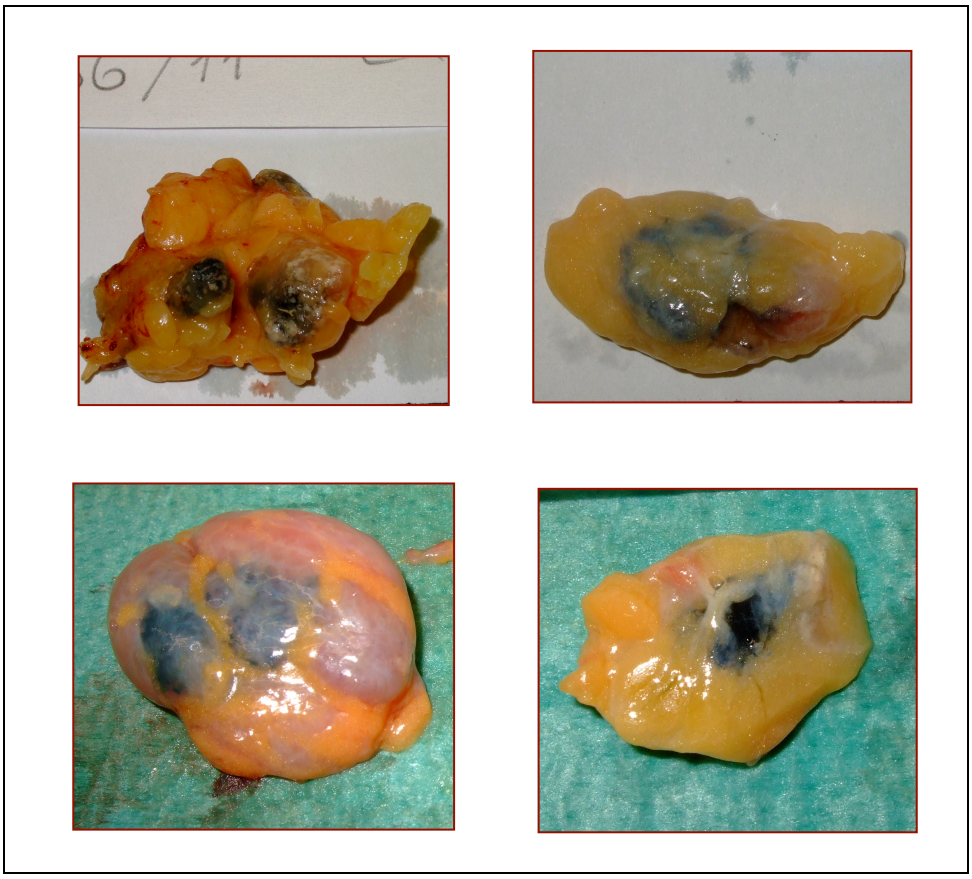

Figure 3: All investigated locoregional lymph nodes contained black color

For extraction of the black particles, adipose tissue was removed and lysis was done as described. The digested samples were analyzed and the concentration of Carbon Black was quantified spectro-photometrical. The amount of black pigment was in the range of 0.11 mg/g for lymph node specimen #7 up to 17.0 mg/g for sample #12. A mean concentration of 6.9 ± 5.2 mg/g Carbon Black was extracted from sixteen investigated lymph node specimens (table 3).

Lymph node	amount CB [mg/LN]	Weight of lymph node specimen [g]	amount CB [mg/g]
#1	0.80	0.06	13.30
#2	0.51	0.04	12.75
#3	0.06	0.05	1.20
#4	0.26	0.04	6.50
#5	0.64	0.05	12.80
#6	0.09	0.02	4.50
#7	0.01	0.09	0.11
#8	0.59	0.08	7.37
#9	0.19	0.04	4.75
#10	0.39	0.14	2.78
#11	0.08	0.02	4.00
#12	0.34	0.02	17.00
#13	0.05	0.03	1.67
#14	0.14	0.02	7.00
#15	1.45	0.12	12.08
#16	0.32	0.12	2.66

Table 3: Amount of CB [mg/g] extracted from the related locoregional lymph nodes #1 - #16.

In a previous study, we could show, that during the tattooing procedure, the pigment concentration for red azo dyes in skin ranges from 0.60 to 9.42 mg/cm^2 (mean value: 2.53 mg/cm^2) directly after tattooing, depending on the pigment concentration applied to the skin surface, the size of the pigment crystals, and the respective method of tattooing [27]. Regarding black tattoos, we estimate about half amount being

punctured into human skin by professionally tattooing artists. Deposition of the pigment into the dermis results in the permanence causing the tattoo. In the present study we were able to show, that the main part is transported away by vascular system, in particular through lymph channels and therefore, the amount of tattoo pigment remaining in the dermis is decreased.

4.5 Conclusion

For the first time, we were able to establish a spectro-photometrical detection method for the quantification of Carbon Black nanoparticles in both, tattooed human skin specimens and related locoregional lymph nodes.

For sixteen investigated tattooed human skin specimens, the amount of Carbon Black was in the range of 21.1 ± 14.9 µg/cm^2 up to 194.9 ± 21.1 µg/cm^2. In a preliminary tattooing experiment using pig skin, a mean CB concentration of 517.2 ± 198.3 µg/cm^2 could be quantified. Therefore, in large part (> 80%), tattoo ink particles are being transported from the site of the tattoo via lymphatic system and frequently found in the lymph nodes. The amount of CB in related sixteen locoregional lymph nodes was in the range of 0.11 mg/g up to 17.0 mg/g.

With specific regard to raising concerns about the potential toxicity of Carbon-based nanoparticles at several levels, including the chemical reactivity of particles and the physical interaction of these with cellular structures involved in the catalysis of biological redox processes [28], induction of reactive oxygen species (ROS) generation may be enhanced upon exposure of cells to particular matter. Since Carbon Black is already listed as possible carcinogenic to humans (group 2 B) [24], there is no legal regulation on black tattoo inks so far. In a first step, declaration of the pigments and auxiliary ingredients used should be an essential part to protect human health.

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5. Polycyclic aromatic hydrocarbons and UVA radiation: potential risk for oxidative stress in skin with black tattoos*

Abstract

Polycyclic aromatic hydrocarbons (PAH) constitute a major class of chemical carcinogens present in the environment. These compounds require metabolic activation to electrophilic metabolites to exert their mutagenic or carcinogenic effects. In addition, upon light irradiation, PAH exhibit phototoxicity.

We previously detected a variety of widespread PAH in commercial available black tattoo inks and hence in tattooed human skin. Since photoirradiation of PAH can be another activation pathway, we investigated 20 PAH and phenol on possible photochemical alterations, which were triggered by UVA radiation. By HPLC measurement and mass spectroscopy, thirteen environmental pollutants could be analyzed generating photo – mediated decomposition products. Therefrom, twelve PAH were detected forming epoxide-derivatives. Oxygen-free working conditions or the addition of specific quenchers clearly showed that the oxidation of PAH is mediated by singlet oxygen generation. *In vitro* conditions showed already cytotoxicity for parent PAH without irradiation. Upon UVA irradiation, the toxicity of PAH depended on the photoproduct.

Since medical literature contains many adverse skin reactions at the site of the tattoo, risk assessment should be an essential part to protect human health to ensure

*Results of this chapter are part of a manuscript: Lehner K, Santarelli F, Vasold R, Sidoroff A, König B, Landthaler M, Bäumler W, "Effects of laser and UV radiation on black tattoo inks", Lasers Surg Med, 2012, in preparation

*Sample preparation was done by F. Santarelli

safety for people with black tattoos exposed to sunlight or any other artificial light source.

5.1 Introduction

Tattooing is very popular worldwide and most tattoos predominantly contain black color [1]. Black tattoo inks mainly consist of Carbon Black, a mixture of different solvents and other ingredients, whereas the actual composition may vary for different ink products. Carbon Black is already listed as possible carcinogenic to humans (group 2 B) [2]. Nevertheless, in the process of tattooing, human skin is punctured with vibrating tiny solid needles, which are moistened with tattoo colorant. Since PAH adsorb onto the large hydrophobic aromatic carbon black surface by strong π–π-stacking interactions, PAH may be additionally injected into skin together with these black nanoparticles. The concentration of 20 different PAH was recently quantified in commercially black ink suspensions by HPLC technique, which ranged from 0.14 to 201 µg per gram dry ink [3].

The adsorbed PAH may stay together with the black ink particles in the dermis for long time or may slowly come off from the particles during the following months. A previously investigation of black tattooed human skin specimens showed a mean PAH value of 6.57 µg/cm^2 (*chapter 3*). Since a single tattoo frequently covers a skin area of about 400 cm^2, we calculate a PAH concentration of 2.6 mg in skin. Generally, most of the foreign pigment is taken up by phagocytes and can be aggregated into crystals [4], or can be located in dermal fibroblasts [5] (figure 1).

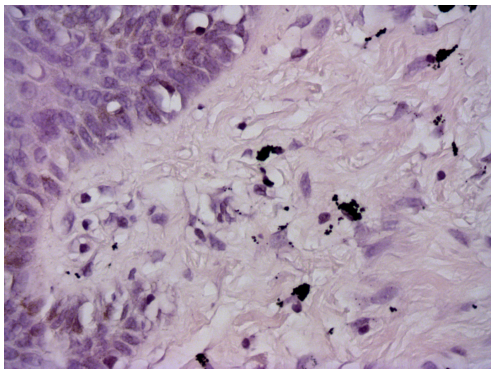


Figure 1: Punch biopsy and histology of black tattooed human skin, embedded in paraffin and after hematoxylin-eosine (H&E) staining, recorded at a Zeiss Axiostar Plus microscope (10 fold optical magnification). The ink particles can be seen as black spots with different sizes that are randomly distributed in the dermis of the skin.

Polycyclic aromatic hydrocarbons (PAH) are a class of genotoxic environmental contaminants [6-8]. They are formed upon incomplete combustion processes during both, natural events and human activities [9-11]. Being ubiquitous in the environment, PAH exposure is associated with multiple adverse health effects inducing tumors, primarily in the lungs, larynx, kidney, bladder and the skin [6, 12-15]. Some PAH are classified by the International Agency of Research in Cancer [16], the United States Environmental Protection Agency (US EPA) and the National Toxicology Program as potential human carcinogens [17-19].

Carcinogenicity and metabolic activation pathways of PAH including carcinogenicity have been well-studied: metabolism into *bay*-region dihydrodiol epoxides by microsomal enzymes, especially human cytochrome (CYP) enzymes, formation of radical-cation by one-electron oxidation and the ortho-quinone formation by dihydrodiol dehydrogenase have been determined *in vivo* [6, 17, 18, 20-25] (figure 2).

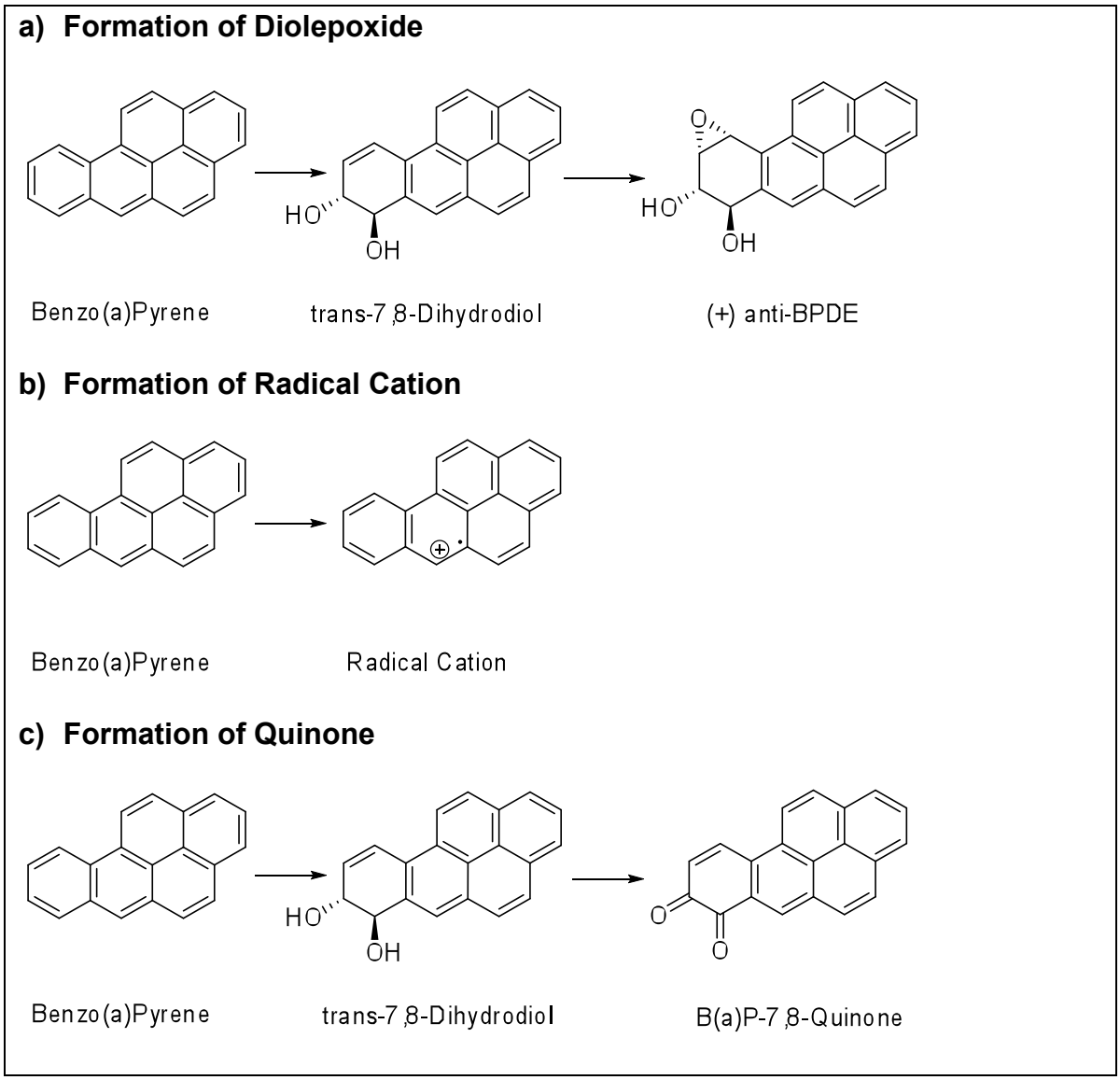
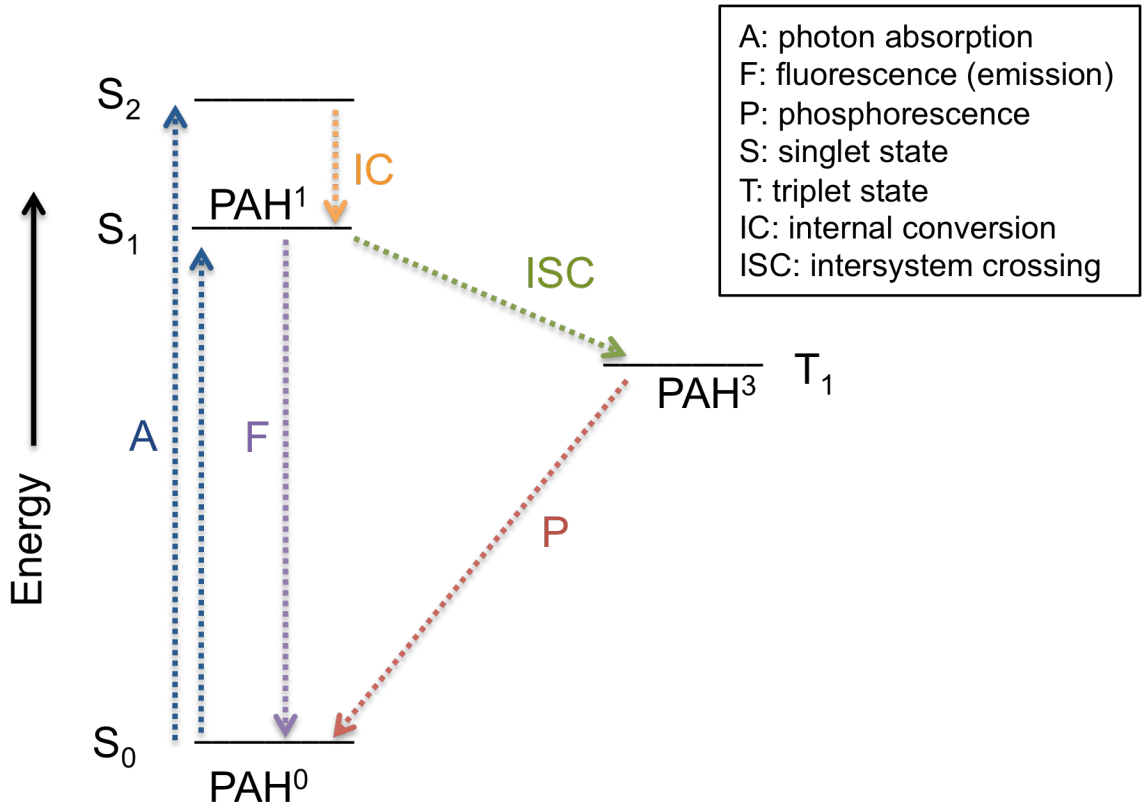


Figure 2: Exemplarily description of the principle metabolic activation pathways of benz[a]pyrene that may lead to tumor initiation

All these pathways result in binding of the ultimate metabolites with cellular DNA to form exogenous DNA adducts leading to cancer formation. Penning et al. described that the activation pathway forming quinones also involves the generation of reactive oxygen species (ROS) leading to endogenous DNA adducts [22].

UV radiation is well absorbed by PAH molecules, which prompts another pathway of inducing phototoxicity [17, 25, 26]. Because of multiple rings, upon light absorption, the PAH molecule is excited to excited energy states (figure 3) that can initiate a

series of reactions yielding ROS such as singlet oxygen ($^1O_2$) and other reactive intermediates.



