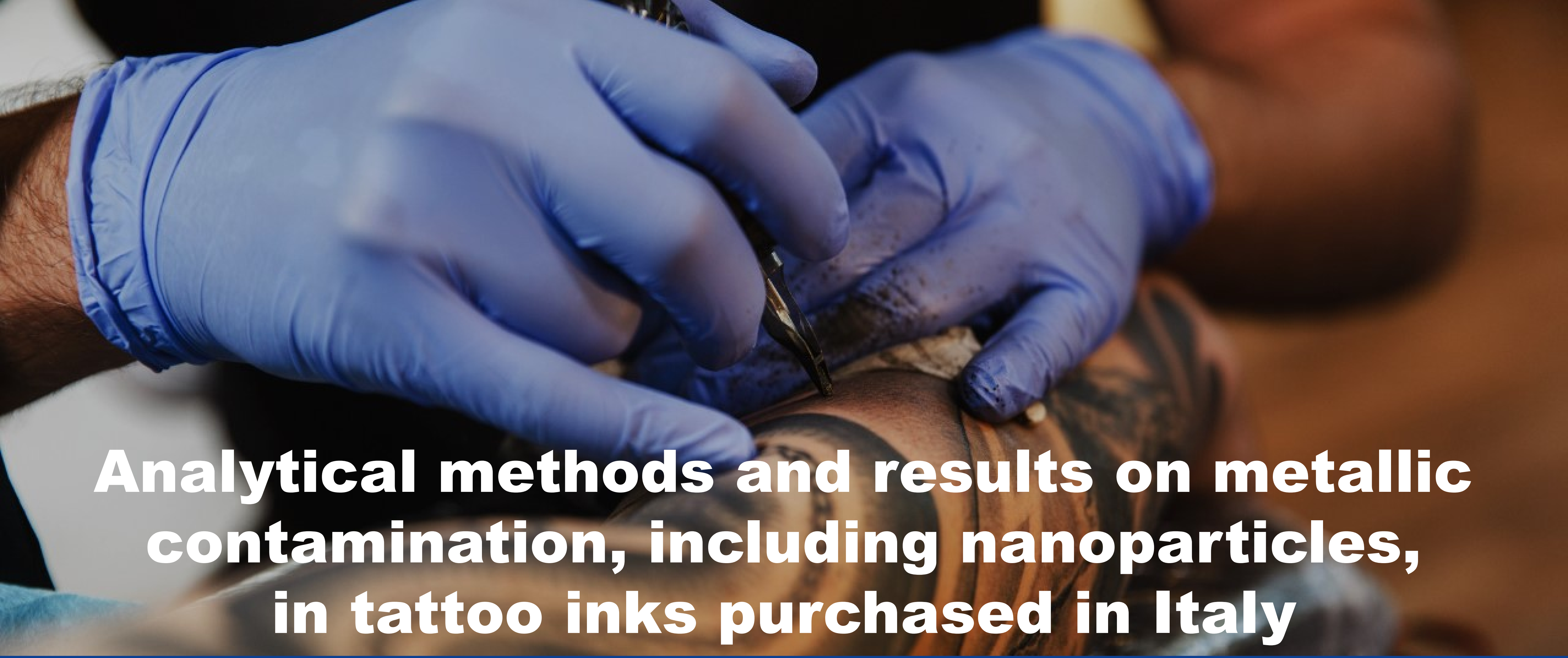


Analytical methods and results on metallic contamination, including nanoparticles, in tattoo inks purchased in Italy

Beatrice Bocca

Beatrice Battistini

Challenges in Public Health Protection in the 21st Century:
2nd International Conference on Tattoo Safety

Metals in tattoo inks

Considering the increasing popularity of tattooing and the possible presence of harmful substances as metals in inks used for tattoos, there is a need for developing and harmonizing methods to detect “unsuitable tattoo inks”

Pigments used in tattoo inks are produced mainly for largescale applications in construction or cosmetic industries, or for automotive coatings or plastics, not specifically for use in injecting into the skin, and they generally show low purity (70%-90%)

Arsenic (As)
Barium (Ba)
Copper (Cu)
Selenium (Se)
Mercury (Hg)
Cobalt (Co)
Tin (Sn)
Cadmium (Cd)
Lead (Pb)
Zinc (Zn)
Chromium (Cr)
Antimony (Sb)
Nickel (Ni)

Metals are used in tattoo inks either as inorganic pigments, such as metal oxides, or as metal-organic complexes

Tattoo inks were also shown to contain metallic impurities, such as Cr(VI) in Cr-oxides, Ni, Cu and Co in Fe-oxides

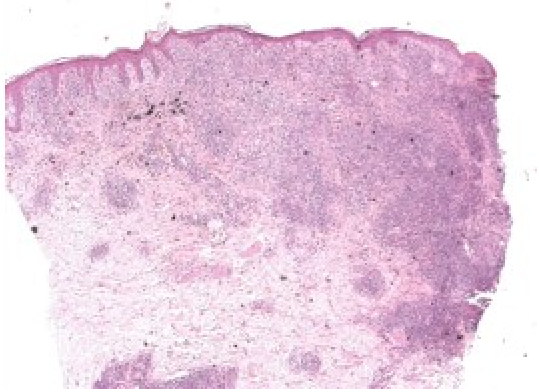
Forte et al., 2009. Market survey on toxic metals contained in tattoo inks. Sci. Total Environ. 407 (23), 5997e6002

Nanoparticles (NPs) in tattoo inks

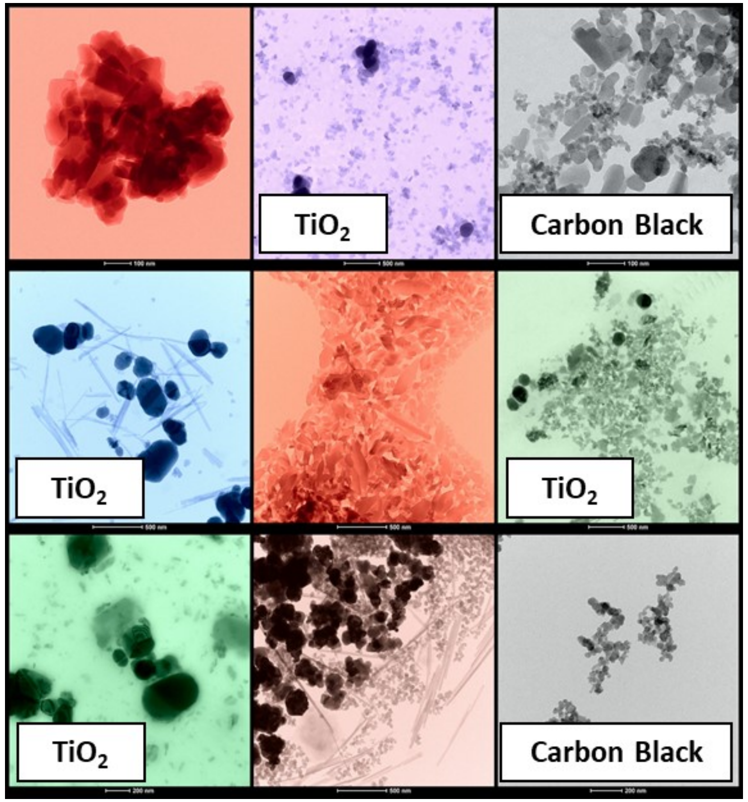
Concern has been raised due to emerging evidence on the occurrence of particles in the nanosize range (<100 nm) in tattoo inks. In particular, carbon black particles were present in black inks and particles of TiO_2 were recognized in blue, green and violet inks

