TATTOO PIGMENTS IN SKIN:

Determination and Quantitative Extraction of Red Tattoo Pigments

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Die Neugier steht immer an erster Stelle eines Problems,

das gelöst werden will.

- Galileo Galilei -

Für Daniel
&
meine Familie

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1. Establishment of an Extraction Method for the Recovery of Tattoo Pigments from Human Skin using HPLC Diode Array Technology*

1.1. Introduction

The number of tattooed individuals increased significantly,[1-3] especially among youth.[3-8] In the United States, ~16% of the population is tattooed, whereas in Europe it is ~10%. In the past, people used inorganic metal salts, containing chromium, manganese, mercury, and cobalt. Due to their brilliance and their great insolubility, today many azo pigments are used for tattooing. A significant number of these azo pigments are organic pigments manufactured primarily for other uses such as printing, painting cars, and coloring other consumer products. Tattoo inks contain many components. Frequently, tattoo inks are pigment mixtures and may contain components such as titanium dioxide for lightening the ink shade. Precursors and byproducts of pigment synthesis may also be present. In addition, diluents used to suspend pigments may be complex mixtures.[9,10]

Tattoo colorants are also used as permanent make-up make up for application on the eyelid, eyebrow, and lip.[11] Many tattoo pigments are manufactured for other intended uses and may not have an established history for safe use in tattooing.[9,10] Currently, there is no legal requirement for listing ingredients, including pigments, on the labeling of tattoo inks. The U.S. Food and Drug administration considers the pigments used in tattoo inks to be color additives, which require pre-market approval. Currently, no pigments have been approved for use in tattoo inks.[12]

In the process of tattooing, the pigment suspension is initially deposited on the skin and then implanted by needle punctures. Some of the deposited pigment may be recognized by macrophages as foreign bodies and carried from the site of the tattoo via the lymphatic or circulatory system. As a result, tattoo pigments

* Results of this chapter have been published: Engel, E.; Santarelli, F.; Vasold, R.; Ulrich, H.; Maisch, T.; König,B.; Landthaler, M.; Gopee, N.V.; Howard, P.C.; Bäumler, W. *Anal. Chem.* **2006**, *78*, 6440.

may frequently be found in lymph nodes.[13] Pigment remaining at the site of the tattoo is usually found in the dermis and may be found intracellularly. Deposition of the pigment into the dermis results in the permanence usually associated with tattoos. Due to their insolubility, tattoo pigments are resistant to enzymatic degradation in the skin. Frequently, pigments in a tattoo are aggregated into crystals with a size ranging from about 0.1 to 10 µm.[14]

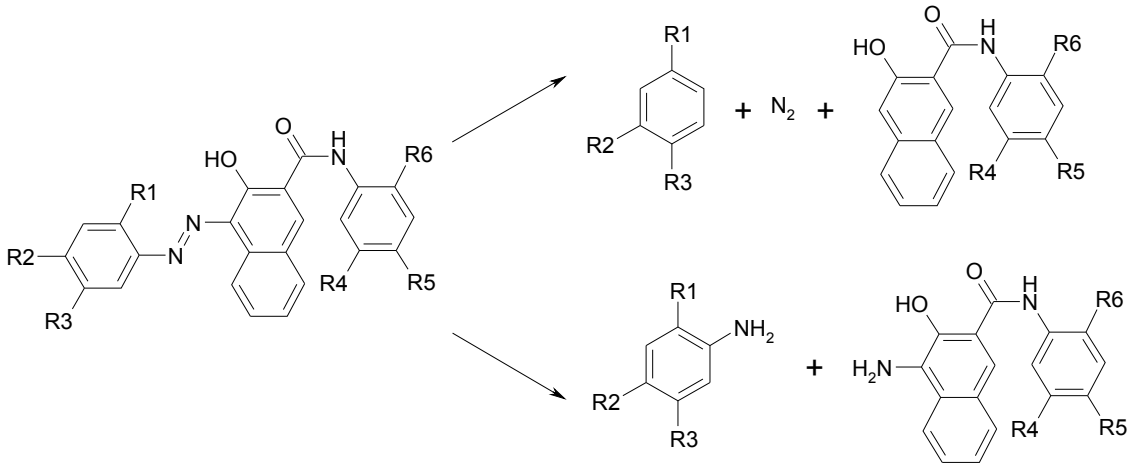
Because of an improved self-image or social stigmatization many tattooed individuals undergo a therapy of tattoo removal by using predominantly Q-switch lasers.[15]

In fact, high intensities and short pulse durations of a laser are necessary to destroy selectively the pigments and not the surrounding skin.[16] After being absorbed in the pigment molecule, the energy of the laser light is converted to heat or breaks chemical bonds inside the molecule. Additionally, the ultrashort heating may lead to the disruption of the pigment particle. The laser irradiation changes the shape and the size of the tattoo particles abruptly as proved by histology.[17]

It is well known that an increase of temperature in a number of azo dyes above 280 °C forms 3,3'-dichlorobenzidine,[18] a proven genotoxin toward human lymphocytes.[19] Laser irradiation of the two widely used azo compounds, Pigment Red 22 (PR 22) and Pigment Red 9 (PR 9) resulted in the photodecomposition products 2-methyl-5-nitroaniline (MNA), 4-nitrotoluene (NT), 2,5-dichloraniline (DCA), and 1,4-dichlorobenzene (DCB)[15] (Figure 1). NT is toxic as shown with human lymphocytes.[20] 5-Nitro-*o*-toluidine, which is also designated to MNA, may cause liver dysfunction as shown with workers from a hair dye factory.[21] Additionally, MNA and other di-nitro-toluenes showed greatest mutagenic activity toward *Salmonella typhimurium* YG as demonstrated by Sayama et al.[22] DCB has been reported to cause tumors in kidney of male rats and in liver of male and female mice,[23] whereas DCA was capable of inducing nephrotoxicity in rats.[24]

However, toxicity and mutagenic activity of molecules is correlated to the respective concentration taken up by a human. Presently, little is known about the concentrations of pigments or decomposition products in tattooed sites of the skin. In light of the millions of people with tattoos, scientific investigations on

cutaneous concentrations of pigments and byproducts are critically needed to evaluate risks associated with tattooing.



R1	R2	R3	R4	R5	R6	name
Cl	H	Cl	H	H	OCH_3	PR 9
CH_3	H	NO_2	H	H	H	PR 22

Figure 1: Chemical structure of PR 22 and PR 9 used as coloring pigments in Cardinal Red (CR) and I8, respectively. For both pigments, the possible decomposition pattern is shown. Additional decomposition products may occur (chlorine, oxidation). The substituents of the pigment molecules are listed in the table portion.

As a first step, we established a method for the quantitative extraction of tattoo pigments and their respective decomposition products from different media. The extraction step was validated through recovery experiments. That is, pigments such as PR 9 and PR 22 synthesized in high pure quality or decomposition products were added to aqueous suspensions or homogenized skin at a known concentration, extracted, and quantified by using high-performance liquid chromatography diode array (HPLC-DAD) technology.

1.2. Materials and Methods

Tattoo Pigments.[*] PR 22 (C.I. 12315, CAS 6448-95-9) and PR 9 (C.I. 12460, CAS 6410-38-4) were synthesized via azo coupling according to Cook et al.[25] The starting material for the synthesis of PR 22 was naphthol AS (NAS; 99%, Sigma-Aldrich, Steinheim, Germany) and MNA (99%, Aldrich Chemical Co., Inc., Milwaukee, WI). PR 9 was synthesized using methoxynaphthol AS (*m*-NAS; 98%, Aldrich Chemical Co., Inc.) and DCA (99%, Acros Organics). Both raw products were purified by slurrying in acetonitrile (LiChroSolv, Merck, Darmstadt Germany).

Stock Solutions. For the stock solutions of the non-volatile compounds MNA, NAS, PR 22, *m*-NAS, and PR 9 with the following concentrations were dissolved in chloroform: MNA (0.5 mg/mL), NAS (0.25 mg/mL), PR 22 (0.3 mg/mL), *m*-NAS (0.2 mg/mL), and PR 9 (0.3 mg/mL). The volatile compounds were also dissolved in chloroform at the following concentrations: NT (5.0 mg/mL) (>98%, Fluka, Buchs, Switzerland), DCA (10.0 mg/mL), and DCB (4.0 mg/mL) (>99%, Fluka). The ISTD stock solution was 9,10-diphenylanthracene (1.0 mg/mL acetonitrile/methylene chloride (1:1) (Oekanal, Sigma-Aldrich, Seelze, Germany). All used solvents were of gradient grade quality for liquid chromatography (LiChroSolv, Merck, Darmstadt, Germany).

Extraction from Solvents. One milliliter of the stock solution of the non-volatile compounds was added to a 15-mL PP test tube (Cellstar, Greiner Bio-one, Frickenhausen, Germany). The solvent was removed by blowing nitrogen (2 bar, 20 min, 60 °C) (nitrogen 5.0, Linde Gas, Höllriegelskreuth, Germany). One hundred microliters of the undiluted stock solution of the volatile compounds was added. The tube was filled with either 5 mL of water, produced by a Milli-Q Ultrapure water purification system (Millipore, Schwalbach, Germany) or 5 mL of phosphate-buffered saline (PBS; Biochrom, Berlin, Germany). Afterwards, 1 mL of methanol (LiChroSolv, Merck) was added. The compounds were extracted with 3 mL of methylene chloride four times.

[*] PR 22 and PR 9 were synthesized by Matthias Gottschalk in his Zulassungsarbeit.

The total volume of the four extraction steps (each with 3 mL of methylene chloride) was collected in a modified Kuderna-Danish concentrator (with attached Snyder column). Then 200 µL of diethylene glycol dimethyl ether (Diglyme) (Fluka, Deisenhofen, Germany), a low volatile liquid, was added. This compound works as keeper; it enables the solvents to evaporate and momentarily prevents vaporization of the volatile compounds (NT, DCA, DCB). The solution was concentrated in the 200 µL of Diglyme under stirring and heating to 60 °C for 20 min. Finally, the remaining solvent mixture of methylene chloride and methanol was completely evaporated under continuing stirring, elevated temperature (60 °C), and a gentle stream of nitrogen (2 bar, 20 min). For the HPLC analysis, each sample, consisting of the extracted compounds concentrated in 200 µL of keeper, was reconstituted in 1.7 mL of chloroform and a 100-mL solution of internal standard. After the final step, the total volume of each samples was 2 mL.

Enzymes. A total of 7500 units of collagenase (type VII, Sigma-Aldrich, Taufkirchen, Germany) were dissolved in 1 mL of PBS (Biochrom). Proteinase K was used as a ready-to-use solution (Dako Corp., Carpinteria, CA). ATL buffer and proteinase K (> 600 AU/mL) were purchased from Qiagen (Hilden, Germany).

Skin Preparation. Human skin was obtained from surgical excisions (Department of Dermatology, University of Regensburg, Germany) and stored at - 80 °C. For further treatment, the tissue was chopped up to slices with size of ~1 cm^2 and adipose tissue was removed by a scalpel. Each sample was powdered in liquid nitrogen and added to Eppendorf cups (Eppendorf, Wesseling-Berzdorf, Germany), and 400 µL of PBS was added. Proteins were denatured by heating at 95 °C for 5 min according to Gaber et al.[26] After cooling to room temperature, 240 µL of collagenase solution (7500 units/mL), a 250-µL aliquot of proteinase K ready-to-use solution (10 units/mL) and 250 µmL of PBS were added. The suspension was stirred at 37 °C for 12 h. Subsequently, the digested skin was centrifuged (10 min, 13000 rpm, 20 °C). A total of 180 µL of buffer ATL and 20 µL of proteinase K were added to the pellet, mixed by vortexing, and incubated at 55 °C for 2.5 hrs until the tissue was completely lysed (flow scheme, see Figure 2).

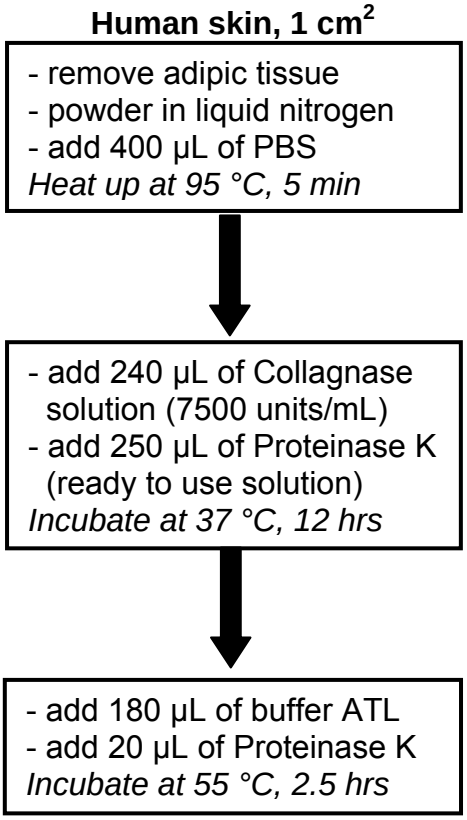


Figure 2: Lysis scheme for the degradation of human skin tissue.

Microscopic Analysis. For vital staining, 20 µL of lysed skin sample was mixed with 60 µL of PBS and 20 µL of Trypan blue (0.5% w/v, Biochrom). Successful lysis and Trypan blue staining of the cells was examined by light microscopy (Zeiss Vario-AxioTech, Goettingen, Germany) (data not shown). As reference primary normal human dermal fibroblasts (NHDF) were incubated with 0.5% trypsin for 30 min (Gibco Life technologies, Eggenstein, Germany). NHDF cells were purchased from CellSystems Biotechnologie (St. Katharinen, Germany). NHDF were propagated in FGM-2 BulletKit (Clonetics BioWhittaker), respectively. Media were supplemented with 10% foetal calf serum (Sigma Chemie, Deisenhofen, Germany), 1% L-glutamine, and 1% penicillin–streptomycin (Gibco, Eggenstein, Germany) in a humidified atmosphere containing 5% carbon dioxide at 37 °C. Cells were washed with PBS and harvested using a treatment with 0.05% trypsin/0.53 mM EDTA (Gibco) in PBS for 10 min. Cells were reseeded at 1 x 10^5 cells/mL in 75-cm^2 tissue culture flasks and were used between passages 2 and 10.

Extraction from Lysed Skin. For extracting skin, 1 mL of the stock solution of the nonvolatile compounds was added to a PP test tube, and the solvent was removed under a stream of nitrogen. A 100-µL sample of the stock solution containing volatile compounds was added. Then, the solution resulting from digestion of 1 cm^2 of human skin was added to the test tube. The volume was increased up to 5 mL with PBS. The aqueous skin phase was stabilized by adding 1 mL of methanol. The resulting mixture was extracted 4 times with 3 mL of methylene chloride.

The total volume of the four extraction steps (each with 3 mL of methylene chloride) was collected in a modified Kuderna-Danish concentrator (with attached Snyder column). Then 200 µL of Diglyme was added. The solution was concentrated in the 200 µL of Diglyme under stirring and heating to 60 °C for 20 min. Finally, the remaining solvent mixture of methylene chloride and methanol was completely evaporated under continuing stirring, elevated temperature (60 °C) and a gentle stream of nitrogen (2 bar, 20 min). For the HPLC analysis, each sample, consisting of the extracted compounds concentrated in the 200 µL keeper, was reconstituted in 1.7 mL of chloroform and 100 µL of a solution of internal standard. After the final step, the total volume of each sample was 2 mL (flow scheme, Figure 3).

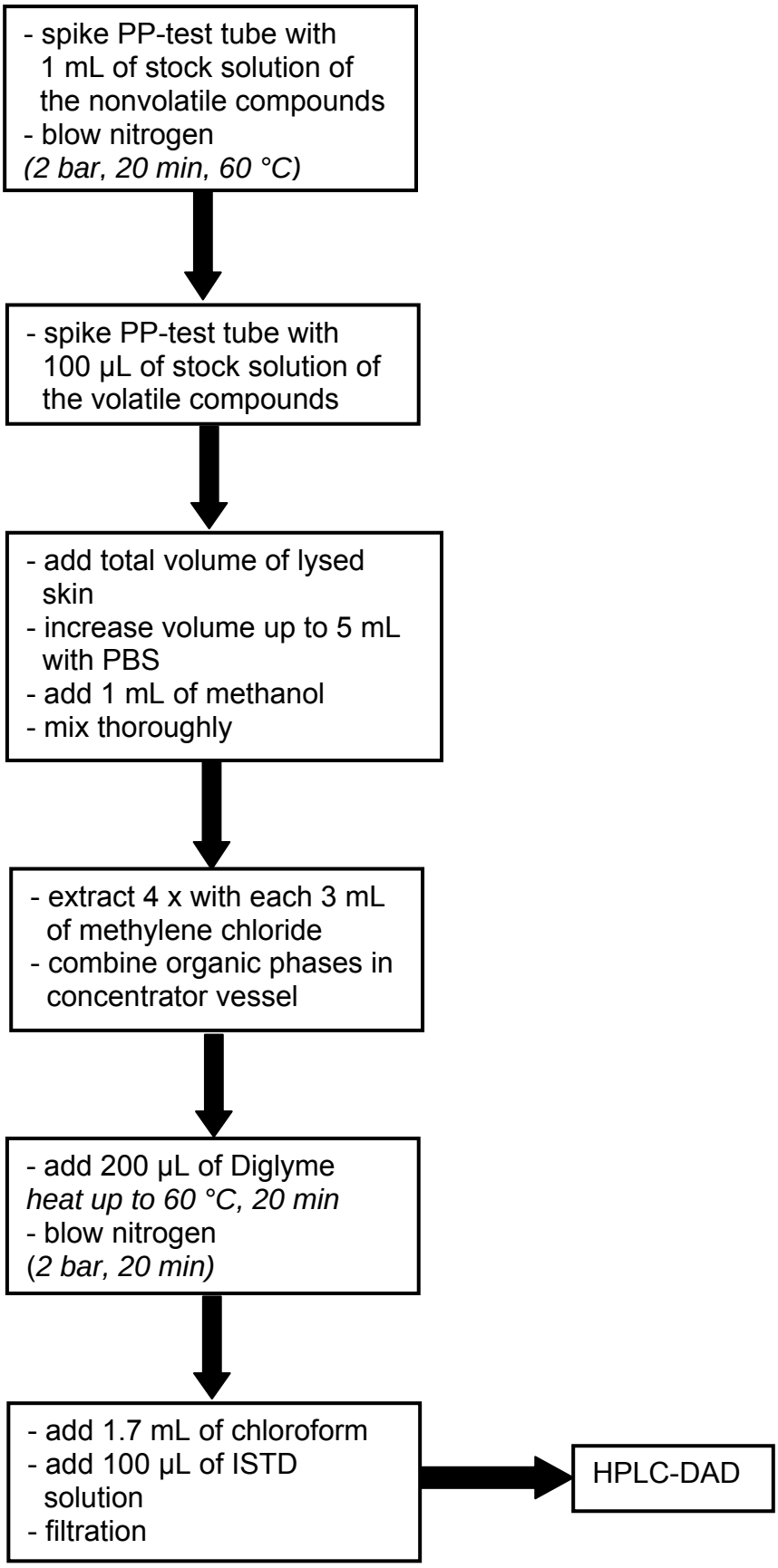


Figure 3: Flow scheme of the extraction from lysed skin.

HPLC Analysis. The samples were filtered using a PTFE filter (Chromafil, O-20/15, organic, pore size 0.2 mm; Machery-Nagel, Düren, Germany). A 10-µL sample was analyzed using a model 1100 HPLC (Agilent Technologies,Waldbronn, Germany) fitted with a C18 analytical column (Phenomenex Luna, particle size 3 µm, 150 x 4.60 mm, Aschaffenburg, Germany) and DAD. The data were analyzed using a HPLC-3D ChemStation Rev. B.01.01. Gradient elution was done with water (0.0059 w/v% trifluoroacetic acid) (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. A gradient profile with the following proportions of solvent B was applied [*t* (min), % B]: (0, 10), (20, 95), (50, 95). The chromatograms were monitored with wavelength switching [t (min), λ (nm)]: (0, 258), (17, 220), (19, 258).

The concentration of PR 22 and PR 9 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*.

1.3. Results and Discussion

Tattoos are popular because they are adornments; on the other hand, they can also show a variety of adverse reactions.[27–31] Besides adverse reactions, there is another risk factor regarding the colorants used for tattooing. Since these compounds are predominantly not produced for tattooing but are also ingredients of paints and varnishes, there are no specific declarations on the ingredients. The colorants consist of starting material and byproducts of the synthesis, titanium dioxide for the lightening of the colorant, and other unspecified compounds in different concentrations. On one hand there are regulations that relate to ingredients in paints and varnishes, but these regulations are different from those regulating cosmetics, foods, and drugs.

In Europe, many of the azo pigments used in tattoos such as PR 22 are not allowed in cosmetics since they can be cleaved, yielding carcinogenic amines.[32] In the United States, the FDA considers the inks used in intradermal tattoos, including permanent make-up, to be cosmetics and considers the pigments used in the inks to be color additives requiring premarket approval under the Federal Food, Drug, and Cosmetic Act. However, because of other public health priorities and a previous lack of evidence of safety concerns, FDA has not traditionally regulated tattoo inks or the pigments used in them. In addition, concerns raised by the scientific community regarding the pigments used in these inks have prompted FDA to investigate the safe use of tattoo inks. FDA continues to evaluate the extent and severity of adverse events associated with tattooing and is conducting research on inks.[33]

A major obstacle for a risk assessment of tattoo pigments is the fact that the amount of tattoo colorants in the skin is unknown. Therefore, the major goal of the present investigations was the development of a procedure that allows the determination of the concentration of tattoo pigments in the skin. First, an extraction method was established to separate the pigment molecule from skin constituents. To quantify the pigment concentration, the method has to be verified using recovery experiments. That is, a certain amount of pigments was added to water, PBS, or skin and the respective recovery rates were determined by HPLC.

Two widespread tattoo pigments (PR 22, PR 9) were used for the present investigations. However, tattoo colorants from tattoo studios exhibit a purity of usually less than 80% (area %, HPLC analysis, data not shown), which is useless for precise recovery experiments. Therefore, both pigments were synthesized, yielding a high purity (> 98%, area %, HPLC, data not shown) that is comparable to pharmaceutical grade.

In laser removal of tattoos, the pigments in the skin are irradiated with very high laser intensities leading to temperatures in the pigments higher than 400 °C. Previous investigations of our group showed that the two pigments, PR 22 and PR 9, are decomposed by laser light and the products could be identified as NT, MNA, NAS, DCB, DCA, and *m*-NAS.[15] During laser irradiation, the concentration of these products increased up to 70-fold. Therefore, the extraction method of pigments was extended to the respective laser-induced decomposition compounds.