$PAH^3 + {}^3O_2 \longrightarrow PAH + {}^1O_2$

PAH^3 or $PAH^1 + O_2 \xrightarrow{\text{e - transfer}} PAH^{+\bullet} + O_2^{-\bullet}$

PAH^3 or PAH^1 + DNA Base $\xrightarrow{\text{e - transfer}}$ PAH & DNA radicals

PAH^3 or $PAH^1 + O_2 \xrightarrow{\text{chemical oxidation}} PAH\text{-}O_2\text{-intermediates}$

PAH^3 or PAH^1 + co-existing molecules $\xrightarrow{\text{excited-state reaction}}$ reactive intermediates

Figure 3: Typical jablonski diagram: the exemplarily diagram illustrates the electronic states of a PAH molecule and some important transitions between the electronic states.

We previously determined the efficacy of singlet oxygen generation upon exposure to ultraviolet radiation [3]. These intermediates can damage DNA, protein and cell membrane, which may lead to acute toxicity and genotoxicity.

Skin is the largest organ in humans that is frequently exposed to solar radiation. In case of black ink particles in skin, the adsorbed PAH is likewise exposed to UV of solar radiation and the generation of reactive oxygen species might add a potential risk for skin cancer [27-29].

The photochemical and phototoxic activities of PAH were reviewed by Yu et al [25]. Irradiation of PAH results in the generation of reactive species, e.g. ROS, reactive aromatic species, radicals or epoxides. These species can damage human cells, whereas cellular DNA is the main target leading to genotoxicity. The possible DNA damage includes single strand cleavage, double strand cleavage, deletion of a base (depurination/depyrimidation), oxidation of guanine to 8-hydroxy – or 8-oxo-guanine, thymine-thymine dimer formation, PAH-DNA covalent adducts, DNA-DNA cross-links, and DNA-protein cross-links. Irradiation in the presence of lipids may induce lipid peroxidation. In general, the genotoxic effects of UVA photons are mostly mediated by photosensitization reactions. Already contact of PAH to intact skin may cause severe health problems.

Consequently, we exposed 20 of the widespread PAH as well as phenol to UVA radiation, which sufficiently penetrates skin (superficial dermis) and hence can be absorbed by the PAH on the ink particles. We applied HPLC – DAD monitoring and mass spectroscopy (LC – MS) to investigate possible photochemical alterations, which were triggered by UVA radiation. Products of the photo-process likely due to the involvement of reactive oxygen species, namely epoxides, were subsequently tested by chemical reaction with n-butylamine. This primary amine acts as strong

nucleophile and is able to open an electrophilic oxirane structure, which can be then detected by a peak shift in HPLC and mass spectroscopy.

5.2 Materials and Methods

5.2.1 Chemicals and Solvents

As reference, 20 well known PAH (purity ~ 99 %) were obtained from Sigma Aldrich (Steinheim, Germany): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene, dibenzo[a,e]pyrene, dibenzo[a,l]pyrene, 5-methylchrysene and benzo[j]fluoranthene. Phenol (purity > 99%) was obtained from Riedel-de Haen. For the internal standard (ISTD), 9,10-diphenylanthracene (purity > 99 %) was obtained from Riedel-de Haen. Acetonitrile as solvent for both, PAH and phenol as well as for solvent B for HPLC–DAD or LC - MS analysis was of gradient grade quality for liquid chromatography LiChro-Solv (Merck, Darmstadt, Germany). Millipore water as solvent A for LC – MS analysis was freshly produced by a novel Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim, Ce´dex). N – butylamine (n-$BuNH_2$) $C_4H_{11}N$ (99%) was obtained from ABCR (Karlsruhe, Germany).

5.2.2 Photoirradiation of PAH with UVA light

For UVA exposure, a 50 µL sample of each PAH and phenol stock solution (1.0 mg/mL) was placed in UV-transparent quarz glass cuvettes (SUPRASIL, type 101-QS, Hellma, Mühlheim, Germany) and filled up with 450 µL acetonitrile. The total volume of each sample was 500 µL. The samples were closed securely and exposed to a broad band UV lamp (OmniCure, Series 2000, IGB-Tech GmBH, Friedelsheim, Germany) at a distance of 1.0 cm. The samples were irradiated with 5, 50 and 300 J/cm^2. For each irradiated sample, a reference sample (control) was kept. Color

change (from colorless to brown) for the irradiated solutions could be noticed (figure 4).

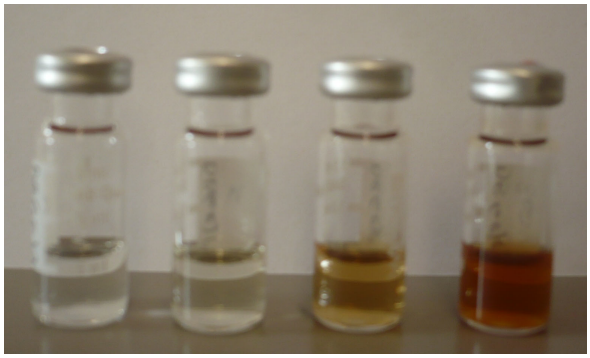


Figure 4: The PAH solution became darker with increasing light dose

The levels of possibly generated intermediates were determined by HPLC peak area compared to the reference sample (control), which can be converted into concentrations, based on the method of internal standard calibration.

5.2.3 UVA irradiation of black tattoo ink

Tattoo inks are commercially available. We selected ink “IC” from 19 purchased black tattoo suspensions for UVA radiation experiments. 2 mL of ink was filled in an UV-transparent cuvette and irradiated with 5, 50, and 300 J/cm^2 UVA light. A reference sample (control, without irradiation) was kept. 500 µL of each irradiation step were subsequently extracted according to our established extraction procedure in a mixture of benzene/acetone and analyzed by HPLC – DAD and HPLC – MS monitoring.

5.2.4 Chemical analysis of epoxide formation

After UVA radiation of single PAH and phenol with a light dose of 5, 50 and 300 J/cm^2 as described each sample was collected in HPLC vials. One equivalent of n-

butylamine (n-$BuNH_2$) was added to each irradiated PAH and control (PAH without irradiation) sample, respectively. The samples were mixed for 12 hours at room temperature in a thermomixer at 6 x 100 cm^{-1} (Minishaker, Eppendorf, Germany). In the presence of an epoxide, n-butylamine should be able to open the oxirane ring due its stronger nucleophilic character to form the amine-intermediate by elimination of oxygen (figure 5).

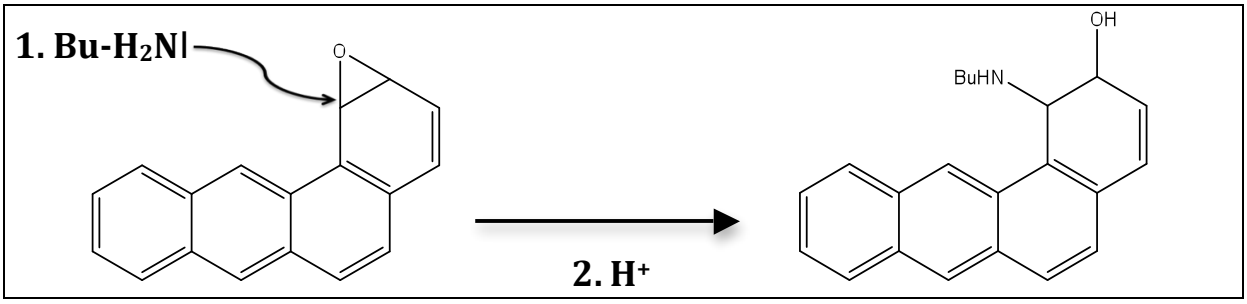


Figure 5: Principle mechanism of epoxide ring opening in the presence of n-$BuNH_2$

The level of potentially photo induced epoxide formation was determined by HPLC peak area compared to a reference sample (irradiated PAH sample without n-butylamine).

5.2.5 Formation of oxygenated intermediates initiated by photo – oxidation of PAH in the presence of a free radical scavenger

Experiments for selected PAH were conducted in the presence and absence of NaN_3 or nitrogen atmosphere. The concentration of NaN_3 was 50 mM. Irradiation under oxygen-free conditions (nitrogen atmosphere) was done in parallel.

5.2.6 Chemical Analysis: HPLC – DAD, HPLC - MS

For HPLC – DAD and HPLC - MS analysis, the samples were filtered using a PTFE filter (Chromafil, O-20/15, organic, pore size 0.2 mm; Machery-Nagel, Düren, Germany), respectively. A 1.0 µL sample was analyzed using a model 1100 HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column for nanoscalic environmental analysis (Phenomenex Environsep PP, particle size 3 µm, 125 x 2.00 mm, Aschaffenburg, Germany) with DAD and MSD (Agilent 6100 Series Single Quadrupol and G1978B Mulltimode Source). The data were analyzed using a HPLC-3D ChemStation Rev. B.04.02. Gradient elution was done with water (0.0059% w/w trifluoroacetic acid) (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.3 mL/min. A gradient profile with the following proportions of solvent B was applied [*t* [min]; % B]: (0, 40), (2, 40), (27, 98), (35, 98). The chromatograms were monitored at 220 nm.

The concentration of PAH in the solutions was determined by the method of internal standard. For each compound (i), the calibration factor (CF_i) was determined in a calibration run (single level calibration). The respective concentration of the standard was chosen to be in the range of the concentration of the tattoo pigment.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K the mass of compound i in the solution k and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

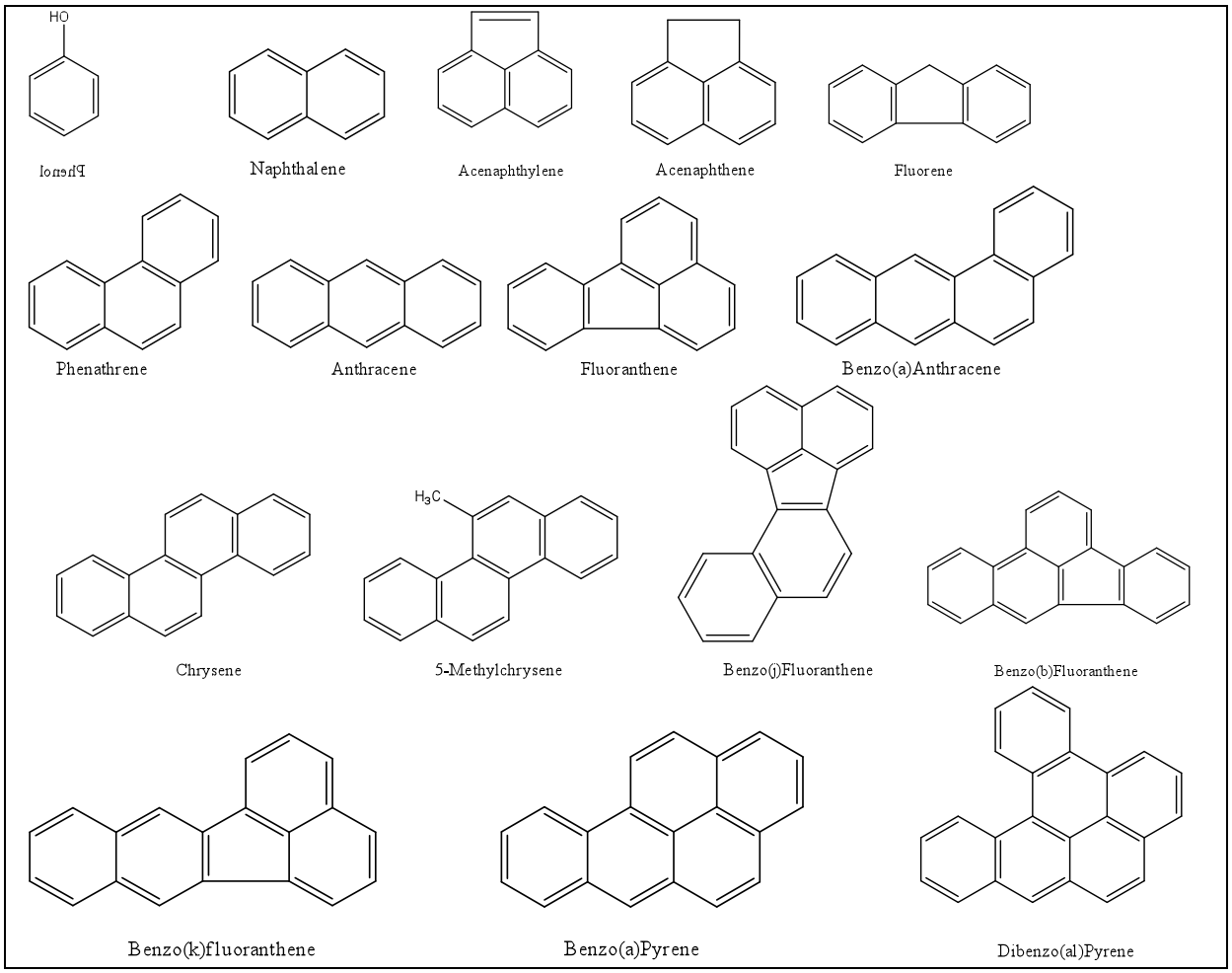
5.2.7 Cell experiments

Primary human dermal keratinocytes (NHEK) were purchased from Cascade Biologics (Invitrogen GmbH, Karlsruhe, Germany). NHEK were propagated in complete Epilife media (Cascade Biologics) in a humidified atmosphere containing 5% CO2 at 37°C. Sub-confluent cells were washed with PBS and harvested using a treatment with 0.05%trypsin／0.02%EDTA in PBS for 10 min; 1 x 10^4 cells／well of a 96-mircotitreplate were used for the phototoxicity experiments (n=3). NHEK were used between passages 3–4. Single PAH were diluted 1:1000 in Epilife media for a final concentration of 100nM. Incubation time of was 300 min. Thereafter, the cells were washed; fresh media was added prior illumination with 0 or 300 J／cm^2 (UVA 700; Waldmann, Schwenningen, Germany). To measure cell viability, MTT assay was performed 24 h and 48 h after irradiation. The OD (optical density) of cells without irradiation and without incubation of PAH (medium only) was normalised to 100%. Staining was performed according to Mosmann et al [30]. Read out of OD was done using an ELISA reader (precision microplate reader, E max, Molecular Devices).

5.3 Results and Discussion

5.3.1 UVA photoirradiation of 20 PAH and phenol

Irradiation of 20 single PAH and phenol was performed with 5, 50 and 300 J/cm^2 of UVA light. The 20 PAH included naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, 5-methylchrysene, benz[j]fluoranthene benz[b]fluoranthene, benz[k]fluoranthene, benz[a]pyrene, dibenz[a,l]pyrene dibenz[a,h]anthracene, benz[g,h,i]perylene, indeno[1,2,3-c,d]pyrene and dibenz[a,e]pyrene (figure 6).



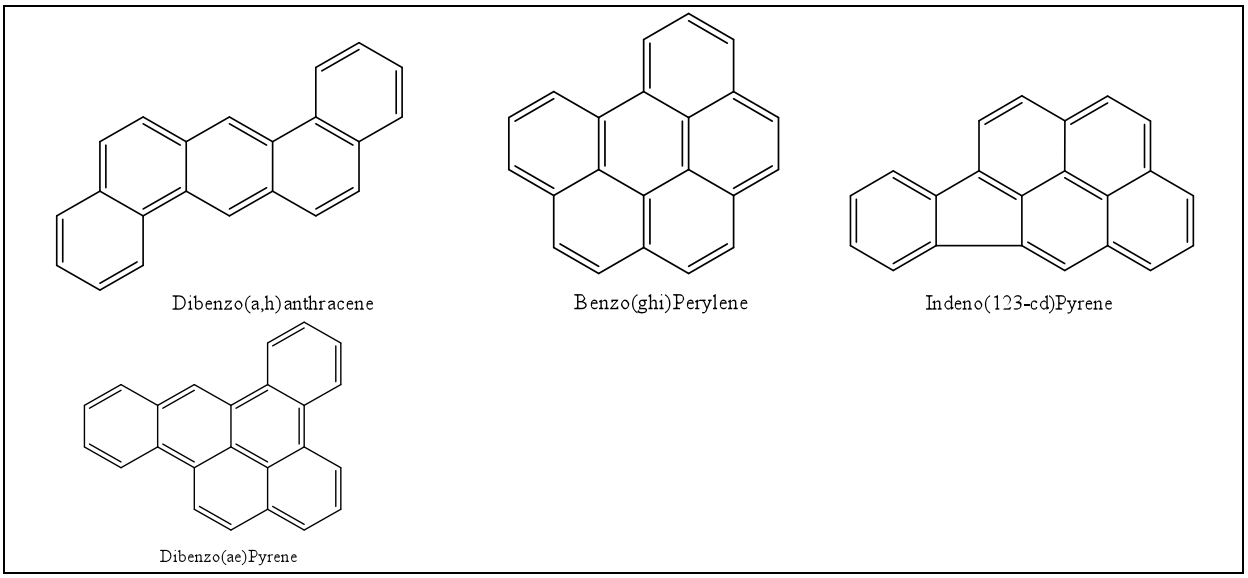


Figure 6: All investigated 21 hazardous environmental pollutants (phenol and 20 PAH): some of the PAH are classified as human carcinogens

The level of PAH decomposition following irradiation was measured by quantitative calibration of the amount of PAH based on the HPLC peak area. The results are shown in table 1. Comparison of irradiated PAH with those from control (single PAH in acetonitrile solution, without light irradiation) indicates that some investigated PAH may generate photo - intermediates. Among these PAH, anthracene and dibenz[a,l]pyrene decompose significantly (70 - 94 %) after a light dose of already 5 J/cm^2 (3.3 s irradiation time, 1.5 W/cm^2). Acenaphthene, phenanthrene, fluoranthene benz[a]anthracene, chrysene, 5-methylchrysene, benz[b]fluoranthene, benz[k]fluoranthene, benz[a]pyrene, dibenz[a,h]anthracene and benz[g,h,i]perylene produced highest level of decomposed species after 300 J/cm^2 UVA, while for naphthalene, acenaphthylene, fluorene, pyrene, benz[j]fluoranthene, indeno[1,2,3-c,d]pyrene, dibenz[a,l]pyrene, and phenol no decomposition reaction could be detected by HPLC at any irradiation time. A typical HPLC chromatogram for the decomposition of a PAH, e.g. dibenz[a,l]pyrene, is shown in figure 7.