Cases of pseudolymphomas observed in tattooed patients do not unambiguously point at one specific substance, but high concentration of Cr and Ni in biopsies taken from the tattooed area and in the applied inks were observed

SKIN LESIONS IN THE RED-COLOURED TATTOOED AREAS

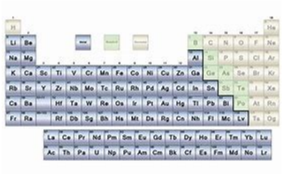


TEM IMAGES OF THE TATTOO INKS



Bocca et al., 2017. Size and metal composition characterization of nano- and microparticles in tattoo inks by a combination of analytical techniques. JAAS 32:616-628
Cristaudo et al., 2012. Permanent tattoos: evidence of pseudolymphoma in three patients and metal composition in the dyes. Europ J Dermatol. 2012, 22, 776-80

Overview of the limits and methods for detection



Metal	CoE ResAP	REACH Annex XVII
As	2	0.5
Ba (soluble)	50	500
Cd	0.2	0.5
Cr(VI)	0.2	0.5
Co	25	0.5
Cu (soluble)	25	250
Hg	0.2	0.5
Ni	ALTA	5
Pb	2	0.7
Sb	2	0.5
Se	2	5
Sn	50	0.5 (organic Sn)
Zn (soluble)	50	2000

ALTA: as low as technically achievable

- In the new REACH restriction (14 December 2020) on hazardous substances in tattoo inks, concentration limits for some metals (As, Co, Pb, Sb, Sn) have been lowered or aligned to the CoE ResAP(2008)
- For other metals (Cd, Cr(VI), Cu, Hg, Se, Zn) the restriction appears to be weaker than CoE ResAP
- For Ni, no limit in CoE ResAP is specified, while it is 5 ppm in REACH restriction
- Analytical methods have not been reported for the detection of metals in both frameworks

CONFUSION BETWEEN MANUFACTURERS AND AUTHORITIES HAPPENS, IF BOTH ARE ANALYZING THE PRODUCTS IN DIFFERENT WAYS, BECAUSE THIS LEADS TO DIFFERENT AND UNCOMPARABLE RESULTS

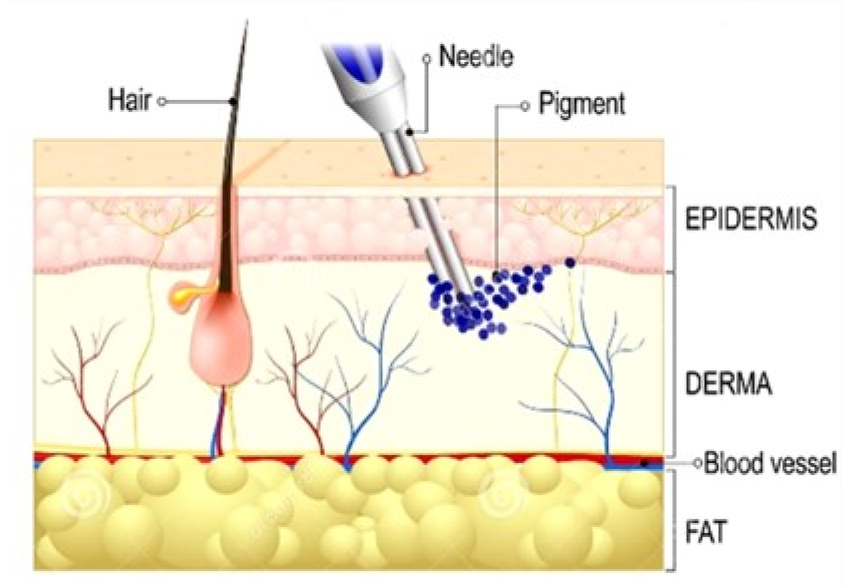
Missing information about NPs

The resolution CoE ResAP did not mention about nano-forms of existing chemicals in tattoo pigments

In the REACH restriction, although the impact of NPs on human health was highlighted, neither a general guidance about their physicochemical characterization nor a limit was included

Only the carbon black NPs were included in the REACH restriction list. In agreement with their inclusion in the Cosmetic Directive (EC/1223/2009), their use in tattoo inks was considered acceptable if their maximal concentration does not exceed 10% weight and their primary particle size is more than 20 nm

Whilst, the Regulation on cosmetic products governs the use of other nanomaterials (TiO_2 and ZnO) in products for dermal application at a maximum allowed concentration of 25% weight as UV-filters in sunscreen cosmetics



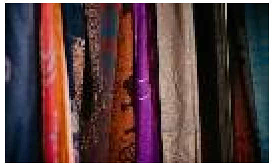
EVEN IF THESE CONCENTRATIONS CAN BE EFFECTIVE TO PRESERVE CONSUMER HEALTH BY NANOMATERIAL EXPOSURE VIA DERMAL ABSORPTION, IT CAN BE NOT IF NPs ARE INJECTED INTO THE DERMIS

Collection of methods

The ISO methods on cosmetics, textiles, leather or environmental matrices may serve as a reference for the detection of metals and leachable metals

ISO/TR 17276:2014 Cosmetics
ISO 12787:2011 Cosmetics

EN ISO 17072-1 Leather
EN ISO 17072-2 Leather
ISO 17075 Leather



EN 16711-1:2015 Textiles
EN 16711-2:2015 Textiles

EPA 3050, EPA 3051, EPA 3052 Solis, sediments and sludges
ISO 17294-2 Water quality
EPA 218.7 Drinking water

BUT, METHODS SHOULD BE VALIDATED SPECIFICALLY FOR THE TATTOO MATRIX AND MINIMUM PERFORMANCES PARAMETERS (LOD/LOQ, RECOVERY, REPEATABILITY, REPRODUCIBILITY) SHOULD BE EVALUATED AND REPORTED TO GUARANTEE THE FIT FOR PURPOSE AND THE RELIABILITY OF RESULTS

Piccinini et al., 2015. Safety of tattoos and permanent make-up: Compilation of information on legislative framework and analytical methods. EUR 27394. Luxembourg (Luxembourg): Publications Office of the European Union; 2015. JRC94760

Technical challenges

Quantify the Cr(VI) form by ensuring that:

- the extraction brings all forms of Cr(VI) into solution
- the extraction conditions do not induce the inter-conversion from Cr(III) to Cr(VI)

The analysis of NPs according to the EU definition on nanomaterials includes different steps:

- the **identification of the nanomaterial**
- the specification of the nanomaterial including **size and size distribution of particles** (mean, median and ±SD in nm)
- an estimation of the **quantity of nanomaterial** contained
- the **number of particles between 1-100 nm** (cumulative number)

THE MAIN TECHNICAL CHALLENGE IS THE NECESSITY OF USING MORE THAN ONE ANALYTICAL METHOD TO OBTAIN A COMPLETE CHARACTERIZATION OF INKS

Definition of nanomaterial (2011/696/EU)

HAS ADOPTED THIS RECOMMENDATION

1. Member States, the Union agencies and economic operators are invited to use the following definition of the term 'nanomaterial' in the adoption and implementation of legislation and policy and research programmes concerning products of nanotechnologies.