Establishment of the Workup Scheme. The workup scheme was established representatively for one pigment (PR 22) and one decomposition product (NT). A 1-mL sample of PR 22 stock solution was diluted with 1 mL of methanol and 10 mL of methylene chloride to obtain the volume of extraction solution. Nitrogen was blown into the flask until PR 22 was dried (2 bar, 35 °C) (for conditions see Table 1, study 1).

Study 1 shows a high recovery rate for PR 22, but the value for NT was below 60% (Table 1). Due to the high volatility of the three compounds NT, DCA, and DCB, a special treatment during the workup of the extraction solution was necessary. High volatility of these substances is shown by their high vapor pressure: NT (0.4 hPa, 20 °C), DCA (0.057 hPa, 20 °C), and DCB (0.8 hPa, 20 °C). Thus, the keeper Diglyme was added to the extraction volume prior to solvent removal. A keeper is a low-volatile liquid and retains the volatile compound while the solvent can evaporate. Nevertheless, evaporation under a gentle stream of nitrogen and elevated temperature is necessary.

The conditions in study 2 with addition of Diglyme as keeper increased the recovery rate of NT up to almost 100%, with a good yield for PR 22. However, some PR 22 was adhered to the flask wall. In study 3, the temperature was increased to 60 °C and the flask wall was rinsed additionally with methylene

chloride after a first step of blowing nitrogen to increase the recovery of PR 22. Therefore, the flask was cooled to room temperature, rinsed with 2 mL of methylene chloride, and heated to 60 °C. To shorten the workup time only, the temperature was increased to 60 °C without rinsing with methylene chloride. Due to the higher temperature, the recovery rate of NT decreased to under 85% (studies 3 and 4). In study 5, the volume of keeper was doubled with the result of recovery rates for NT of > 90%. Using the conditions of study 5, the recovery rates and workup scheme are a reliable basis for quantitative extraction from water, PBS, and lysed skin later on.

study	com-pound	keeper [µL]	press. [bar]	temp. [°C]	time	recovery rate	comment
1	PR 22	-	2	35	60	96.9 ± 4.01	no keeper
1	NT	-	2	35	60	55.8 ± 7.46	no keeper
2	PR 22	100	2	35	60	90.4 ± 3.11	PR 22 sticks to flask wall
2	NT	100	2	35	60	104.0 ± 1.72	
3	NT	100	2	60	135	84.9 ± 3.56	rinse flask wall
4	NT	100	2	60	40	83.3 ± 0.87	
5	NT	200	2	60	40	96.5 ± 1.72	200 µL of keeper

Table 1: Modified conditions for the work up of the extracted compounds.

Recovery from Water or PBS. As a first step, the extraction scheme was established with water and PBS as precursors of lysed skin. The quantification of the tattoo pigments PR 22 with its starting material, decomposition products NT, MNA, NAS, and PR 9 with their starting material, and decomposition products DCB, DCA, and *m*-NAS was performed by HPLC. Therefore, the chromatography was calibrated for these six compounds (see Figure 4).

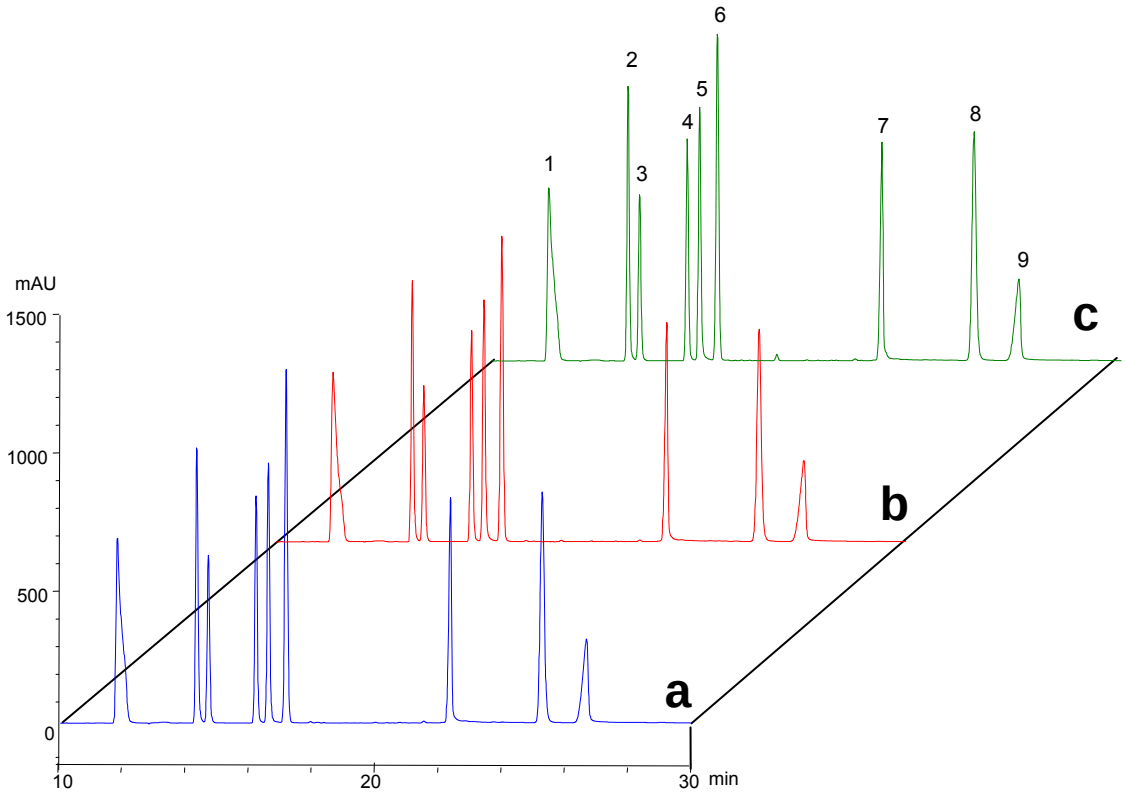


Figure 4: Standard HPLC chromatogram (a). Tattoo compounds extracted from water (b) and PBS (c): MNA (1), NT (2), DCA (3), m-NAS (4), NAS (5), DCB (6), PR 22 (7),10-DPA (8), PR 9 (9).

Except for DCB, the extraction method yielded high recovery rates for both solvents ranging between 92 and 100% (Table 2). The results show that the pigments and all decomposition compounds can be quantitatively extracted from an aqueous environment regardless of the different chemical properties. The recovery rate of DCB is also an acceptable value. Thus, the workup scheme seemed to be appropriate and the recovery experiments were performed for the skin.

Recovery from Lysed Skin. When people are tattooed, the pigments are injected into the dermis, which is the major part of the skin showing a thickness of up to 5 mm. After tattooing, a portion of the implanted pigment is taken up by phagocytes that attempt to remove the pigments from skin via the lymphatic or blood vessel system. However, much of the implanted pigment agglomerates into crystallites that are too large for phagocytic removal by macrophages. Therefore, a major part of the pigments remains intracellular in the dermis inside so-called secondary lysosomes.[34]

	water	PBS	lysed skin
PR 22	96.3 ± 3.06	93.2 ± 3.33	96.6 ± 1.41
NT	98.6 ± 8.46	92.3 ± 8.64	94.9 ± 1.98
MNA	99.5 ± 2.94	95.6 ± 2.71	91.5 ± 1.42
NAS	99.2 ± 0.73	95.1 ± 2.75	94.2 ± 1.34
PR 9	92.1 ± 1.82	94.9 ± 4.41	94.8 ± 1.92
DCB	74.5 ± 12.63	69.8 ± 13.96	86.6 ± 2.68
DCA	101.2 ± 4.37	99.7 ± 5.69	95.7 ± 2.22
m-NAS	92.9 ± 2.22	95.3 ± 5.75	94.8 ± 2.17

Table 2: Recovery rates (RSD, (n=3) of the red tattoo pigments PR 22 with its decomposition products MNA, NT and NAS and PR 9 with DCB, DCA and m-NAS.

To determine the amount of pigments in a complex matrix like human or animal skin, different methods can be applied. First, the matrix is completely disintegrated leading to a suspension. The pigment molecules are extracted from suspension and quantified by HPLC. Second, supercritical fluid extraction and microwave-assisted extraction is utilized. That has been recently performed to determine the concentration of harmful azo dyes in leather.[35,36] The amines are extracted and finally determined by HPLC with diode array detection. This extraction method can be managed with a small amount of organic solvents, but it provides hardly any information about the pigments used. Azo dyes are usually determined indirectly by measuring their corresponding amines, formed after chemical reduction by use of sodium dithionite or tin(II) chloride.[36]

Therefore, we decided to disintegrate the skin and all cellular structures such as the secondary lysosomes, which may contain the pigment crystallites. The lysis of human skin was tested using reagents with different pH. First, complete lysis of human skin is possible using NaOH[37] or pepsin in 10% HCl. Lysis with 5 M NaOH is a quite simple and effective method. However, in combination with

high temperature (50 °C), the tattoo pigments are cleaved and the structural information is lost. In addition, the treatment of skin with pepsin in 10% HCl leads to a satisfying tissue degradation, but the low pH makes the extraction of pigments impossible. Therefore, the disintegration of skin at neutral pH was applied.

Our extraction scheme provides a degreasing and extraction method of human skin for determining the azo compound, corresponding amines, and other decomposition products. The advantage of our established degradation is the sensitivity; skin is dissolved without destroying the chemical substances. The degradation is divided in several steps. High temperature is used for denaturing the proteins. Collagenase breaks down collagen and elastin and sets cells free from the extracellular matrix. Proteinase K, an endolytic protease, cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic, or hydrophobic amino acids. The smallest peptide to be hydrolyzed is a tetrapeptide. Thus, proteinase K destroys cell proteins resulting in damaged cell membranes. Finally, the ATL buffer also contributes toward tissue lysis. These four steps guarantee a complete and gentle lysis of human skin without destroying the structure of the molecules that should be extracted quantitatively.

As mentioned above, the pigments of a real tattoo are intracellularly localized. Complete disintegration of the cells in the skin is proven by Trypan blue staining. Trypan blue penetrates through damaged cell membranes into the cytoplasm of dead cells. Its anions bind to cell proteins and stain the cells blue. Living cells exclude the dye and appear in the microscope transparent. The results of lysed skin were verified and compared with living NHDF cells (bright under the microscope) and NHDF cells incubated with trypsin (blue under the microscope). On the microscope slide with the lysed skin, no bright and no blue-stained spots (<5%) could be seen. That evidenced the total lysis of human skin tissue. No individual cells showing a typical cellular shape were detected. In contrast, 98 ± 3% blue stained cells were detected upon trypsin treatment of NHDF cells, revealing nonviable cells containing a damaged cell membrane but retaining their cellular shape.

For the extraction of chemical substances from human or animal tissue, several methods are described in the literature. The recovery for pontamine sky blue and evans blue from skin has been performed for the measurement of capillary permeability. Nitta et al. recovered pontamine sky blue from the skin of adult male albino rabbits with recovery rates from 72.5 to 93.5% depending on the injected concentration.[38] Ankier et al. extracted pontamine sky blue from mouse pinna with over 96% (96.1 ± 4.7%).[39] Harada et al. recovered azovan blue (evans blue) with recoveries of 96% and trypan blue with > 90% from the skin of rats.[40] These three groups based their calculations on photometric measurements of the supernatant of extracted skin. Liquid-liquid extraction and photometric measurements was the chosen method of the following groups for the recovery of evans blue and pontamine sky blue. Suzuki and Arai extracted evans blue from skin of adult albino rabbits with 96.84% recovery.[41] Katayama et al. also used evans blue for recovery experiments from skin of guinea pigs and rats (95%).[42] Humphrey recovered evans blue from rat skin in almost complete recovery.[43] The recovery of pontamine sky blue could be optimized to almost 96% and of evans blue to almost quantitiative recovery rates.

However, the advantages of pontamine sky blue and evans blue are their known chemical and physical properties and their high solubility in aqueous solutions. In contrast to that, the pigments PR 22 and PR 9 are declared as insoluble, showing a poor solubility in organic solvents such as dichloromethane and chloroform. At the same time, the recovery must operate for the respective decomposition products, which are partially volatile.

Yeganeh and McLachlan recovered terbinafine from skin at ~ 60%, from liver, adipose, and muscle at more than 76%.[37] Villain et al.[44] established a procedure to screen for benzodiazepines and hypnotics in human hair. The extraction recovery of these compounds ranged from 32 to 76%. Kim et al. demonstrated an analysis of hair samples of cannabis abusers. They established an extraction method for the detection of cannabidiol, cannabinol, and Δ-9-tetrahydrocannabinol with recovery rates from 37.9 to 94.5%.[45] Gratacos-Cubarsi et al. detected residues of sulfamethazine, a sulphonamide chemotherapeutic agent, in cattle and pig hair. Recovery rates varied from 70 to 85%.[46]

Using the disintegration method of the skin and our extraction scheme, we were able to recover PR 9 at ~ 95% and PR 22 at ~ 97% (see Table 2, Figure 5). The recovery rates of the decomposition products ranged from about 87 to 96%. In light of the very different chemical properties of the substances to be extracted, the whole procedure seems to be suitable to determine the concentration of pigments and the laser-induced decomposition products in skin. Our extraction scheme is suitable for both, the poorly soluble pigments and the highly volatile decomposition products. Combining both demands, we present a challenging way to determine the resulting amount of these compounds in skin. The recovery rates are in each case leading or comparable with recovery rates of other working groups.

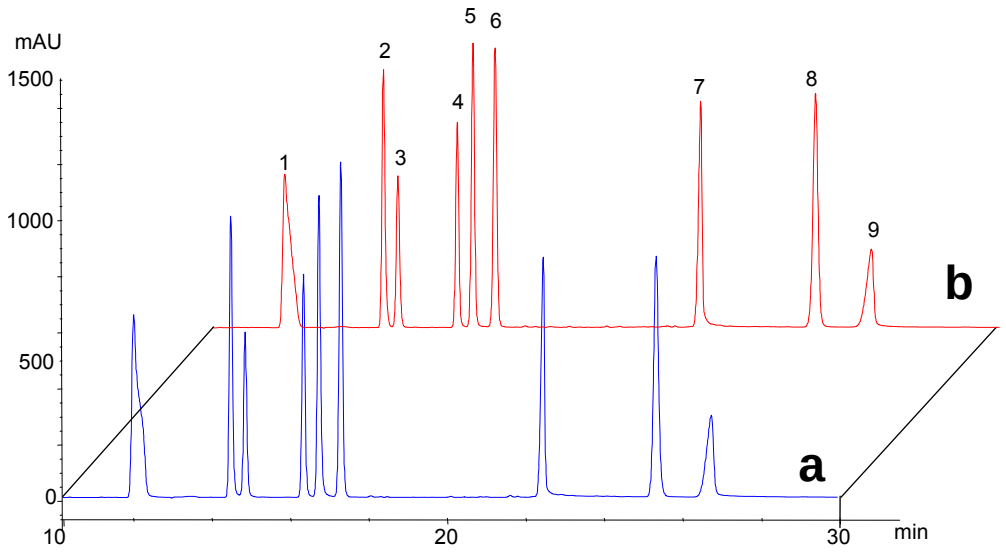


Figure 5: Standard HPLC chromatogram (a). Tattoo compounds extracted from lysed skin (b): MNA (1), NT (2), DCA (3), m-NAS (4), NAS (5), DCB (6), PR 22 (7),10-DPA (8), PR 9 (9).

1.4. Conclusions

Despite the poor solubility of the azo pigments and the high volatility of some of the decomposition products, our method provides an effective scheme for the extraction of tattoo pigments from human skin. Our extraction results in the recovery of all nonvolatile and volatile compounds, except for DCB, from water, and PBS shows recovery rates of more than 91%. The RSD (n = 3) for extraction of the nonvolatile compounds from all matrixes was in the range of 1.34 - 5.75%. These values demonstrate the reliability of the extraction and workup method. The volatile compounds NT, DCB, and DCA show higher RSD (n = 3), because of their high vapor pressure. Extraction of DCB from water and PBS could be obtained with RSD (n = 3) 12.63 and 13.96%. Nevertheless, extraction from lysed skin resulted in 86.5% with RSD (n = 3) of 2.68%. For the extraction from lysed skin, the RDS (n = 3) of each compound does not exceed 3 %. Thus, we have established a reliable extraction scheme of tattoo pigments and their decomposition products from water, PBS, and lysed skin as the basis for the extraction of pigments from real tattoos.

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2. Modern Tattoos Cause High Concentrations of Hazardous Pigments in Human Skin*

2.1. Introduction

In recent years, the number of tattooed individuals has increased significantly,[1,2] especially among young people.[3] In the United States, up to 24% of the population are tattooed,[2] whereas in European countries like Germany approximately 9% of the population and about 12% in the United Kingdom have tattoos.[4,5] Nowadays, azo pigments are frequently used for tattooing because of their colour intensity and their longevity. However, azo pigments are primarily manufactured for other purposes such as printing, the painting of cars, and the staining of various consumer products. Tattoo colorants are mixtures of pigments (colour) and multiple other ingredients. These colorants usually contain titanium dioxide for lightening the shade,[6] precursors and by-products of pigment synthesis, as well as diluents that are used for pigment suspension.[7,8] Tattoo colorants are also applied for permanent make-up on eyelids, eyebrows, and lips.[9]

Despite the high incidence of tattoos worldwide, no common legal requirement for listing ingredients has been introduced so far. In Europe, many azo pigments employed in tattoos (e.g. Pigment Red 22) are not allowed for use in cosmetics because they may be decomposed yielding carcinogenic amines.[10]

In the process of tattooing, pigment suspension is deposited in the dermis by piercing the skin with tiny solid needles that are moistened with tattoo colorant. On closer examination, tattooing is a complex procedure that includes various risks for the skin and maybe even for the human body. Pigments and impurities may cause adverse skin reactions at the site of the tattoo.[11-22] In addition, part of the colorants are transported to other anatomical locations such as lymph nodes.[23,24] Laser light could cleave pigments in the skin during tattoo removal[25] or pigment decomposition may be caused by ultraviolet radiation during solar

* Results of this chapter are submitted: Engel, E.; Santarelli, F.; Vasold, R.; Maisch, T.; Howard, P.C.; Ulrich, H.; Prantl, L.; König, B.; Landthaler, M.; Bäumler, W. *Br. J. Dermatol.* **2007**
* Sample preparation was done by F. Santarelli.

light exposure; both procedures have been known to cause hazardous compounds such as carcinogenic amines.[26]

To estimate the risk of any health problems that tattooing might involve for the skin, the pigment concentration in tissue should be determined – a procedure that has not been attempted so far.

2.2. Materials and Methods

Pigments. The red tattoo pigment PR 22 (C.I. 12315, CAS 6448-95-9) was either synthesized in pure quality (> 98%)[27] or purchased as original tattoo pigment (purity ~ 80%, data not shown).[7] The pigments were suspended in concentrations of 10% (w/v) and 25% (w/v) in a vehicle of 10% of glycerol (87%, Merck) in water (Milli-Q® Ultra-pure Water-Purification System, Millipore) with the addition of 100 µL of isopropanol as solubility enhancer.

Skin. Pigskin was purchased from a local butchery. Human skin was obtained from skin excisions for other reasons. Excision sites were either abdomen or upper arms. The fatty tissue was removed; skin thickness measured approximately 3 to 4 mm depending on the excision site. Researchers used the tattoo machine, type "*new lightning*" (Deep Colours GmbH, Germany), and typical tattooing needles ('liners, shaders') to inject the colorant into the skin. On round needles ('liners'), tips are arranged in a circle, whereas flat needles ('shaders') have linear tips. All needles were solid needles with either four flat (4F), four round (4R), eight flat (8F), or eight round (8R) tips. Tattoo artists tend to use round needles with nine tips (9R). Both needles and the tattoo machine are frequently used for tattooing worldwide.

Tattooing. We applied different methods for skin tattooing. In method (A), researchers tattooed pigskin either with synthesized PR 22 or with commercial PR 22 (method B). In method (C), professional tattoo artists tattooed pigskin with synthesized PR 22. In method (D), researchers tattooed human skin either with commercial PR 22 or with synthesized PR 22 (method E).

A rectangular skin area of about 1 by 3 cm was tattooed (Figure 1a). We made three punch biopsies measuring 5 mm in diameter, extracted the pigment of these samples separately and calculated the mean value for the resulting three values.[27] The concentration of skin pigment was calculated as follows: Using a 5 mm circular punch biopsy, the skin volume is a cylinder with a skin area of 19.63 mm^2 times the height of the cylinder. However, only skin material stained with tattoo pigment may be used for analysis. Hence, we histologically determined the cylinder height in order to be able to calculate the pigmented material for each sample. For this height determination, we performed an

additional punch biopsy taken from each specimen using standard histological staining (H&E) and light microscopy. The concentration of skin pigments was calculated as the ratio of the amount of extracted pigment and the volume stained by the pigment (see Figure 1c). For better depiction, we then calculated the pigment concentration as amount of pigment per cm^2 of tattooed skin. For each needle and applied pigment concentration, experiments were performed in triplicate and results were averaged. The use of skin was approved by the local IRB.