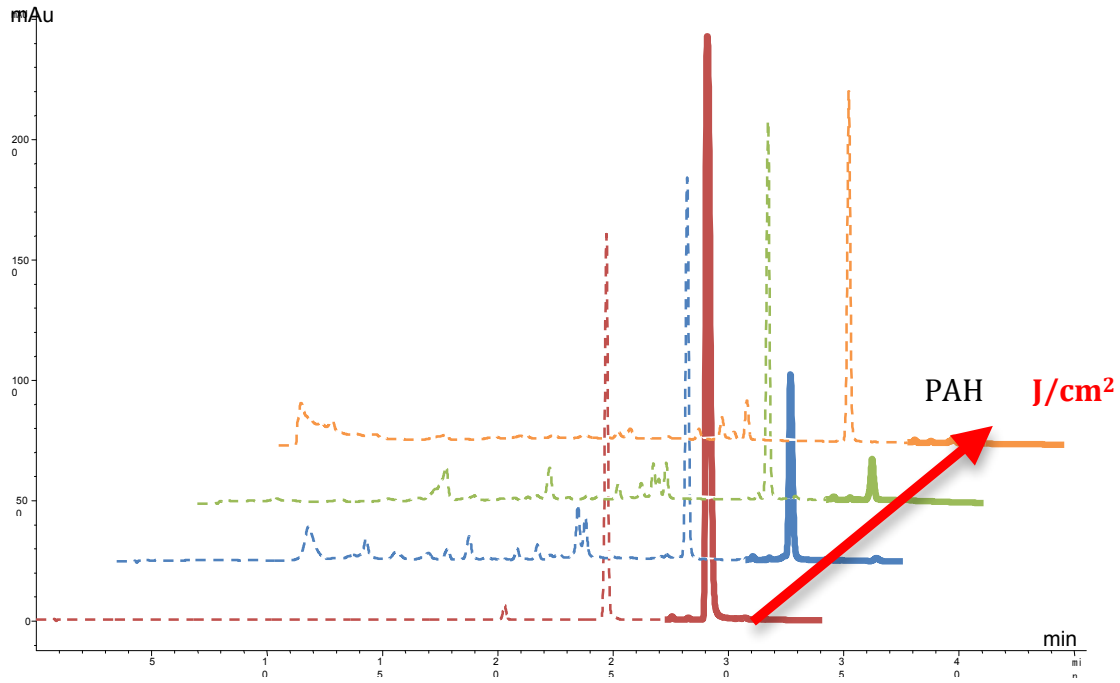


Figure 7: HPLC chromatograms of PAH dibenz[a,l]pyrene (red) after 5 (blue) , 50 (green) and 300 (orange) J/cm^2 UVA light irradiation. With increasing light dose, the PAH peak decreases until complete decomposition

HPLC peak area of dibenz[a,l]pyrene decreases with increasing irradiation time until complete decomposition.

The concentration of phenol, naphthalene, acenaphthylene, fluorene, pyrene, benz[j]flouranthene, indeno[1,2,3-c,d]pyrene and dibenz[a,e]pyrene remain constant, actually after 300 J/cm^2 UVA light (table 1).

UVA	[J/cm^2]	[J/cm^2]	[J/cm^2]	[J/cm^2]
	0	**5**	**50**	**300**
PAH	**Decomposition [%]**			
1 Phenol	0	0	0	0
2 Naphthalene	0	0	0	0
3 Acenaphthylene	0	0	0	0
4 Acenaphthene	0	0	7	99.9
5 Fluorene	0	0	0	0
6 Phenanthrene	0	0	0	43
7 Anthracene	0	94	94	99.9
8 Fluoranthene	0	0	0	20
9 Pyrene	0	0	0	0
10 Benz[a]Anthracene	0	0	9	99.9
11 Chrysene	0	0	0	90
12 5-Methylchrysene	0	0	0	99.9
13 Benz[j]Fluoranthene	0	0	0	0
14 Benz[b]Fluoranthene	0	0	0	61
15 Benz[k]Fluoranthene	0	0	4	11
16 Benz[a]Pyrene	0	2	18	99.9
17 Dibenz[a,l]Pyrene	0	70	99.9	99.9
18 Dibenz[a,h]Pyrene	0	0	28	94
19 Benz[g,h,i]Perylene	0	0	4	99.9
20 Indeno[1,2,3-c,d]Pyrene	0	0	0	0
21 Dibenz[a,e]Pyrene	0	0	0	0

Table 1: amount [%] of decomposition of phenol (1) and PAH (2-21) after UVA light dose of 0, 5, 50, 300 J/cm^2. No decomposition reaction for phenol (1) and PAH (2), (3), (5), (9), (13), (20) and (21) at any irradiation time.

5.3.2 Decomposition of PAH in black tattoo inks after UVA irradiation

A commercially available black tattoo ink was selected from 19 purchased ink suspensions, which contained high amounts of PAH and phenol [3]. Black ink “IC” was irradiated as described with an UVA light dose of 5, 50, and 300 J/cm^2. A reference sample (control) was kept. After extraction of PAH from the ink samples according to our established extraction procedure in benzene/acetone, the extracts were analyzed by HPLC – DAD and HPLC – MS detection. With increasing light dose, the amount of single PAH decreases significantly, e.g. PAH benz[g,h,i]perylene

(- 40 % after 300 J/cm^2) and indeno[1,2,3-c,d]pyrene (-50 % after 300 J/cm^2) (figure 8).

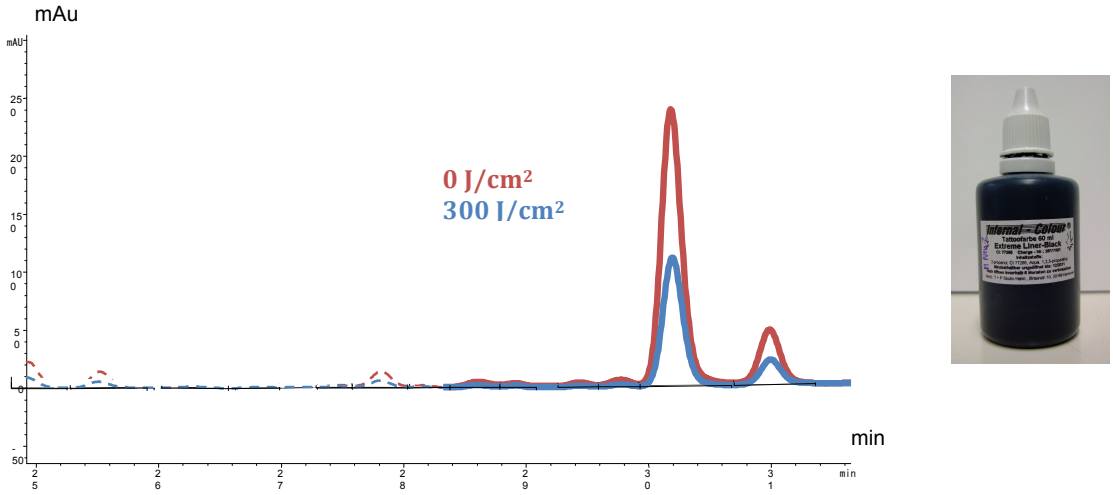


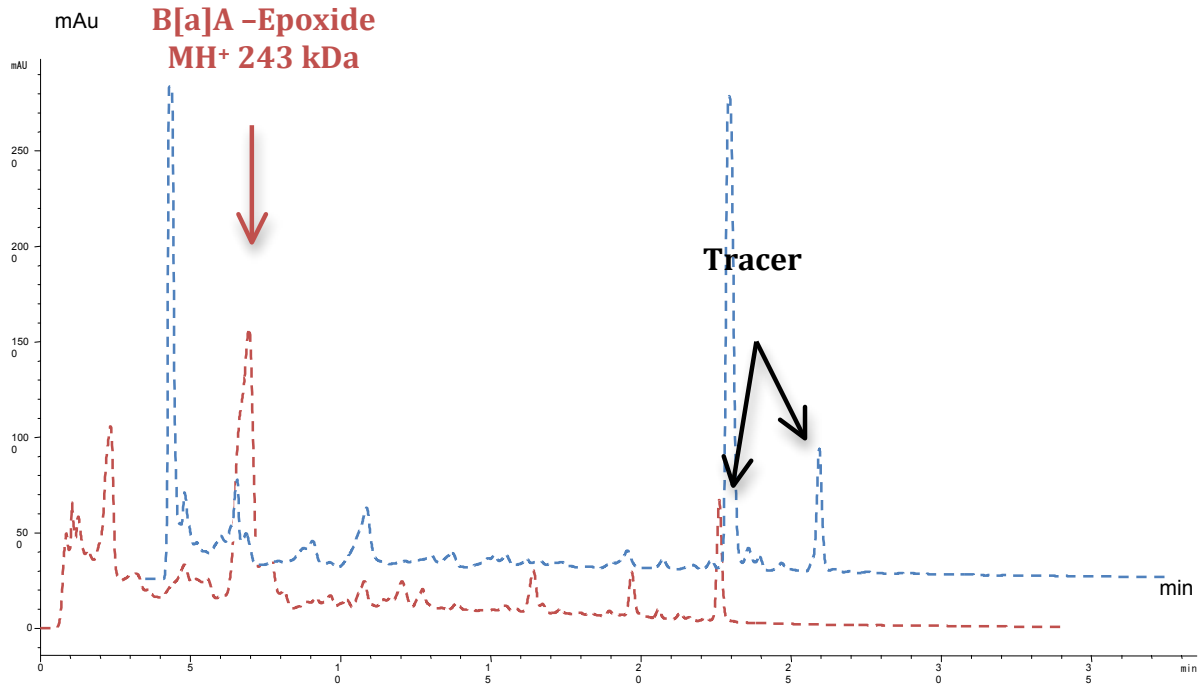
Figure 8: Commercial available black tattoo ink "IC" after UVA irradiation with 300 J/cm^2 (blue) and control (red, without light). HPLC chromatogram of PAH showed decreasing peak area with increasing UVA light irradiation

The total amount of PAH in the ink decreased by about 60 % after a light dose of 300 J/cm^2 in comparison to control ink (sample without irradiation). As it is a well studied photochemical oxidation reaction for PAH to form epoxides [31-34], we subsequently focused on this type of photo – modified product in order to analyze the chemical trigger of PAH photo – decomposition.

The theory of bay region dihydrodiol epoxide mechanism *in vivo* is well accepted by scientists whereby the diol-epoxides are electrophiles capable of binding to DNA. It has been thoroughly studied that B[a]P requires metabolic activation by cytochrome P450 enzymes through covalent binding to DNA (DNA adduct formation) and the active metabolite benz[a]pyrene-7,8-diol-9,10-epoxide (BPDE) represents probably the ultimate carcinogen [35, 36]. Generally, for PAH with relatively planar highly conjugated aromatic structures, metabolic activation is required to exert their mutagenic/carcinogenic effects. In most cases, oxidation of PAH by P450 enzymes is

an initial step in the activation process to produce the polar biochemically reactive electrophilic species capable of interacting with surrounding molecules. Therefore, in an experimental setup we analyzed the photo – induced formation of epoxides of investigated 13 PAH, which decomposed partly or even completely after UVA irradiation in solution, in the presence of one equivalent of n – butylamine. As a result, for all PAH except fluoranthene, epoxide formation could be demonstrated after UVA irradiation. An example is shown in figure 9a.

(a)



(b)

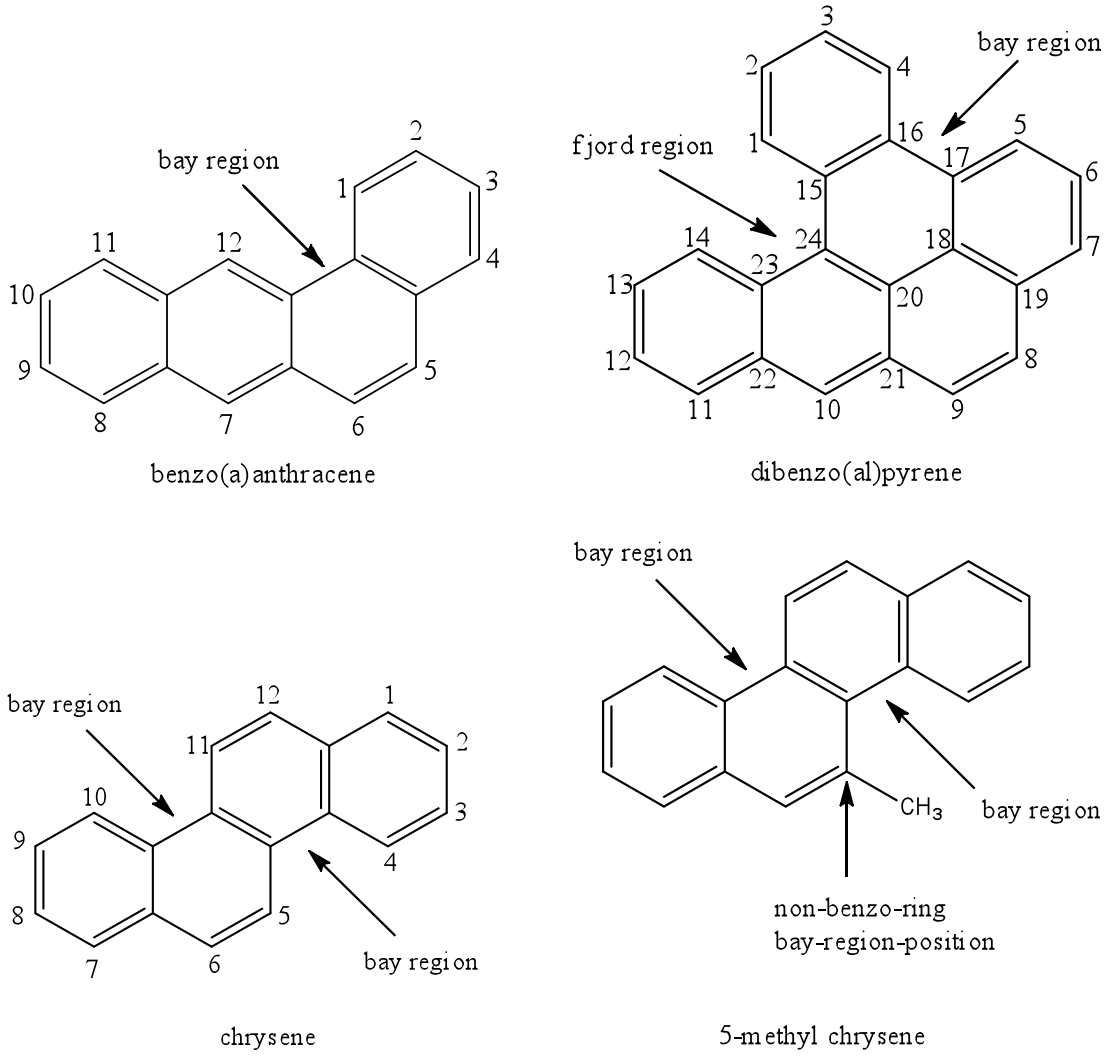


Figure 9: (**a**) HPLC chromatogram of benz[a]anthracene (B[a]A) after 300 J/cm^2 UVA illumination prior (red) and after addition of n – $BuNH_2$ (blue). B[a]A – epoxide could be detected using mass spectroscopy (LC – MS) with MH^+ = 243 kDa after 300 J/cm^2. The peak disappeared after reaction with n-$BuNH_2$. (**b**) Chemical structure and atomic numbering for benz[a|anthracene (bay-region), dibenz[a,l]pyrene (bay-region and fjord-region), chrysene (two bay-regions) and 5-methylchrysene (two bay-regions and methyl-group in non-benzo-ring-bay-region position)

Addition of a primary amine (n-$BuNH_2$) results in nucleophilic substitution and due to high ring strain opening of the oxirane to form the PAH – amine intermediate: as seen for B[a]A, the detected HPLC – peak for the prior PAH – epoxide disappeared in the chromatogram. This effect came to an agreement regarding the overall

molecular weight for B[a]A – epoxide by mass spectroscopic (MS) analysis with ESI positive ionisation (electron spray ionisation).

As shown for many *in vivo* studies, generally, the reactivity of the aromatic ring systems can be fundamentally different. Napthalene for example, the simplest PAH, is far less accessible to epoxidation than benz[a]pyrene, which possess a bay-region comprising an ortho-fused benzene ring: Upon its reaction to the ultimate carcinogen the aromatic system is less influenced, leaving a stabilized, unaffected pyrene system. As a matter of principle, a bay region occurs in a PAH when an angularly fused benzene-ring is present. In case of B[a]A the bay region is between carbon atoms 1 and 12 (figure 9b). Molecular and steric overcrowding in this region leads to distortion of the molecule resulting in larger external C-C-C angles and torsion about the respective C-C bond. Another investigated PAH, dibenz[a,l]pyrene, additionally contains a fjord-region and is also considerably distorted as a result of atomic overcrowding: the hydrogen atoms on C1 and C14 would be too close if the molecule were planar. The overcrowding across the fjord region is also relieved by increases in the external C-C-C bond angles and by extensive torsion. Chrysene and its substituted derivative 5-methylchrysene are an example for PAH containing two bay regions. Thus, all these non-planar structures either with bay-region(s) or/and fjord-region, are highly reactive at that positions and therefore the main target for electrophilic attack, e.g. by ROS, since they rapidly form dihydrodiol epoxides through metabolic activation *in vivo* [34, 37-39].