2. 'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

Laboratory of ICP-MS at ISS

1. High Resolution HR-ICP-MS → **MULTI-ELEMENTAL QUANTIFICATION**

2. Ion Chromatographic IC-ICP-MS → **QUANTIFICATION OF Cr(VI)**

3. Single Particle SP-ICP-MS → **DETERMINATION OF METAL NPs CONCENTRATION AND SIZE**

4. Asymmetric Field Flow Fractionation and Multi-Angle Light Scattering FFF-MALS-ICP-MS → **DETERMINATION OF SIZE, COMPOSITION AND CONCENTRATION OF METAL NPs**

1. Total metal by HR-ICP-MS: METHOD

2009: no. 56 inks (from USA and EU) purchased online
2019: no. 20 inks (from USA, EU and China) purchased in tattoo shops

Acid-digestion →
HNO_3
HF
H_2O_2

Microwave reactor

Water dilution →

HR-ICP-MS

Principle: **Microwave-assisted acid digestion of tattoo inks and analysis of 18 metals** (Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Se, Sn, Ti, Zn) **by HR-ICP-MS** using standard addition calibration for quantification and internal standardization to account for instrumental drifts

Description: A representative sample (0.25 g) is digested in **4 mL HNO_3, 1 mL HF and 1 mL H_2O_2** for 20 min and increasing MW power (250 W to 600 W). After cooling, the vessel content is diluted with high purity deionized water and analysed by HR-ICP-MS

Validation: The **LoD** ranged from 0.012 (Cd) to 0.18 µg/g (Al and Fe) and the **LOQ** varied from 0.04 (Cd) to 0.6 µg/g (Al and Fe). The **between-day reproducibility** ranged from 1.58% to 5.33%, while the **repeatability** between 2.67% and 7.05%. The **recovery** by adding certified reference standards to tattoo inks was between 92% and 109%

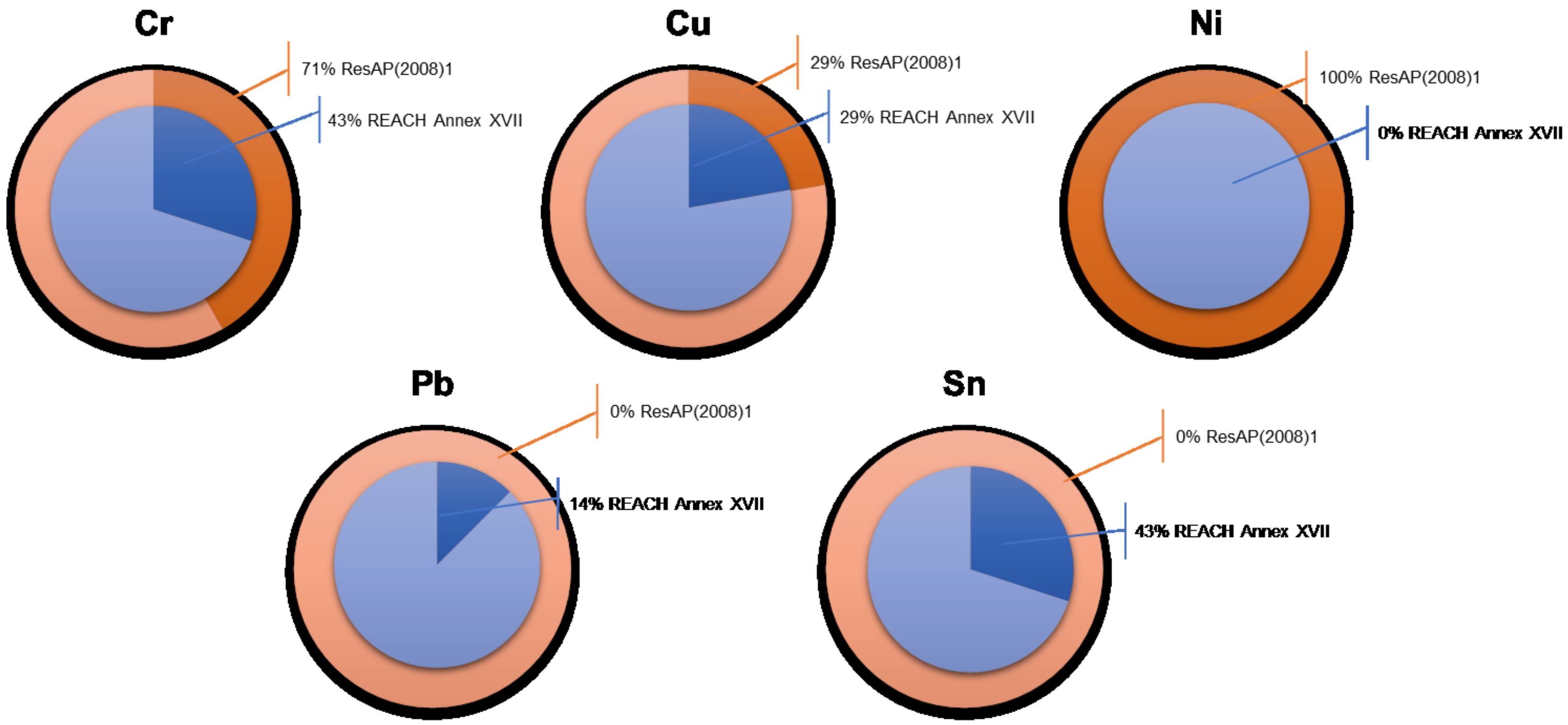
Forte et al., 2009. Market survey on toxic metals contained in tattoo inks. Sci. Total Environ. 407 (23), 5997e6002
Battistini et al., 2020.Quantitative analysis of metals and metal-based nano- and submicron-particles in tattoo inks. Chemosphere 245, 125667