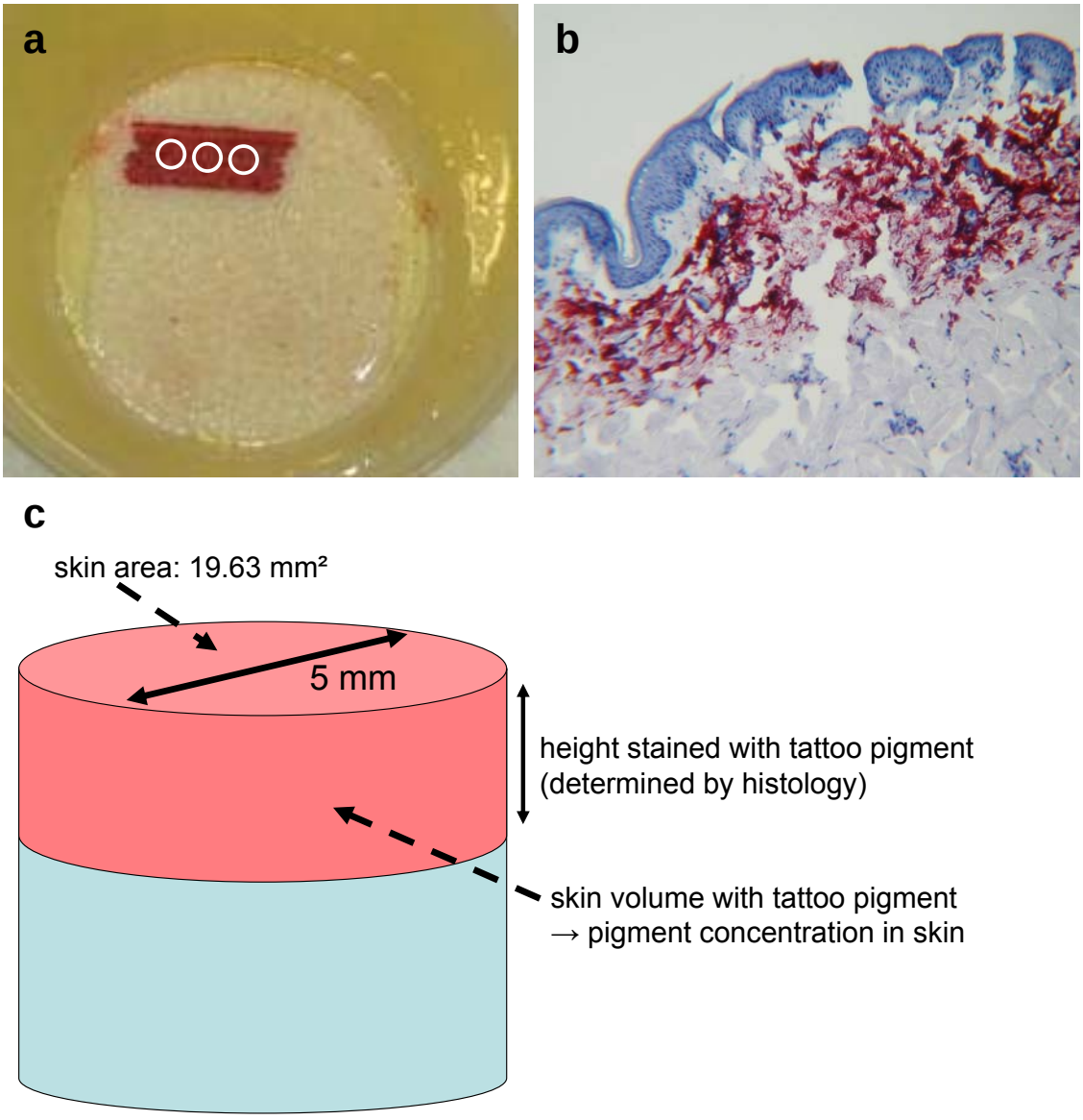

Figure 1: Skin specimen and histology. The images show recently tattooed pigskin in a stainless steel holder (a), a histological slide of tattooed pigskin (b), and a sketch that illustrates the determination of pigment concentration in skin (c). The white circles in (a) indicate the site of the three punch biopsies.

Disintegration and Extraction. In contrast to our previous report[27] and according to Gaber et al.[28], we inserted each sample in 400 µL of PBS (Phosphate Buffered Saline, Biochrom) at 95 °C for 20 min. After the samples had cooled to room temperature, we added 180 µL of tissue lysis buffer (buffer ATL) and 15 µL of Proteinase K (QIAGEN). Samples were stirred at 55 °C for 30 min until complete lysis of the tissue. Each process was carried out as previously described.[27] The concentrated residue was reconstituted in methylene chloride (LiChroSolv®, Merck). We used transmission electron microscopy (TEM) for evaluating pigment size and shape.

2.3. Results and Discussion

Based upon a very recently established procedure,[27] we quantitatively extracted pigments from tissue after tattooing and, for the first time, determined their respective concentration in the skin. However, the investigation of tattooing on a scientific level represents a challenge owing to the large variety of tattooing procedures available. Therefore, we used different pigment suspensions and different needles, and both researchers and tattoo artists performed human and pigskin tattooing. This laborious procedure should help to avoid the generation of random values for pigment concentration in skin. Since red tattoo pigments frequently cause allergic skin reactions,[11] we used the widespread red pigment PR 22 in our experiments.

Usually, vertically vibrating needles are used for tattooing that inject pigments into the skin with an initial penetration depth of up to 2 mm. Special machines produce this vibration at a frequency of about 30 Hz. Needles exhibit different shapes and number of tips. For tattooing we initially used original tattoo colorants from the tattoo market.[7] However, these colorants usually exhibit a purity of less than 80%. Since these impurities may affect the precise recovery experiments, we additionally synthesized PR 22 in a high purity of about 98%.

To determine the pigment concentration in skin, we first used pigskin that is available in a standardized manner at all times. After performing the same experiments with human skin, we compared extraction results to pigskin experiments. To consider different concentrations of pigments as applied in routine tattoo practice, we used PR 22 at concentrations of 10% (w/v) or 25% (w/v).

Synthesized PR 22 in Pigskin. For synthesized PR 22, values for pigment concentration are shown in Table 1 (method A) as amount of pigment per 1 cm^2 of tattooed skin. Values range from 0.63 mg/cm^2 to 5.19 mg/cm^2 depending upon the different concentration applied to the skin as well as the type and shape of needles used. Histology showed the depth of tattoo pigments to depend upon skin properties like surface tension. In accordance with our previous report,[27] relative standard deviation (RSD) does not depend upon the

digestion and extraction scheme but on the properties of the applied pigment suspension.

method	needle size	applied concentration (w/v)	amount per tattooed area [mg/cm^2]	RSD [%]
A	8R	10%	0.63	13.5
	8R	25%	1.42	7.8
	4R	10%	1.75	5.9
	4R	25%	5.19	15.8
	8F	10%	1.02	30.0
	8F	25%	2.60	21.6
	4F	10%	2.49	4.9
	4F	25%	3.44	13.4
B	8R	10%	1.90	32.9
	8R	25%	3.59	14.1
	4R	10%	2.90	45.3
	4R	25%	9.42	11.8
C	9R	25%	0.60	14.7
D	8R	25%	0.95	23.9
E	8R	25%	1.69	7.4
mean value			**2.53**	**17.9**

Table 1: Concentrations of pigments in skin. The amount of PR 22 deposited in 1 cm^2 pigskin and human skin each. Researchers obtained concentration values in experiments with (A) synthesized and (B) commercial PR 22 in pigskin. The values in (C) stand for experiments performed by tattoo artists using synthesized PR 22 in pigskin. The values for human skin are displayed using commercial (D) or synthesized (E) PR 22. Relative standard deviation (RSD) is given for each experimental setting. The last line of the table shows the respective mean of the values for each different setting.

A needle in a group of four tips (4R) results in higher values because the area covered by one puncture of 4R is smaller than the area covered by 8R, i.e. more needle injections are necessary for tattooing a certain area of skin when using 4R. Flat needles with 8 tips (8F) result in slightly higher values than 8R. Flat needles show the same correlation between the number of tips and the amount of tattoo pigment injected into the skin.

Comparison of Synthesized and Commercial PR 22. The injection of commercial PR 22 resulted in higher amounts of pigment in the skin as compared to synthesized PR 22 (Table 1, conditions A and B), although the commercial colorant contained not only pigment but also impurities up to 20%. Azo pigments tend to agglomerate requiring additional procedures after synthesis. Thus, chemical companies optimise their manufacturing processes that leads to a lower aggregation susceptibility.[29]

The pigment synthesized in our laboratory[30] was highly pure PR 22 and did not receive any further treatment. This could explain the different agglomeration and aggregation of primary crystallites. These differences are shown in the TEM pictures (Figure 2) of commercial or synthesized PR 22 with different mean particle diameters of about 154 nm and 202 nm respectively. Hence, the commercial and our synthesized pigments showed a different sedimentation behaviour in suspension. We measured a decrease in pigment concentration by 30% in the supernatant of suspension for the synthesized sample, whereas, in the commercial sample, the concentration remained unchanged.

This difference suggests a different amount of pigment attached to the needle when dipped into such suspensions. This clearly affects the concentration of pigments injected into the skin but should reflect the various conditions in routine tattooing. The mixture of ingredients in tattoo colorants is neither regulated nor standardized. Despite these facts, the resulting concentrations of pigments in skin are in a confined range regardless the methods used for tattooing (see Table 1).

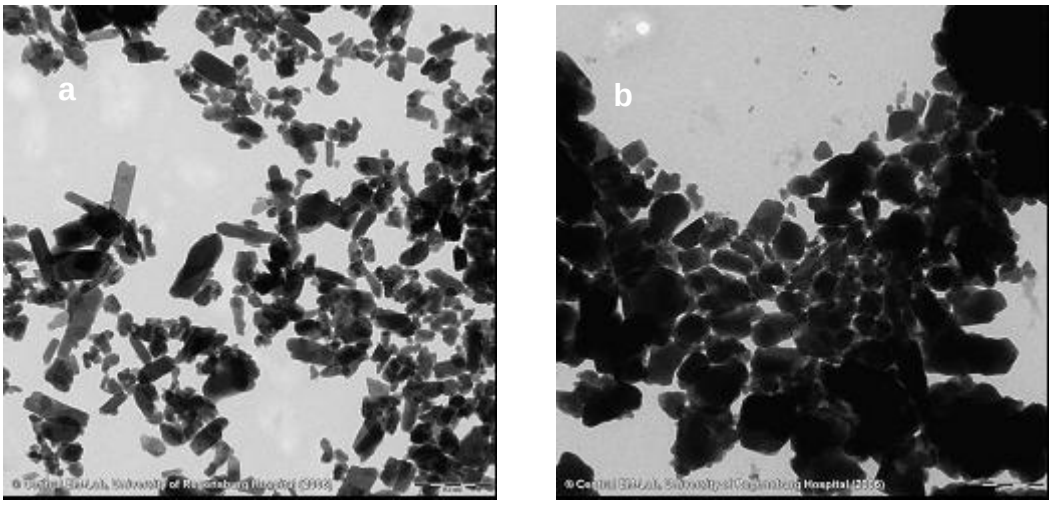

Figure 2: Size and shape of pigments. Transmission electron microscopy (TEM) images of commercial (a) and synthesized (b) PR 22 (Scale bars = 500 nm).

Tattoo Artists Tattoo Pigskin. To compare our scientific procedure with real tattooing, two professional tattoo artists were asked to tattoo pigskin with a suspension of pure PR 22 in the concentration of 25% (w/v) using their own equipment including machine and needles. Table 1 (method C) shows less pigment concentrations in the skin tattooed by tattoo artists but still comparable to the values achieved by researchers.

Human Skin. We tattooed various specimens of human skin from different patients. The pigment concentrations determined by extraction matched the range of PR 22 that had been tattooed into pigskin (Table 1, methods D and E). This provides evidence that the nature of tattooed skin has only a minor effect on pigment concentration in skin.

2.4. Potential Health Problems

For the first time, the concentration of tattoo pigment in skin has been determined ranging from 0.60 to 9.42 mg/cm^2. Values and their RSD depend upon the different methods used in our experiments, which should reflect the different conditions in the daily practice of tattooing.

Regardless the different methods used, these values yield an overall mean concentration of 2.53 mg of pigment in 1 cm^2 of tattooed skin (Table 1). Thus, about 253 mg of azo pigment PR 22 are deposited in the dermis for a typical tattoo covering a skin area of 100 cm^2. This is an alarming fact because, in Europe, many azo pigments such as Pigment Red 22 are forbidden to be used in cosmetics, which are only applied to the skin surface.[8] This prohibition is based upon the assumption that such pigments are to some extent absorbed by skin. This absorption may cause health problems, in particular when carcinogenic amines are formed owing to the decomposition of azo pigments. However, in tattooing, hundreds of milligrams are directly injected into the skin.

The medical literature contains multiple case reports[16] on adverse skin reactions after tattooing such as cutaneous pseudolymphoma,[15] granulomatous tattoo reactions,[12,17] allergic reactions,[19,21,22] pseudoepitheliomatous epidermal hyperplasia,[13] and even non-melanoma skin cancer[14,17] or malignant melanoma[18,20] - albeit skin cancer is assumed to occur only occasionally. The extent to which these adverse reactions are caused by pigments, by other ingredients, or by impurities including bacteria or viruses remains unclear. Unfortunately, physicians are not obliged to report health problems caused by tattoo colorants. As a first step, dermatologists could report on the incidence and the possible reasons of skin problems after tattooing.

Long-term health problems in either skin or other organs could be evaluated by epidemiological studies that are lacking so far. More detailed information on this topic is important since, according to our calculation, the frequent use of azo pigments has only started 10 to 15 years ago. Such studies are definitely necessary to assess whether health problems caused by tattoo colorants are only individual cases without any relevance for public health. However, if tattooing involves any major health risk, it could affect not only the skin but also

other organs because of the transportation of colorants inside the human body. The exact mechanisms of transportation and the extent of pigment transportation are yet unknown. Thus, pigments are injected into the skin and are transported inside the body similar to medical drugs, which necessitate years of clinical trials with regard to possible side effects and health risks. However, unlike medical drugs, these colorants do not have an established history of safety use.[7,8]

In our investigation, we aimed to determine the concentration of a typical tattoo pigment in the skin immediately after tattooing. This is an important step towards risk assessments with regard to potential health problems caused by tattoo pigments, in particular for the skin. Since approximately 20% of colorants represent impurities, these substances can be included in risk assessment. Other risks may result from the possible light-induced decomposition of tattoo pigments in skin. The ultraviolet part of the solar light spectrum may decompose tattoo pigments as shown for Pigment Yellow 74.[26]

Many tattooed individuals change their mind and request the removal of their tattoo after some time. A widespread method of tattoo removal is the application of short laser pulses at high intensities.[31] Laser light penetrates the skin and is selectively absorbed in pigments. The high absorption coefficient of pigments and the high laser intensities lead to temperatures well above 400 °C.[25] In previous investigations, we showed pigments PR 22 and PR 9 to be decomposed by laser light. The products of this decomposition were identified as 4-nitrotoluene, 2-methyl-5-nitroaniline, naphthol AS, 1,4-dichlorobenzene, 2,5-dichloroaniline, or methoxy-naphthol AS[25], which are proven to be toxic or even carcinogenic. In the worst case, each pigment deposited in the skin is decomposed into carcinogenic amines during laser light exposure. Thus, more research is necessary in order to find out if this decomposition causes any major health problems for tattooed individuals.

2.5. References

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3. Photochemical Cleavage of a Tattoo Pigment by UVB Radiation or Natural Sunlight*

3.1. Introduction

UVB radiation (280 - 320 nm) is absorbed well by many biological macromolecules such as proteins, lipids and DNA. The transformation of radiant energy into photochemical energy can be damaging to the cell.[1] When UVA radiation is absorbed by tissue, reactive oxygen species (ROS) such as oxygen radicals or singlet oxygen are produced. These, too, can damage cellular components leading to premature aging of the skin or skin cancer.[2]

In addition to the endogenous substances in the skin, exogenous materials can absorb UV radiation. These can include pigments applied into the skin as a tattoo or permanent make-up (PMU). On the one hand, tattoos can serve to willingly isolate an individual from society; on the other hand, in recent times decorative tattoos and permanent make-up (tattooed eyeliner, eye shadow and lip contours) have become enormously popular. In the USA 16% of the population possess tattoos; the numbers are similar in Europe. According to a survey by the Demoscopic Institute of Allensbach in 2003, about 9% of the population in Germany have at least one tattoo, among younger people (age 16 – 29 years) 23% (Table 1).[3] In recent years the number of people with tattoos has further risen.

In the past, inorganic pigments such as titanium dioxide (white), cadmium sulfide (yellow), chromium oxide (green), cadmium selenide (red) and iron oxides (black) were employed.[4] Today, mostly dye-based pigments are used for colored tattoos. Chemical analyses have shown that these include industrial organic pigments such as azo dyes or polycyclic compounds.[5] These pigments are usually used to dye or paint consumer goods (for example, car paints). The tattoo artist enjoys using these pigments, because they are very durable and almost insoluble and thus provide for a brilliant, permanent tattoo. For

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* HPLC analysis was performed by A. Spannberger in her Zulassungsarbeit.

permanent make-up, mineral pigments are also used in addition to organic pigments. Black tattoos can consist of a mixture of pigments giving a very dark color. In the simplest case, inks based on carbon or iron oxide can be employed.

Tattoos und Piercings

Question: „Do you have a tattoo?“
Question: „Are your pierced?“ (without earlobe piercing)

German Population

	total %	16-29 years %	30-44 years %	45-59 years %	> 60 years %
I have one or more tattoos	**9**	23	10	6	2
I am pierced	**6**	26	5	1	-

population 16 years or older in percent

source: Allensbacher Archiv, IfD-Umfrage 7046

Table 1: Results of the survey of the Demoscopic Institute Allensbach from August 28th to September 9th 2003 (number of respondents: 2126, representation: Germany, population 16 years and older, archive-number of the survey: 7046; source: Körperkult bei den Jüngeren: Tattoos und Piercings, Institut für Demoskopie Allensbach, Allensbacher Berichte, Nr. 24, 2003, 1-4)

Once introduced into the skin, these pigments form small crystals usually located intercellularly, as histological studies have shown.[6,7] In the case of organic pigments, UV radiation in the range of 250 - 400 nm can be absorbed by these compounds, especially UVB. Just as with endogenous absorbers, the absorbed energy can be transformed into heat or cause a photochemical reaction. The generation of heat plays a key role in the removal of tattoos. Short impulses of light of a few nanoseconds in duration and light intensities in the megawatt range heat the pigment crystals to high temperatures, destroying them and even cleaving individual molecules.

UVB radiation is considered high energy light which can initiate many photochemical processes. Therefore, some time ago the National Center for Toxicological Research (NCTR), commissioned by the Food and Drug

Administration (FDA), examined cleavage of tattoo pigments by UV radiation. UVB-induced cleavage of a popular yellow pigment (Pigment Yellow 74: PY 74) *in vitro* was found.[8] Toxic decomposition products were identified with likely involvement of reactive oxygen species. Red tattoo pigments can be involved in toxic allergic or granulomatous skin reactions.[9–12] One of the most common red pigments is Pigment Red 22 (PR 22) which we have already examined with regard to laser-induced cleavage.[13] In this study, possible photochemical cleavage of this red pigment, which has appropriate absorption in the UVB range, was examined using chromatography (HPLC) and mass spectrometry. Using the same methods in a long-term experiment, the effects of sunlight on this pigment were also studied.

3.2. Materials and Methods

Pigments and Chemicals. Pigment Red 22 (PR 22, CAS 6448-95-9, C.I. 12315) is a widely used azo dye belonging to the group of naphthol-AS dyes. It is synthesized by azo coupling according to Cook et al.[14] and after purification displays a purity of over 98% (area %, data not shown[15]). This purity is comparable to pharmaceutical purity.

Preparation of the Solutions. Highly purified PR 22 was dissolved in tetrahydrofuran (0.19 mg/mL [THF, p.a., Merck, Darmstadt, Germany]), dioxane (0.06 mg/mL [p.a., Merck, Darmstadt, Germany]), dichloromethane (0.2 mg/mL [HPLC Gradient Grade, Mallinckrodt Baker, Deventer, The Netherlands]) and chloroform (0.20 mg/mL [LiChrosolv, Merck, Darmstadt, Germany]).

Reference Substances. Reference substances for PR 22 were dissolved in acetonitrile in the following concentrations: 2-methyl-5-nitroaniline (0.25 mg/mL [99%, Aldrich Chemical Company, Inc., Milwaukee, USA]), 4-nitrotoluene (0.25 mg/mL [> 99%, Fluka, Buchs Switzerland]) and naphthol-AS (0.1 mg/mL [99%, Sigma- Aldrich, Steinheim, Germany]).

UVB Exposure. For UVB exposure precision test tubes of quartz glass (SUPRASIL, Hellma, 110-QS, thickness 2 mm, Müllheim, Germany) were filled with 600 µL pigment solution. To reduce oxygen partial pressure, the tubes were rinsed before and after filling with argon gas for 10 minutes. The tubes were closed securely and exposed to a broad band UV lamp (280 - 320 nm, intensity 0.0015 W/cm^2, Type UV 800, Waldmann Lichttechnik, Villingen-Schwenningen, Germany) at a distance of 25 cm. The samples were irradiated for at least 4 hours or to a color change from orange to yellow. For each irradiated sample, a reference was incubated in the dark at 4 °C.

Sunlight Exposure. A 1.0 mL portion of the pigment solution was filtered using a PTFE filter (Chromalfil, O-20/15, organic, pore size 0.2 µm, Machery-Nagel, Düren, Germany) into an injection flask (DAB- 10 quality, Chromatographiehandel Müller, Fridolfing, Germany). To reduce oxygen partial pressure, the injection flasks were also rinsed with argon gas for 10 minutes before and after being filled. The injection flasks were securely sealed and

placed on the inside of a window on the west side of the building in natural sunlight for 110 days (May - August 2005). For each sample a reference was kept at 4 °C in the dark.

Preparation and HPLC Analysis. For HPLC analysis all samples were filtered using a PTFE filter and injected into a modular HPLC system (Hewlett- Packard Ltd., Waldbronn, Germany). The system consists of an HP 1050 quaternary pump (Mod. Nr. 79852AX), an HP 1050 autosampler (Mod. Nr. 79855A), an HP 1050 4-channel online degasser (Mod. Nr. G1303AX), an Agilent 1100 column thermostat (Mod. Nr. 61316a) and an Agilent 1100 photo diode array detector (Mod. G1315b, Agilent Technologies Ltd., Waldbronn, Germany). Evaluation was done by the ChemStation Version HPLC-3DChemStation Rev. A.08.04 (1008). The mixture to be analyzed was separated by a C18 column (Phenomenex luna, particle size 3 µm, 150 x 4.60 mm, Aschaffenburg, Germany). A binary eluent mixture consisting of water (0.0059% [w/v] trifluoroacetic acid) (eluent A) and acetonitrile (eluent B) was pumped with a constant flow of 1.0 mL/min. The following gradient profile was used (t [min], % B: [0, 10], [20, 95], [50, 95]). The chromatograms were registered at 258 nm. The injected volume was 10 µL.

LC/MS online Coupling. Using a triple stage mass spectrometer (TSQ 7000, Thermoquest Finnigan, Toronto, Canada) the masses of the compounds, especially the UVB- and sunlight-induced decomposition products, were determined.

3.3. Results and Discussions

A research team of the National Center for Toxicological Research (NCTR) of the FDA recently showed that a pigment (PY 74) in solvent is cleaved into toxic products photochemically when exposed to UV radiation in a sunlight simulator.[8] These authors concluded form their *in vitro* study that these substances can be generated in tattooed skin when this skin is exposed for a longer period of time to sunlight or other sources of UV radiation. This could also be the case for medical UV radiation sources.