5.3.3 Chemical analysis of photo – modified PAH products, in particular epoxides, after UVA light irradiation, using n-BuNH_2

UVA radiation showed, that thirteen of 21 pollutants decomposed partly or even completely with increasing light dose, namely anthracene, acenaphthene,

fluoranthene, phenanthrene, benz[a]anthracene, chrysene, 5-methylchrysene, benz[b]fluoranthene, benz[k]fluoranthene, benz[a]pyrene, dibenz[a,l]pyrene, dibenz[a,h]anthracene, and benz[g,h,i]perylene. Since the theory of bay region dihydrodiol epoxide formation is widely accepted as the dominant mechanism of chemical carcinogenesis of PAH, in an experimental setup we investigated oxygenated decomposition products, in particular PAH – epoxide intermediates. Therefore, one equivalent of n – butylamine was added directly after irradiation of PAH solution and mixed over night. Due to high ring strain and reactivity of epoxides, the primarily amine should form the amine – PAH – derivative by a nucleophilic substitution mechanism. This reaction could be detected qualitatively for all investigated 13 PAH after UVA irradiation, except for fluoranthene by HPLC – DAD monitoring. The respective molecular weight of the epoxide – PAH derivative was detected by mass spectroscopy. This investigation leads to the assumption that reactive oxygen species (ROS), possibly singlet oxygen $^1O_2$, are involved in the oxidation process as mechanistically described in figure 3: light absorption can promote PAH to their higher electronic states. The excited state energy can be released by emitting light or heat or can be transferred to, amongst others, molecular oxygen, to generate ROS, reactive intermediates or photo – modified PAH derivatives. To prove this theory, the type of oxygen species involved must be determined.

5.3.4 Photoinduced formation of modified PAH derivatives in the presence of nitrogen (N_2) or singlet oxygen scavenger NaN_3

The involvement of singlet oxygen during UVA irradiation of PAH (300 J/cm^2) was investigated. The used UVA lamp shows an emission maximum in the spectral range from 350 to 380 nm, in which PAH show a sufficient absorption. Spectral overlap of

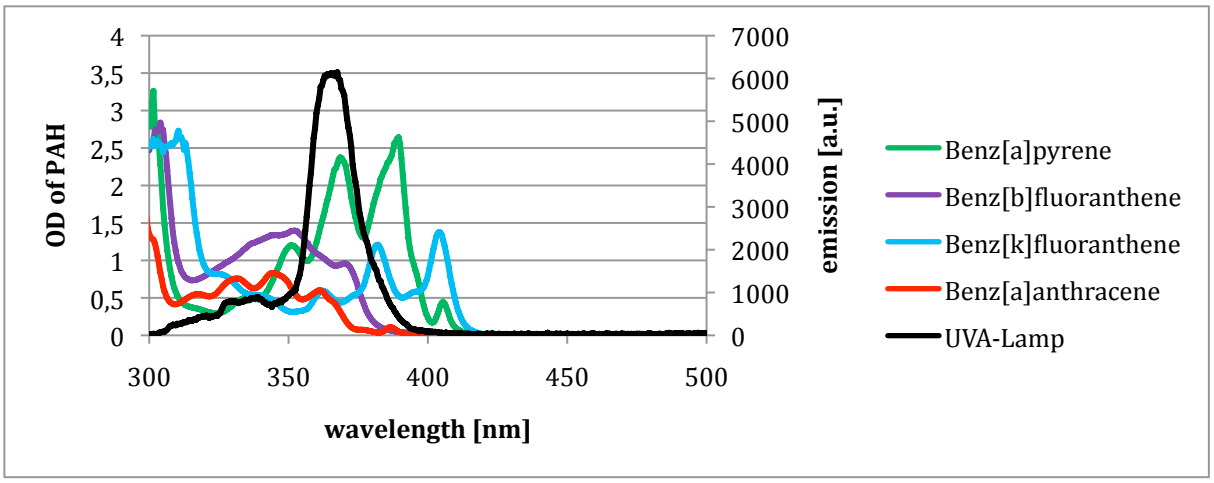
UVA lamp and selected four PAH benz[a]anthracene (B[a]A, red), benz[a]pyrene (B[a]P, green), benz[b]fluoranthene (B[b]Fl, violet) and benz[k]fluoranthene (B[k]Fl, blue) at concentrations of 50 µM are shown in figure 10a.

Sodium azide NaN_3, a common scavenger for singlet oxygen $^1O_2$, was employed for this study. In parallel, the experiments were also done under nitrogen conditions, for which reason the UV-transparent cuvettes were rinsed before and while UVA irradiation to create an oxygen-free atmosphere. The results are shown in figure 10, the values are given in table 2.

PAH	ΦΔ	decomposition [%] after 300 J/cm^2	decomposition [%] after 300 J/cm^2; + N_2	decomposition [%] after 300 J/cm^2; + NaN_3
B[a]A	0.85	99.9	50.0	85.0
B[a]P	0.66	99.9	0.0	15.0
B[b]Fl	0.45	61.0	0.0	2.0
B[k]Fl	0.27	11.0	0.0	0.0

Table 2: Singlet oxygen quantum efficiency Φ_Δ for selected PAH in comparison with the percentage amount of UVA decomposition after 300 J/cm^2 UVA irradiation and in presence of N_2 or NaN_3

(a)



(b)

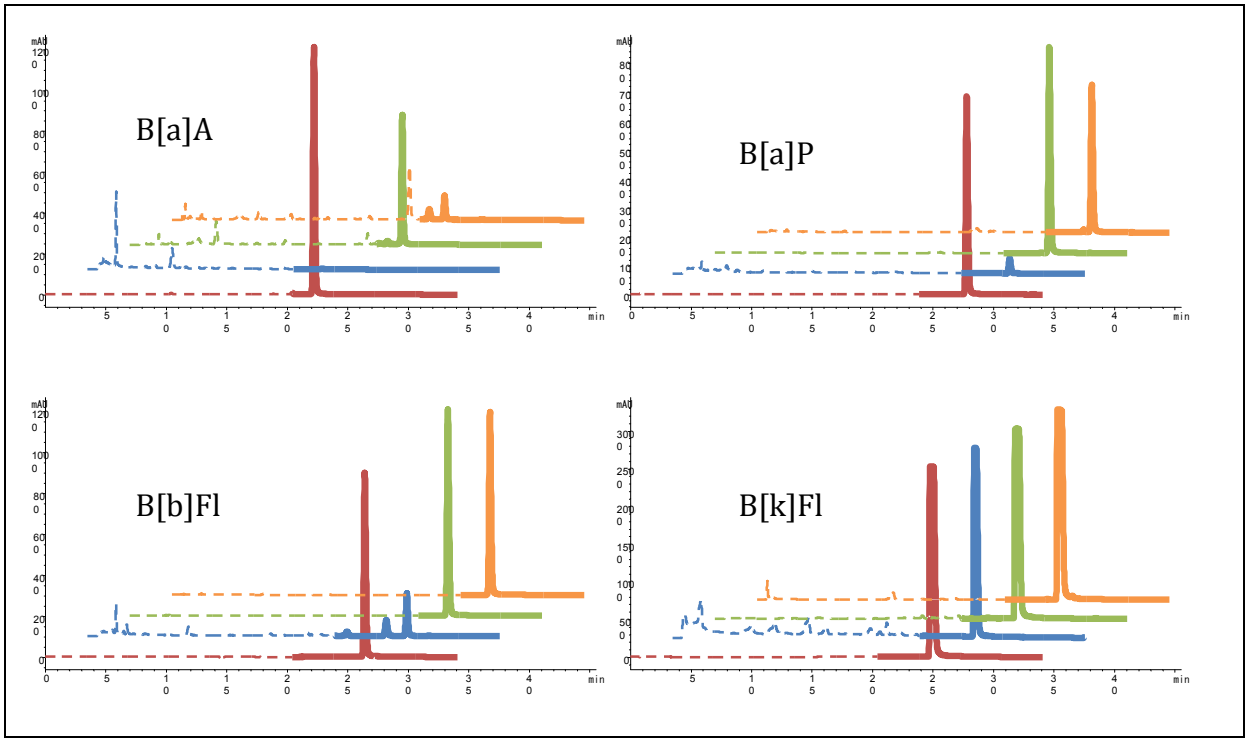


Figure 10: (**a**) Spectral overlap of UVA lamp "OmniCure" and PAH benz[a]anthracene, benz[a]pyrene, benzo[b]fluoranthene and benzo[k]fluoranthene; OD: optical density (**b**) typically HPLC chromatograms of PAH benzo[a]anthracene (top left), benzo[a]pyrene (top right), benzo[b]fluoranthene (bottom left) and benzo[k]fluoranthene (bottom right). Without irradiation (control, red), after 300 J/cm^2 UVA light (blue) and in presence of N_2 (green) or NaN_3 (orange); x-axis: absorption [mAu], y-axis: retention time [min|

B[a]A decomposed nearly completely in the presence of oxygen after UVA exposure. Due to a high Φ_Δ (0.85), under nitrogen atmosphere, even a minimal residual amount of oxygen causes degradation of the PAH molecule. In case of a scavenger (NaN_3) generated singlet oxygen molecules by B[a]A could not be sufficient quenched. Furthermore in case of B[a]P (Φ_Δ = 0.66) and B[b]Fl (Φ_Δ = 0.45), irradiation in the presence of oxygen yielded decomposition of PAH of 99.9% for B[a]P and 61.0% for B[b]Fl. Under oxygen-free working conditions (nitrogen-atmosphere), singlet oxygen generation did not occur for both PAH. Regarding addition of NaN_3-scavenger,

produced $^1O_2$ can be nearly completely quenched. For B[k]Fl (Φ_Δ = 0.27) only less degradation of PAH was detected. Due to moderate $\Phi\Delta$, singlet oxygen was not produced under oxygen-free working conditions. In case of NaN_3, the produced $^1O_2$ could be efficiently quenched. The higher the singlet oxygen quantum yield of the PAH molecule, the less the quenching efficacy of scavenger. Again the higher the singlet oxygen quantum yield of the PAH molecule, the higher the decomposition of these molecules occur even at very low oxygen concentrations.

The results finally clearly indicate the involvement of singlet oxygen in the photo – mediated decomposition process. In terms of carcinogenic activity exhibited by a PAH the competition between carcinogenesis and detoxification depends on the pathway, in which the PAH participates.

5.3.5 PAH incubated with NHEK without and with UVA irradiation

Since ROS such as singlet oxygen ($^1O_2$) and other reactive intermediates has been reported to damage cellular constituents, an *in vitro* experimental setup with human keratinocytes (NHEK), incubated with selected PAH after UVA light exposure was carried out. UVA irradiation of NHEK incubated with PAH was conducted with a light dose of 0 and 300 J/cm^2. Cell viability was measured by MTT assay 24 or 48 h after treatment. The results are shown in figure 11. The experiment was performed in triplicate.

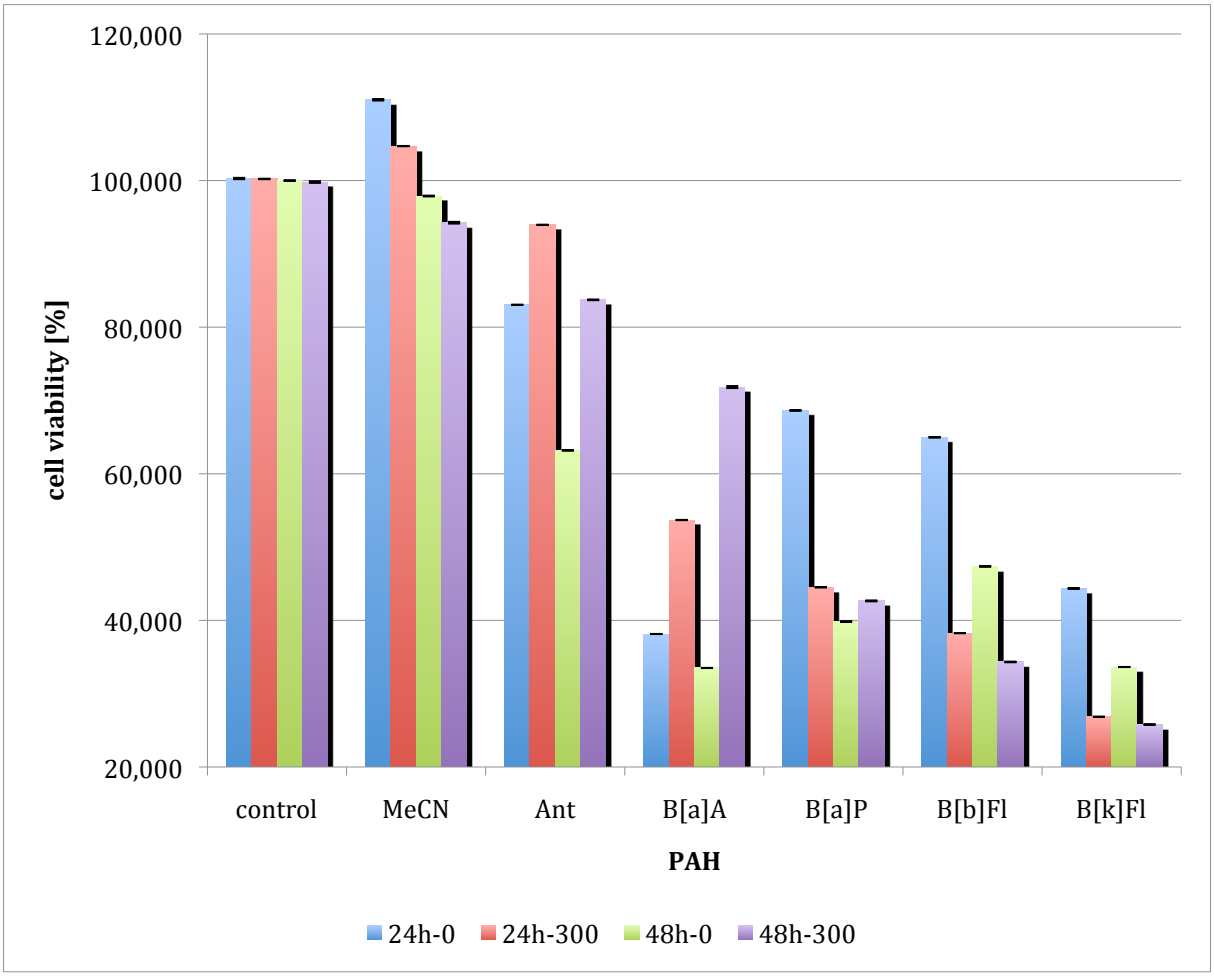


Figure 11: MTT assay: PAH anthracene (Ant), B[a]A, B[a]P, B[b]Fl and B[k]Fl incubated with NHEK for 24 h / 48 h without irradiation and after 300 J/cm^2 UVA light

For the control sample (NHEK with media only) cell viability was calculated at 100%. There is no significant influence on the solvent MeCN at any incubation or irradiation time. For investigated PAH without irradiation, incubation on NHEK for 24 or 48 h showed already cytotoxic effect with decreasing cell viabilty (< 70 – 50 %). After UVA irradiation, PAH B[b]Fl and B[k]Fl turned out to be even more toxic. Anthracene (Ant) and B[a]A showed higher cell viability after illumination than before and cell vitality for B[a]P (300 J/cm^2) remained constant.

The difference in cytotoxicity of PAH without and after UVA irradiation could be explained due to the possibly formation of different photooxidation products. It has

been reported that upon light irradiation, amongst others, anthracene, benz[a]anthracene and benz[a]pyrene react with oxygen to form endoperoxides as intermediates and the corresponding diones or quinones as the stable end photoproducts [25, 40, 41]. In terms of carcinogenesis, photooxidation of PAH to PAH – diones is considered a detoxification pathway of PAH [42]. This phenomenon would explain the higher cell viability of Ant and B[a]A after UVA exposure. Also B[a]P tend to detoxify after irradiation.

These results show that PAH present in cells generate ROS or reactive intermediates, especially epoxides and singlet oxygen $^1O_2$. This may indicate that such ROS or reactive intermediates possibly appear also in real tattoos when the black ink in skin is exposed to UV of solar radiation.

5.4 Conclusion

In present investigation we demonstrate that upon UVA irradiation, thirteen of 21 environmental pollutants, previously quantified in black tattoo suspensions, generate photo – mediated decomposition products. After chemical reaction of respective irradiated PAH samples with n-butylamine, formation of epoxides was detected in all cases except fluoranthene. Oxygen-free working conditions or the addition of specific quenchers showed that the oxidation of PAH is clearly mediated by singlet oxygen generation. Under *in vitro* condition, the decreasing cell viability indicates already cytotoxicity for the parent PAH without irradiation. The toxicity of PAH after UVA irradiation depended on the photoproduct: detoxification could be clearly demonstrated for B[a]A and B[a]P. Further studies on the analysis of the exact oxygenated species should be done in the near future.

In the process of tattooing, PAH are injected into human skin together along with the black ink. UVA exposure may form alteration of PAH and then damage cellular tissue, in particular by one important pathway that proceeds through a three-step sequence resulting in the formation of diol epoxides. This may react with DNA to produce adducts that might lead to mutations and possibly initiate a carcinogenic process. Medical literature contains many adverse skin reactions at the site of the tattoo [43], but finally the intrinsic chemical trigger is unknown. To ensure safety for people with black tattoos exposed to sunlight or any other artificial light source, risk assessment should be an essential part to protect human health.

For those people, who are going to receive a tattoo, we strongly recommend that black tattoo inks should be manufactured yielding PAH-free Carbon Black, e.g. by combustion of pure acteylene-gas without PAH. This could help to avoid such PAH related risks for the tattooed skin.

5.5 References

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6. Polycyclic aromatic hydrocarbons (PAH) and Carbon Black nanoparticles present in black tattoo inks as potential generators of deleterious decomposition products after laser tattoo removal*

Abstract

Laser tattoo removal is one of the most commonly used indications for medical laser. Tattoo inks contain a multitude of potentially immunogenic ingredients, e.g. PAH, that can be released or modified by laser treatment. Since the biomechanics of laser removal of tattoo particles at the cellular level are incompletely understood, we studied the interaction of black tattoo ink and intense laser pulses (Q-switched Nd:YAG) with regard to possible chemical alterations of PAH molecules and Carbon Black nanoparticles.