1. Total metal by HR-ICP-MS: RESULTS

	Al (µg/g)	As (µg/g)	Ba (µg/g)	Cd (µg/g)	Co (µg/g)	Cr (µg/g)	Cu (µg/g)	Fe (µg/g)	Hg (µg/g)	Mn (µg/g)	Mo (µg/g)	Ni (µg/g)	Pb (µg/g)	Sb (µg/g)	Se (µg/g)	Sn (µg/g)	Ti (µg/g)	Zn (µg/g)
Black	7.93	0.02	0.77	nd	0.28	2.09	nd	11.4	nd	0.15	0.11	0.28	1.21	0.11	1.66	0.10	nd	0.42
Red	9.25	nd	0.10	nd	nd	2.10	nd	6.35	0.17	0.10	0.28	1.54	0.21	0.11	1.69	nd	6.28	2.65
Yellow	12.6	nd	7.77	nd	nd	0.70	nd	6.77	0.06	0.07	0.17	0.84	0.29	nd	1.38	nd	227	0.41
Green	11.4	nd	18.1	0.06	nd	0.22	3882	20.8	0.06	0.24	2.15	0.14	0.17	nd	1.66	nd	378	0.98
Blue	19.1	nd	6.29	nd	0.14	0.35	13158	10.5	0.06	0.05	7.65	1.40	0.15	0.28	1.45	0.50	3972	1.54
Violet	26.1	0.03	0.52	nd	0.14	0.03	nd	12.2	0.11	0.29	0.11	0.56	0.31	0.51	1.63	0.75	10579	2.37
White	51.5	0.07	0.32	0.06	nd	0.19	nd	5.47	0.06	0.10	nd	1.40	0.38	0.79	2.09	0.80	21716	7.54
Min-Max	7.93-51.5	nd-0.07	0.10-18.1	nd-0.06	nd-0.28	0.03-2.10	nd-13158	5.47-20.8	nd-0.17	0.07-0.29	nd-7.65	0.14-1.54	0.15-1.21	nd-0.79	1.38-2.09	nd-0.80	nd-21716	0.41-7.54

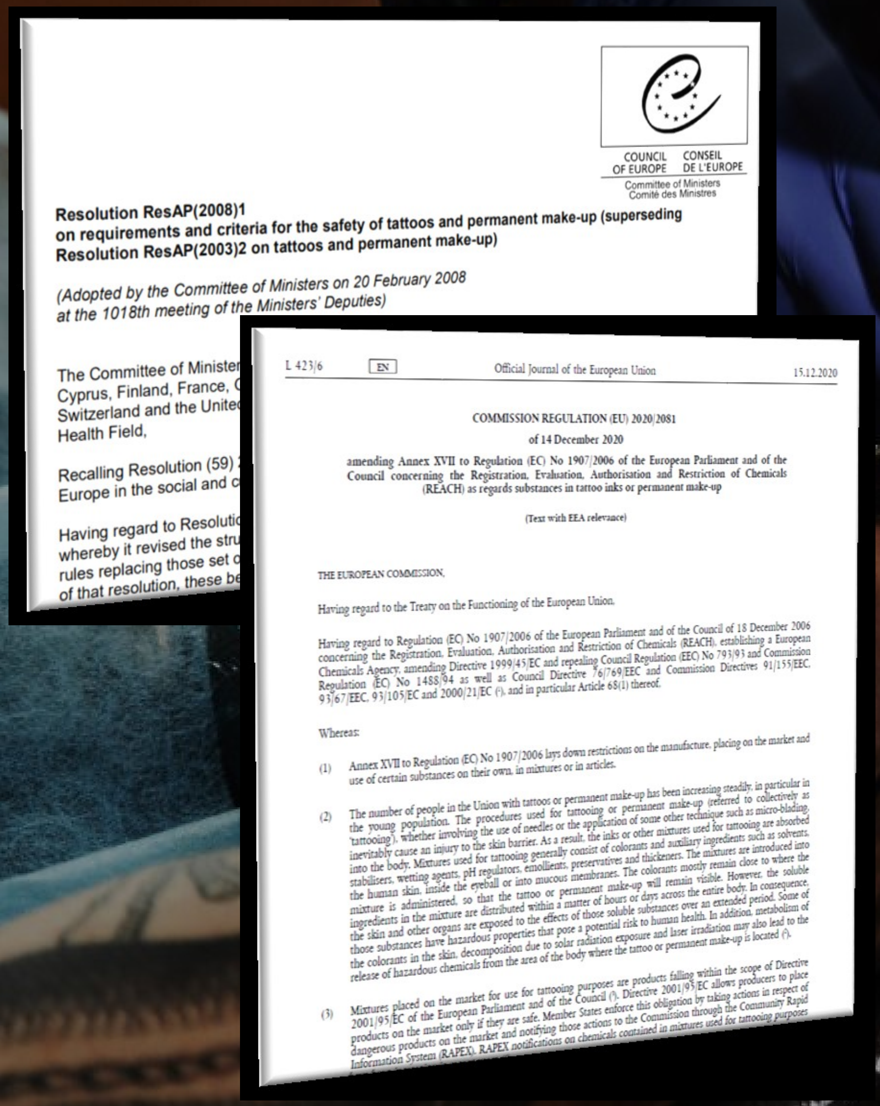
- Ti was the highest concentrated metal mainly in white inks but also in the other colours, except in black
- Cu was observed only in blue and green colours, accordingly to uses of Cu-phthalocyanine colorants
- Al > Ba > Fe > Zn were also contained at prominent quantities in inks
- Traces of metals were observed for As, Cd, Co, Hg, Mn, Mo, Pb, Sb, Se, Sn and only in a few inks

1. Total metal respect to CoE ResAp and REACH



- Cr was above the CoE ResAP (0.2 ppm) in 71% of inks, and above the REACH restriction (0.5 ppm) in 43% of inks
- Cu exceeded both restrictions in the 29% of inks
- Ni was above the LoQ of the method in all inks, but lower than 1.5 ppm (five-times lower than REACH restriction of 5 ppm)
- Pb and Sn were above the REACH limits (more restrictive than CoE ResAP) in 14% and 43% of inks, respectively

1. Total metal by HR-ICP-MS: DISCUSSION


COUNCIL OF EUROPE CONSEIL DE L'EUROPE
Committee of Ministers
Comité des Ministres

Resolution ResAP(2008)1
on requirements and criteria for the safety of tattoos and permanent make-up (superseding Resolution ResAP(2003)2 on tattoos and permanent make-up)

(Adopted by the Committee of Ministers on 20 February 2008 at the 1018th meeting of the Ministers' Deputies)

The Committee of Minister
Cyprus, Finland, France,
Switzerland and the United
Health Field,

Recalling Resolution (59)
Europe in the social and c

Having regard to Resoluti
whereby it revised the stru
rules replacing those set o
of that resolution, these be

L 423/6 EN Official Journal of the European Union 15.12.2020

COMMISSION REGULATION (EU) 2020/2081
of 14 December 2020
amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards substances in tattoo inks or permanent make-up

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC, and in particular Article 68(1) thereof,

Whereas:

(1) Annex XVII to Regulation (EC) No 1907/2006 lays down restrictions on the manufacture, placing on the market and use of certain substances on their own, in mixtures or in articles.

(2) The number of people in the Union with tattoos or permanent make-up has been increasing steadily, in particular in the young population. The procedures used for tattooing or permanent make-up (referred to collectively as 'tattooing'), whether involving the use of needles or the application of some other technique such as micro-blading, inevitably cause an injury to the skin barrier. As a result, the inks or other mixtures used for tattooing are absorbed into the body. Mixtures used for tattooing generally consist of colorants and auxiliary ingredients such as solvents, stabilisers, wetting agents, pH regulators, emollients, preservatives and thickeners. The mixtures are introduced into the human skin, inside the eyeball or into mucous membranes. The colorants mostly remain close to where the mixture is administered, so that the tattoo or permanent make-up will remain visible. However, the soluble ingredients in the mixture are distributed within a matter of hours or days across the entire body. In consequence, the skin and other organs are exposed to the effects of those soluble substances over an extended period. Some of those substances have hazardous properties that pose a potential risk to human health. In addition, metabolism of the colorants in the skin, decomposition due to solar radiation exposure and laser irradiation may also lead to the release of hazardous chemicals from the area of the body where the tattoo or permanent make-up is located.