Many organic pigments used in tattooing or for PMU do indeed absorb light from the UV spectrum. In order to estimate a possible risk of photochemical cleavage of these pigments in the skin, certain factors must be known. One is the dose of UV radiation on the tattooed skin. Further, how deep the radiation penetrates the skin must be known. Considering the absorption coefficient of the pigments in this spectral range, the energy available for photochemical cleavage can be estimated. Knowledge of the pigment concentration in the skin and thus the amount of possible decomposition products would be a further basis for estimating the possible health risk.

In this study, we sought to determine if the widely used Pigment Red 22 can be photochemically cleaved by UVB radiation or even sunlight. At present, it is not possible to perform this experiment in skin. Therefore, a quantitative study in solutions is a first and indicative step. Most pigments are poorly soluble and, in the skin, exist as small crystals. Such chemical analyses are best done using solvents capable of adequately dissolving these pigments. The solvents should not themselves influence photochemical cleavage of the pigments, i.e. they should be inert. Pigment Red 22 is an azo dye out of the group of naphthol-AS pigments, used in the chemical industry as a dye, for example, in paints. The use of this pigment on the skin surface is not allowed by the cosmetic laws (Annex IV of the Cosmetics Directive of the EU).[16] Due to lack of legal regulation, such pigments can be used for tattooing or PMU (trade name e.g. Devil's Red). As such pigments are produced for industrial purposes, they can display impurities of 10% or more. These impurities can disturb chemical analytic tests. For this reason, PR 22 used in this study was synthesized in

highly purified form at the Institute or Organic Chemistry. Analysis of the synthesized pigment using HPLC revealed a purity of > 98%.

Red naphthol-AS pigments display clear absorption maxima in the ranges 300 – 330 nm and 480 - 580 nm,[5] almost reaching the high degree of light absorption of metals. Due to this strong absorption of PR 22 in the UVB range, studies on photochemical cleavage were done using a UVB radiation source with emission from 290 - 320 nm (Figure 1).

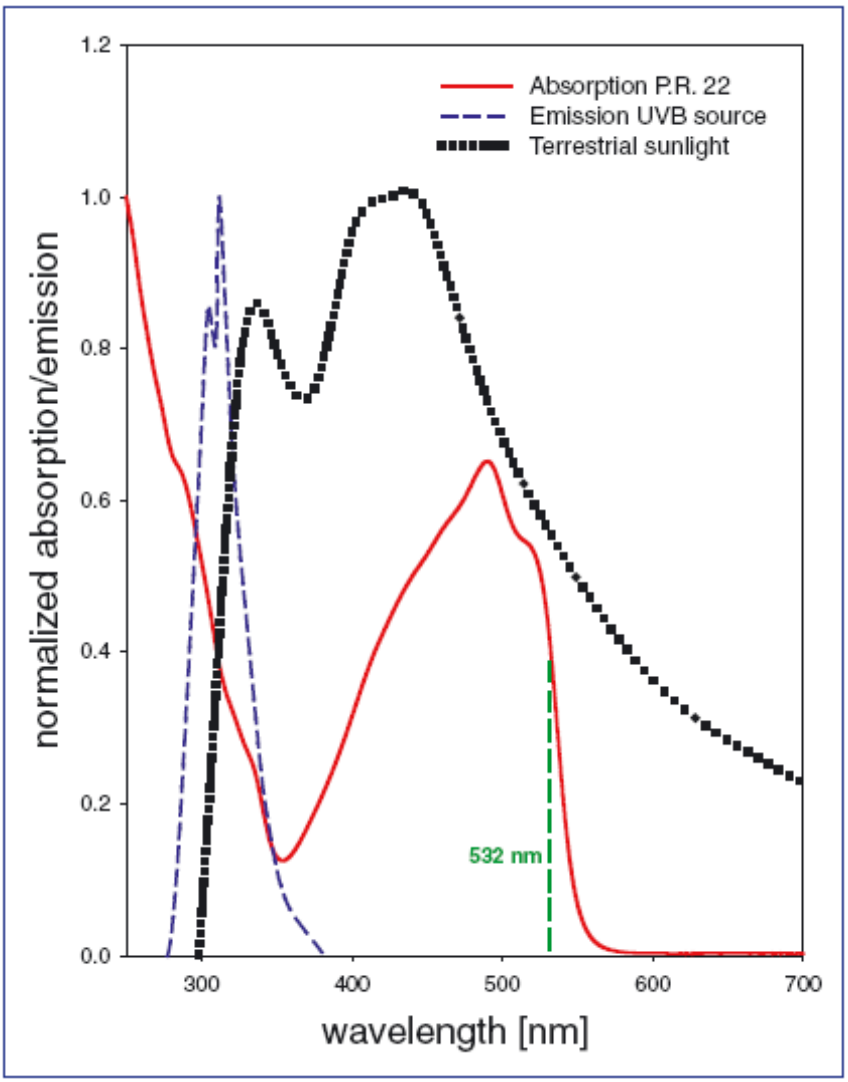

Figure 1: The emission spectrum of the UVB-lamp is overlaid on the absorption spectrum of PR 22. The spectral distribution of natural sunlight is added for comparison (taken from: http://www.lichttechnik.tu-berlin.de). The green line indicates the wavelength (532 nm) of the frequency-doubled Nd:YAG Laser. All spectra are normalized to their respective maximal value.

In comparison to the UVB experiments, the dissolved pigment was also exposed to natural sunlight in a long-term experiment. In both irradiation experiments the solvents THF and dioxane (cyclic ethers) as well as dichloromethane and chloroform as halogenated solvents were used. These solvents were chosen as thay could dissolve the poorly soluble azo pigments in a quantifiable manner. To do so, the pigment was dissolved in various solvents up to the maximal possible concentration. Past studies by our group have shown that both common tattoo pigments PR 22 and PR 9 can be cleaved by laser light and that toxic and carcinogenic products can occur, among them 4-nitrotoluene (NT), 2-methyl-5-nitroaniline (MNA), naphthol-AS (NAS), 1,4-dichlorobenzene (DCB), 2,5-dichloroaniline (DCA) and methoxy-naphthol-AS (m-NAS) (Figure 2).[13, 17–21] During laser irradiation, the concentration of several of these substances increased up to 70-fold.[13] We also examined the study samples for these typical decomposition products.

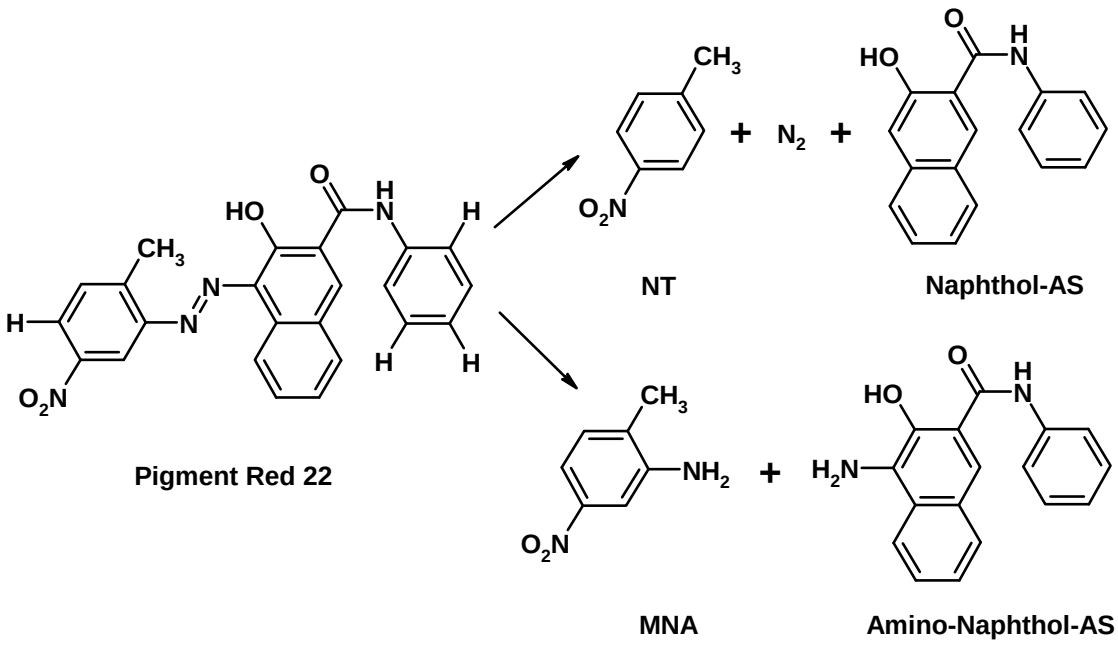

Figure 2: Chemical structure of PR 22, chromophoric pigment in Cardinal Red. For the pigment a possible decomposition pattern and the possible decomposition products are shown. Additional change of the decomposition products by oxidation is possible.

Analysis of the UVB irradiated Pigments.

Radiation intensity was measured before each experiment and was 1.5 mW/cm^2. The duration of irradiation of each pigment solution was 4 - 8 hours. That corresponds to a radiation dose of 21.6 - 43.2 J/cm^2. Duration of irradiation was chosen so that an optically visible color change in the pigment solution was observable and thus the analysis for photochemical decomposition products would be successful.

All samples were analyzed by HPLC and LC/MS coupling; resulting decomposition products could be detected and identified (Table 2). Pigments in the various solvents (dark controls), which were stored for the duration of UVB irradiation (2.5 - 8 hrs) in darkness at 4 °C were analyzed as references.

solvent	duration of UVB irradiation [hrs]	decomposition products	remaining amount of pigment [%]
THF	2.5	MNA, NT	23
dioxane	4	MNA, NT	4
$CHCl_3$	4	MNA, NT	98
CH_2Cl_2	8	NT	80

Table 2: PR 22 – decomposition products and remaining amount of pigment after UVB-radiation.

Solvent THF. The most reactive solvent was THF, which use resulted in cleavage into the two products MNA and NT. After only 150 min UVB irradiation, the color of the solution changed from orange to yellow (Figure 3, Table 3). At the same time, the amount of pigment declined to about 23% of the original amount in a non-irradiated, fresh reference sample. Even in the dark control (150 min darkness) traces of the cleavage product NT could be detected analytically.

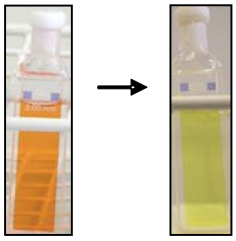

Figure 3: PR 22 – color change in the solvent THF after 150 min of UVB radiation. This results in a dose of 13.5 J/cm^2. Color changes from orange to yellowish.

Solvent Dioxane. In the solvent dioxane PR 22 is also instable and is cleaved to NT and MNA. For a color change from orange to yellow 240 min UVB irradiation is necessary (Table 3). After this time about 96% of the original amount of pigment was cleaved. In the dark control (240 min darkness) the decomposition products NT and MNA could not be detected.

color change

none	**slow**	**rapid**
PR 22 in $CHCl_3$ 240 min (21.6 J/cm^2)	**PR 22 in dioxane 240 min (21.6 J/cm^2)**	**PR 22 in THF 150 min (13.5 J/cm^2)**
PR 22 in CH_2Cl_2 480 min (43.2 J/cm^2)		

Table 3: PR 22 – relative velocity of the color change in different solvents after UVB radiation.

Solvent Chloroform. PR 22 dissolved in chloroform was irradiated with UVB for 4 hours, the color remained a constant orange and only 3% of the original pigment was cleaved (Table 2). Nonetheless, both cleavage products NT and MNA were produced in small quantities. In the dark control (240 min darkness) no decomposition products or changes in the amount of pigment were found.

Solvent Dichloromethane. Even after 8 hours of UVB irradiation PR 22 in dichloromethane showed no bleaching of color (Table 3). The amount of pigment sank to 80% of the original amount and NT could be detected as a decomposition product. The dark control (480 min darkness) showed no trace of decomposition products or change in amount of pigment.

Analysis of the Pigment Solutions after Sunlight Exposure.

In addition to the UVB light source, samples of PR 22 in the four solvents THF, dioxane, chloroform and dichloromethane were exposed to sunlight for 110 days. Table 4 shows the color change of PR 22 in THF after 110 days as an example. The original orange color was totally bleached until a colorless solution resulted. Even in the halogenated solvent, the samples were colorless after 110 days exposed to sunlight (Figure 4). These illustrations show that natural sunlight is capable of destroying tattoo pigments.

color change

slow → **rapid**

PR 22 in $CHCl_3$	PR 22 in CH_2Cl_2	PR 22 in dioxane	PR 22 in THF
110 days	50 days	32 days	10 days

Table 4: PR 22 - relative velocity of the color change to colorless in different solvents after exposition to solar light for 110 days. The time in the figures indicates the duration of the color change to colorless.

Figure 4: PR 22 – color change in the solvent dichloromethane after exposition to solar light for 110 days. Color changes from orange to colorless.

Solvents THF and Dioxane. PR 22 in THF and dioxane is cleaved completely and the resulting decomposition products are further cleaved. At the end, no substance at all, neither tattoo pigment nor decomposition products, could be detected. Sunlight causes complete mineralization in these solvents (Figure 5).

In the dark control (110 days darkness) the pigment was also cleaved and both cleavage products could be identified. In THF only about 6% of the original amount of pigment remained, in dioxane 17%. The results of the dark controls suggest a definite additive effect due to the solvent.

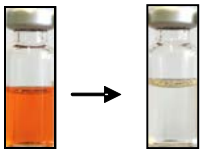

Figure 5: PR 22 – color change in the solvent THF after exposition to solar light for 110 days. Color changes from orange to colorless.

Solvents Chloroform and Dichloromethane. Cleavage was not as progressed in the halogenated solvents, so that the decomposition product NT could be identified (Table 5, Figure 4). The remaining amount of pigment was far below 10% of the original amount. In the dark control (110 days darkness) no decomposition products or changes in the amount of pigment were found. It can therefore be assumed that the destruction of pigment was only induced by sunlight.

Sunlight has a significantly broader band than the UVB radiation employed (Figure 1). The pigment absorbs very well in the UVB range, also contained in sunlight, but there is also strong absorption in the visible spectrum. To remove this red pigment a laser at 532 nm could also be utilized.[22]

solvent	natural sunlight [days]	decomposition products	remaining amount of pigment [%]
THF	110	---*	0
dioxane	110	---*	0
$CHCl_3$	110	NT	3
CH_2Cl_2	110	NT	0.3

* no longer detectable

Table 5: PR 22 – decomposition products and remaining pigment after exposure to solar light.

Comment on the Solvents.

As most pigments are nearly insoluble, solvents need to be found that can produce solutions of these pigments of adequate concentration. The search for appropriate solvents for UV experiments was motivated by the work of Howard.[8] His group used THF for incubation of PY 74 in simulated sunlight and could identify many decomposition products.

In the research for this study, it was important not only to generate and demonstrate various decomposition products, it was important to exclude influences not stemming from UV irradiation or natural sunlight. Effects of the solvents are of particular importance in this regard. Therefore, THF and dioxane are less adequate; chloroform and dichloromethane are most suitable.

The results show that both processes, UVB exposure and exposure to natural sunlight, are capable of cleaving the examined tattoo pigment. We could demonstrate without doubt, that UVB irradiation or sunlight can destroy the tattoo pigment and lead to the formation of the same toxic and carcinogenic decomposition products as the use of laser light.

In solubility tests on PR 22, THF as well as dioxane, chloroform and dichloromethane were suitable to dissolve quantifiable amounts of the pigments. Incubation of PR 22 in the four solvents for 110 days in darkness at 4 °C shows that they are only of limited suitability for UV studies. The cyclic

ethers THF and dioxane were so reactive, that even without UV irradiation a portion of the pigment was destroyed. THF and dioxane are thus not suitable for photochemical studies of the pigment, as it cannot be established which reactions are mediated by the solvent and which are purely induced by UV radiation.

The halogenated solvents chloroform and dichloromethane, in contrast, have no influence on the stability of the pigment and the amount of dye remained unchanged during 110 days in darkness. Both solvents are well suited for photochemical studies of pigments *in vitro*.

Comment on the Radiation Doses.

Unfortunately, as far as we know, no scientific reports on the chemical stability of tattoo pigments in the skin after light exposure exist. Patients do report repeatedly of fading of tattoos or even almost total disappearance, especially in cases of PMU. These reports are, unfortunately, not subject to scientific analysis.

The light doses employed may at first appear somewhat high. They therefore have to be correlated to natural UVB radiation on earth or to medical-therapeutic light doses (Table 6). Depending on the angle of incidence of the sun and geographic latitude, highly variable UVB light intensities reach the skin and the tattoo pigments therein. The cumulative UVB dose in Germany is about 330 J/cm^2.[23] In the treatment of psoriasis, artificial UVB light sources are employed that can apply an average cumulative dose of 20 J/cm^2 in the tattooed area in a matter of a few weeks.[24]

In comparing the dose of natural or medically applied UVB light with the dose used in our *in vitro* experiments, the following must be kept in mind: histology shows that the pigment occurs in crystalline from usually intracellularly in the dermis. These pigment crystals are found at a depth of about 0.25 - 1.7 mm in the papillary as well as the reticular dermis.[6] The intensity of UVB radiation decreases dramatically at these depths. The effect of UVB light is probably limited to pigment in the papillary dermis. Due to the very high absorption coefficient of the pigments, the little amount of UVB which reaches the pigment

is absorbed very well. The light-induced destruction of pigment in the skin naturally occurs in a much more delayed manner than Table 6 suggests.

pigment solution: original color: red	**color after irradiation**	**duration of UVB irradiation [hrs]**	**dose [J/cm²]**	**equivalente sun exposure* [days]**
PR 22 in THF	yellow	**2.5**	13.5	15
PR 22 in dioxane	yellow	**4**	21.6	24
PR 22 in $CHCl_3$	orange	**4**	21.6	24
PR 22 in CH_2Cl_2	orange	**8**	43.2	48

*** in relation to the total annual dose of UVB in Germany of abount 330 J/cm^2 [23] and assuming that the spectral emission of the lamp corresponds to the UVB of the sun.**

Table 6: Comparison of the duration of UVB-radiation with the theoretical duration of solar light exposure in Germany.

3.4. Conclusions

Our results show for the first time that the tattoo pigment PR 22 is chemically altered when exposed to sunlight or broad band UVB radiation. For *in vitro* studies, the solvents chloroform and dichloromethane appear most suitable, as they adequately dissolve the pigment and hardly affect the light-induced cleavage process. For PR 22, the decomposition products MNA and NT postulated from laser treatment could be detected. Further degradation of the detected decomposition products cannot be ruled out. The toxicological assessment based on available literature shows that both MNA and NT can have cumulative effects on the organism and can be toxic on inspiration, swallowing or contact with the skin. MNA is also a mutagen and appears in category 2 of cancer-causing substances. NT also possesses genotoxic potential. To which extent these results apply to the *in vivo* situation must be clarified by further studies.

An assessment of the health hazard cannot be made on the basis of the current data. Toxicity and carcinogenicity of chemical compounds as well as their light-induced decomposition products depends, among other factors, on their concentration in the skin. Considering the large number of people with tattoos that spend time in the sun or receive medically indicated UVB therapy, it is important to determine the concentration of tattoo pigments in the skin in order to evaluate the associated risk. Further studies on tattooed skin are needed.

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4. Tattoo Pigments in Skin: Concentration, Transportation and Light Induced Decomposition of an Azo Pigment using SKH-1 Mouse Model*

4.1. Introduction

Puncturing tattoo pigments into the skin can be compared with an injury of the upper skin layers. As soon as the pigments are injected into the dermis they are recognized by the body as foreign particles. By means of phagocytosis, the tattoo pigments are removed from the site of tattooed skin and are transported by the lymphatic system to other anatomical locations. As described in literature lymph nodes located next to the tattoo show frequently black pigmentation mimicking metastatic malignant melanoma or positive sentinel lymph node in melanoma.[1,2]

Based upon sentinel node biopsy, these pigmented lymph nodes are removed and analyzed by histopathology. However, histopathological examination of the lymph nodes does not detect metastases. In such cases, the pigmentation of the lymph nodes is caused by decorative tattoos of the skin area next to these lymph nodes. In addition, exposure of pigments in the skin to solar light or laser radiation during tattoo removal can cause decomposition of the pigments yielding hazardous compounds such as carcinogenic amines.[3-5]

To investigate transportation of tattoo pigments after tattooing, we established an animal model using SKH-1 hairless mice. The mice received tattoos with Pigment Red 22 (PR 22) on their back. The extraction of pigments immediately after tattooing yielded the concentration of pigments that is placed in the body. In contrast to that, the extraction of pigments from skin six weeks (42 days) after tattooing should elucidate the extent of pigment transportation in the mice. To investigate the decomposition of pigments by laser or solar light, laser irradiates tattooed skin (*ex vivo*) or living mice are exposed to solar light for 31 days.

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* Tattooing of the mice was performed by P.C. Howard.
* Samle preparation was done by F. Santarelli.

4.2. Materials and Methods

Pigment. Highly pure PR 22 was synthesized via azo coupling according to Cook et al.[6] and purified by slurring in acetonitrile (purity > 98%, area %, HPLC, data not shown). The starting material for the synthesis of PR 22 was naphthol AS (NAS; 99%, Sigma-Aldrich, Steinheim, Germany) and MNA (99%, Aldrich Chemical Company, Inc., Milwaukee, USA).

PR 22 was suspended leading to 25% (w/v) emulsion that is composed of 10% glycerol in water. The vehicle was sterilized by passage through a 0.2-µm filter prior to use.

Animals. Female Crl/SKH-1 (hr^-/hr^-) hairless mice were obtained from Charles River (Boston, MA) at 5 weeks of age. The mice were housed for 2 weeks in the NCTR Quarantine facility and acclimated in the animal room for 1 week prior to use. The treatment of the mice conformed to Animal Care and Use Committee guidelines at this American Association for Laboratory Animal Careapproved facility.

At 8 weeks of age, mice were anesthetized intraperitoneally with sodium pentobarbital (25 mg/kg body weight) prior to treatment. The mice were tattooed with PR 22. The tattoos were made by a single pass longitudinally on the dorsum with a 14-pt long-tapered tattoo needle (AIMS Inc, Hornell, NY) using a commercial tattoo machine (AIMS Inc). The tattoo device was adjusted to allow exposure of only ~1 - 2 mm of the needle tip beyond the barrel guide. Gentle pressure was used to facilitate penetration of the needle into the skin resulting in deposition of the pigments in the dermis. The mice received four single pass ‘‘stripes’’ (Figure 1a). Five mice were asphyxiated using carbon dioxide at 1 day post tattooing (Figure 2a).