We could demonstrate, that single PAH in solution may not be affected by laser treatment, since PAH do not absorb laser light at 532 nm. In the presence of Carbon Black nanoparticles, nearly all investigated 20 PAH decomposed in the range of 15.2% up to 99.9%. With special concern to Carbon Black, we were able to detect a 90% disruption of ink nanoparticles by Trans-Electron-Microscopy (TEM) and Dynamic-Light-Scattering (DLS).

We conclude, that tattoo pigments, in particular Carbon Black and PAH, may decompose by laser therapy and may trigger formation of ROS and possibly deleterious modified photo-derivatives of hazardous parent PAH.

*Results of this chapter are part of a manuscript: Lehner K, Santarelli F, Vasold R, Sidoroff A, König B, Landthaler M, Bäumler W, "Effects of laser and UV radiation on black tattoo inks", Lasers Surg Med, 2012, in preparation

6.1 Introduction

Tattooing is a worldwide phenomenon that exists since the early beginnings of modern civilization. As part of human culture, this art of body decoration has reached significantly popularity, especially among young people [1-5]. In the past, inorganic metal salts containing chromium, cobalt, manganese, or mercury has been used in tattoo colorants. Today, tattoo inks are mixtures of many components including precursors and by-products of pigment synthesis as well as various diluents for pigment suspension, whereas the actual composition may vary for different ink products.

Millions of people in the western world have at least one tattoo [2] and 60 % predominately contain black color [1, 6]. The inks used for black tattoos are manufactured for other intended uses like printing, painting cars and coloring consumer products. Up to now, regulation and legislation for listing ingredients on the labeling of tattoo inks are rare or incomplete. Black tattoo suspensions mainly consist of Carbon Black (CB), which is manufactured by incomplete combustion [7].

CB is already listed as possible carcinogenic to humans (group 2 B) [8, 9]. Besides, our chemically analysis including GC – MS measurement yielded remarkable amounts of auxiliary ingredients such as dibutyl-phthalate and various solvent admixtures in a variety of commercial available black tattoo suspensions [10]. Due to our established extraction procedure and HPLC – DAD monitoring, furthermore, we were also able to detect polycyclic aromatic hydrocarbons (PAH) in the black inks [11]. The concentration of PAH in the inks ranged from 0.14 to 201 µg per gram dry ink. Due to their hydrophobic structure, PAH may be adsorbed onto the large conjugated aromatic carbon black skeleton as a result of strong $\pi - \pi -$ interactions.

PAH are well studied and some of them are known to be carcinogenic, mutagenic or teratogenic [12, 13].

During the tattooing procedure, such black ink suspensions are placed in the dermis. Some of the deposited Carbon Black – possibly together with PAH - may be recognized by macrophages as foreign bodies and carried from the site of the tattoo via the lymphatic system. The black ink particles, which remain in the dermis, represent the black color of a tattoo. Frequently, pigments in a tattoo are aggregated into crystals by the assistance of macrophages showing a size that ranges from about 0.1 to 10 µm [14]. In addition, ink particles were located within dermal fibroblasts [15], which could be also demonstrated for PAH in the present study in an experimental *in vitro* setup. *In vitro* and *in vivo* studies with combustion-derived nanoparticles (CDNP), such as Carbon Black, reported associations between these ultrafine particles and adverse health implications, depending on type of personal exposure and mechanisms of cellular uptake of nanoparticles [16, 17].

As the incidence of tattooing continues to increase, so does the demand of people for tattoo removal. Due to various reasons, often aesthetic-, social- or employment-related, many tattooed individuals undergo a therapy of tattoo removal [5, 18]. In order to destroy the pigment selectively while minimizing the risk of side-effects to the skin, e.g. permanent pigmentary alteration or scarring, the use of Q-switched lasers (i.e. alexandrite, ruby and 532 nm/1064 nm Nd:YAG) is recommended by experts and appeared to be the “gold standard” [14, 19-24]. “Q-switched” (“Quality-switched”) laser treatment requires high intensities and ultra-short pulse duration (nanoseconds). Histopathologic and electron- microscopic investigations of laser treated skin showed, that after being absorbed in the pigment molecule, the energy of the laser light is converted to heat or triggers structural changes inside the molecule. During ultra-short heating, the pigments will reach very high temperatures

of several hundred degrees Celsius and may then, as proved by histology, lead to disruption of the pigment particle. Especially reduction in both pigment size and density clearly indicates tattoo color fading [14, 15, 25-27].

We already found that PAH are still attached to black ink particles, even years after tattooing (*chapter 3/4*). Therefore, we aimed to analyze possible chemical alterations of PAH molecules that might be triggered by the interaction of intense laser pulses and CB particles. We exposed PAH in solution, PAH/CB suspension, and commercial black tattoo ink to laser pulses of a commercial Q-switched Nd:YAG laser at 532 nm (frequency-doubled). Disruption of CB particle size was detected by Trans-Electron-Microscopy (TEM) and Dynamic-Light-Scattering (DLS). Possible photoproducts were investigated by using HPLC – DAD and mass spectroscopy.

6.2 Materials and Methods

6.2.1 Chemicals and Solvents

As reference, 20 well known PAH (purity ~ 99 %) were obtained from Sigma Aldrich (Steinheim, Germany): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benz[b]fluoranthene,benz[k]fluoranthene, benz[a]pyrene, dibenz[a,h]anthracene, benz[g,h,i]perylene, indeno[1,2,3-c,d]pyrene, dibenz[a,e]pyrene, dibenz[a,l]pyrene, 5-methylchrysene and benz[j]fluoranthene. Phenol (purity > 99%) was obtained from Riedel-de Haen. For the internal standard (ISTD), 9,10-diphenylanthracene (purity > 99 %) was obtained from Riedel-de Haen. Acetonitrile as solvent for both, PAH and phenol as well as for solvent B for HPLC–DAD or LC - MS analysis was of gradient grade quality for liquid chromatography LiChro-Solv (Merck, Darmstadt, Germany). Millipore water as solvent A for LC – MS analysis was freshly produced by a novel Milli-Q Advantage A10 system (TOC 5 ppb, Millipore, Molsheim, Ce´dex). Carbon Black was purchased from Sigma Aldrich (Steinheim, Germany). N – butylamine (n-$BuNH_2$) $C_4H_{11}N$ (99%) was obtained from ABCR (Karlsruhe, Germany).

6.2.2 Sample preparation

Each PAH and phenol was diluted in MeCN to obtain a 1.0 mg/mL single PAH stock solution.

Single PAH stock solution: 1 mL of each stock solution was filled in a laser glass test tube with magnetic stir bar. The samples were closed securely using special fabricated glass caps (glassblowing, University of Regensburg, Germany) and placed on a magnetic stirrer.

Carbon Black suspension: 300 mg of pure Carbon Black was suspended with 10 mL acetonitrile/isopropanol (10:1) in a round-bottom flask to obtain a 30 mg/mL CB stock solution. 0.5 mL of CB suspension was combined with 0.5 mL of single PAH stock solution, filled in a laser glass test tube, closed and stirred, respectively. The total volume of each sample was 1.0 mL.

Black tattoo ink: Previously investigated commercial ink (Infernal Color) was selected, which contained high amounts of PAH [11]. 1 mL of ink was filled in a laser glass test tube, closed and placed on a magnetic stirrer.

Laser system: The samples were exposed to a Nd:YAG laser (Spectron Laser Systems, Germany). Irradiation was done at a wavelength of 532 nm, repetition rate of 10 Hz, radiant exposure of 3 J/cm^2 and pulse duration of 6 ns. The total exposure time was 10 min while stirring. The principle construction is shown in figure 1. For each laser sample, a reference (control) was kept.

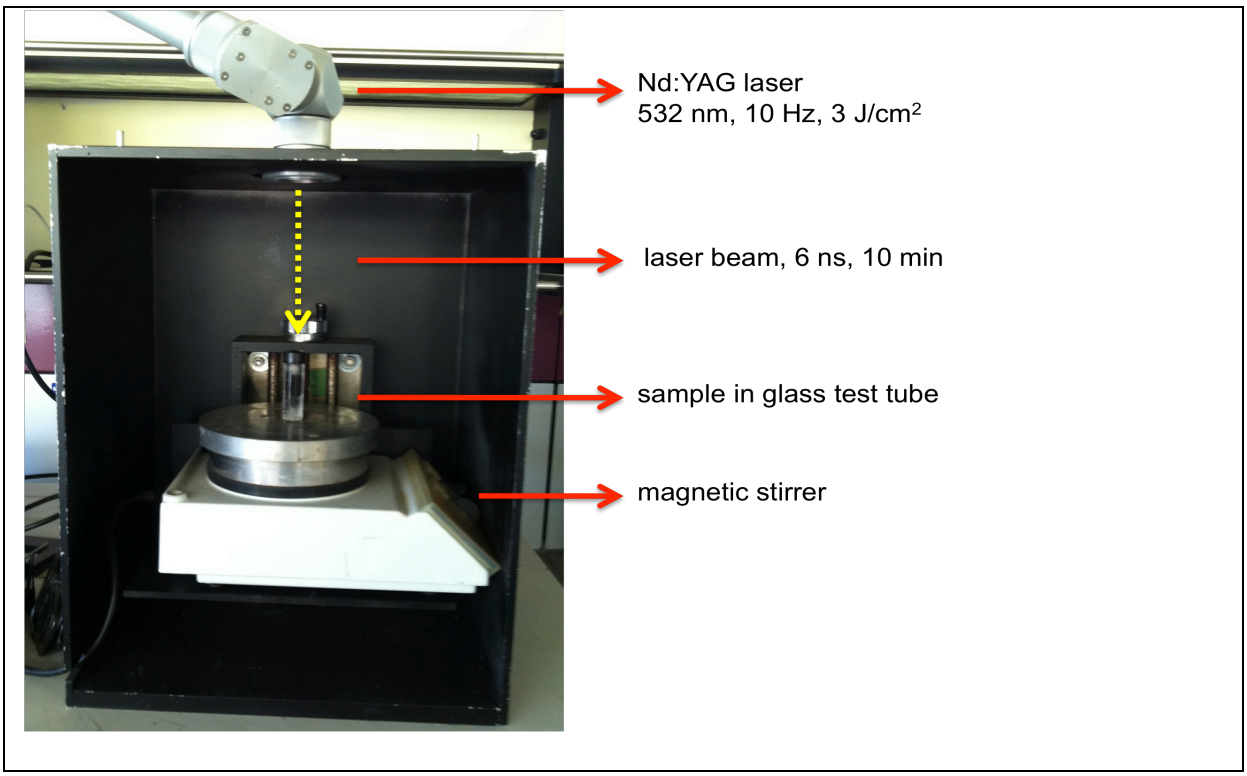


Figure 1: Principle laboratory laser construction using a Nd-YAG laser system (532/1064 nm, 10 Hz, 3 J/cm^2)

Epoxide formation: One equivalent of n-butylamine was added to each irradiated sample and control, respectively. The samples were mixed for 12 hours at room temperature in a thermomixer at 6 x 100 cm^{-1} (Minishaker, Eppendorf, Germany). In the presence of an epoxide, n-butylamine should be able to open the oxirane ring due its stronger nucleophilic character to form the amine-intermediate.

6.2.3 Energy-dispersive X-ray spectroscopy (EDX), Transmission electron microcopy (TEM) and Dynamic Light Scattering (DLS)

EDX analysis: In principle, EDX analysis is an analytical technique, which is used for the elemental analysis (atomic structure) or chemical characterization of a specimen. Selected black tattoo ink (Infernal Color) was lyophilized and EDX spectra were recorded by EDX-laboratory (Institute of Dentistry, University of Regensburg, Germany).

TEM analysis: In principle, TEM analysis yields information on the internal structure of materials. TEM analysis of black tattoo ink sample before and after laser treatment was recorded at “Zentrales Labor für Elektronenmikroskopie“ (Institute of Pathology, University of Regensburg, Germany).

DLS analysis: Dynamic Light Scattering, also referred to as Photon Correlation Spectroscopy (PCS), is a non-invasive technique, used to determine the size distribution profile of molecules and particles typically in the submicron region. The size distribution of Carbon Black in acetonitrile was recorded before and after laser treatment using a Zetasizer Nano ZS (Malvern Instruments Ltd., Zetasizer Ver. 6.01).

6.2.4 Cell experiments

Normal human dermal fibroblasts were obtained by standard culture explants from excised skin (Department of Dermatology, Regensburg University Hospital). Cells

were grown at 37°C in culture flask (Falcon UK) in complete DMEM media (PAN Biotech GmbH) with 10%FCS in a humidified atmosphere containing 5 % CO_2 until 50% of confluence. A sample of selected black tattoo ink (Infernal Color) was extracted by our established extraction procedure [11]. The residual PAH extract was diluted by a factor 1000 with DMEM media and incubated with human dermal fibroblasts for 240 min. For reference (control), MeCN was diluted by a factor 1000 and also incubated with human dermal fibroblasts for 240 min. Thereafter, the cells were washed and fresh media was added. Fluorescence was detected using a Zeiss Axiotech Vario fluorenscence microscope containing an appropriate dual-band filter set (Omega opticals, Battleboro, VT, USA) for absorption (at 365 nm) and emission (at 450 nm).

6.2.5 Extraction procedure

Samples without (control) and after laser treatment were extracted according to our established extraction procedure in a mixture of benzene/acetone [11].

6.2.6 Chromatographic Analysis: HPLC – DAD & GC – MSD

For HPLC – DAD and GC – MSD analysis, the samples were filtered using a PTFE filter (Chromafil, O-20/15, organic, pore size 0.2 mm; Machery-Nagel, Düren, Germany), respectively. A 1.0 µL sample was analyzed using a model 1100 HPLC (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column for nanoscalic environmental analysis (Phenomenex Environsep PP, particle size 3 µm, 125 x 2.00 mm, Aschaffenburg, Germany) with DAD and MSD (Agilent 6100 Series Single Quadrupol and G1978B Mulltimode Source). The data were analyzed using an HPLC-3D ChemStation Rev. B.04.02. Gradient elution was done with water (0.0059% w/w trifluoroacetic acid) (solvent A) and acetonitrile (solvent B) at a

constant flow rate of 0.3 mL/min. A gradient profile with the following proportions of solvent B was applied [*t* [min]; % B]: (0, 40), (2, 40), (27, 98), (35, 98). The chromatograms were monitored at 220 nm.

Analysis was also done using Agilent Technologies GC/MS-System, consisting of 7890 A GC and 5975C Inert XL EI/CI MSD with a CTC Pal Autosampler. Method settings: Injection Volume 1.00 µL, He-Flow 1 mL/min. Oven Program: 40 °C for 3 min, then 15 °C/min to 280 °C for 5 min, then 25 °C/min to 300 °C for 5 min with Splitless mode (1 min); heater 250 °C; Transferline temperature of 300°C. The used column was an Agilent HP-5MS (30 m x 250 µm x 0.25 µm). Interpretation was done using the Agilent MSD ChemStation E.02.00.493 and NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0f, July 23 2008. For Matchfactor NIST >900, a substance was regarded as clearly identified.

The concentration of PAH in the solutions was determined by the method of internal standard. For each compound (i), the calibration factor (CF_i) was determined in a calibration run (single level calibration). The respective concentration of the standard was chosen to be in the range of the concentration of the tattoo pigment.

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

Where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K the mass of compound i in the solution k and m_{Tr}^K the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k and a_i^K the area of compound i in solution k.

6.3 Results and Discussion

When black tattoos should be removed, physicians apply intense and very short laser pulses to skin surface. The laser light penetrates the skin and is preferentially absorbed by the black ink particles in the dermis. The morphology of such ink particles is shown in figure 2. EDX analysis provides evidence that commercial inks mainly consist of carbon, agglomerated in graphite-like sheet structures as exemplarily shown after 3000 fold magnification of a lyophilized ink sample.