(3) Mixtures placed on the market for use for tattooing purposes are products falling within the scope of Directive 2001/95/EC of the European Parliament and of the Council. Directive 2001/95/EC allows producers to place products on the market only if they are safe. Member States enforce this obligation by taking actions in respect of dangerous products on the market and notifying those actions to the Commission through the Community Rapid Information System (RAPEX). RAPEX notifications on chemicals contained in mixtures used for tattooing purposes

2009

14% of tattoo inks showed metals (Ni, Pb, Ba, As, Cd, Zn, Cr, Co and Cu) above the CoE ResAP levels

Cr, Ni and Co exceeded the safe allergological limit of 1 ppm in 62%, 16% and 2% of inks, respectively

2019

100% of tattoo inks were conform to CoE ResAP levels, indicating ink production has shifted to purer materials and best manufacturing practices

A lower percentage of exceedances respect to dermatological limit of 1 ppm were observed for the allergens Cr (28%), Ni (15%) and Co (0%) in inks, respect to ten-years ago

2. Ion Chromatographic (IC)-ICP-MS: METHOD

2018: no. 29 inks from USA and EU purchased online

Alkaline extraction

Na_2CO_3 + NaOH, pH 13

IC

Anion exchange chromatography

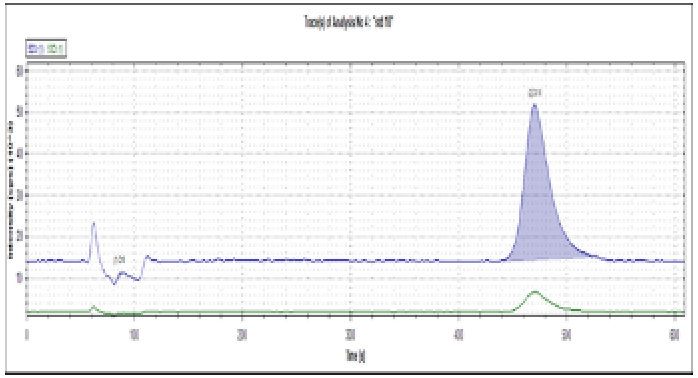
Mobile phase: $(NH_4)_2SO_4$/ NH_4OH

iCAPQ-ICP-MS

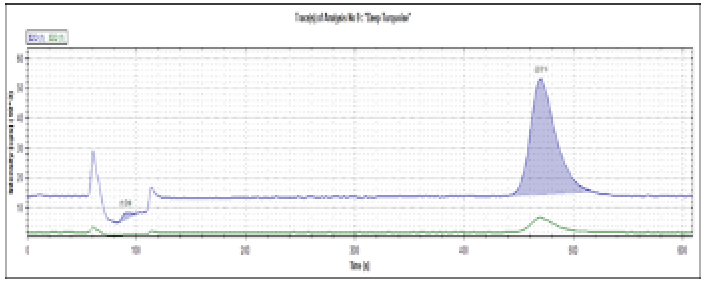
Principle: **first alkaline extraction step followed by ion chromatographic (IC) separation using anion exchange columns and on-line detection by ICP-MS** equipped with He collision cell so any interfering specie on the Cr signal was removed. **In the presence of a strong alkaline environment, Cr(III) precipitates as insoluble hydroxides**

Description: ca. 0.5 g of inks were extracted with 25 mL of alkaline extraction solution (Na_2CO_3 + NaOH, pH ~ 13). The solutions were heated, under stirring, at 90-95°C for 60 min. The sample was then centrifuged, and the supernatant solution was filtered and analysed. For elution of the Cr species, 0.25 M $(NH_4)_2SO_4$ and 0.1 M NH_4OH as the mobile phase (0.4 mL/min isocratic) was used

Validation: **99% recovery** by spiking Cr(VI) standard solution to tattoo ink before extraction. The **LoD and LoQ were 0.02 mg/kg and 0.07 of Cr(VI)** in tattoo ink, and **intra-day repeatability** of 8.1%



Cr(VI) IN ALKALINE EXTRACTION SOLUTION



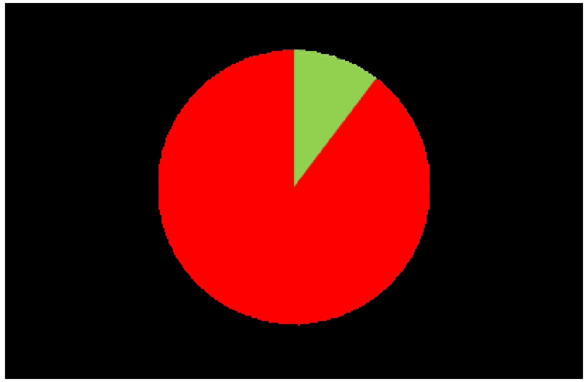
Cr(VI) IN TATTOO INK

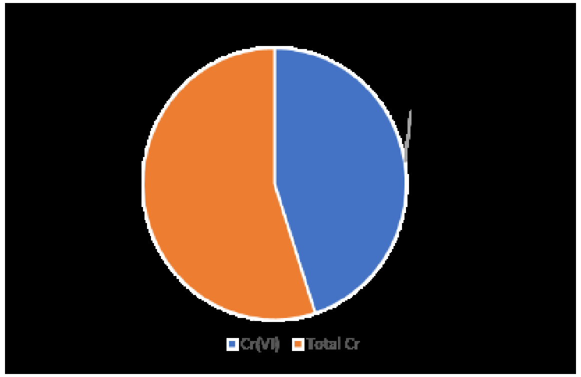
Bocca et al., 2018. Hexavalent chromium in tattoo inks: Dermal exposure and systemic risk. Contact Dermatitis 79:218–225

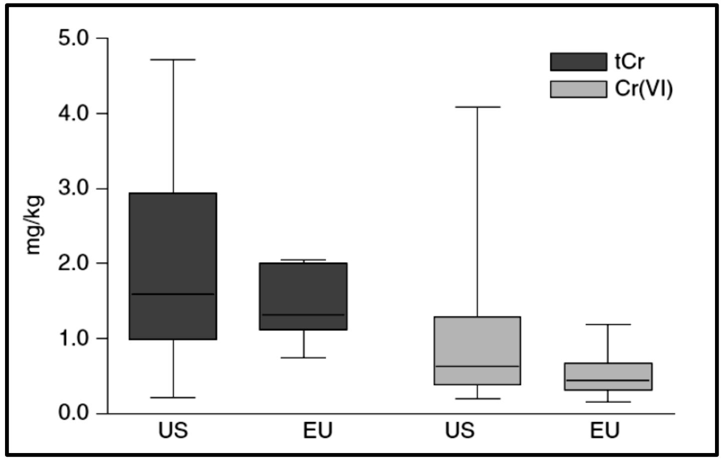
2. Ion Chromatographic (IC)-ICP-MS: RESULTS