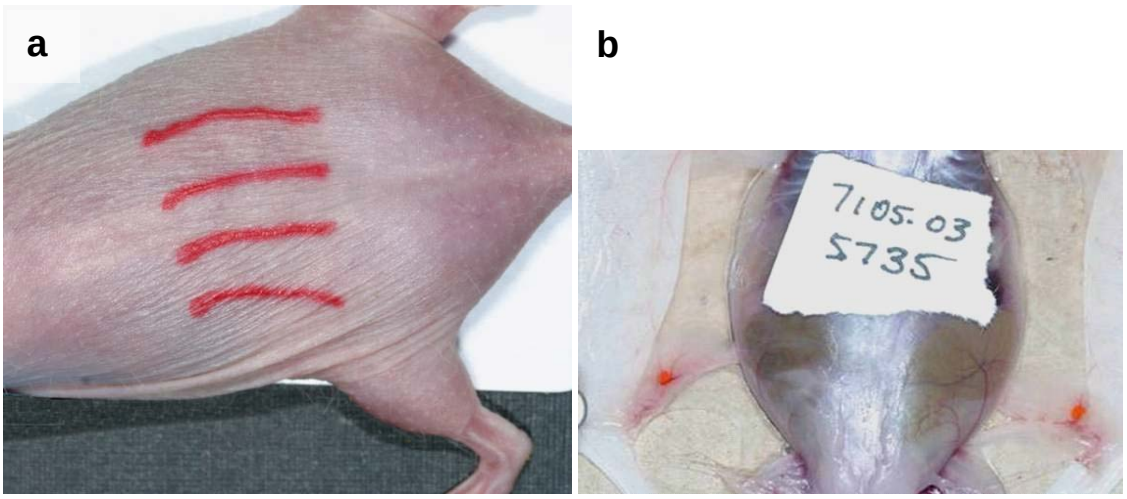

Figure 1: Mice were tattooed with highly pure synthesized PR 22 (25% w/v) as shown by the four single pass tattoo ‘‘stripes’’ (a). PR 22 has been transported to the lymph nodes causing a reddish coloration (b).

Exposure to Simulated Solar Light (ssl). The remaining 14 mice were held for 2 weeks to allow the tattooed skin to recover, then half of the mice were exposed to simulated solar light (1.4 SED/day ssl) for 4.5 weeks (31 days) while the remainder were held without light exposure. The same sacrifice procedure was repeated after 4.5 weeks (Figure 2b).

Tattooed skin area was dissected at each time point and frozen at – 80 °C to store for further preparation.

Exposure to Laser Light. Excised stripes of mice held without light exposure were exposed to a frequency-doubled Nd:YAG laser (Wavelight, Erlangen, Germany) at a wavelength of 532 nm, which is absorbed in PR 22 (Figure 2c). The tattooed stripes were irradiated with a pulse duration of 6 ns yielding a total radiant exposure of 165 J/cm^2.

Preparation and Disintegration of the Skin Samples. One stripe per animal was sampled by a punch (∅ = 5 mm) and disintegrated based on the steps as previously reported.[7]

Extraction. The extraction and work up was performed based on the recent investigations (chapter 2.2.).

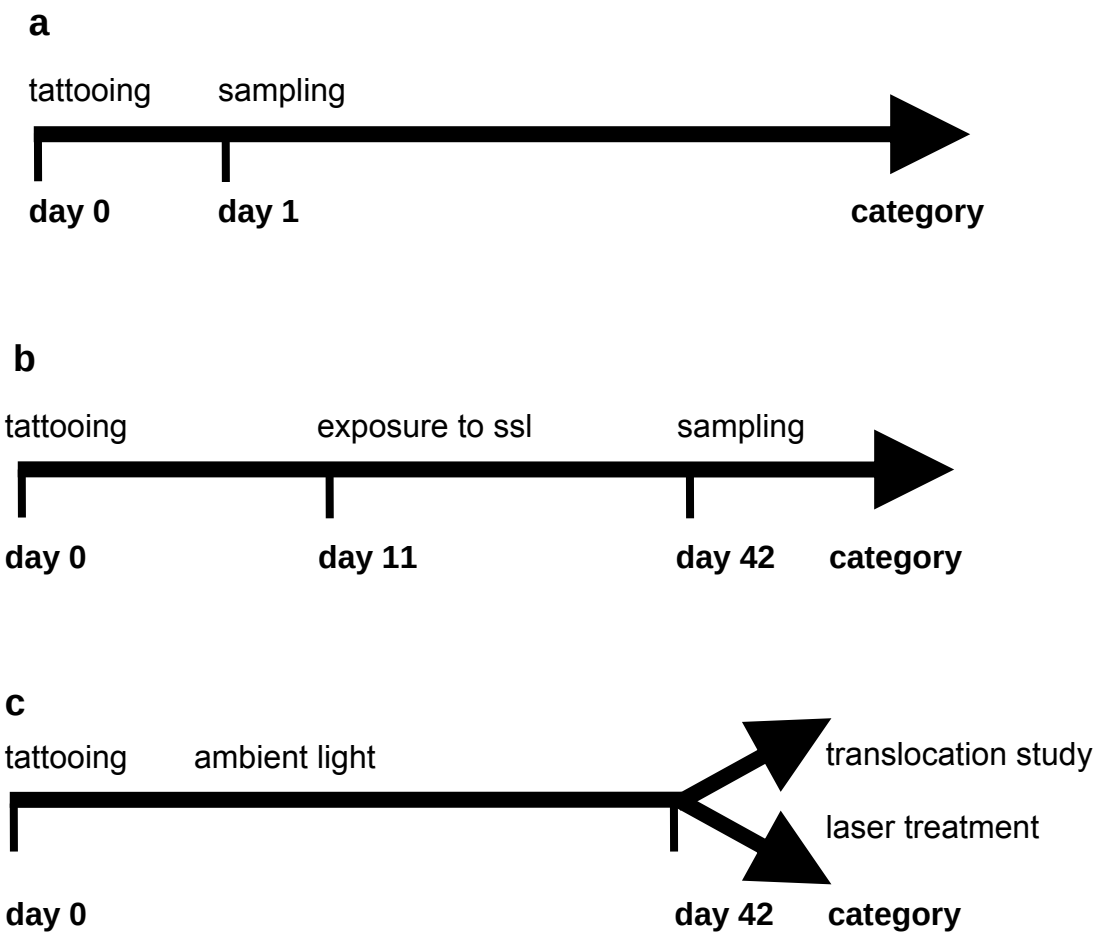

Figure 2: The time scale shows the points for tattooing, sampling, exposure to simulated solar light (ssl) and laser treatment. One day after tattooing the amount of PR 22 punctured into skin can be determined (a). Eleven days after tattooing seven mice were exposed to ssl until day 42 (b). The seven remainder were held with ambient light until day 42 (c). One stripe per animal was used for the determination of the translocation study, the other stripe was irradiated by laser light.

HPLC Analysis. The samples were filtered using PTFE-filter (CHROMAFIL®, O-20/15, organic, pore-size 0.2 µm, Machery-Nagel, Düren, Germany). 20 µL were analyzed using a HPLC model 1100 (Agilent Technologies, Waldbronn, Germany) fitted with a C18 analytical column (Phenomenex luna, particle size 3µm, 150 x 4.60 mm, Aschaffenburg, Germany) and DAD. The data were analyzed using a HPLC-3D-ChemStation Rev. B.01.03. Gradient elution was done with water [0.0059 (w/v%) trifluoroacetic acid] (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. A gradient profile with the following proportions of solvent B was applied [t (min), % B]: (0, 10), (20, 95), (50, 95). The chromatograms were monitored with wavelength switching [t

(min), λ nm]: (0, 258), (11.85, 228), (12.70, 280), (15.77, 228), (19.60, 280), (24.50, 266).

The concentration of PR 22, 2-methyl-5-nitroaniline (MNA), 4-nitrotoluene (NT) and Naphthol AS 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.

4.3. Results

Based on a very recently established procedure,[7] we extracted quantitatively pigments from skin tissue 1 day and 42 days after tattooing and determined the respective concentration of PR 22 remaining in the skin after transportation. We tattooed mice skin using a widespread tattoo pigment PR 22 (high pure quality, > 98%, area %, HPLC, data not shown) with a concentration of 25% (w/v) and with a 14-pt long-tapered tattoo needle (Figure 2a).

Extraction after 1 Day. Five samples were extracted and resulted in an average amount of 34.53 µg per punch (RSD 35.8%; Table 1). As previously shown, the RSD does not depend on the extraction mode, but on the tattooing process. Mainly in this case manually tattooing and tattooing of living animals causes a certain deviation. We calculated a value of 138.12 µg per stripe and 552.48 µg per tattooed animal (4 stripes per animal).

condition	amount per punch [µg]	amount per stripe [µg]	amount per animal [µg]	RSD [%]
1 day	34.53	138.12	552.48	35.8
42 days	24.86	99.44	397.76	24.4
42 days + laser	12.82	51.28	205.12	11.6
42 days + solar light	9.87	39.48	157.92	60.8

Table 1: The values show the amount of PR 22 extracted after 1 day, after 42 days without laser light, after 42 days with laser light and after 42 days with solar light. The respective relative standard deviation of the values (RSD) is added to the table.

Extraction after 42 Days. Skin samples of seven mice were punched and analyzed. The average amount of PR 22 per punch was 24.86 µg (RSD 24.4%, Table 1), which is less as compared to extraction after the first day. We calculated a value of 99.44 µg per stripe and 397.76 µg per tattooed animal (4 stripes per animal). The comparison with the extraction after 1 day exhibits a pigment reduction of 28% during the 42 days (Figure 3).

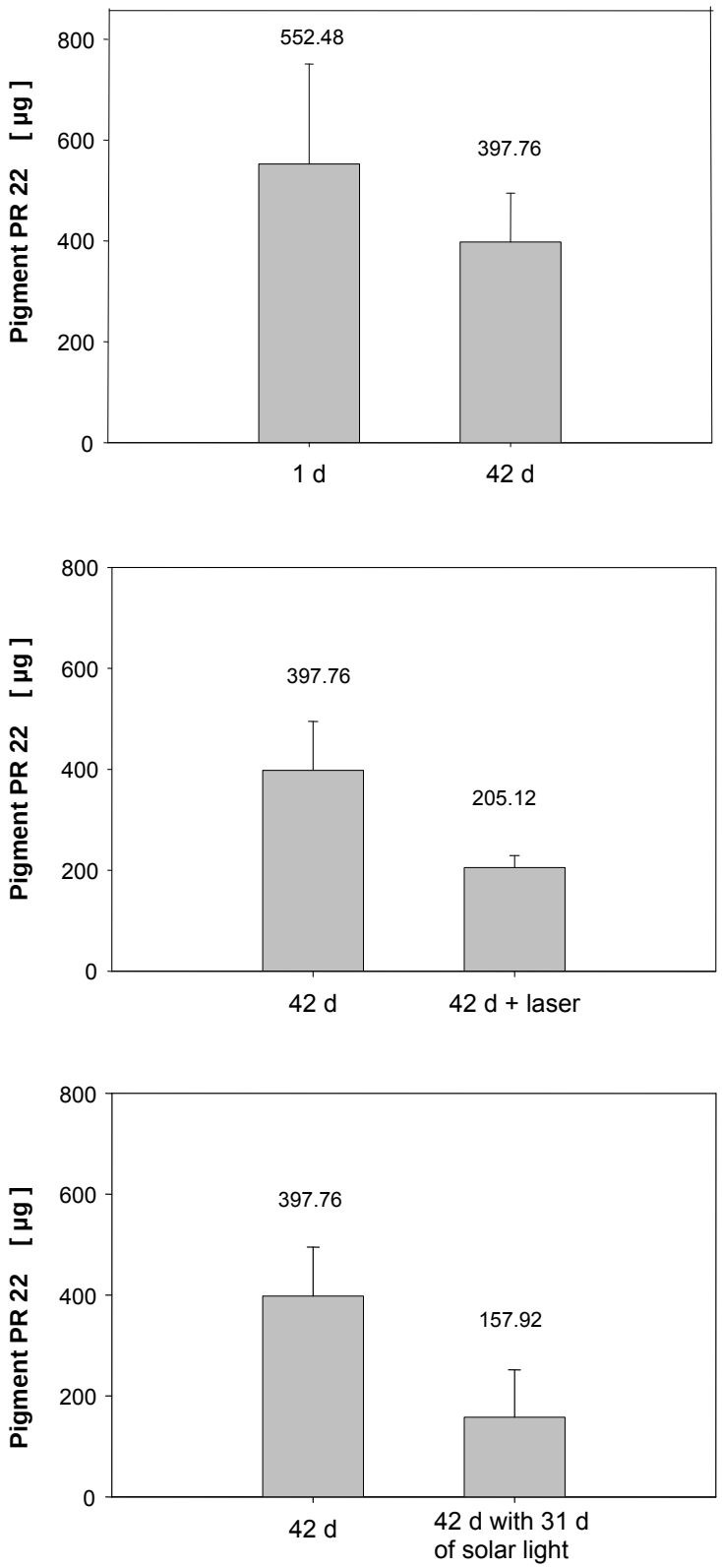

Figure 3: The values show the total amount of PR 22 in the mouse skin 1 day or 42 ays after tattooing (top). The exposure to laser light (middle) or solar light (bottom) of the mouse skin caused pigment decomposition. This is shown by the decrease of the amount of extracted pigment as compared to the untreated control.

Extraction after 42 Days and Laser Irradiation. After laser irradiation, seven punches were extracted and resulted in an average amount of PR 22 with 12.82 µg per punch, 205.12 µg per animal respectively (RSD 11.6%, Table 1). Thus, the laser irradiation reveals a reduction of pigment mass of 48.4% as compared to the samples without laser (Figure 3). This is a difference of 192.64 µg of PR 22 per animal that should be cleaved by laser light. That means 192.64 µg (4.52×10^{-7} mol) of PR 22 are available for decomposition into MNA, NT and NAS.

The decomposition products were also extracted from the skin samples after laser irradiation. NAS could be detected with 0.11 µg per punch (RSD 14.8%, Table 2), MNA with 0.24 µg per punch (RSD 23.0%, Table 2). NT was extracted from 5 punches with 0.11 µg per punch (RSD 28.5%, Table 2).

In one tattooed mouse, laser irradiation yielded NAS with 1.76 µg (6.69×10^{-9} mol), MNA with 3.84 µg (2.53×10^{-8} mol) and NT with 1.76 µg (1.28×10^{-8} mol). Thus, only 8% of the decomposed PR 22 was directly detected with its products MNA und NT.

decomposition product	amount per punch [µg]	amount per stripe [µg]	amount per animal [µg]	RSD [%]
NAS	0.11	0.44	1.76	14.3
MNA	0.24	0.96	3.84	23.0
NT	0.11	0.44	1.76	28.5

Table 2: The values show the amount of the laser induced decomposition products NAS, MNA, and NT. The respective relative standard deviation of the values (RSD) is added to the table.

Extraction after 42 Days with Solar Light Exposure. After tattooed skin of live mice had been irradiated for 31 days, the extraction of PR 22 and its decomposition products was performed. The results show that 9.87 µg PR 22 per punch were remaining (average of 7 punches, RSD 60.8%, Table 1). However, the decomposition products were below the detection limit of our system.

After 42 days of exposure to simulated solar light, up to 157.92 µg of PR 22 stay inside the dermis of an animal. That is, up to 60% of the pigment is cleaved by the simulated solar light (Figure 3) during irradiation.

4.4. Discussion

A major prerequisite for a health risk assessment is the knowledge of the pigment concentration in the human body. Tattooing is an archaic procedure and there is no information about the amount of pigments punctured in the skin, which is currently a major obstacle for such a risk assessment. Since the concentration of pigments in tissue is unknown, there is a critical need for scientific investigations. Since it is difficult to study tattooing and pigment transportation in humans, we applied the SKH-1 mouse model for these investigations.

Transportation of Pigments. In the process of tattooing, the pigment suspension is simply punctured in the skin using tiny needles. Deposition of the pigment in the dermis results in the permanence usually associated with tattoos. Tattoo colorants are injected into the skin and are partly transported to other anatomical locations, whereas the exact mechanisms of transportation and distribution remain unknown. Among others, the local lymph nodes show the same color as the tattoos in the skin (Figure 2b).[8] Thus, tattoo colorants are administered like medical drugs, but without any medical reason.

The lack of information might be due to the complex pigment chemistry, the high insolubility of pigments and the difficulty to extract pigments quantitatively from tissue. Based on a simple consideration, the amount of pigment placed in the body must be equal to the amount in the skin directly after tattooing. After that, one part of the pigments is transport away, in particular via lymphatic system. The other part stays in the skin and causes the respective tattoo color.

Our results for the SKH-1 mice show that immediately after tattooing an average amount of 34.53 µg of PR 22 was extracted from one punch. This corresponds to 552.48 µg of tattoo pigment per tattooed animal (4 stripes per animal).

The used tattoo needle showed a size of 14 pt (4.94 mm), that defines the width of one single pass stripe. Based on this consideration each mouse received a concentration of PR 22 of 176 mg/ 100 cm^2. This value matches very well the

previously reported amount of 253 mg/ 100 cm^2, which is a mean value of different methods used (chapter 2.3.).

The reduction of the amount of PR 22 in the mouse skin is demonstrated by the decrease of PR 22 from 34.53 µg to 24.86 µg within 42 days. With regard to the size and number of stripes, 397.76 µg PR 22 per animal remained in the skin. This leads to a concentration of 127 mg/ 100 cm^2 considering the needle size of 14 pt. The comparison with the extraction after 1 day exhibits a pigment reduction of 28% during the 42 days (Figure 3).

Laser Treatment. After several years people often decide to remove their tattoos. Beside adverse reactions[9-13] of the pigments itself, the main reasons for removing tattoos are improved self-image or social stigmatization. Traditional modalities are the removal of the pigment-containing skin using salabrasion,[14] cryosurgery,[15] surgical excision[16] or CO_2-laser application.[17] However, these methods induce permanent scarring.

Using Q-switch lasers, tattoo removal is associated with significantly lower risk of scarring.[18] The majority of tattoo pigment is found within cells, and not free, within the dermis. While many pigment particles measure “a few microns”, others are significantly larger[19] or when accumulated within cells may act as larger aggregate bodies. According to the principles of selective photothermolysis[20] the laser impulses show a high intensity and ultra short pulse durations of a few nano seconds (Q-switched lasers).

After being absorbed in the pigment molecule, the energy of the laser light is converted to heat or breaks chemical bonds inside the molecule. Additionally, the ultrashort heating may lead to the disruption of the pigment particle. The laser irradiation changes the shape and the size of the tattoo particles abruptly as proved by histology.[19]

It is well known that an increase of temperature in a number of azo dyes above 280 °C forms 3,3’-dichlorobenzidine,[21] a proven genotoxin towards human lymphocytes.[22] Laser irradiation of the widely used azo compound Pigment Red 22 (PR 22) and resulted in the photodecomposition products 2-methyl-5-nitroaniline (MNA), and 4-nitro-toluene (NT) (Figure 4).[5] NT is toxic as shown with human lymphocytes.[23] 5-nitro-*o*-toluidine, which is also designated to MNA, may cause liver dysfunction as shown with workers from a hair dye factory.[24]

Additionally, MNA and other di-nitro-toluenes showed greatest mutagenic activity towards Salmonella typhimurium YG as demonstrated by Sayama et al.[25]

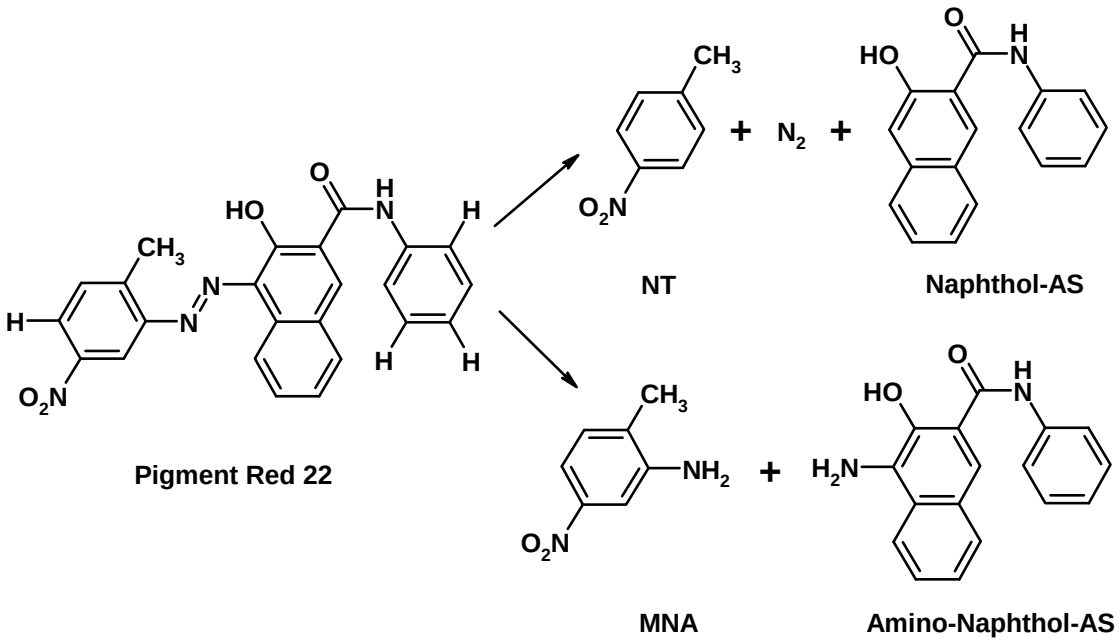

Figure 4: The chart shows the decomposition of PR 22 molecule that is caused by either solar or laser light.

To proof these *in vitro* results, we used mice as a model for the human body. We tattooed mice as described above, kept them for six weeks with ambient ligh exposure and excised the tattoo stripes. These stripes were treated with laser light. The laser induced decomposition products and the remaining PR 22 were extracted and quantified.

We found an average amount of PR 22 with 12.82 µg per punch. That corresponds to 205.12 µg per animal and 6.5 mg/ 100 cm^2 skin area, respectively. In comparison to the sample before laser irradiation laser light causes a decrease of the pigment mass of 48.4% (Figure 3). This is a difference of 192.64 µg of PR 22 per animal that can be cleaved by laser light. That means 192.64 µg (4.52 x 10^{-7} mol) of PR 22 could be decomposed to MNA, NT and NAS.