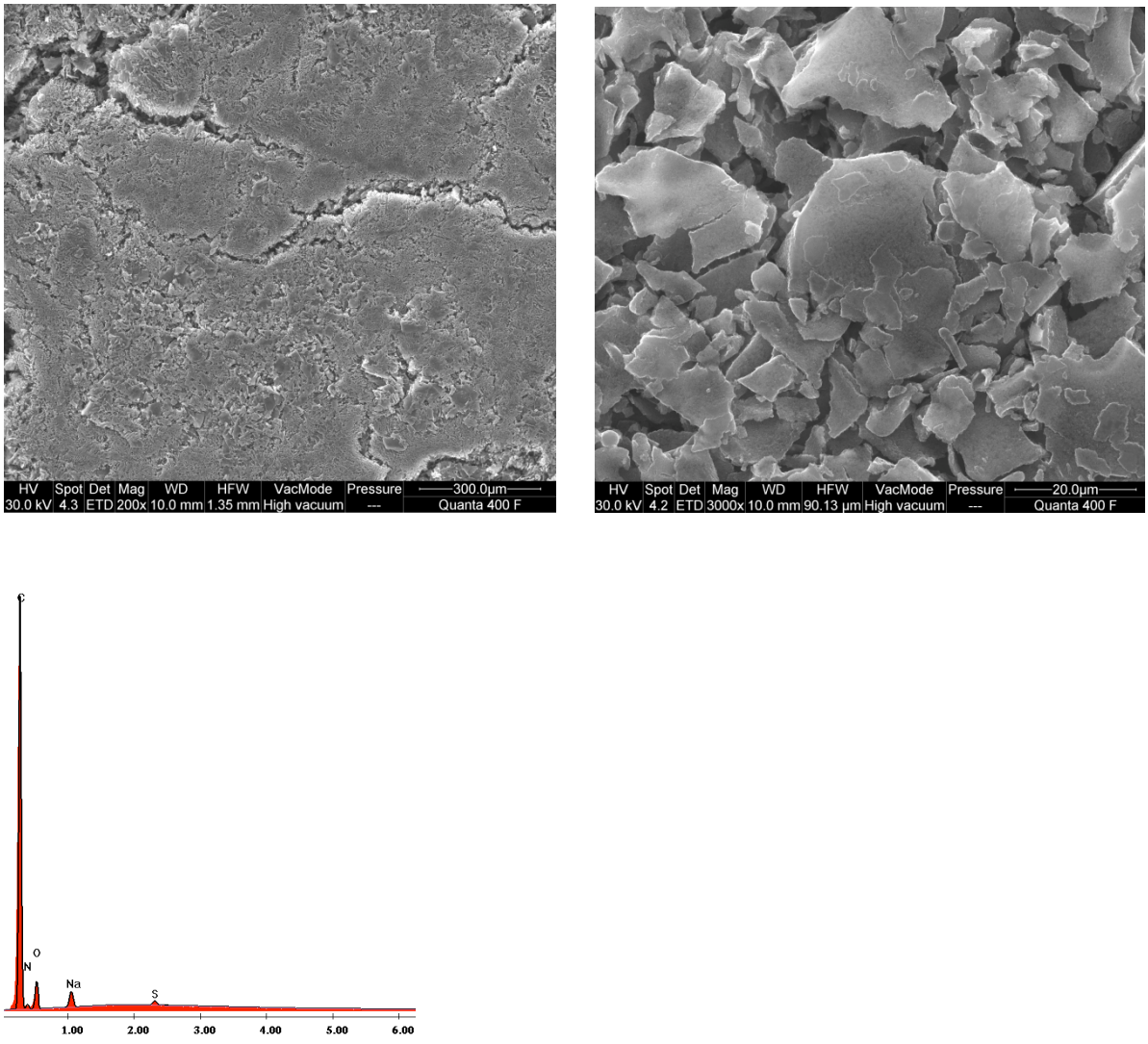


Figure 2: 200 fold (top, left) and 3000 fold (top, right) magnification of black ink. Carbon Black consists of a mixture of agglomerated cluster-like structures. Black tattoo ink mainly consists of carbon atoms, EDX analysis of selected black ink (Infernal Color) (bottom).

According to our extraction procedure for human skin and HPLC – DAD monitoring (*chapter 2*), we found CB together with PAH in originally black tattooed human skin specimens (*chapter 3/4*). Therefore, we investigated the interaction of laser light, CB and PAH.

6.3.1 Laser treatment of PAH in solution

PAH can absorb the UVA part of solar radiation (220 - 400 nm) and may form deleterious reactive oxygen species (ROS), in particular singlet oxygen ($^1O_2$), which may then affect human tissue [11]. Many PAH investigated are reported to form carcinogenic bay region dihydrodiol epoxides by a metabolic activation pathway *in vivo*, mediated by human cytochrome (CYP) enzymes [28-35].

When using Q-switched lasers for tattoo removal in clinical practice, the applied laser light shows wavelengths of 532, 694, 755, or 1064 nm, depending on the laser type used. Light at these wavelengths is not absorbed by PAH.

Each of investigated 20 PAH or phenol diluted in MeCN was exposed to laser radiation at 532 nm, 10 Hz, 3 J/cm^2 for 10 min, respectively. As a result, the amount of PAH after laser treatment remained constant for every 21 substances. An example is shown in figure 3.

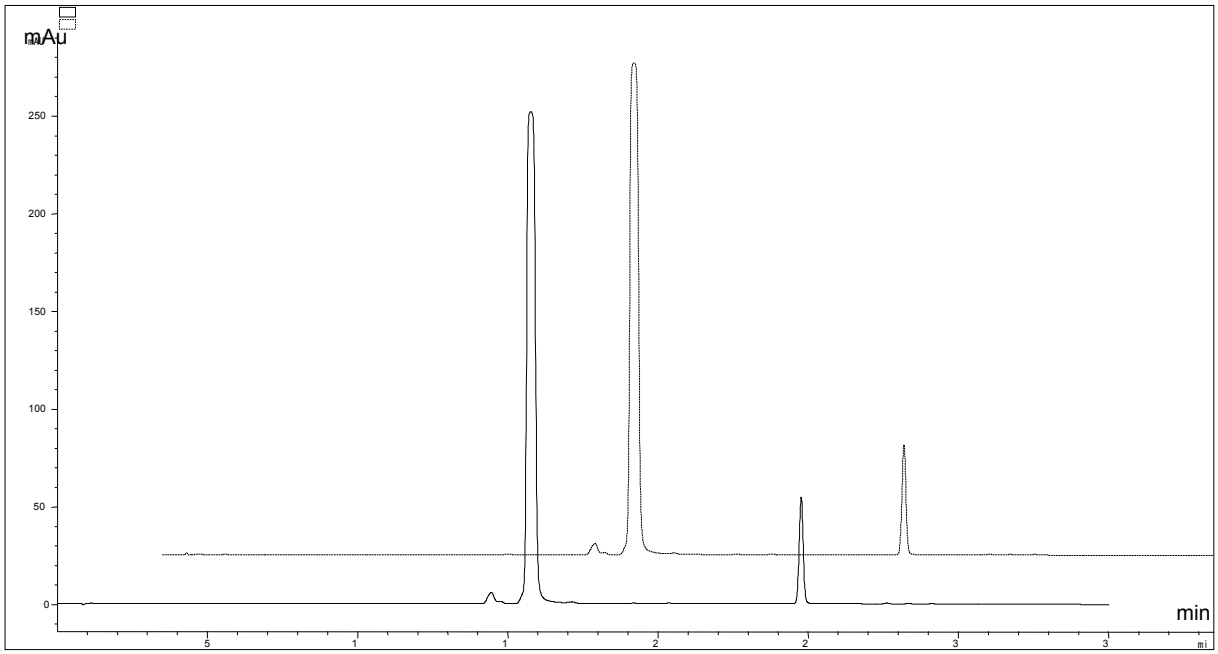


Figure 3: HPLC chromatogram of PAH phenanthrene in acetonitrile solution without (control) and exposed to laser, after established extraction procedure: no decomposition reaction occurred, the concentration of PAH remained constant

There is no change in peak area of PAH in the HPLC chromatogram, when comparing the results prior and after laser irradiation. No decomposition products occurred. The substances remained stable.

6.3.2 PAH in Carbon Black suspension exposed to laser

Since commercially available black tattoo inks mainly consist of Carbon Black, in this experiment, we tried to obtain conditions comparable to black inks. Fine powder of pure Carbon Black was suspended and combined with single PAH solution, respectively. The CB/PAH suspension was irradiated with laser light as above. PAH decomposition occurred as proven by quantitative HPLC calibration using internal standard procedure. An example for the decomposition of single PAH in CB suspension after laser irradiation is shown in figure 4. The HPLC peak area

decreased significantly.

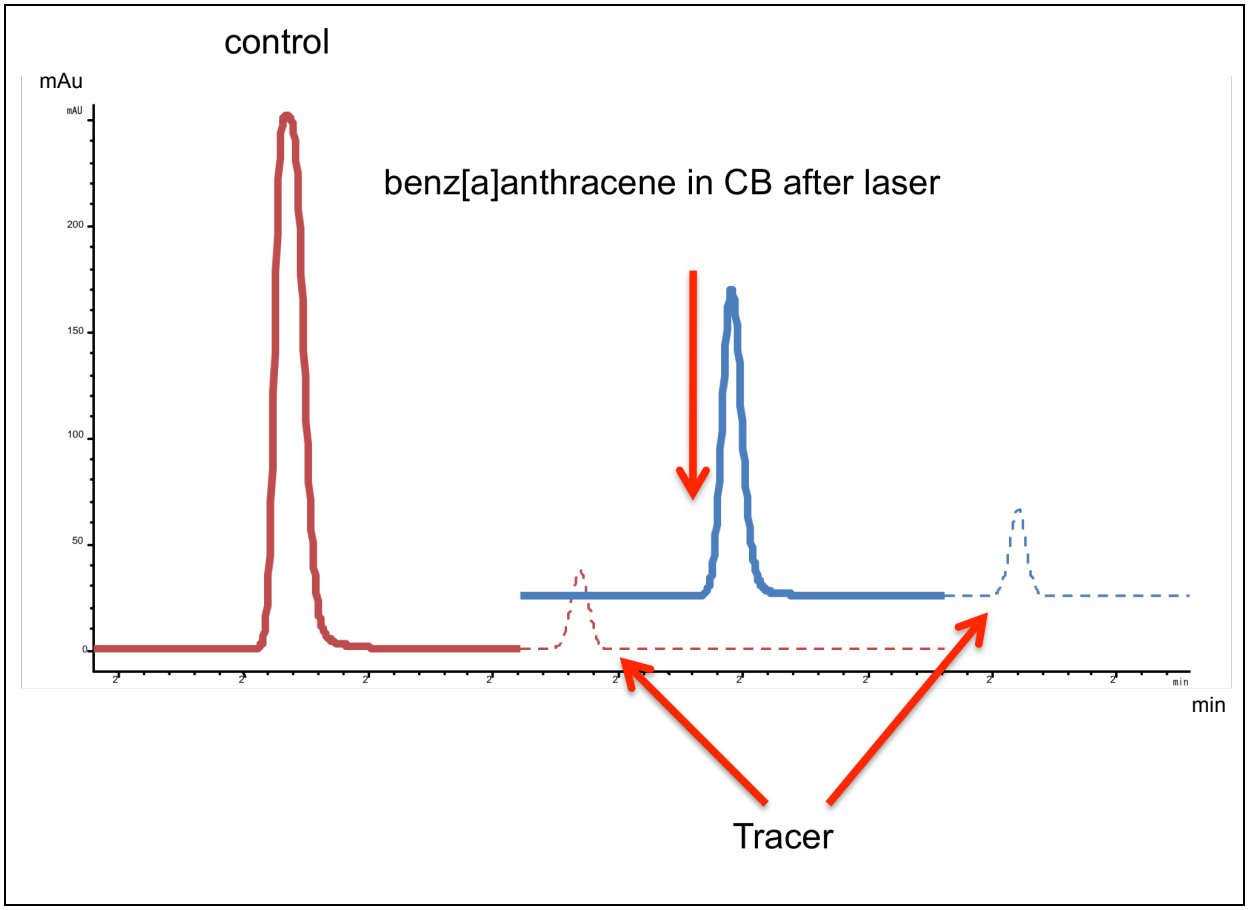


Figure 4: Exemplarily HPLC chromatogram of PAH benz[a]anthracene in CB suspension without (control) and exposed to laser, after extraction procedure. A clear reduction of the PAH peak area after laser treatment could be seen.

In principle, Carbon Black particles have been identified as an impure form of near-elemental carbon with graphite-like sheet structures that fold among themselves to form agglomerates of primary spherules around 10 – 50 nm in diameter, which coagulate and form loose aggregates, which are their most stable form [36].

CB strongly absorbs laser light in the entire visible spectrum and is traditionally viewed as the most important light absorber for solar radiation [37, 38]. Regarding interaction with solar energy, CB is suspected as a driver for global warming through

heating the atmosphere [39-44]. By converting laser energy into heat, chemical reactions between CB nanoparticles and surrounding molecules [41-43], in particular oxygen, may be triggered, e.g. to form reactive oxygen species such as singlet oxygen $^1O_2$ [45, 46]. Since strong sorption of PAH to Carbon Black e.g. by $\pi-\pi$ interactions [47, 48], energy transfer may be also eased by their proximity, resulting in thermal reaction of PAH forming photo-induced decomposition products. Table 1 summarizes investigated 21 aromatic substances on their percental decomposition after laser irradiation.

PAH	decomposition after laser [%]
Phenol	18.2
Naphthalene	57.6
Acenaphthylene	0.0
Acenaphthene	15.2
Fluorene	0.0
Phenanthrene	71.8
Anthracene	82.1
Fluoranthene	18.2
Pyrene	0.0
Benz[a]Anthracene	42.4
Chrysene	93.6
5-Methylchrysene	48.5
Benz[j]Fluoranthene	32.4
Benz[b]Fluoranthene	27.3
Benz[k]Fluoranthene	69.7
Benz[a]Pyrene	33.3
Dibenz[a,l]Pyrene	99.9
Dibenzo[a,h]Pyrene	98.1
Benz[g,h,i]Perylene	45.5
Indeno[1,2,3-c,d]Pyrene	59.3
Dibenz[a,e]Pyrene	60.6

Table 1: Percental [%] decomposition of investigated 20 PAH and phenol in CB suspension after laser treatment

Decomposition of PAH in CB suspension after laser treatment was in the range of

15.2 % for acenaphthene up to 99.9 % for dibenzo[a,l]pyrene. PAH acenaphthylene, fluorene and pyrene remained stable. For anthracene, laser irradiation in CB suspension resulted in decomposition of 82 % of the three-ring molecule under formation of the photo-dimer, which is connected by a pair of new carbon-carbon bonds due to thermal activation. In addition, formation of 9,10 anthracenedione (anthraquinone) could be identified. The position 9 and 10 are preferentially attacked by oxygen with anthraquinone as the most abundant intermediate, as extra stability was gained for the intermediate formed upon attack at these positions by interaction with adjacent delocalization systems [49]. Also, formation of 1,2 benzenedicarboxylic acid (phthalic acid) could be detected as intermediate of photolysis of parent anthracene with oxygen supply.

The common intermediates found after laser treatment of PAH in CB suspension were alcohol-, ketone-, quinone- and aldehyde-derivatives. Independent measurements showed no indication for estimated formation of hazardous epoxides after laser treatment as seen for UVA irradiation of PAH (*chapter 5*).

In conclusion, the intermediates and degradation pathways suggested were not complete, since some intermediates were short-lived and could not be detected, either by applied HPLC – DAD or GC – MS detection. Further studies e.g. in acetone-solution are needed since the presence of 16 % acetone is reported to show higher concentrations and more types of photo degradation intermediates [50] to hence propose a more complete degradation pathway.

6.3.3 Black tattoo ink after laser treatment

Originally black tattoo ink containing high amounts of PAH [11] was exposed to laser light according to our experimental setup. HPLC analysis showed decreasing PAH peaks as exemplarily seen in figure 5.

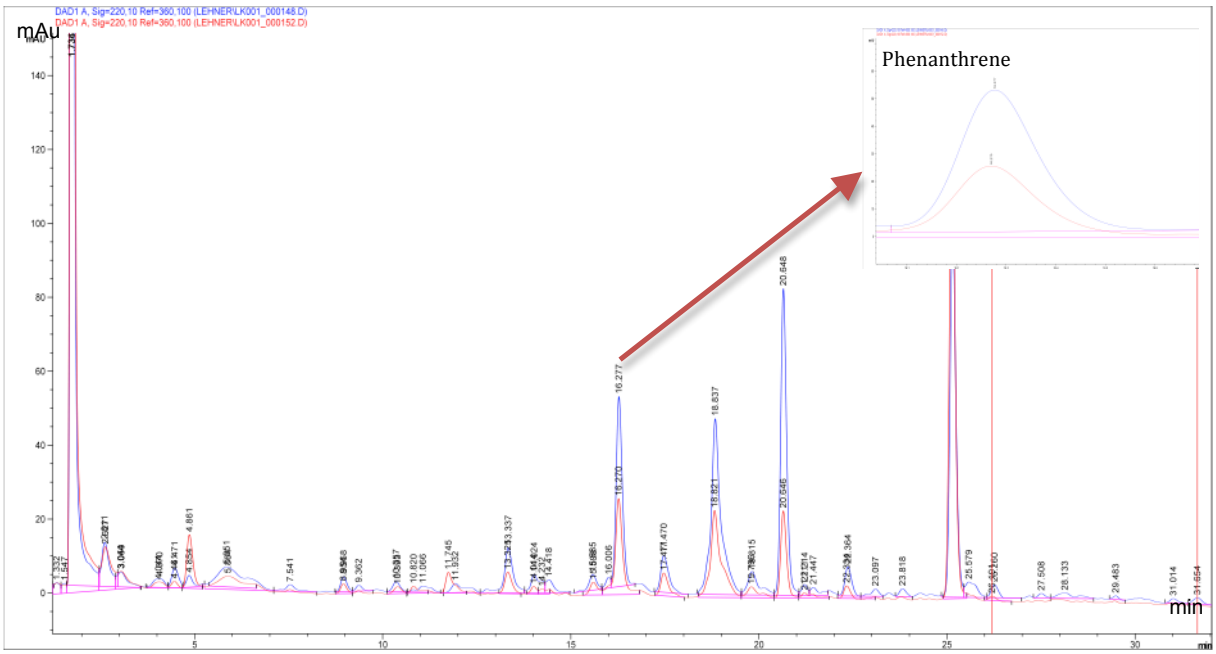
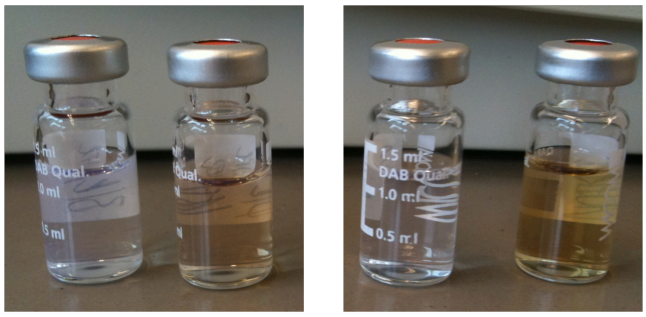


Figure 5: Exemplarily HPLC chromatogram of black tattoo ink before (blue) and after (red) laser: PAH peaks decreased, e.g. phenanthrene, while formation of new, more polar peaks at shorter retention times

Characterization of PAH after laser treatment showed a qualitatively decreasing PAH concentration of 36.4 % (blue) compared to control (red). New formed substances with shorter retention time could not yet be exactly identified according to our database.