	Cr(VI) (µg/g)	Cr(VI)/tCr (%)
Deep blue	0.22	16
Country blue	0.30	58
Brite Orange	2.94	62
Red scarlet	0.46	43
Deep violet	2.38	59
Deep Tourquoise	0.38	21
Lime green	0.42	23
Green grasshopper	1.15	45
True magenta	3.91	84
Baby blue	0.60	94
Violet grape	1.33	91
Cherry bomb	4.09	99
True black	1.25	81
Acquamarine	0.20	91
Hunter green	0.68	31

	Cr(VI) (µg/g)	Cr(VI)/tCr (%)
Ice blue	0.52	16
Violent violet	0.98	57
Doo doo Brown	0.43	28
Mean green	0.68	63
Power white	0.35	41
Light purple	0.65	73
Crimson red	0.40	25
Blue turquoise	0.40	28
Grey Grey	0.67	33
Basic green	0.45	23
Ultramarine	0.16	15
Black	1.19	91
Blue	0.32	43
Chocolate Brown	0.63	57







90% of inks contained Cr(VI) (range: 0.22-4.09 mg/kg) **above the maximum allowed CoE ResAP levels**
28% of inks showed Cr(VI) levels (range: 1.15-4.09 mg/kg) **above the limit of dermal sensitization (1 ppm)**
Cr(VI) levels were lower in samples produced in **EU (0.45 µg/g)** than in **US (1.31 µg/g)**
None of the ink bottles **had labelling** indicating the presence of Cr(VI), as recommended by CoE ResAP

2. Ion Chromatographic (IC)-ICP-MS: RESULTS

	SED (mg/kg bw/d)	MoS (NOAEL/SED)
Deep blue	1.67×10^{-7}	49615
Country blue	2.32×10^{-7}	35722
Brite Orange	2.28×10^{-7}	3645
Red scarlet	3.54×10^{-7}	23450
Deep violet	1.85×10^{-7}	4497
Deep Tourquoise	2.96×10^{-7}	28054
Lime green	3.25×10^{-7}	25577
Green grasshopper	8.90×10^{-7}	9327
True magenta	3.03×10^{-7}	2742
Baby blue	4.65×10^{-7}	17832
Violet grape	1.03×10^{-7}	8040
Cherry bomb	3.17×10^{-7}	2620
True black	9.68×10^{-7}	8573
Acquamarine	1.55×10^{-7}	53584
Hunter green	5.27×10^{-7}	15760

	SED (mg/kg bw/d)	MoS (NOAEL/SED)
Ice blue	4.03×10^{-7}	20609
Violent violet	7.61×10^{-7}	10913
Doo doo Brown	3.31×10^{-7}	25098
Mean green	5.27×10^{-7}	15737
Power white	2.70×10^{-7}	30795
Light purple	5.03×10^{-7}	16487
Crimson red	3.10×10^{-7}	26792
Blue turquoise	3.07×10^{-7}	27062
Grey Grey	5.20×10^{-7}	15948
Basic green	3.51×10^{-7}	23657
Ultramarine	1.26×10^{-7}	65747
Black	9.22×10^{-7}	9006
Blue	2.48×10^{-7}	33490
Chocolate Brown	4.88×10^{-7}	17011

All inks	SED (mg/kg BW/d)	MoS (NOAEL/SED)
Median	4.65×10^{-7}	17832
Minimum	1.26×10^{-7}	2620
Maximum	3.17×10^{-7}	65747

Systemic Exposure Dose (SED):
pigment used for tattooing = 2.53 mg/cm^2
tattoed area = 900 cm^2
absorption rate = 100%
period = 42 days
bw = 70 kg

Margin of Safety (MOS):
a NOAEL of 2.5 mg/kg bw/d for systemic effects in rats was used as a starting point, to which an uncertainty factors were applied for the intraspecies and interspecies variability and to compensate for the less-than-lifetime exposure duration in the animal study

The SED for Cr(VI) (range: 1.26×10^{-7} - 3.17×10^{-6} mg/kg bw/d), for the 100% absorption scenario, **was much lower than the acceptable daily intake (ADI)** for Cr(VI) (8.3×10^{-3} mg/kg bw/d)

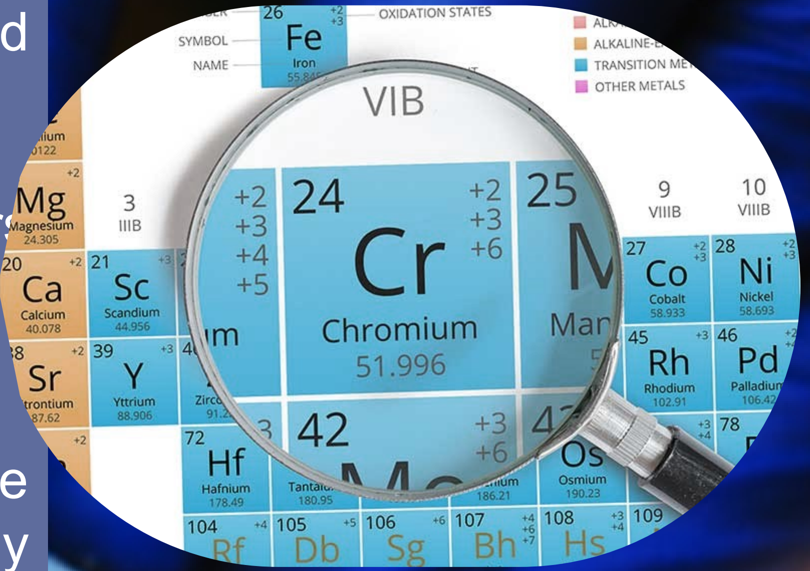
The **MoS values** for Cr(VI) (range: 2620 - 65,747) were **much higher than 100** indicating not significant systemic risk

2. Ion Chromatographic (IC-ICP-MS): DISCUSSION

Based on SED and MOS values, the exposure to Cr(VI) contained in tattoo inks is not expected to increase the risk for tattooed individuals

Notwithstanding this, Cr(VI) data showed tattoo ink manufacturers still do not achieve Cr(VI) levels lower than regulatory thresholds in the final products

For a number of inks, Cr(VI) content may represent a possible cause of skin sensitization, especially if consumers have already been sensitized to Cr(VI) from other sources (Cr(VI) is not permitted in the Cosmetic Regulation!)