The extraction of the decomposition products revealed for 12.82 µg of PR 22 (one punch) an amount of 0.11 µg NAS, 0.24 µg MNA and 0.11 µg NT. This

yields in one tattooed mouse (192.64 µg of PR 22; 4.52 x 10^{-7} mol) NAS with 1.76 µg (6.69 x 10^{-9} mol), MNA with 3.84 µg (2.53 x 10^{-8} mol) and NT with 1.76 µg (1.28 x 10^{-8} mol). Thus, only 8% of the decomposed PR 22 was directly identified by its products such as MNA und NT. The major part 12.82 µg of PR 22 could have been decomposed by other pathways. However, it is more likely that these major decomposition products has been metabolized to other products and could have been therefore not detected by our analysis.

Irradiation with Solar Light. Recently a research team of the National Center for Toxicological Research (NCTR) of the FDA showed that a yellow tattoo pigment (PY 74) in solution is cleaved into toxic products photochemically when exposed to UV radiation in a sunlight simulator.[4] These authors concluded form their *in vitro* study that these substances can be generated in tattooed skin when this skin is exposed for a longer period of time to sunlight or other sources of UV radiation.

For the first time we could show that solutions of PR 22 are chemically altered when exposed to natural sunlight or broadband UVB radiation. The decomposition products MNA and NT postulated from laser treatment could be detected.[3]

To verify these *in vitro* results, the mouse model was used again. In contrast to experiments with laser irradiation, the skin of the mice could heal from tattooing for 10 days. Then the mice were exposed to simulated solar light (1.4 SED/day ssl) for 32 days. After solar light exposure, the tattooed stripes were excised and analyzed.

An average amount of 9.87 µg of PR 22 was extracted from one punch (5.02 mg/ 100cm^2). That is, up to 157.92 µg of PR 22 stay in side the dermis. When comparing this value with the initial amount of pigment in the skin, up to 60% of the pigment is cleaved by the simulated solar light (Figure 3) during irradiation. The concentrations of the decomposition products were below the detection limit of our system. Obviously, PR 22 decomposed and the products disappeared during the period of irradiation (32 days) either by metabolization or by lymphatic transportation.

Time Scale. With the results of this study we can show now a time scale regarding the concentration of tattoo pigment in skin and its changes.

The starting point of this graph is the day, when the mice were tattooed (Figure 2). Immediately after tattooing (1 day later, Figure 2a) the concentration of PR 22 punctured into skin was determined to be 34.53 µg per punch (100%) (Figure 5a). This amount can be reduced through different pathways. Firstly, in the process of wound healing the epidermis is regenerated and part of pigments could be lost due to transportation by wound liquid via skin surface. This process should have stopped a few hours after tattooing and the amount of pigment lost by this process could be not determined.

Secondly, pigment is transported by phagocytosis through the vascular system to the lymph nodes located next to the site of the tattoo (Figure 1b, 5b). This transportation should last several weeks or months, whereas most of the pigment should have been transported during the first weeks after tattooing due to the inflammatory reactions in the skin. Our results show that 42 days after tattooing 28% of pigment disappeared by either wound healing within a few hours or by transportation in the mouse body within several weeks (Figure 2c, translocation study).

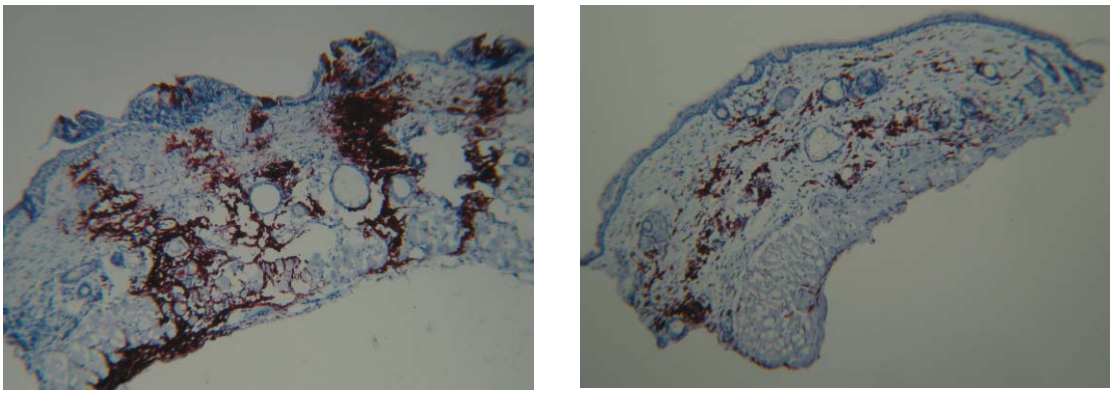

Figure 5: Histology shows the pigment distribution in the mouse skin 1 day (a) or 42 days (b) after tattooing. One day after tattooing, the punctures of the tattoo needle are still visible. 42 days after tattooing the pigment is rather randomly distributed in the dermis due to transportation mechanisms.

Since the most active processes are more or less completed, the reduction of pigment in the skin should decrease and a certain amount of pigments remain in the skin causing the colored tattoo. That is evident also for human tattoos that last for years.

Whenever a tattoo is exposed to solar light, e.g. outside during the summer or in a tanning booth, the pigment could decompose. In our study we could prove this thesis and showed, that 32 days of solar light reduced the concentration of PR 22 by 50% to 12.82 µg per punch. This reduction can be explained by the decomposition of PR 22 by UV and solar light (Figure 2b).[3]

Usually people do not recognize any fading or bleaching of the colour of the tattoo, since azo pigments have tremendous colour strength and small amount of pigment is able to hide the normal skin colour. Thus, a possible cleavage of the tattoo pigment mostly remains unnoticed.

Extrapolation to a Human Tattoo with a Size of 100 cm^2. The amount of PR 22 directly after tattooing is 100% and for a standard human tattoo 253 mg/ 100 cm^2. Thus with a reduction of 28% by removal through the vascular approximately 70 mg of PR 22 are transported to the lymph nodes next located to the tattoo.

If also in humans up to 60% of the pigment are cleaved by solar light, 152 mg might be decomposed. In mice the laser irradiation causes a decrease of the pigment mass of 48.4%. Transferring this decomposition rate to a human tattoo, 123 mg of PR 22 will be cleaved by laser light. The conversion of 8% PR 22 would yield 2.31 mg of NAS, 5.05 mg of MNA and 2.31 mg of NT (Table 3). As explained above, these values could demarcate only the lower limit for these decomposition products and the real numbers could be even higher.

compound	amount per 100 cm^2 [mg]	amount per animal [mmol]	turnover [%]
PR 22	253	5.94×10^{-1}	100
NAS	2.31	8.79×10^{-3}	1.48
MNA	5.05	3.32×10^{-2}	5.59
NT	2.31	1.69×10^{-2}	2.84

Table 3: The values show the expected amount of the decomposition products NAS, MNA and NT for the laser treatment of a tattoo with a standard size of 100 cm^2 containing 253 mg of PR 22.

Comment on Radiant Exposure. The radiant exposure used in the SKH-1 mouse model differs from that radiant exposure applied in clinical practice. Here tattoo removal is done in several sessions witch each 3 - 5 J/cm^2 yielding a lower total radiant exposure. Nevertheless our results are more than a proof of evidence and might have an impact on clinical practice.

4.5. Conclusion

We could show for the first time that after tattooing and healing of the wound 28.0% of the injected pigment is removed from the site of the tattoo. This corresponds to approximately 150 µg of tattoo pigment.

Up to 50% of PR 22 located inside the mouse skin can be cleaved by laser light. Fortunately we could show, that only 8% of PR 22 are decomposed into the hazardous compounds MNA and NT.

Regarding the exposure of tattoos to solar light we found that up to 60% of the pigment is cleaved. The *ex vivo* postulated decomposition products (NAS, MNA, NT) could not be detected. The reason might be, that the decomposition products are removed by the lymphatic system from the site of the tattoo as soon as they are generated.

4.6. References

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5. Modern Tattoos Contain Azo Pigments: an *in-vivo* Proof of Pigment Red 22 and Pigment Red 170*

5.1. Introduction

The determination of the amount of tattoo pigment inside the skin is a first and important step towards risk assessment of tattooing. In the past years, we have analyzed tattoo pigments *in vitro*,[1] in animals (chapter 4.) and in *ex vivo* skin specimen (chapter 2.). We detected decomposition of pigments caused by exposure to laser or solar light and analyzed the respective decomposition products.[2-4]

With regard to risk assessment, a major step is the analysis of tattoos in human skin, in particular the quantification of pigment concentration in real tattoos. Since it is difficult to perform studies with humans, we analyzed tattooed skin of humans that are provided by forensic medicine. The tattoos of these individuals have existed for a long time and all transportation processes in the skin should have been more or less finished at the time of excision. That is, the extraction should yield the concentration of pigments that is present in a typical tattoo.

A major obstacle is the lack of information about the pigment used for tattooing. Usually, neither the tattooist nor the tattooed individual knows anything about the chemicals that are punctured in the skin. Therefore, we focused on red pigments (red tattoos) and established a chemical database for those pigments that are worldwide in use. After extraction, the identity of the pigment is initially unknown. However, if the pigment is in our database, we should be able to identify the pigment and to quantify its concentration in the skin specimen.

* This chapter is part of a manuscript, in preparation: Engel, E.; Gastl, K.; Santarelli, F.; Vasold, R.; Maisch, T.; Penning, R.; Ulrich, H.; König, B.; Landthaler, M.; Bäumler, W. *Toxicol. Appl. Pharmacol.* **2007**.

* The database was established by K. Gastl as described in her Zulassungsarbeit.

5.2. Materials and Methods

Pigments. The red tattoo pigment Pigment Red 22 (PR 22, C.I. 12315, CAS 6448-95-9) was synthesized in pure quality (> 98%).[5] Pigment Red 170 (PR 170, C.I. 12475, CAS 2786-76-7) was purchased from Simon-und-Werner (Clariant Products GmbH (Germany) in standard organic pigment quality.

Tattooed Skin. Tattooed skin was obtained from skin excisions for other reasons (Dept. of Forensic Medicine, Munich) and stored at - 80 °C. Excision site was the left forearm. The fatty tissue was removed; skin thickness measured approximately 2 to 3 mm. We made three punch biopsies measuring 5 mm in diameter and extracted the pigments as previously reported (chapter 2.2.).

HPLC Analysis. The samples were filtered using a PTFE filter (Chromafil, O-20/15, organic, pore size 0.2 mm; Machery-Nagel, Düren, Germany). A 10-µL sample was analyzed using a HPLC model 1100 (Agilent Technologies,Waldbronn, Germany) fitted with a C18 analytical column (Phenomenex Luna, particle size 3 µm, 150 x 4.60 mm, Aschaffenburg, Germany) and DAD. The data were analyzed using a HPLC-3D ChemStation Rev. B.01.01. Gradient elution was done with water (0.0059 w/v% trifluoroacetic acid) (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. A gradient profile with the following proportions of solvent B was applied [t (min), % B]: (0, 10), (20, 95), (50, 95). The chromatograms were monitored at 258 nm.

The concentration of PR 22 in the solution 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.

5.3. Results and Discussion

Spectra Library. As shown by Bäumler et al. in 2000, today red tattoo pigments belong to the class of naphthol AS pigments.[1] In the run-up to the analysis of real existing tattoos several red naphthol AS pigments were screened by HPLC-DAD technology regarding their retention time and UV spectrum. With these data, a database was created for identifying unknown pigments from real existing tattoos by their retention time and UV spectrum.

Identification. Based on a very recently established procedure[5] we extracted the red tattoo pigments from real existing tattoos in human skin tissue. We were able to identify the two red pigments coloring a tattoo at the left forearm of a person (Figure 1).

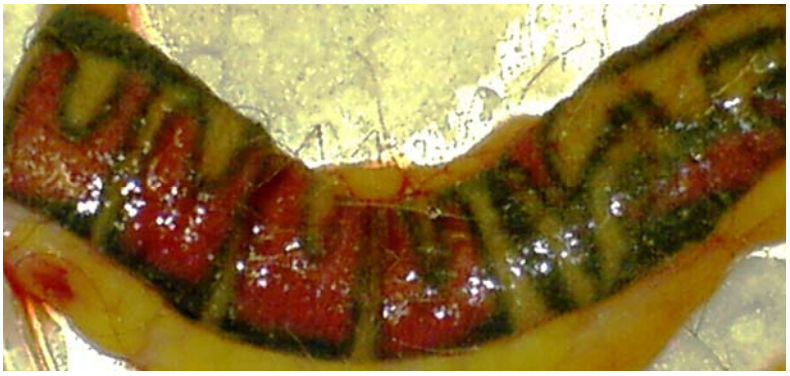

Figure 1: The tattoo exhibits a reddish and black pattern. Three punches were taken from red spots of the tattoo.

The HPLC chromatogram of the extracted tattoo revealed two peaks with an absorption maximum ~ 500 nm (Figure 2). The UV spectrum of peak 2 (RT 22.7 min) has a shoulder at 330 nm, whereas the UV spectrum of peak 1 (RT 18.4 min) shows a shoulder at 302 nm and a bathochrome shift in the range of 500 nm (Figure 3). Based on the comparison of retention time and UV spectrum peak 1 could be identified as Pigment Red 170 and peak 2 as Pigment Red 22 (Figure 4).

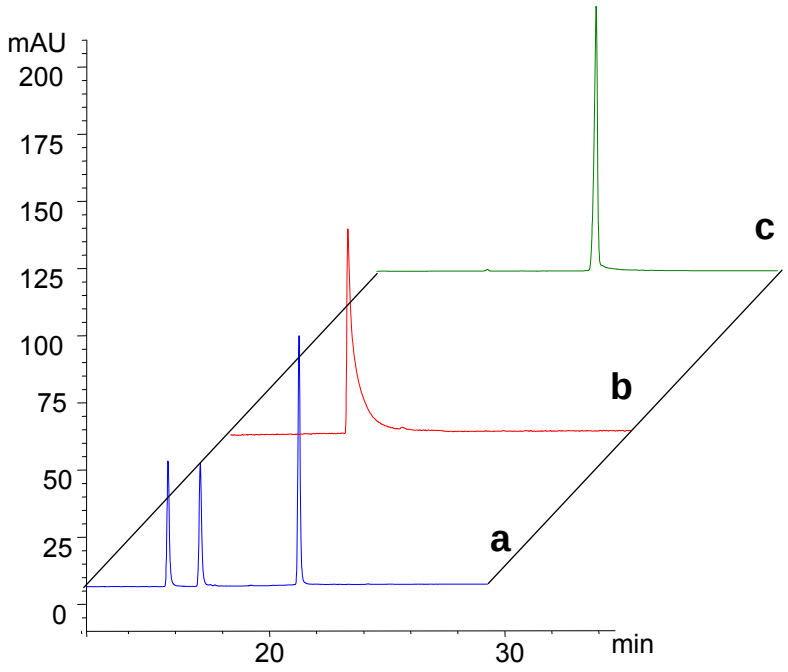

Figure 2: HPLC chromatogram of the extracted tattoo (a), reference PR 170 [0.2 mg/mL DMSO] (b) and reference PR 22 [0.3 mg/mL dichloromethane] (c).

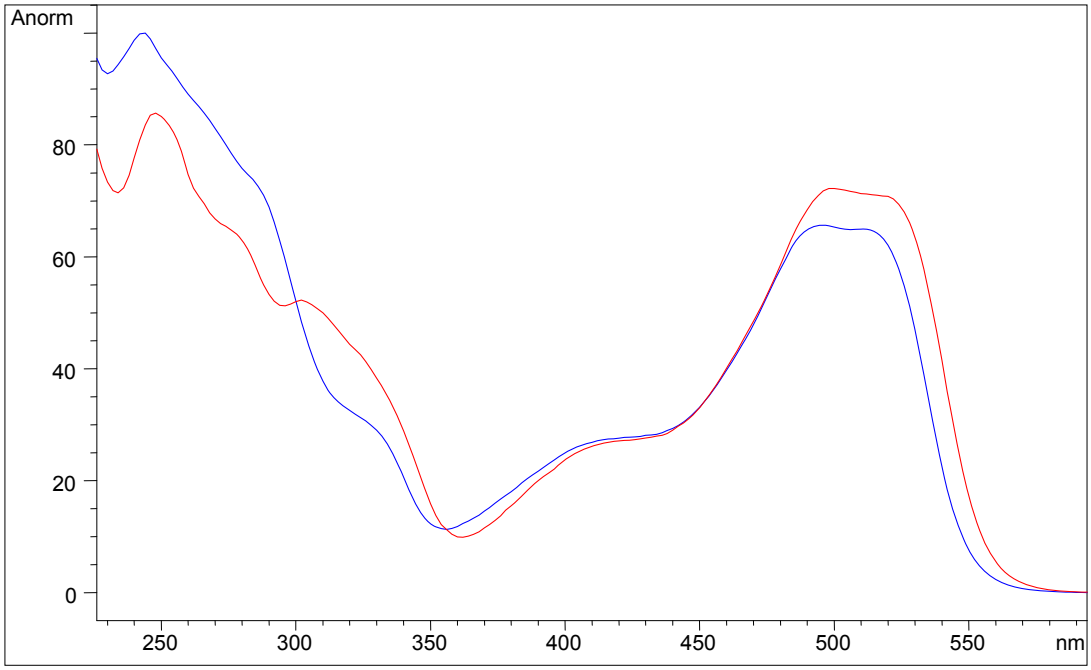

Figure 3: UV spectra of PR 22 (blue line) and PR 170 (red line). The UV spectrum of PR 22 has a shoulder at 330 nm, whereas the UV spectra of PR 170 shows a shoulder at 302 nm and a bathochrome shift in the range of 500 nm.

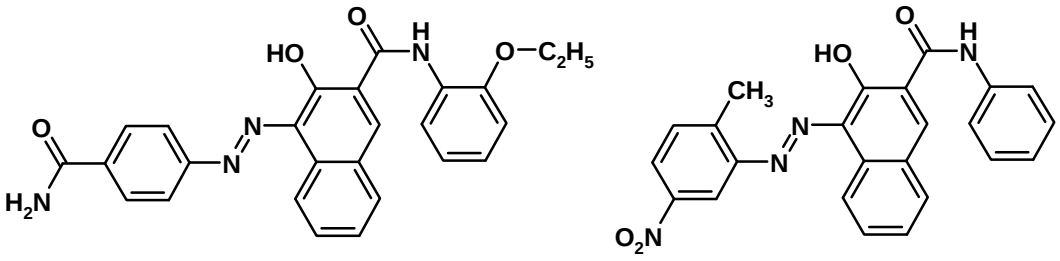

Figure 4: Chemical structure of PR 170 and PR 22. Both pigments belong to the class of naphthol AS pigments and show a different substitution pattern.

Quantification. Three punch biopsies were extracted and resulted in an average amount of 21.25 µg of PR 22 per punch (RSD 24.8%). This amount corresponds to a concentration of 0.11 mg/cm^2 (Figure 5).

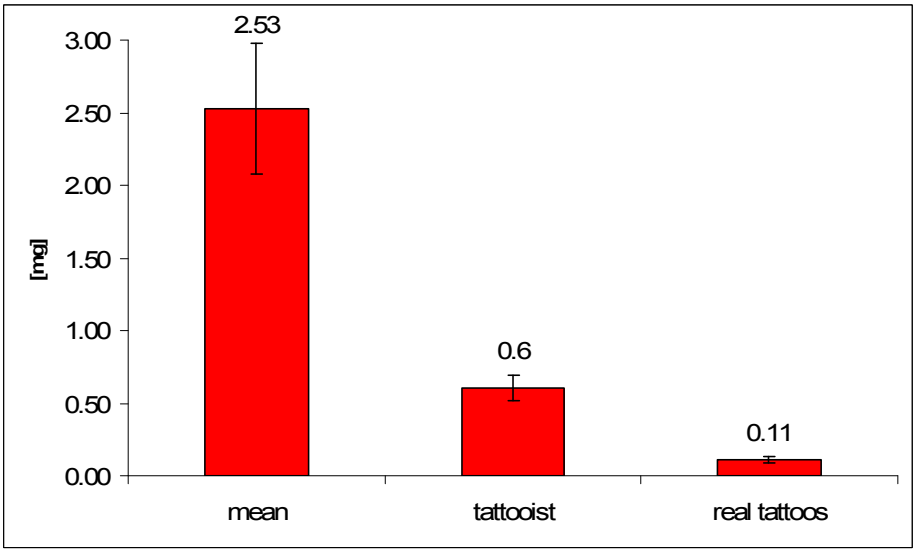

Figure 5: The values show the amount of PR 22 punctured into skin in total, regardless the different methods used (left), the amount tattooed into skin by tattoo artists (middle), the amount of PR 22 remaining inside the skin after several years (right). The decrease of the amount is due to translocation inside the body and possibly decomposition by sun light.

Previously we could show, that the tattooing procedure yields an overall mean concentration of 2.53 mg of PR 22 in 1 cm^2 skin (chapter 2.3.). The comparison with that amount exhibits a reduction of 96% during several years. In this tattooing study professional tattoo artists punctured 0.6 mg of PR 22 into 1 cm^2 skin, which results in a decrease of 82% during several years (Figure 5).

Assuming a mean value of 0.6 mg/cm^2 of PR 22 as starting amount, 0.49 mg/cm^2 are removed from the site of the tattoo.

The pigments are injected into the skin by the use of solid needles. A part of the pigments stays in the dermis causing the tattoo of the skin. Another part is transported away through the vascular system, in particular through lymph channels. Therefore, the colorants can be found in lymph nodes located next to the tattoo [6-9] and the amount of tattoo pigment remaining in the dermis is decreased.

Moreover, a reduction of pigment in the skin is additionally caused by light induced pigment decomposition. Recently, we have shown, that tattoo pigments are decomposed in solution by UV light or solar light.[2,3] This process can contribute to the clear reduction of pigment concentration that is obvious in our investigations. The respective decomposition products should be not detectable in the tattooed area since these products should have been metabolized or removed via vascular system. Occasionally patients report colour fading of tattoos over the years.

Characteristics of PR 170 and PR 22. Previously Steinbrecher et al. described a case report of a 30-year-old man who showed an adverse reaction on the red color of his tattoo.[10] Originally Pigment Red 170 was produced as lacquer for tools, machines, farm machines and especially cars, but also as printing ink for letterpress and stain of PVC. PR 170 is very favored in industry because of its fastness to weathering. The shade of this color is bluish red.[11]

Pigment Red 22 is also known as Cardinal Red, Dragon Red or Spanish Red and shows a yellowish color. It is very popular in the US or Japan. The field of application is also very broad. PR 22 is used in textile industry or for graphical printing.[11]

The use of Pigment Red 22 on the skin surface is not allowed by the cosmetic laws (Annex IV of the Cosmetics Directive of the EU).[12] Due to lack of legal regulation, such pigments can be used as colorants for tattooing or PMU (trade name e.g. Devil's Red) being punctured in the skin of millions of people. As such pigments are produced for industrial purposes, they can display impurities

of 10% or more. These impurities may be components such as titanium dioxide for lightening the ink shade, precursors and byproducts of pigment synthesis.