Since PAH were extracted from the ink before and after laser, the irradiated sample yielded a brown extract from a prior colorless solution (without laser treatment) (Figure 6).



control / PAH in CB
after laser

control / black ink
after laser

Figure 6: PAH samples treated with laser in CB suspension (left) or ink (right) appeared as clear brown solution, compared to control without laser treatment (clear colorless solution)

Characterizing carbon black particles in the ink suspension before and after laser treatment by TEM analysis, the scale bar represents a mean diameter of about 50 to 70 nm before and about 7 nm after laser irradiation (figure 7).

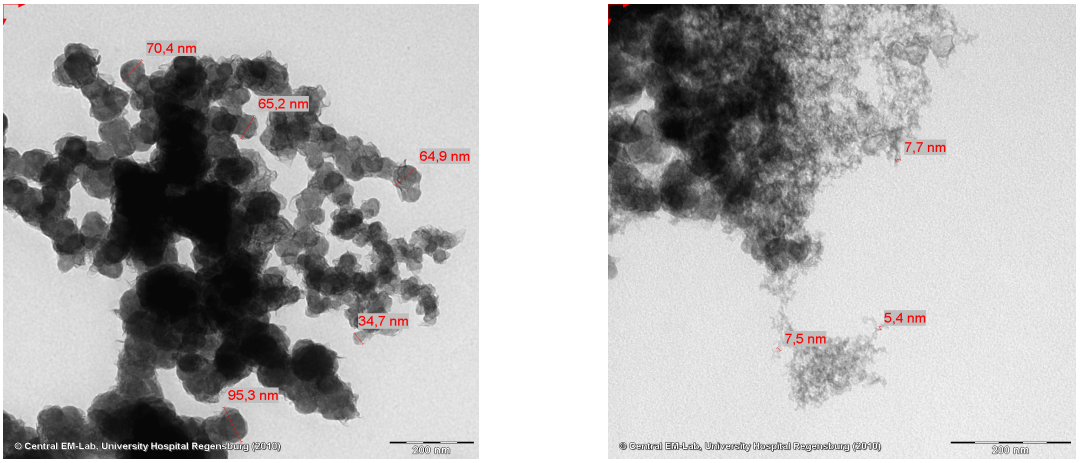


Figure 7: TEM analysis of selected black tattoo ink before (left) and after (right) laser treatment: disruption of particle size after high laser pulses

Regarding size distribution recorded by Dynamic-Light-Scattering, the ink particles had mass median aerodynamic diameters of 308.8 nm before and 140.8 nm after laser treatment (figure 8).

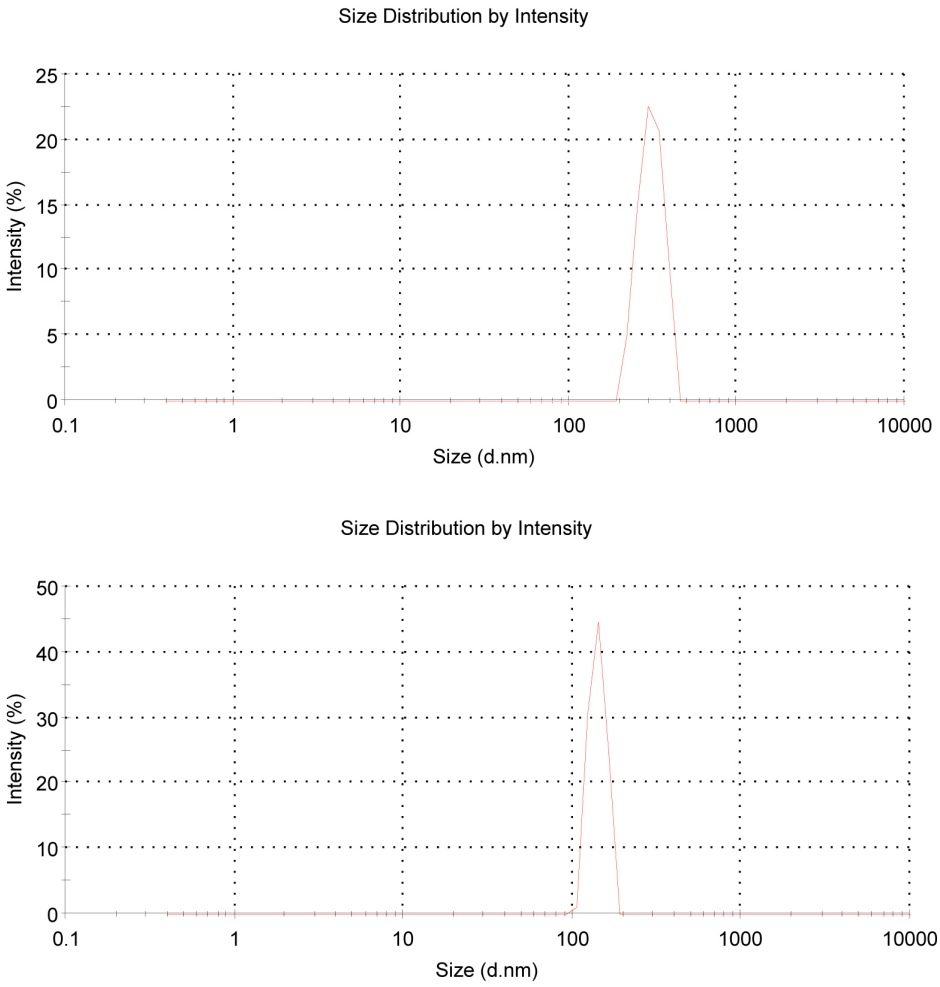


Figure 8: Particle diameter distribution of tattoo ink in suspension examined by Dynamic-Light-Scattering before (left) and after (right) laser treatment

Since disruption of tattoo ink particles in human skin is the common indication for laser tattoo removal, these ultrafine Carbon Black NPs can be transported away from the site of the tattoo via lymphatic system. We could already demonstrate, that Carbon Black present in black tattooed human skin is being transported together with adsorbed PAH to related locoregional lymph nodes (*chapter 3/4*) years after tattooing. In case of laser tattoo removal, we even suspect a much more faster transportation process.

By converting laser energy into heat we assume thermal and maybe triggered photo-mediated reactions, since the ink samples in the glass tubes heated up while

exposed to laser. For a clear understanding of the reaction mechanism, additional measurements are necessary. Up to present data, the exact molecular structure of degraded parent CB and PAH molecules remain unknown according to the applied methods.

6.3.4 CB nanoparticles associated with possible adverse health implications

Tattoos are increasingly popular in today´s society, especially with the advent of laser tattoo removal. Professional tattoos contain a multitude of potentially hazard chemicals that can be additionally altered by laser treatment, resulting in unknown possibly deleterious decomposition products. With specific regard to raising concerns about their potential toxicity, Carbon-based nanoparticles were found to be taken up and localized within secondary lysosomes, along the outer and nuclear membrane as well as inside the nucleus [15, 51]. In an experimental setup, we could demonstrate, that PAH are taken up by human dermal fibroblasts as shown in figure 8.

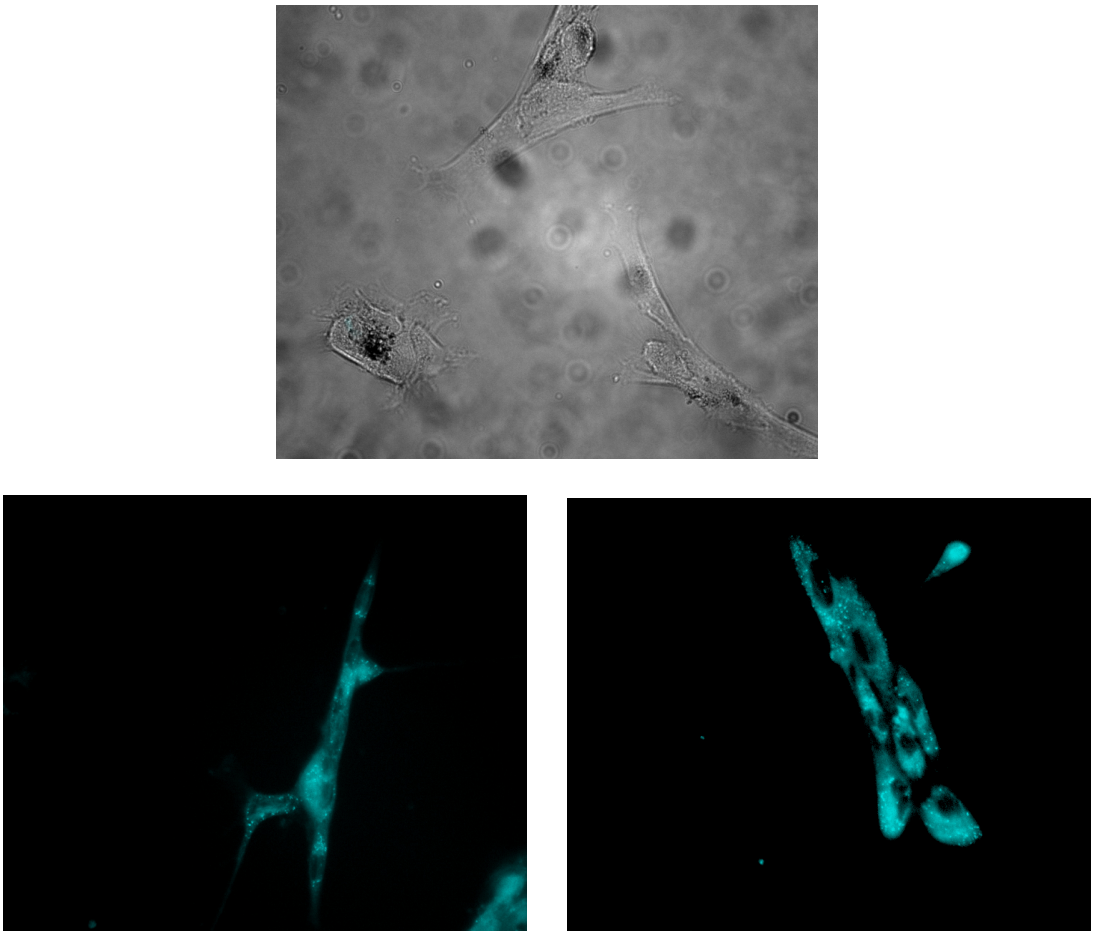


Figure 8: Human dermal fibroblasts incubated without (control, top) and with PAH extract (bottom). The blue fluorescence of PAH, which were taken up by fibroblasts, was imaged by fluorescence microscopy.

PAH located within human dermal fibroblasts showed typical blue fluorescence compared to control. In case of laser tattoo removal, disruption of CB particles and also PAH may form deleterious decomposition products at the nanoscale. Due to lymphatic transportation, the resulting ultrafine, possibly hazardous, nanoparticles may be distributed in human body. The enhanced induction of reactive oxygen species (ROS) generation upon exposure of cells to particular matter contributes to NP toxicity occurring at several levels, including the chemical reactivity of particles and the physical interaction of these with cellular structures involved in the catalysis of biological redox processes [52]. Beyond oxidative damage to biomolecules,

exposure of cells to NP may affect, via ROS formation, cellular signaling cascades that control cell proliferation, inflammatory processes and cell death, depending on physicochemical properties of respective nanomaterials [53-56].

Since some of investigated parent PAH are known to be carcinogenic, mutagenic or teratogenic [12, 13] and Carbon Black is already listed as possible carcinogenic to humans (group 2 B) [8, 9], further investigations on detailed reaction mechanisms and toxicity of produced nanomaterials are urgently recommended.

6.4 Conclusion

Since laser tattoo removal is a very common procedure, in present study we investigated the ability of 20 PAH and phenol, frequently found in black tattoo inks, to decompose after laser treatment. We could show that single PAH in acetonitrile solution was not affected by laser irradiation (Nd:YAG, 532 nm), because PAH do not absorb at this laser wavelength. In contrast, laser irradiation of single PAH in the presence of Carbon Black yielded decomposition of the PAH molecule in nearly every case, in a range between 15 and 99 percent. The list of photo-mediated decomposition products is still incomplete, since some of them are short-lived and could not be detected according to our analytical methods. Laser treatment of black tattoo ink resulted in decomposition of PAH in currently unknown derivatives. Up to present data, it remains unclear, whether decomposed nanomaterials after laser treatment are as cytotoxic, carcinogenic or mutagenic to human tissue as parental PAH and CB. Since lots of people undergo such laser tattoo therapy, with special regard to protect human health, further studies on the chemical analysis of decomposed nanoparticles formed have to be done, in particular *in vivo*.

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7. Abbreviations

ACN	acetonitrile
Ant	Anthracene
ATL buffer	tissue lysis buffer
B/A	benzene/acetone
B[a]A	benz[a]Anthracene
B[a]P	benz[a]pyrene
B[b]Fl	benz[b]Fluoranthene
B[k]Fl	benz[k]Fluoranthene
BP	benzophenone
BPDE	benz[a]pyrene-7,8-diol-9,10-epoxide
CAS-No.	Chemical abstract service number
CB	Carbon Black
CDNP	combustion-derived nanoparticles
CF i	calibration factor of the compound i
C.I.	colour index
cm^2	square centimeter
DAD	diode array detector
DBP	dibutyl phthalate
DF	dibenzofuran
Diglyme	diethylene glycol dimethyl ether
DLS	Dynamic Light Scattering
DNA	deoxyribonucleic acid
DPA	9,10-diphenyl- anthracene
EDX	Energy-dispersive X-ray spectroscopy

EFSA	European Food Safety Authority
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Environmental Protection Agency
ESI	electrospray ionisation
FDA	Food and Drug Administration
9-F	9-Fluorenone
g	gramm
GC	gas chromatography
HCBD	hexachlorobutadiene
H&E	haematoxylin and eosin staining
HET	hexamethylenetetramine
HPLC	high performance liquid chromatography
Hz	hertz
IARC	International Agency of Research in Cancer
IC	Infernal Color (Tattoo Ink)
ICBA	International Carbon Black Association
ISTD	internal standard
J	Joule
LC	liquid chromatography
LN	lymph node
LOD	limit of detection
LOQ	limit of quantification
M	molecule
mAu	milli absorption units
MeCN	acetonitrile
min	minute

mL	milliliter
mg	milligram
µg	microgram
µL	microliter
MS	mass spectroscopy
MW	molecular weight
Nd:YAG	neodymium-doped yttrium-aluminium-garnet
NHEK	normal human dermal keratinocytes
NIST	National Institute of Standards and Technology
nm	nanometer
NP	nanoparticle
ns	nanosecond
n-$BuNH_2$	n-butylamine
OD	optical density
$^1O_2$	singlet oxygen
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PMU	permanent make-up
PTFE	polytetrafluorethylene
Q-switched	Quality-switched
ROS	reactive oxygen species
RSD	relative standard deviation
RT	retention time
rt	room temperature
s	second
TEM	transmission electron microscopy

temp.	temperature
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TOC	total organic carbon
TSCA	Toxic Substances Control Act
US	United States
UV	ultraviolet
W	watt

8. Summary

Tattooing is a worldwide phenomenon that exists since the early beginnings of our modern civilization. As part of human culture, this art of body decoration has reached significantly popularity, especially among young people under 30 years of age. In the past, inorganic metal salts containing chromium, cobalt, manganese, or mercury has been used in tattoo colorants. Today, tattoo inks are mixtures of many components including precursors and by-products of pigment synthesis as well as various diluents for pigment suspension, whereas the actual chemical composition may vary for different ink products.

Millions of people in the western world have at least one tattoo and 60 % predominately contain black color. Actually, these inks are manufactured for other intended uses like printing, painting cars and coloring consumer products. Up to now, there is no legal requirement or regulation for listing ingredients on the labeling of tattoo inks and they may not have an established history for safe use in tattooing. In Germany, a first tattoo regulation appeared in 2009; it interdicts the use of azo pigments that can be cleaved to hazardous aromatic amines.

Despite the high number of individuals with black tattoos, we mainly focused on the chemical analysis of black tattoo inks. Since black inks are produced by imperfect combustion, they mainly consist of Carbon Black. It is, therefore, unsurprising that such black inks contain high amounts of polycyclic aromatic hydrocarbons (PAH) and phenol.

With regard on the process of tattooing, pigment suspension is initially punctured into the dermis with vibrating tiny solid needles (tattoo machines). Some of the deposited ink particles may be recognized by macrophages as foreign bodies and carried from the site of the tattoo via the lymphatic system. Colorant that remains in the dermis

represents the permanence, which is usually associated with tattoos. This procedure has been also associated with various types of cutaneous eruptions and different incidences of adverse reactions. In case of black tattoo inks, PAH and other substances can be introduced into skin, which might be responsible for health problems associated with tattoos, but up to now, the intrinsic chemical trigger is unknown.

To estimate the risk of any health problems of tattooing, the ingredients used and their concentration must be determined in both, ink suspension and in the skin and the human body. This has not been performed so far and was now firstly investigated by our research group.

In a previous study, we established an extraction procedure for determination and quantification of 20 different PAH in various commercial available black tattoo suspensions using liquid chromatography detection (HPLC – DAD). We detected total concentrations of PAH in the different inks ranging from 0.14 to 201 µg/g. Even benz[a]pyrene, a known carcinogen, was found in four ink samples at a mean concentration of 0.3 ± 0.2 µg/g. Regarding auxiliary ingredients, we investigated black tattoo inks in more detail by using GC – MS analysis to identify potential irritants or allergens, which might have the potential to be harmful for humans (*chapter 1*). We found the substances hexachlorobutadiene (HCBD), hexamethylenetetramine (HET), dibenzofuran (DF), dibutyl phthalate (DBP), 9-Fluorenone (9F), and benzophenone (BP), which are classified as (possible) genotoxic (HCBD, HET, DBP, BP), teratogenic (DBP) or as a possible human carcinogen (group C, HCBD). GC analysis and NIST database revealed the presence of other substances in the black inks, which were detected qualitatively, beneath 1,6-hexandiole, oleamide, 7-hexyl-2-oxepanone, propylene glycol, carbitol

cellosolve and others. Many of the inks contain more than one ingredient at the same time, thus, the described health problems are more likely to be caused by some of the ingredients listed in this investigation, whereas the listing might be still incomplete.