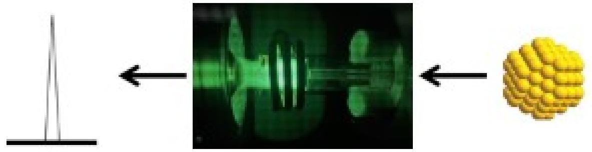
3. Single Particle (SP)-ICP-MS: METHOD

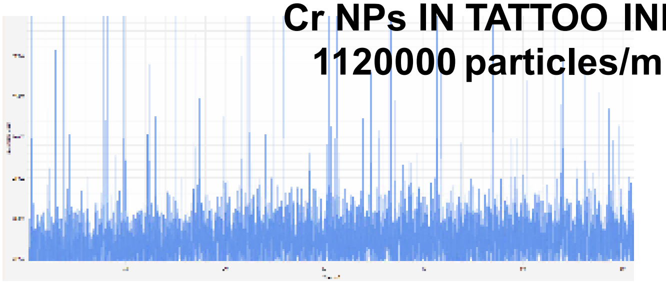
2019: no. 20 inks from USA, EU and China provided by professional tattoo artists

Water dilution

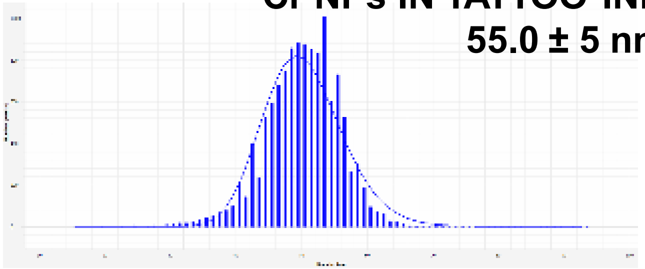
Sonication by ultrasound ice cold water bath

SP-ICP-MS





Cr NPs IN TATTOO INK
1120000 particles/mL



Cr NPs IN TATTOO INK
55.0 ± 5 nm

Principle: when suspended NPs are carried to the plasma they generate an «event» (that appears as a «spike»). **The frequency of the events is directly proportional to the number of particles entering the plasma, while the intensity (height) of the events to the diameter of particles.** Thus, particle number concentrations (particle/mL) and particle sizes (nm) are simultaneously evaluated

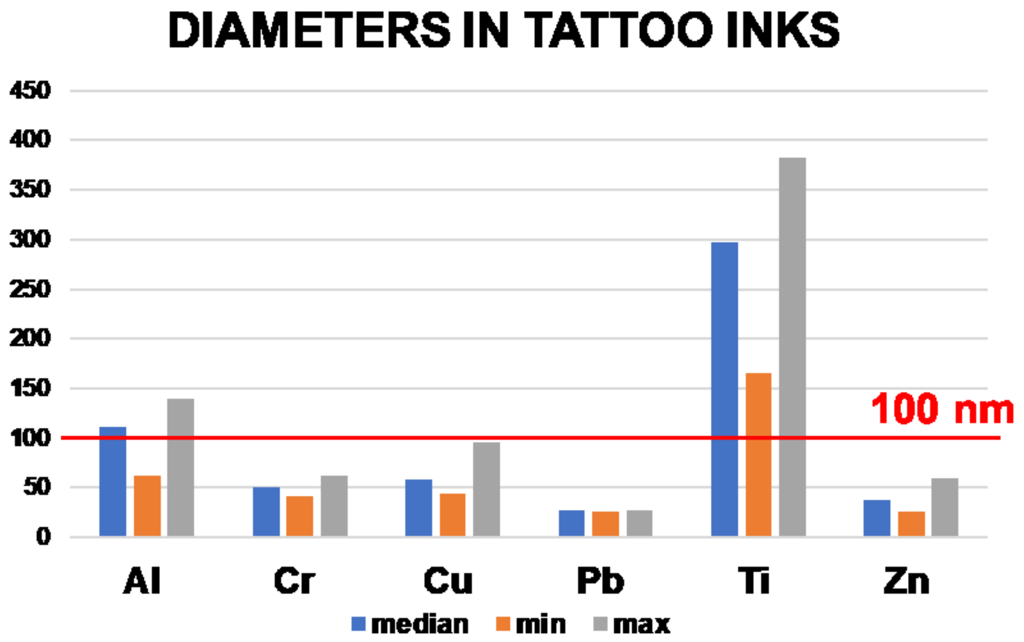
Description: tattoo ink **were diluted with ultrapure deionized water**, through successive steps. After each dilution step, the inks were vortexed for 1 min and **sonicated for 10 min by ultrasound ice cold water bath to prevent subsequent agglomeration**

Validation: **Accuracy** on size (96-112%), **repeatability** (RSD% < 11%) and **linearity** (R^2 > 0.99) in the range of 1-50 µg/L were assessed **by analyzing certified reference NPs standards** of Al_2O_3 (30 nm), Co_3O_4 (15 nm), Cr_2O_3 (60 nm), CuO (25-55 nm), NiO (18 nm), TiO_2 (18 nm and <100 nm) and ZnO (50-80 nm and 30-40 nm)

Battistini et al., 2020.Quantitative analysis of metals and metal-based nano- and submicron-particles in tattoo inks. Chemosphere.

3. Single Particle (SP)-ICP-MS: DIAMETERS

Colours	Ink name	Al (nm)	Cr (nm)	Cu (nm)	Pb (nm)	Ti (nm)	Zn (nm)
Black	Liner Black	nd	56 ± 3	nd	nd	nd	nd
	True Black	140 ± 13	55 ± 5	nd	28 ± 3	nd	nd
	Black Hole	nd	nd	nd	nd	nd	nd
Red	Light Red	138 ± 20	55 ± 5	nd	nd	166 ± 34	nd
	Bright Red	80 ± 29	nd	nd	27 ± 7	nd	26 ± 6
Yellow	Golden Yellow	118 ± 16	43 ± 4	nd	nd	332 ± 32	nd
	Dark Yellow	62 ± 10	42 ± 7	nd	nd	282 ± 43	45 ± 4
Green	Light Green	115 ± 13	62 ± 4	46 ± 13	nd	260 ± 30	nd
	Everglades Green	77 ± 12	nd	60 ± 6	nd	323 ± 54	33 ± 3
	Galapagos Green	77 ± 10	43 ± 5	62 ± 14	nd	321 ± 63	39 ± 2
Blue	Mario's Blue	115 ± 12	45 ± 7	57 ± 9	nd	228 ± 53	nd
	Sea Blue	110 ± 17	46 ± 8	96 ± 11	nd	346 ± 66	49 ± 8
	Frostbite Blue	129 ± 18	nd	44 ± 13	nd	383 ± 62	32 ± 3
Violet	Grape	111 ±11	nd	nd	nd	293 ± 65	nd
	Carol's Pink	113 ± 17	nd	nd	nd	301 ± 43	48 ± 7
White	Snow White	69 ± 15	nd	nd	nd	264 ± 47	36 ± 4
	Pure White	119 ± 27	50 ± 6	nd	nd	333 ± 35	59 ± 3
Brown	Coco	105 ± 17	nd	nd	26 ± 7	nd	27 ± 3
	Dark Brown	nd	52 ± 10	nd	nd	nd	30 ± 4
Grey	Silver	78 ± 11	nd	nd	nd	294 ± 51	43 ± 4



Al (median, 111 nm) present in 85% of inks
Cr (median, 50 nm) observed in 55% of inks
Cu (median, 59 nm) observed only in green and blue colours
Zn (median, 38 nm) contained in 60% of inks
Ti present at submicron sizes (**median, 298 nm**) in 70% of inks (white and coloured)

Pb (median, 27 nm) found only in 0.15% of inks (black, brown and red)
Co, **Hg**, **Ni** were not detected as particles in inks