The medical literature reports on malignant skin lesions such as basal cell carcinoma[13,14] or melanoma[15,16] in tattoos, but without providing evidence for causality.

5.5. Conclusion

For the first time we could analyze real existing tattoos from human skin tissue. We identified two widespread red tattoo pigments Pigment Red 22 and Pigment Red 170. PR 22 was analyzed quantitatively and its concentration in the skin was determined to be 0.11 mg/cm^2. As previously reported tattooists puncture up to 0.60 mg/cm^2 of PR 22 into the skin.

The comparison with this amount exhibits a reduction of 82% of tattoo pigment during several years. Partly, the amount of tattoo pigment is decreased by the transport through the vascular system to the lymph nodes next located to the tattoo.

Due to health concerns, most of the azo pigments are not allowed to be used in cosmetics. This prohibition is based upon the assumption that such pigments are to some extent absorbed by skin. This absorption may cause health problems, in particular when carcinogenic amines are formed owing to the decomposition of azo pigments.

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6. Azo Pigments and a Basall Cell Carcinoma at the Thumb*

6.1. Introduction

Basal cell carcinoma is the most common malignancy in humans. Skin cancer, predominantly basal cell carcinoma and squamous cell carcinoma, have accounted for about 40% of all cancers in the United States and their frequency has been increasing.[1] However, the nail unit is a very uncommon site for basal cell carcinoma to develop.[2-4] In 2006, *Martinelli et al.* reviewed the English literature and found only 17 patients with a basal cell carcinoma at this location.[2]

The cause of basal cell carcinoma located at the fingers remains unclear. Comparable to other locations, ultraviolet radiation, chronic actinic skin damage, suppression of the immune system or chemical compounds like arsenic may play a role.

6.2. Case Report

A 58-year-old male patient presented an aching lesion at the left thumb that persists since three years. The clinical inspection of the lesion showed an erosive, erythematic lesion (1 x 0.5 cm) at the left thumb including the lateral nail fold.

In addition, the patient reported that he is an enthusiastic angler. He has recognized that successful fishing is improved when the fishing baits are colored. Therefore, he dips the baits into a colorant prior to fishing and each time the color gets into contact with the left thumb. To elucidate the role of the colorant, chemical analysis was performed to identify the unknown chemical sample.

* Results of this chapter have been accepted for publication: Engel, E.; Ulrich, H.; Vasold, R.; König, B.; Landthaler, M.; Süttinger, R.; Bäumler, W. *Dermatology* **2007**, in press.

6.3. Materials and Methods*

A biopsy specimen was taken from the skin lesions of the patient and was formalin fixed and stained with hematoxylin-eosin (Figure 1).

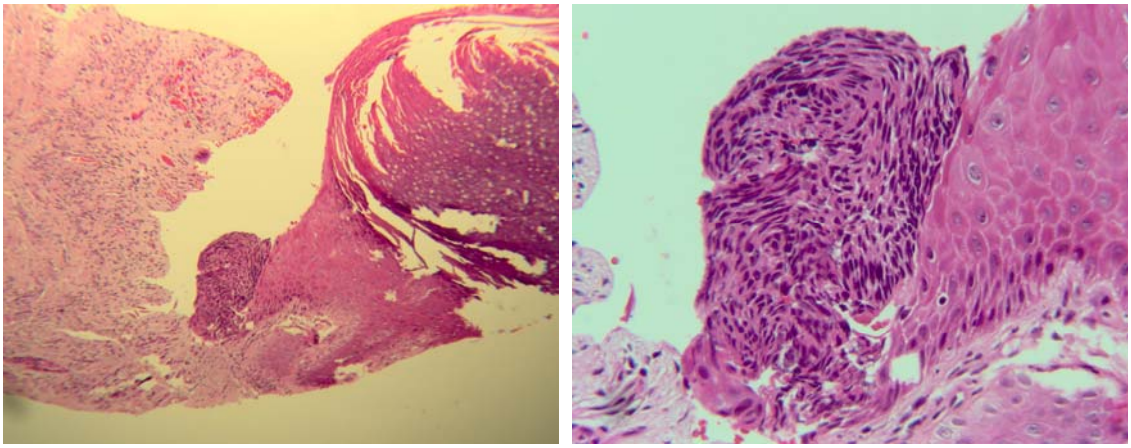


Figure 1: Histology with H&E-staining (a) and enlarged picture showing the basaloid cells (b).

In a first step **High Pressure Liquid Chromatography** (HPLC) was used for analyzing the provided sample. The substance was dissolved in methanol [1.0 mg/mL] (LiChrosolv®, Merck KGaA, Darmstadt, Germany) and filtered using a PTFE-filter (CHROMAFIL®, O-20/15, organic, pore-size 0.2 µm, Machery-Nagel, Düren, Germany). 20 µL of this solution were injected into an HP 1090 HPLC system (Hewlett-Packard GmbH, Waldbronn, Germany) fitted with a normal phase column (Agilent LiChrosphere, Si 60, particle size 5 µm, 250 mm x 4 mm, Merck, Darmstadt, Germany). The data were analyzed using the ChemStation Version HPLC-3D-ChemStation Rev. B.01.01 [164]. Gradient elution was done with hexane (solvent A) and isopropanol (solvent B) at a constant flow rate of 0.8 mL/min. A gradient profile with the following proportions of solvent B was applied [t (min), % B]: [0, 10], [40, 90], [50, 90]. The chromatograms were monitored at 240 nm.

* IR Spectra were recorded by Dr. Andreas Lange.

Electrospray Mass Spectrometry (ESI-MS) was applied collecting further information. Using a triple stage mass spectrometer (TSQ 7000, Thermoquest Finnigan, Toronto, Canada) the mass of the respective compound was determined.

In a next step the substance was analyzed by **Infrared Spectroscopy** (IR). The identification was done by comparing the fingerprint of the compound with an IR spectra database. FTIR spectra (3200 – 400 cm^{-1}) of KBr pellets were recorded via a FTS 800 spectrometer (Scimitar Series, DigiLab, Varian, Darmstadt, Germany) and background correction.

6.4. Results

The epidermis was clearly altered and the dermis showed fibrosis and many ectatic vessels. Using pancytokeratin staining the specimen showed an aggregation of basaloid cells adhered to the epidermis. The tumor was excised using the technique of Mohs micrographic surgery.

HPLC Analysis. The HPLC chromatogram of the unknown colorant shows a peak at RT 27.4 min with an absorption maximum at 512 nm (Figure 2a). Peaks eluting earlier than RT 7 min times are impurities. By applying electrospray ionisation (ESI) the mass of the molecule ion $[(M\text{-}H^{+})^{-}]$ could be determined to be 726.3 Da. The isotope distribution [m/z (%) = 724.3 (5), 726.3 (100), 727.3 (50), 728.3 (16), 729.3 (3), 730.3 (1)] indicates the presence of a chromium complex.

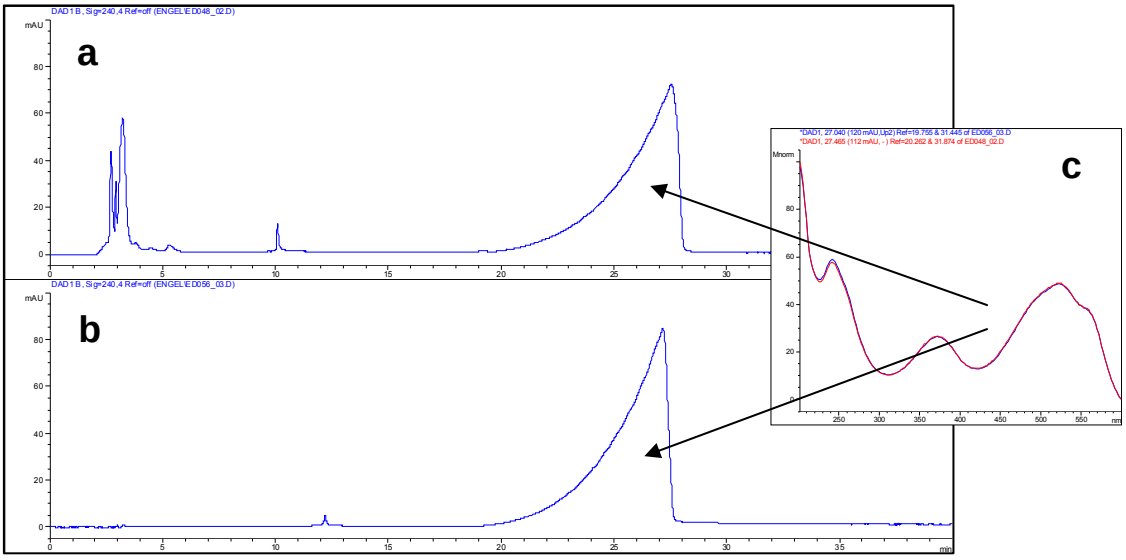


Figure 2: HPLC chromatograms are shown. The chromatogram (a) corresponds to the unknown colorant and shows a main peak at RT 27.4 min. The reference compound C.I. Solvent Red 8 elutes at RT 27.1 min (b). Both compounds show an identical UV-spectrum with an absorption maximum at 512 nm (c).

IR Data. Based on IR data [(KBr pellet, cm^{-1}): 2926, 2854, 2656, 2258, 2129, 1636, 1600, 1555, 1513, 1467, 1434, 1370, 1318, 1303, 1274, 1248, 1202, 1168, 1077, 1050, 1036, 1004, 956, 870, 819, 758, 687, 658, 601, 483] BASF was able to screen an IR library. According to this screening, the unknown colorant consists of a pigment that is a 1:2 chromium complex azo pigment, very similar to C.I. Solvent Red 100 or C.I. Solvent Red 8 (Figure 3, 4).

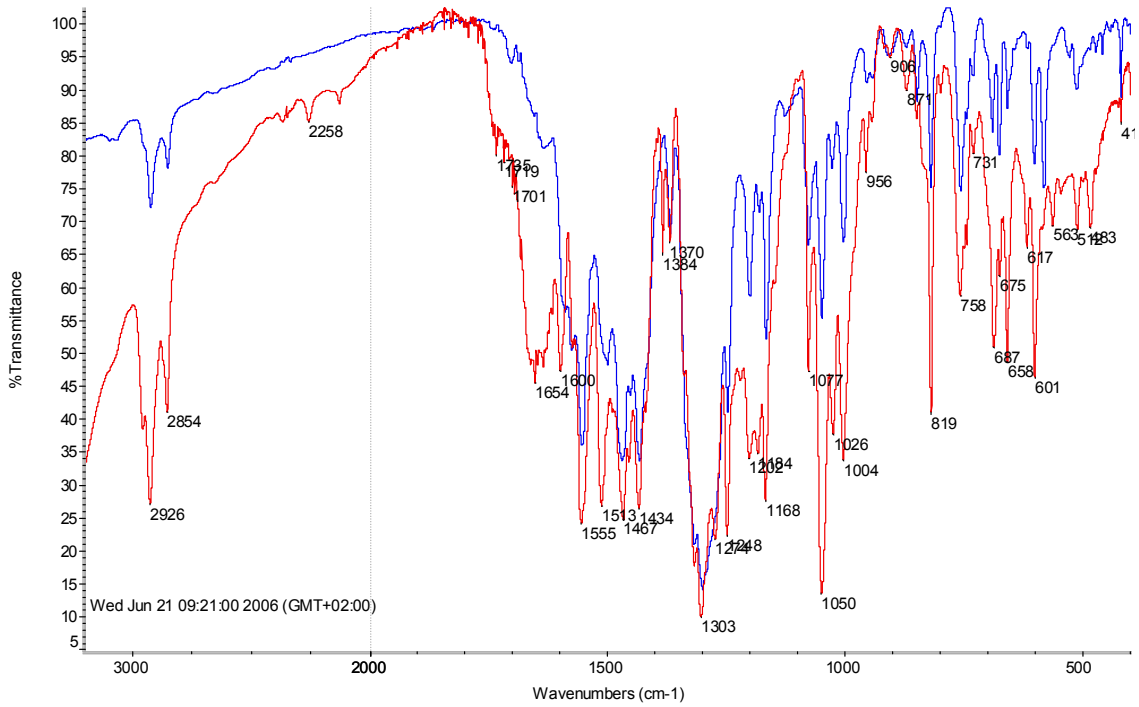
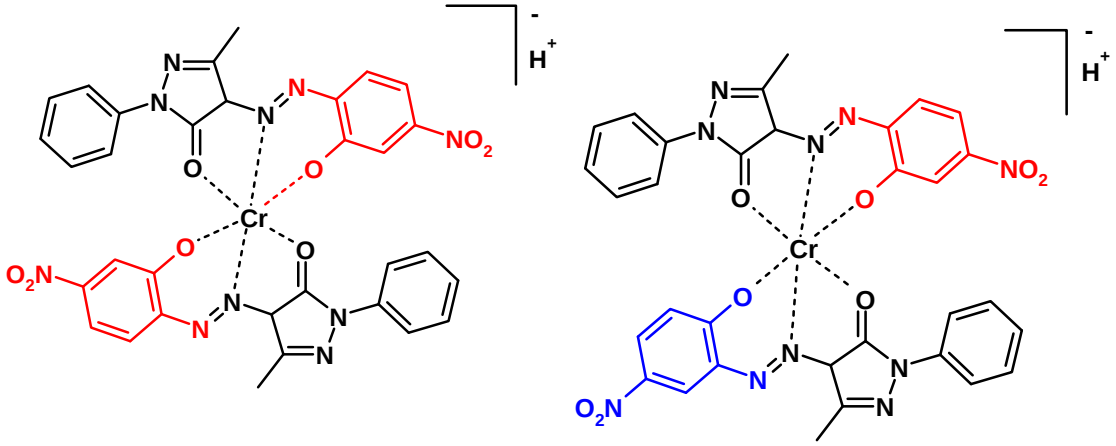


Figure 3: Infrared spectroscopy shows a good agreement of the unknown colorant (red line) to C.I. Solvent Red 100 (blue line), which was found by the screening of an IR library. According to the fingerprint (< 1500 cm^{-1}), which is specific for every compound, the provided compound is very similar to C.I. Solvent Red 100. Both, C.I. Solvent Red 100 and C.I. Solvent Red 8 show very similar IR spectra.

Both, Solvent Red 8 and Solvent Red 100 are 1:2 chromium azo complexes. They vary in their coordination to the central chromium(III)-ion. In the case of Solvent Red 8 the chromium(III)-ion coordinates two molecules of the azo pigment C.I. 127145, whereas in the case of Solvent Red 100 the coordination sphere consists of azo pigment C.I. 127145 and C.I. 127135. The only difference between both azo pigments is the substitution of the nitro group located in meta-position (C.I. 127135) or in para-position (C.I. 127145) to the azo group. The calculated molecular weight of both compounds matches the molecular weight obtained for the provided colorant sample.



Solvent Red 8 Solvent Red 100

Figure 4: Chemical structure of the two possible azo pigments. Solvent Red 8 and Solvent Red 100 vary in their coordination to the central chromium(III)-ion. The difference between both azo pigments is the substitution of the nitro group located in meta-position (C.I. 127135) or in para-position (C.I. 127145) to the azo bound.

To verify these results the reference sample Solvent Red 8 (provided by Clariant Frankfurt, Germany) was analyzed under the same conditions. The HPLC chromatogram also shows a peak at RT 27.1 min (Figure 2b) with an UV-spectrum identical to the unknown substance (Figure 2c). ESI-MS measurements resulted in the same molecular weight and the same isotope distribution.

6.5. Discussion

Basal cell carcinoma located at the fingers (Figure 4) is rare and the cause for the tumor at this site remains usually unclear. Our patient reported a frequent contact of his finger to the colorant he is using to stain the fishing baits. The origin of the colorant was unclear and a list of ingredients was not available. Therefore, no warning notices were on the bottle containing this colorant.

Due to this fact, the colorant was suspected to be associated with the basal cell carcinoma at the thumb. Therefore, we prompted the patient to provide a bottle with the colorant for further chemical analysis. Since there was no indication for the chemical nature of the colorant, the liquid, red colorant was dried and investigated by the means of three different analytical methods.

The results of three methods in all likelihood suggest Solvent Red 8 being the red color in the colorant. Azo pigments such as Solvent Red 8 are usually applied to stain consumer goods. Due to health concerns, most of the azo pigments are not allowed to be used in cosmetics, that is, to get into contact with skin.[5]

In contrast to cosmetics, tattooing is not regulated and therefore such azo pigments are used as tattoo colorants being punctured in the skin of millions of people.[6-8] The medical literature reports on malignant skin lesions such as basal cell carcinoma[9,10] or melanoma[11,12] in tattoos, but without providing evidence for causality. Part of the pigments and their impurities are transported from the skin into the lymph nodes staining them for years.[13]

Solvent Red 8 is a wide spread azo pigment used in paints for cars and wood, whereas Solvent Red 100 is used for textiles such as leather, silk or wool. The chemical synthesis of Solvent Red 8 requires the aromatic amine 2-amino-5-nitrophenol. The azo pigments of tattoo colorants also contain such amines.[7] The pigments are simply manufactured to stain consumer goods but not to be used as pharmaceutical compounds in humans. Therefore, the azo pigments contain a high percentage of impurities like the aromatic amines, which are

frequently toxic or carcinogenic. Additionally, the pigments can be cleaved by light and can release these amines.

2-amino-5-nitrophenol has been examined by US National Cancer Institute (NTP-studies). The compound was mutagenic in *Salmonella typhimurium* strains. 2-Amino-5-nitrophenol induced forward mutations in mouse L5178Y lymphoma cells. An increase in chromosomal aberrations and sister chromatid exchanges was observed in cultured Chinese hamster ovary (CHO) cells following incubation with 2-amino-5-nitrophenol. Rats that received 2-amino-5-nitrophenol responded with an increased incidence of acinar cell adenomas of the pancreas.[14] Comparable findings are reported for 2-amino-4-nitrophenol that is used fort he synthesis of Solvent Red 100.[15]

Based on these facts and the results of our chemical analysis, there might be a certain probability that the carcinogenic amines in the colorant Solvent Red 8 have induced the basal cell carcinoma in our patient. Consequently, the role of azo pigments that are punctured in the skin for tattooing should be also highlighted in the near future.

6.6. References

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7. Abbreviations

ACN	acetonitrile
ATL buffer	tissue lysis buffer
C.I.	colour index
CAS-No.	chemical abstract service number
CF i	calibration factor of the compound i
Crl/SKH-1	Charles River / Skin and Cancer Hospital
DAD	diode array technology
DCA	2,5-dichloroaniline
DCB	1,4-dichlorobenzene
Diglyme	diethylene glycol dimethyl ether
DNA	deoxyribonucleic acid
DPA	9,10-diphenyl-anthracene
ESI	electrospray ionisation
FDA	Food and Drug Administration
FTIR	Fourier transformed infrared spectroscopy
H&E	haematoxylin and eosin staining
HPLC	high performance liquid chromatography
hr^-/hr^-	hairless strain
hrs	hours
Hz	hertz
IR	infrared spectroscopy
ISTD	internal standard
M	molecule
min	minute
mL	milliliter
µL	microliter
MNA	2-methyl-5-nitroaniline
m-NAS	methoxy-NAS
MS	mass spectroscopy
naphthol-AS	arylide of the 2-hydroxy-3-naphthoic acid
NAS	naphthol-AS
NCTR	National Center for Toxicological Research
Nd:YAG	neodymium-doped yttrium-aluminium-garnet
NHDF	normal human dermal fibroblasts
NT	4-nitrotoluene
NTP	national toxicology program
PBS	phosphate buffered saline
PMU	permanent make-up
PP	polypropylene
PR 22	Pigment Red 22

PR 9	Pigment Red 9
press.	pressure
pt	points
PTFE	polytetrafluorethylene
PY 74	Pigment Yellow 74
ROS	reactive oxygen species
RSD	relative standard deviation
RT	retention time
SED	standard erythema dose
ssl	simulated solar light
TEM	transmission electron microscopy
temp.	temperature
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UV	ultraviolet
w/v	weight/volume

8. Summary

The number of tattooed individuals increased significantly, especially among youth. In the United States, up to 24% of the population has tattoos, whereas in European countries like Germany about 9% and the United Kingdom about 12% are tattooed. Today frequently azo pigments are used for tattooing since they are brilliant and provide a long-lasting tattoo in the skin. These azo pigments are manufactured primarily for other purposes like printing, painting cars and coloring various consumer products. Tattoo colorants are mixtures of pigments (color) and multiple other ingredients. These colorants may contain titanium dioxide for lightening the shade, precursors and byproducts of pigment synthesis, as well as diluents that are used to suspend the pigments. Tattoo colorants are also used for permanent make-up at the eyelid, eyebrow or lip.

Despite the millions of people with tattoos, there is currently no common legal requirement for listing ingredients, including the pigments. That is, for tattooing non-FDA-approved pigment is introduced into skin to produce indelible designs. In Europe, many of azo pigments used in tattoos (e.g. Pigment Red 22) are not allowed in cosmetics since they can be decomposed yielding carcinogenic amines. The FDA continues to evaluate the extent and severity of adverse events associated with tattooing and is conducting research on colorants.

On closer examination, tattooing is a complex procedure that includes different risks for the skin and even for the human body. The pigments and the impurities could cause adverse reactions of the skin at the site of tattooing. In addition, part of the colorants are transported away to other anatomical locations like the lymph nodes.

Moreover, tattoos may be exposed to solar light or - in case of tattoo removal - to laser light. Both procedures have been known to result in the decomposition of such pigments *in vitro* causing hazardous compounds such as carcinogenic amines.

To estimate the risk of any health problems of tattooing, the colorant concentration in the skin and the human body after tattooing of the colorants must be determined. This has not been performed so far and was now firstly investigated by our research group.

Therefore, an extraction method was established to determine the concentration of tattoo pigments and decomposition products quantitatively (*chapter 1*). The extraction of two widely used azo compounds, Pigment Red 22 (PR 22) and Rigment Red 9 (PR 9) and their laser induced decomposition products 2-methyl-5-nitroaniline (MNA), 4-nitro-toluene (NT), 2,5-dichloroaniline (DCA) and 1,4-dichlorobenzene (DCB) were accomplished using recovery experiments and HPLC-DAD technology. Despite the poor solubility of the pigments, a nearly complete recovery from aqueous suspension (> 92%) or lysed skin (> 94%) was achieved. The decomposition products were extracted from aqueous suspension or skin showing a recovery of up to 100%, except for the very volatile DCB.