To elucidate the role of PAH in skin, the concentration of PAH or phenol in tattooed skin should be determined (*chapter 2*). We established an extraction method to recover PAH quantitatively from digested human skin. The extraction of phenol as well as 20 PAH was accomplished using recovery experiments and HPLC – DAD technology. PAH and phenol could be almost completely recovered from digested human skin with recovery rates of 96 to 99 %. Even the very volatile smaller aromatic two- and three-ring structures like naphthalene, acenaphthene, and acenaphthylene were successfully extracted using a combined vortex and ultrasonic procedure and a keeper compound. The presence of Carbon Black in the digested skin did not affect the recovery rate.

The use of the established procedure should now allow a quantitative extraction of PAH from tattooed skin specimens. (*chapter 3*). Sixteen tattooed human skin specimens and related locoregional lymph nodes were digested and tested for 20 different PAH. The amount of these substances was quantified by using HPLC – DAD technology. PAH were found in twelve of the sixteen tattoos and in eleven locoregional lymph nodes. The PAH concentration ranged from 0.07 – 0.57 µg/cm^2 in the tattooed skin and 0.05 – 11.75 µg/g in the lymph nodes. Our results provide evidence that PAH can be found in tattooed skin months or years after tattooing. In addition, Carbon Black obviously transported the PAH to the locoregional lymph nodes.

Since all investigated specimens contained black color, for the first time, we were able to establish a spectro-photometrical detection method for the quantitative

recovery of Carbon Black nanoparticles from tattooed human skin samples and the related locoregional lymph nodes (*chapter 4*). In sixteen investigated tattooed human skin specimens, the overall mean amount of Carbon Black was 110.8 ± 48.3 µg/cm^2. In a preliminary tattooing experiment using pig skin, a mean CB concentration of 517.2 ± 198.3 µg/cm^2 was punctured into skin. Therefore, in large part (> 80%), tattoo ink particles are being transported from the site of the tattoo via lymphatic system and frequently found in the lymph nodes. The amount of CB in related sixteen locoregional lymph nodes was in the range of 0.11 mg/g up to 17.0 mg/g. Since Carbon Black is already listed as possible carcinogenic to humans (group 2 B), there is no legal regulation on black tattoo inks so far.

Sunlight exposure to people with black tattoos might pose another risk and affect human skin integrity (*chapter 5*). It is known, that some of PAH previously detected in black tattoo inks can absorb UV radiation and generate reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$). We were able to demonstrate that upon UVA irradiation, thirteen of 21 investigated environmental pollutants generate photo – mediated decomposition products: formation of epoxides was detected in all cases except fluoranthene. Oxygen – free working conditions could show, that the oxidation of PAH is clearly mediated by $^1O_2$ generation. *In vitro* conditions showed already cytotoxicity for parent PAH without irradiation. Upon UVA irradiation, the toxicity of PAH depended on the photoproduct.

As the incidence of tattooing continues to increase, so does the demand of people for tattoo removal (*chapter 6*). Tattoo inks contain a multitude of potentially immunogenic ingredients, e.g. PAH, that can be released or modified by laser treatment. We studied the interaction of black tattoo ink and intense laser pulses (Q-switched Nd:YAG) with regard to possible chemical alterations of PAH molecules and Carbon Black nanoparticles. Single PAH in solution may not be affected by laser treatment,

since PAH do not absorb laser light at 532 nm. In the presence of Carbon Black nanoparticles, nearly all investigated 20 PAH decomposed in the range of 15.2% up to 99.9%. Photo-mediated decomposition products could not be clearly identified, since some of them are short-lived and could not be detected according to our analytical methods. With special concern to Carbon Black, we were able to detect a 90% disruption of ink nanoparticles by TEM and DLS. Up to present data, it remains unclear, whether decomposed nanomaterials after laser treatment are as cytotoxic, carcinogenic or mutagenic to human tissue as parental PAH and CB.

In light of these overall results, we urgently recommend regulation of tattoo inks so that only those inks without hazardous substances may be used. This could be started with a first step: substances that are not permitted for the use in cosmetics should be prohibited from being punctured into skin. Moreover, lack of knowledge should be removed by requiring complete listing of the ingredients as for cosmetics.

9. Zusammenfassung

Tätowierungen existieren weltweit schon seit den frühen Anfängen moderner Zivilisation. Dabei gewann dieser Körperkult in heutiger Zeit vor allem bei den unter 30 Jährigen zunehmend an Popularität. Während früher Tätowierungsfarben auf Basis anorganische Metallsalze mit Chrom-, Cobalt-, Mangan- oder Quecksilber-Einschlüssen verwendet wurden, so bestehen sie heute aus einer Vielzahl verschiedener organischer Komponenten wie Azoverbindungen oder polyzyklischen aromatischen Kohlenwasserstoffen (PAK).

In den USA sind bis zu 24 % der Bevölkerung tätowiert, in Deutschland sind es etwa 10 %. Eine kürzliche Studie im deutschsprachigen Raum hat ergeben dass etwa 60 % der rund 3400 Befragten ein hauptsächlich schwarzes Tattoo tragen. Die dafür verwendeten Farben werden eigentlich zum Lackieren von Konsumgütern wie z.B. in der Automobilindustrie, hergestellt, sie besitzen jedoch keine Zulassung für die Anwendung auf der Haut, eine gesetzliche Regulierung fehlt vollständig. Weder Herstellerangaben noch pharmakologische- oder toxikologische Informationen über die verwendeten Inhaltsstoffe werden deklariert. Seit 2009 gibt es in Deutschland immerhin schon eine Tätowiermittelverordnung, die den Gebrauch von Azoverbindungen, welche in karzinogene Amine gespalten werden können, untersagt.

Schwarze Tätowierungsfarben bestehen hauptsächlich aus Carbon Black. Dieser, als Ruß bekannte schwarze, pulverförmige Feststoff, entsteht vor allem durch unvollständige Verbrennungsprozesse, wodurch sich polyzyklische aromatische Verbindungen wie PAK oder auch Phenol als Nebenprodukte bilden. Betrachtet man den komplexen Tätowierungsvorgang nun näher, so werden Pigmente zusammen mit den jeweiligen Verunreinigungen in die Dermis eingestochen. Dass es bei

Tätowierungen dann zu Problemen kommen kann, liegt zum einen an der mechanischen Traumatisierung und an Infektionen oder aber auch an der chemischen Zusammensetzung der Farben. In der kürzlich veröffentlichten Umfrage klagten 2/3 der Teilnehmer über Blutungen, Schwellungen und brennenden Schmerzen direkt nach dem Tätowierungsprozess, immer noch über 200 Teilnehmer gaben Juckreiz, Infiltration, Papeln und Ödeme als dauerhafte Probleme im Bereich des Tattoos an. In einer ersten Studie lag unser Fokus auf der chemischen Analyse von 20 PAK, die von der amerikanischen Umweltbehörde US-EPA und der Europäischen Kommission aufgrund Ihrer Toxizität und ihres mutagenen Potentials gelistet sind. Als wohl bekanntester Vertreter dieser gefährlichen Stoffgruppe sei hier das Benzo[a]Pyren genannt. Durch die Etablierung eines speziellen Extraktionsverfahrens konnten wir mittels HPLC Analyse PAK Konzentrationen in verschiedenen schwarzen Tätowierungsfarben im Bereich von 0.14 bis 201 µg/g nachweisen, darunter auch das erwähnte Karzinogen.

In einer erweiterten Studie wurden die Farben nun mittels Gaschromatographischer Analyse auf potentielle Allergene oder reizende Zusatz- und Inhaltsstoffe untersucht (*Kapitel 1*). Darunter fanden sich mit bis zu 710 µg/g erhebliche Mengen an zum Teil gentoxischen oder teratogenen Substanzen wie hexachlorbutadien (HCBD), hexamethylentetramin (HET), dibenzofuran (DF), dibutyl phthalat (DBP), 9-Fluorenon (9F) und benzophenon (BP). Qualitativ konnten noch einige weitere Stoffe wie 1,6-hexandiol, oleamid, propylen-glycol und andere detektiert werden. Oftmals wurden sogar mehrere dieser bedenklichen Inhaltsstoffe gleichzeitig in nur einem Farbpräparat nachgewiesen.

Um nun ein eventuelles Risiko für den menschlichen Organismus abschätzen zu können, ist es notwendig, die Konzentration an Tätowierungspigmenten in der Haut

zu kennen (*Kapitel 2*). Das bereits etablierte Verfahren für die Extraktion von PAK aus schwarzen Tätowierungsfarben musste nun auf organisches Gewebe angepasst werden. Die vormals langen und aggressiven Extraktionsbedingungen (60 min Ultraschallbad, 60 °C) wurden zunächst auf die Extraktion aus exzidierter Schweinehaut angepasst. Durch Zusatz einer schwer-flüchtigen chemischen Verbindung, dem sog. „keeper“, konnte ein Verlust an leicht-flüchtigen PAK wie Naphthalin, Acenaphthen und Acenaphthylen verhindert werden. Mit diesem neuen Extraktionsschema (vortex und Ultraschallbad alternierend 1/5/1/5/1 min, RT) war es uns nun möglich, eine Recovery von 20 PAK und Phenol aus menschlichem Hautlysat mit 96 – 99 % zu erreichen.

Mit diesem etablierten Verfahren konnten nun 20 PAK aus real tätowierter Menschenhaut extrahiert werden (*Kapitel 3*). 16 verschiedene schwarze Hautpräparate wurden lysiert und analytisch mittels HPLC – DAD auf PAK untersucht. In 12 schwarzen Tattoos konnten wir PAK im Bereich von 0.07 – 0.57 µg/cm^2 quantifizieren. Die PAK Phenanthren, Acenaphthen, Fluoren, Anthracen, Benzo[j]fluoranthen und Naphthalin seien hier als Hauptvertreter genannt. Da ein Teil der Pigmente in der Haut als Fremdkörper über das Lymphsystem abtransportiert wird, wurden ebenfalls die lokoregionären Lymphknoten untersucht. Elf davon enthielten PAK mit Konzentrationen im Bereich von 0.05 – 11.75 µg/g wobei größtenteils auch diejenigen PAK detektiert wurden welche zuvor in den jeweiligen Tätowierungen gefunden wurden.

Da sowohl alle 16 untersuchten Tätowierungen als auch die jeweiligen Lymphknoten schwarz gefärbt waren, wurde nun ein spektrophotometrisches Verfahren entwickelt, um den Gehalt an Carbon Black Nanopartikel in menschlichem Gewebe zu untersuchen (*Kapitel 4*). Carbon Black ist bereits als potentielles Karzinogen (Gruppe 2B) eingestuft. Exzidierte Schweinehaut wurde nun mit sowohl eigens hergestellter

als auch mit originaler Tattoofarbe in verschiedenen Konzentrationen und verschiedenen Nadelformen und Nadelgrößen tätowiert. Je nach Größe der Pigmentkristalle, der verwendeten Konzentration der Suspension und des jeweiligen Tätowierungsprozesses belief sich der Gehalt an Carbon Black auf Werte zwischen 0.40 – 0.75 mg/cm^2. In den untersuchten Hautpräparaten konnte das schwarze Pigment in einem Konzentrationsbereich von 21.1 ± 14.9 µg/cm^2 bis zu 194.9 ± 21.1 µg/cm^2 (im Mittel 110.8 ± 48.3 µg/cm^2) nachgewiesen werden. Geht man nun davon aus, dass ein Tätowierer im Mittel 0.52 mg/cm^2 an Carbon Black in die Haut einbringt so müsste im Laufe der Zeit etwa 80 % des schwarzen Pigments abtransportiert sein. Die spektrophotometrische Analyse der jeweiligen lokoregionären Lymphknoten lieferte eine Carbon Black Konzentration in Höhe von 0.11 mg/g bis zu 17.0 mg/g.

Von zunehmendem Interesse ist auch der Einfluss an UV – Strahlung auf die Tätowierungspigmente (*Kapitel 5*). Viele in den Tattoofarben vorkommenden PAK absorbieren UV – Licht und können reaktive Sauerstoffspezies (sog. ROS), vor allem den hochreaktiven Singulett Sauerstoff $^1O_2$ erzeugen. *In vitro* Messungen hatten bereits in vorangegangenen Experimenten ergeben, dass die Quantenausbeute an Singulett Sauerstoff für PAK zwischen 46 % und 82 % liegen und damit zum Teil höher sind als für Photosensibilisatoren in der Photodynamischen Therapie (PDT). PAK könnten somit unter dem Sonnenlicht auf photodynamischem Wege den hochreaktiven Singulett Sauerstoff auch in der Haut erzeugen. Bei 13 aus 20 PAK konnten wir vor allem die Bildung von PAK – Epoxid – Derivaten analytisch mittels HPLC – MS und chemischer Reaktion mit n-butylamin nachweisen. Unter Sauerstoffausschluss konnte dann letztendlich auf die Generierung des hochreaktiven Singulett Sauerstoff $^1O_2$ rückgeschlossen werden. Wie gefährlich diese Sauerstoff Spezies sein können, wird am enzymatisch gesteuerten Mechanismus von Benzo[a]Pyren im menschlichen Körper deutlich. Das

entstehende, sog. „Ultimative Karzinogen“ kann mit der DNA reagieren und so deren Struktur verändern, was Zellteilung verhindern oder Mutationen begünstigen kann. Dieser Polyzyklus ist bereits auch für andere PAK, wie z.B. Dibenzo[a,l]Pyren, beschrieben, welches wir ebenfalls in den Tätowierungsfarben quantifizieren konnten.

Aus verschiedensten Gründen unterziehen sich viele Menschen einer Lasertherapie, um die Tätowierungen letztendlich wieder entfernen zu lassen (*Kapitel 6*). Die Interaktionen zwischen Laser Energie und schwarzen Tätowierungsfarben wurden nun näher untersucht. Während freie PAK bei Lasereinwirkung von 532 nm nicht absorbierten, zersetzten sie sich in Gegenwart von Carbon Black weitestgehend (15.2% bis 99.9%). Neben der Entstehung reaktiver Sauerstoff Spezies vermuten wir auch die Bildung von PAK-Sauerstoff-Derivaten, thermisch induziert durch die räumlich Nähe zwischen absorbierendem Carbon Black und PAK. Spektrophotometrisch konnte gezeigt werden, dass sich diese Carbon Black Nanopartikel mit einem Partikeldurchmesser von etwa 50 nm – 70 nm nach Lasereinwirkung auf etwa 7 nm reduzieren. Diese Nanopartikel werden dann vom Lymphsystem abtransportiert was weitere Untersuchungen vor allem aber *in vivo* Studien, erforderlich macht. Aufgrund extremer Kurzlebigkeit konnten jedoch weitere Zersetzungsprodukte mit unseren analytischen Methoden vorerst nicht detektiert werden.

Die letztendliche Toxizität und Karzinogenität der Tätowierungsfarben und deren Inhaltsstoffe sowie Licht-induzierten Spaltprodukten hängt unter anderem natürlich von deren Konzentration in der Haut ab. Angesichts der großen Zahl an tätowierten Menschen möchten wir mit unseren einschlägigen Ergebnissen die Gesetzgeber dringend dazu auffordern, einheitliche Regulierungen der Tätowierungsfarben zu veranlassen.

10. Appendix

10.1 Publications

- **Lehner K,** Santarelli F, Vasold R, Sidoroff A, König B, Landthaler M, Bäumler W, "Effects of laser and UV radiation on black tattoo inks", Lasers Surg Med, **2012**, in preparation
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ORAL PRESENTATIONS

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- **K. Lehner,** “Polycyclic Aromatic Hydrocarbons and Softener present in Black Tattoo Inks may additionally interact with UVA or Laser Radiation“, 18. Tagung der Deutschen Gesellschaft für Lasermedizin (DGLM), Ulm, 05/**2011**
- **K. Lehner,** “Schwarze Tattoofarben als Trojanisches Pferd für PAK”, 46. Jahrestagung der Deutschen Dermatologischen Gesellschaft (DDG), Dresden, 04/**2011**
- **K. Lehner,** “PAK, Phenol, Weichmacher und Anderes in Schwarzen Tattoofarben”, 46. Tagung der Deutschen Dermatologischen Gesellschaft (DDG), Dresden, 04/**2011**
- **K. Lehner,** “Black Magic – Magic Black? The dark side of Tattoos”, Weihnachtscolloquium des Instituts für Organische Chemie, Universität Regensburg, 12/**2008**

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- **K. Lehner**, F. Santarelli, R. Vasold, A. Sidoroff, B. König, M. Landthaler, W. Bäumler; "Schwarze Tattoofarben als Trojanisches Pferd für PAK - Detektion in schwarzen Tätowierungen und Lymphknoten", Jahrestagung der Österreichischen Gesellschaft für Dermatologie und Venerologie (ÖGDV), Linz, Österreich, 11/**2011**
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ADDITIONAL CONFERENCES AND EDUCATIONS

- Workshop “Psychologie des Verkaufens”, Strategische Partnerschaft Sensorik e.V., Biopark Regensburg, 02/20**12**
- Tagung DermoEuregio, “Entzündliche Hauterkrankungen im Erwachsenen und Kindesalter“, Terme di Comano, Trient, Italy, 12/**2011**
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- Inhouse – Seminar “Patentrecherche”, TÜV Rheinland Akademie GmbH Niederlassung Nürnberg, Strategische Partnerschaft Sensorik e.V., Biopark Regensburg, 03/**11**
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- 22. Münchner Fortbildungswoche für praktische Dermatologie und Venerologie, München, 07/**2010**
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- Tagung des Collegium Helveticum der ETH Zürich / Universität Zürich “Wider den Methodenzwang – Systematische Ansätze für neue Therapieformen”, Zürich, Schweiz, 03/**2010**
- 45. Jahrestagung der Deutschen Dermatologischen Gesellschaft (DDG), Dresden, 05/**2009**

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