Based on this extraction procedure we could determine the amount of tattoo pigment punctured into skin (*chapter 2*). We tattooed excised pigskin and human skin with Pigment Red 22 under various conditions. After tattooing, we quantitatively extracted the pigment in order to determine the pigment concentration in skin. The concentration of pigments ranged from about 0.60 to 9.42 mg per cm^2 of tattooed skin (mean value 2.53 mg/cm²) depending upon the size of the pigment crystals, the pigment concentration applied to the skin surface, and the respective procedure of tattooing.

It is known from literature, that a yellow tattoo pigment (Pigment Yellow 74) is cleaved by simulated solar light into toxic compounds. Since PR 22 is cleaved by laser light into toxic or even carcinogenic compounds we investigated the influence of UVB radiation and natural sun light on PR 22 (*chapter 3*). PR 22 was dissolved in different solvents (tetrahydrofuran, dioxane, chloroform and dichloromethane). The solutions were irradiated with either UVB-radiation or with natural sunlight. An evident cleavage of the pigment was detected in all

solvents, when exposed to UVB radiation or natural sunlight. The primary decomposition products (NAS, MNA, NT) were identified. In tetrahydrofuran and dioxane the pigment concentration decreased significantly during UVB irradiation, whereas the pigment was completely destroyed during sunlight exposure. In chloroform and dichloromethane the concentration of PR 22 decreased only slightly during UVB irradiation, whereas during sunlight exposure the pigment was almost completely destroyed. We found that PR 22 is cleaved in tetrahydrofuran and dioxane without any influence of radiation. Since chloroform and dichloromethane do not affect the cleavage process, these solvent are optimal for such *in vitro* experiments. Last but not least we demonstrated that PR 22 is cleaved by natural sunlight and broad band UVB radiation into toxic and carcinogenic compounds.

Puncturing tattoo pigments into the skin can be compared with an injury of the upper skin layers. As soon as the pigments are injected into the dermis they are recognized by the body as foreign particles. By means of phagocytosis, the tattoo pigments are removed from the site of tattooed skin and are transported by the lymphatic system to other anatomical locations like lymph nodes located next to the tattoo.

To investigate transportation of tattoo pigments after tattooing, we established an animal model using SKH-1 hairless mice (*chapter 4*). The mice received tattoos with PR 22 on their back. The extraction of pigments immediately after tattooing yielded the concentration of PR 22 of 34.53 µg per punch (1.76 mg/cm^2) that is placed in the body. In contrast to that, the extraction of PR 22 from skin six weeks (42 days) after tattooing elucidates the extent of pigment transportation in the mice. Only 24.86 µg of PR 22 per punch remain inside the dermis (1.27 mg/cm^2). That corresponds to a removal of 28% of tattoo pigment.

To investigate the decomposition of pigments by solar light, tattooed living mice were exposed to solar light for 31 days. We found that up to 60% of the pigment is cleaved (PR 22 remaining: 0.50 mg/cm^2). The *ex vivo* postulated decomposition products (NAS, MNA, NT) could not be detected. The reason

might be, that the decomposition products are removed by the lymphatic system from the site of the tattoo as soon as they are generated.

Ex vivo laser treatment of tattooed mouse skin showed that up to 50% of PR 22 located inside the mouse skin can be cleaved by laser light (PR 22 remaining: 0.65 mg/cm^2). Fortunately we could show, that only 8% of PR 22 are decomposed into the hazardous compounds MNA and NT.

For the first time we could quantitatively analyze real existing tattoos in human skin tissue (*chapter 5*). The extraction of a tattoo revealed PR 22 and PR 170 to be the red tattoo pigments used by the artist. Several years after tattooing the concentration of PR 22 inside the skin is 0.11 mg/cm^2. Concerning the concentration of 0.6 mg/cm^2 punctured into skin by tattooist 82% of tattoo pigment are transported from the site of the tattoo during several years.

The last chapter (*chapter 6*) deals with an interesting additional problem of azo dyes. We describe a case report of a 58-year-old patient with a periungual basal cell carcinoma at the thumb. Basal cell carcinoma is the most common malignant neoplasm of the skin, whereas the localization at the nail unit is very rare. The specific feature of the reported case is the frequent exposure to fishing baits that he had stained with an unknown colorant. The use of chromatography, mass spectrometry and infrared spectroscopy revealed the colorant as the 1:2 chromium complex azo pigment Solvent Red 8. Solvent Red 8 is a widespread synthetic azo pigment that is applied to stain consumer products. Compounds such as Solvent Red 8 can be cleaved to carcinogenic amines under e.g. light exposure, in particular after incorporation into the human body. As a result of the frequent skin contact to this azo pigment, this hazard compound might have induced the basal cell carcinoma in our patient.

9. Zusammenfassung

Die Popularität von Tätowierungen hat in die letzten Jahren, vor allem unter den Jugendlichen, stark zugenommen. In den Vereinigten Staaten sind bis zu 24 % der Bevölkerung tätowiert, die Zahlen in Europa sind ähnlich. In Deutschland tragen rund 9 % der Bevölkerung eine Tätowierung, in England sind es schon 12 %. Für die farbigen Tätowierungen werden inzwischen mehrheitlich Farben auf der Basis von industriellen Pigmenten eingesetzt. Chemische Analysen haben ergeben, dass es sich häufig um organische Pigmente wie Azoverbindungen oder polyzyklische Verbindungen handelt. Diese Pigmente werden eigentlich zum Färben oder Lackieren von Konsumgütern produziert (z.B. Autolacke). Die Tätowierer setzen diese Pigmente gerne ein, weil sie sehr beständig und nahezu unlöslich sind. Dies sind genau die Eigenschaften, die für ein brillantes, beständiges Tattoo in der Haut sorgen. Wie eigene Recherchen ergeben haben, werden die Farbstoffe in Pulverform oder als Emulsionen von einigen Großhändlern in USA oder Europa an Tätowierer ohne Angaben zu den Inhaltsstoffen verkauft. Diese Substanzgemische enthalten in wechselnder Zusammensetzung neben der eigentlichen farbgebenden Komponente, Vor- und Zwischenprodukte aus dem Syntheseprozess des Pigments sowie große Mengen an Titandioxid (Farbaufhellung) und weitere nicht spezifizierte Zusatzstoffe. Tätowierungsfarbstoffe kommen in großem Umfang auch bei Permanent Make-up im Bereich der Lippenkonturen, des Augenlids oder der Augenbrauen zum Einsatz.

Trotz der großen Anzahl von mehreren Millionen Menschen, die eine Tätowierung tragen, gibt es derzeit keine einheitlichen Regelung zur Deklarierung der Pigmente und Inhaltsstoffe. Im Rahmen von Tätowierungen werden also Substanzen in die Haut eingebracht, die von der US FDA zu diesem Zweck nicht zugelassen sind. Auch in Deutschland unterliegt die Verwendung von Tätowierungspigmenten derzeit keiner gesetzlichen Kontrolle, da es sich weder um Kosmetika noch um Arzneimittel handelt. Interessanterweise ist der Einsatz vieler Pigmente (z.B. Pigment Red 22) auf der Hautoberfläche durch die Kosmetikverordnung verboten (Annex IV of the

Cosmetics Directive of the EU), da sie in karzinogene Amine gespalten werden könnten. Werden sie aber in die Haut eingestochen, gibt es keine Einschränkungen. Mit Blick auf die Millionen von Tätowierten in Deutschland ist der Gesetzgeber hier dringend gefragt, entsprechende Regelungen zu erlassen.

Bei näherer Betrachtung ist Tätowieren ein komplexer Prozess, der verschiedene Risiken für die Haut, bzw. für den menschlichen Körper birgt. Die Pigmente und die darin enthaltenen Verunreinigungen können zu unerwünschten Hautreaktionen führen. Ein Teil des eingebrachten Substanzgemisches, vor allem Verbindungen mit kleinem Molekulargewicht, wird innerhalb kurzer Zeit über das Gefäßsystem der Haut abtransportiert. Ein deutlicher Hinweis darauf ist häufig die Färbung der lokoregionären Lymphknoten.

Von zunehmendem Interesse ist auch der Einfluss von Sonnenstrahlung, oder im Falle einer Entfernung, des Laserlichts auf Tätowierfarbstoffe. In beiden Fällen kann es *in vitro* zur Spaltung der Tätowierungspigmente in karzinogene Amine kommen.

Die Toxizität und Karzinogenität von chemischen Verbindungen, wie auch deren Licht induzierten Spaltprodukten, hängen unter anderem von deren Konzentration in der Haut ab. Bedenkt man, dass sich viele Menschen mit ihrem Tattoo in der Sonne aufhalten bzw. sich einer medizinisch indizierten Therapie mit UVB-Licht unterziehen etc., ist es wichtig, die Konzentration von Tätowierungspigmenten in der Haut zu bestimmen, um damit eventuell verbundene Risiken einschätzen zu können. Bisher war die Menge an Pigment, die beim Tätowieren in die Haut eingebracht wird, völlig unklar.

Zur Bestimmung des Gehalts an Tätowierungspigmenten in der Haut wurde erstmals ein Extraktionsschema etabliert, mit dem sowohl die Pigmente als auch deren Spaltprodukte quantitativ extrahiert werden können (*Kapitel 1*). Die Extraktion zweier weit verbreiteter Azopigmente, Pigment Red 22 (PR 22) und Pigment Red 9 (PR 9), und deren Laser-induzierten Spaltprodukte 2-Methyl-5-nitroanilin (MNA), 4-Nitrotoluol (NT), 2,5-Dichloranilin (DCA) und 1,4-

Dichlorbenzol (DCB) wurde mittels Recovery-Experimenten und quantitativer HPLC (DAD-Technologie) etabliert. Trotz der nur geringe Löslichkeit der Pigmente konnten diese fast vollständig aus wässriger Suspension (> 92 %) oder lysierter Haut (> 94 %) extrahiert werden. Bis auf das sehr flüchtige DCB wurden die Spaltprodukte nahezu vollständig (~ 100 %) extrahiert.

Dieses Extraktionsschema war die Basis für die anschließenden Tätowierungsstudien (*Kapitel 2*). Durch fachgerechtes Tätowieren wurde exzidierte Schweinehaut und Menschenhaut mit PR 22 in verschiedenen Konzentrationen und verschiedenen Nadelformen und Nadelgrößen tätowiert. Somit konnte erstmals die Menge an Farbstoff quantitativ bestimmt werden, die durch Tätowieren in die Haut eingebracht wird. Der Gehalt an Pigment belief sich, je nach Größe der Pigmentkristalle, der verwendeten Konzentration der Suspension und des jeweiligen Tätowierungsprozesses, auf Werte zwischen 0.60 und 9.92 mg/cm^2 (Durchschnitt 2.53 mg/cm^2).

Aus der Literatur ist bekannt, dass ein gelbes Tätowierungspigment (Pigment Yellow 74) durch Sonnenlicht in toxische Produkte gespalten werden kann. Da PR 22 durch Laserlicht in toxische, z.T. sogar karzinogene Substanzen gespalten werden kann, wurde der Einfluß von UVB-Strahlung und natürlichem Sonnenlicht auf PR 22 untersucht (*Kapitel 3*). Dazu wurde PR 22 in verschiedenen Lösungsmitteln (Tetrahydrofuran, Dioxan, Chloroform und Dichlormethan) gelöst und die Lösungen entweder mit UVB-Strahlung oder mit natürlichem Sonnenlicht bestrahlt. In allen Lösungsmitteln konnte zweifelsfrei die Licht induzierte Spaltung des Pigments nachgewiesen und die primären Spaltprodukte (NAS, MNA, NT) identifiziert werden. In den Lösungsmitteln Tetrahydrofuran und Dioxan nimmt die Konzentration des Pigments durch die UV-B Strahlung deutlich ab, während es durch das Sonnenlicht völlig zerstört wird. In Chloroform und Dichlormethan nimmt die Konzentration von PR 22 durch die UV-B Strahlung nur etwas ab, Sonnenlicht zerstört es jedoch nahezu vollständig. Wir konnten zeigen, dass PR 22 in Tetrahydrofuran und Dioxan schon ohne den Einfluss von Licht zerstört wird. Da sich die Lösungsmittel Chloroform und Dichlormethan selbst gegenüber den ablaufenden chemischen

Spaltprozessen neutral verhalten, sind sie für *in vitro* Untersuchungen am besten geeignet. Es konnte gezeigt werden, das Pigment Red 22 unter dem Einfluss natürlichen Sonnenlichts und breitbandiger UV-B-Strahlung in toxische oder kanzerogene Substanzen gespalten wird.

Diese Umverteilung der Tätowierungspigmente wurde im Tiermodel an haarlosen SKH-1 Mäuse studiert (*Kapitel 4*). Jedem Tier wurden PR 22 in 4 Streifen auf den Rücken tätowiert. Einen Tag nach dem Tätowieren wurde die Menge an Farbstoff bestimmt, die in den Körper eingebracht werden kann (34.53 µg an PR 22 pro Stanze (1.76 mg/cm^2). Nach 6 Wochen (42 Tage) wurde das Ausmaß der Umverteilung im Körper ersichtlich. Nur noch 24.86 µg pro Stanze (1.27 mg/cm^2) verblieben in der Haut. Demnach wurden 28 % der eingebrachten Pigmentmenge umverteilt.

Zur Untersuchung, welchen Einfluss Sonnenlicht auf bestehende Tätowierungen hat, wurden tätowierte Mäuse 31 Tage lang im simulierten Sonnenlicht ausgesetzt. Es zeigte sich, dass bis zu 60% der vorhandenen Pigmentmenge gespalten wurde und die Konzentration nur noch 0.5 mg/cm^2 betrug. Die *ex vivo* postulierten Spaltprodukte konnten nicht nachgewiesen werden. Womöglich wurden sie sofort nach der Entstehung durch das lymphatische System abtransportiert.

Im Rahmen einer *ex vivo* Laserbehandlung tätowierter Mäusehaut wurde ~ 50 % der vorhandenen Pigmentmenge gespalten. Davon wurden wiederum nur 8 % in die postulierten Spaltprodukte MNA und NT gespalten. Die Konzentration an PR 22 betrug nach der Laserbehandlung nur noch 0.65 mg/cm^2.

Im Rahmen dieser Dissertation ist es erstmals gelungen, den Gehalt an Tätowierungspigment in einer seit mehreren Jahren am Menschen bestehenden Tätowierung zu bestimmen (*Kapitel 5*). Aus einer roten Tätowierung konnten PR 22 und PR 170 eindeutig als die beiden farbgebende Pigmente extrahiert werden. Der Gehalt an PR 22 in dieser Tätowierung betrug 0.11 mg/cm^2. Geht man davon aus, dass Tätowierer im Mittel 0.6 mg/cm^2 an

Tätowierungsfarbstoff in die Haut einbringen, müßten in diesem Fall 82 % des Pigments abtransportiert worden sein.

Im letzten Kapitel (*Kapitel 6*) wird ein zusätzliches Problem von Azofarbstoffen beschrieben. In der Klinik und Poliklinik für Dermatologie stellte sich ein 58-jähriger Patient mit einem periungualen Basalzellkarzinom an seinem linken Daumen vor. Basalzellkarzinome sind die häufigsten malignen Neoplasien der Haut. Die Lokalisation im Bereich des Fingernagels ist jedoch sehr ungewöhnlich. Die Besonderheit dieses Falles ist, dass der Patient häufige Kontakt zu Anglerködern hatte, die er mit einem unbekannten Farbstoff einfärbte. Mittels HPLC, Massenspektroskopie und Infrarotspektroskopie konnte die Substanz als 1:2-Chromkomplex-Azofarbstoff Solvent Red 8 identifiziert werden. Solvent Red 8 ist ein weitverbreiteter synthetischer Azofarbstoff, der zum Färben von Konsumgütern eingesetzt wird. Substanzen wie Solvent Red 8 können z.B. durch Licht in karzinogene Substanzen gespalten werden, u.a. auch nachdem sie in den Körper eingebracht wurden. Durch den häufigen Hautkontakt mit diesem Azofarbstoff, kann das Basalzellkarzinom bei unserem Patienten entstanden sein.

10. Appendix

PUBLICATIONS

- Modern tattoos contain azo pigments: an in vivo proof of Pigment Red 22 and Pigment Red 170, Engel. E.; Gastl, K.; Santarelli, F.; Vasold, R.; Maisch, T.; Penning, R.; Ulrich, H.; König, B.; Landthaler, M.; Bäumler, W. *Toxicol. Appl. Pharmacol.*, **2007**, in preparation.
- Tattoo pigments in skin: concentration, transportation and light induced decomposition of an azo pigment using SKH-1 mouse model, Engel. E.; Santarelli, F.; Vasold, R.; Maisch, T.; Howard, P.C.; Ulrich, H.; König, B.; Landthaler, M.; Bäumler, W. *Toxicol. Appl. Pharmacol.*, **2007**, in preparation.
- Tätowierungspigmente im Fokus der aktuellen Forschung, Engel. E.; Bäumler, W.; Vasold, R. *Nachrichten aus der Chemie*, **2007**, in press.
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Conferences and Presentations

ORAL PRESENTATIONS

- Engel, E. “Photochemical cleavage of a widely used tattoo pigment by UVB radiation and natural sunlight”, *2. World Congress on Work-related and Environmental Allergy, Weimar*, 06/**2007**
- Engel, E. “The archaic procedure tattooing – a first indication of the amount of tattoo pigment in skin“, *2. World Congress on Work-related and Environmental Allergy, Weimar*, 06/**2007**
- Engel, E. “Analyse von Tätowierungspigmenten in Hautgewebe mittels Pyrolyse-GC“, *1.Pyrolyse-Workshop, SIM, Oberhausen*, 05/**2007**
- Engel, E. “Quantifizierung von Tätowierungspigmenten in Hautgewebe“, *44. Tagung der Deutschen Dermatologischen Gesellschaft, Dresden*, 04/**2007**
- Engel, E. “Entwicklung einer Methode zur Extrahierung von Pigmenten aus der Haut“, *44. Tagung der Deutschen Dermatologischen Gesellschaft, Dresden*, 04/**2007**
- Engel, E. “Tätowierungspigmente: Quantitative Extraktion aus der Haut”, *Dermatologisches Fortbildungskolloquium am Klinikum der Universität Regensburg*, 12/**2006**
- Engel, E. “The neverending story: tattoos in human skin”, *Weihnachtskolloquium des Institut für Organische Chemie, Universität Regensburg*, 12/**2005**
- Engel, E. “Tätowierungspigmente, UV-Licht und Laser”, *Dermatologisches Fortbildungskolloquium am Klinikum der Universität Regensburg*, 06/**2005**

POSTER PRESENTATIONS

- Engel, E.; Santarelli, F.; Vasold, R.; Maisch, T.; Ulrich, H.; König, B.; Landthaler, M; Bäumler, W. „Photochemische Spaltung von Tätowierungspigmenten durch UV-B-Strahlung oder Sonnenlicht“, *44. Tagung der Deutschen Dermatologischen Gesellschaft, Dresden*, 04/**2007**
- Engel, E.; Santarelli, F.; Vasold, R.; Maisch, T.; Ulrich, H.; König, B.; Landthaler, M; Bäumler, W. „Entwicklung einer Methode zur Extrahierung von Pigmenten aus der Haut“, *44. Tagung der Deutschen Dermatologischen Gesellschaft, Dresden*, 04/**2007**
- Engel, E.; Santarelli, F.; Maisch, T.; Ulrich, H.; König, B.; Landthaler, M., Vasold, R.; Bäumler, W. „Concentration of tattoo pigment in pig skin after artificial and manually tattooing“”, *58th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), Chicago, USA, 02*/**2007**
- Engel, E.; Santarelli, F.; Vasold, R.; Maisch, T.; Ulrich, H.; Bäumler, W.; König, B.; Landthaler, M. „Tattoos in skin: tattooing and quantitative extraction of a widely used tattoo pigment“, *Skin and Forrmulation 2nd Symposium, Versailles, France, 09*/**2006**
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- Bäumler W.; Engel, E.; Vasold R., Ulrich H.; Landthaler M. “Zerstörung von Tätowierungspigmenten durch UV-Licht oder Laser“, *43. DDG-Tagung, Dresden*, 04/**2005**

ADDITIONAL CONFERENCES

- Seminar “Probenvorbereitung – ein entscheidender Schritt in der Analytik”, Supelco, *Munich, Germany*, 04/**2007**
- GC/SPME-Seminar, Supelco, *Munich, Germany*, 11/**2005**
- 44th Annual Meeting of the Society of Toxicology (SOT), *New Orleans, USA*, 03/**2005**
- HPLC/GS-Seminar, Agilent Technologies, *Little Rock, USA*, 02/**2005**

Curriculum Vitae

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Name:	Eva Engel
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Education

08/2004 – 09/2007	PhD at the section of analytics (Dr. Rudolf Vasold) under Prof. Dr. Burkhard König (Dpartment of Organic Chemistry at the University of Regensburg)
09/2002 – 03/2003	Certification in Chemistry (Zulassungsarbeit) at the section of analytics (Dr. Rudolf Vasold) under Prof. Dr. Burkhard König (Dpartment of Organic Chemistry at the University of Regensburg), thesis on the “chemical analysis of tattoo pigments in human skin”.
11/1999 – 06/2004	study, Chemistry and Biology, University of Regensburg

School Education

06/1999	Abitur (A levels), majors in Biology and Latin.
09/1990 – 06/1998	Gymnasium (grammar school) in Dillingen/ Donau.
09/1986 – 07/1990	Volksschule (primary school) in Wittislingen.

Stay Abroad

01/2005 – 03/2005	Research at the National Center for Toxicological Research (NCTR) of the US Food and Drug Administration in Jefferson, Arkansas, in the working group of Dr. Paul C. Howard.

Honors

05/2007	Poster award for the best poster, 44. Tagung der Deutschen Dermatologischen Gesellschaft, Dresden, Germany, 04/2007
08/2006	Fellowship of the GDCh for the 1st European Chemistry Congress, Budapest, Hungary, 08/2006
01/2005 – 03/2005	Fellowship of postgraduate research programs, Oak Ridge Institute for Science and Education (ORISE), US Department of Energy
09/2002 – 03/2003	Scholarship for lectureship candidates founded by the German Association of Chemical Industry (VCI)