3. Single Particle (SP ICP-MS): CONCENTRATIONS

Colours	Ink name	Al NPs (µg/g)	Cr NPs (µg/g)	Cu NPs (µg/g)	Ti (µg/g)	Zn (µg/g)
Black	Liner Black	nd	0.62	nd	nd	nd
	True Black	0.92	1.51	nd	nd	nd
	Black Hole	nd	nd	nd	nd	nd
Red	Light Red	0.14	1.54	nd	1.44	nd
	Bright Red	2.26	nd	nd	nd	0.50
Yellow	Golden Yellow	0.14	0.07	nd	107	nd
	Dark Yellow	0.71	0.08	nd	540	0.35
Green	Light Green	0.15	0.16	581	97	nd
	Everglades Green	1.00	nd	721	1194	0.12
	Galapagos Green	0.36	0.68	699	578	0.10
Blue	Mario's Blue	0.13	0.28	3287	1093	nd
	Sea Blue	2.29	0.28	492	5270	0.40
	Frostbite Blue	2.29	nd	604	5912	1.59
Violet	Grape Violet	0.12	nd	nd	1053	nd
	Carol's Pink	2.31	nd	nd	5968	0.40
White	Snow White	1.52	nd	nd	6177	0.41
	Pure White	0.29	0.16	nd	2889	0.40
Brown	Coco	1.15	nd	nd	nd	0.12
	Dark Brown	nd	0.71	nd	nd	0.79
Grey	Silver	2.10	nd	nd	3997	0.38

Al particles (0.12 to 2.31 µg/g) were the 1% of total Al

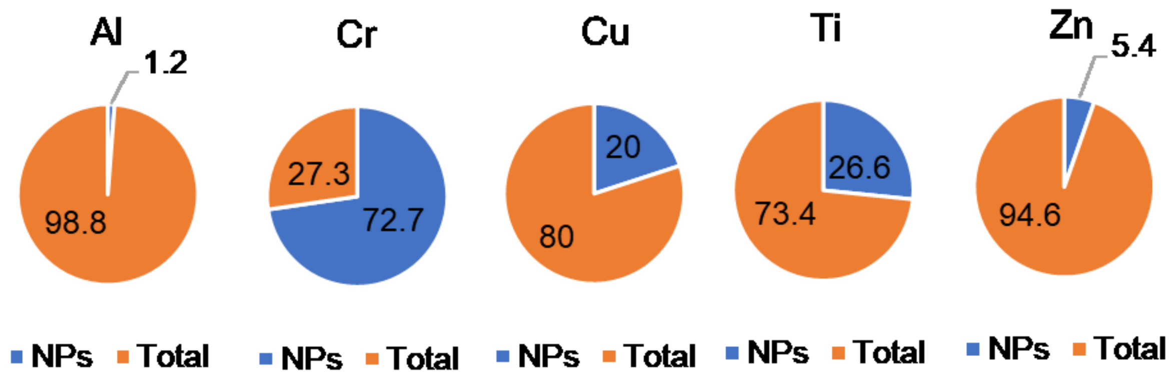
Cu NPs (492-3287 µg/g) represented the 20% of total Cu in green and blue

Cr NPs (0.07 and 1.54 µg/g) represented the 70% of total Cr

Ti particles content varied from low in colored inks to high (6177 µg/g) in white inks

Zn NPs (0.10-1.59 µg/g) were the 5% of total Zn

PERCENTAGES OF NPs RESPECT TO TOTAL METALS



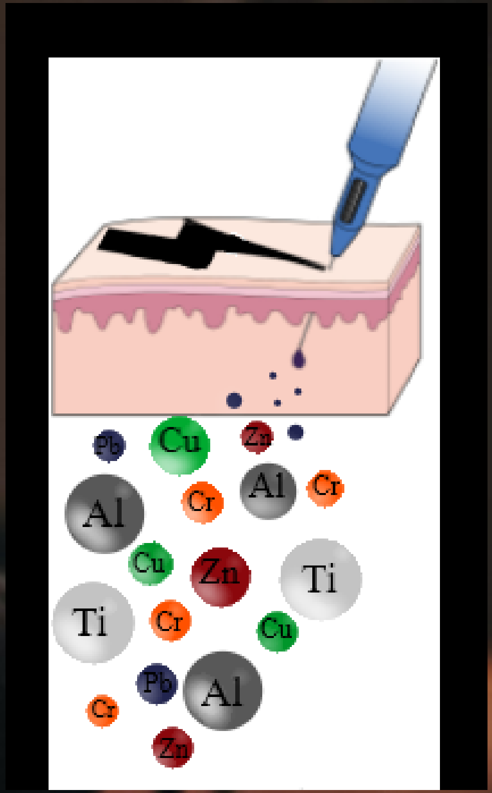
3. Single Particle (SP)-ICP-MS: DISCUSSION

A mixture of several metals at nano-level were observed in tattoo inks, with smaller particles pertaining to Pb and Zn (27-38 nm), intermediate sizes were observed for Cr and Cu (50-59 nm) and diameters between 100-300 nm for Al and Ti particles

The percentages of particles in tattoo inks varied a lot depending on the metal and on the ink (from low percentages to very high percentages)

The presence of particles at sizes <100 nm in tattoo inks might provoke distribution in other organs, behind the skin

Some NPs may be more stable (Al, Ti) while others (Cu, Zn) may be prone to chemical dissolution that may increase the bioavailability of metals and their toxicity



4. Asymmetric Flow Field Fractionation and Multi-Angle Light Scattering (FFF-MALS)-ICP-MS: METHOD

2017: no. 4 inks from USA, EU, Canada purchased online

Water dilution

Sonication in ice cold water bath

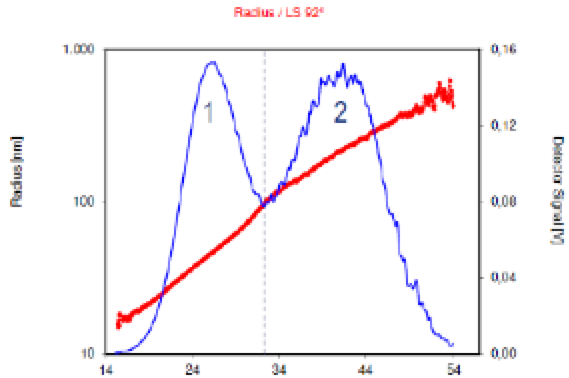
AF4

AF4 fractionation

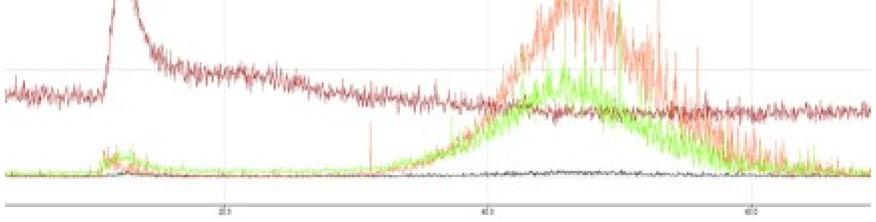
Mobile phase: H_2O

MALS

iCAPQ- ICP-MS



DEEP VIOLET INK



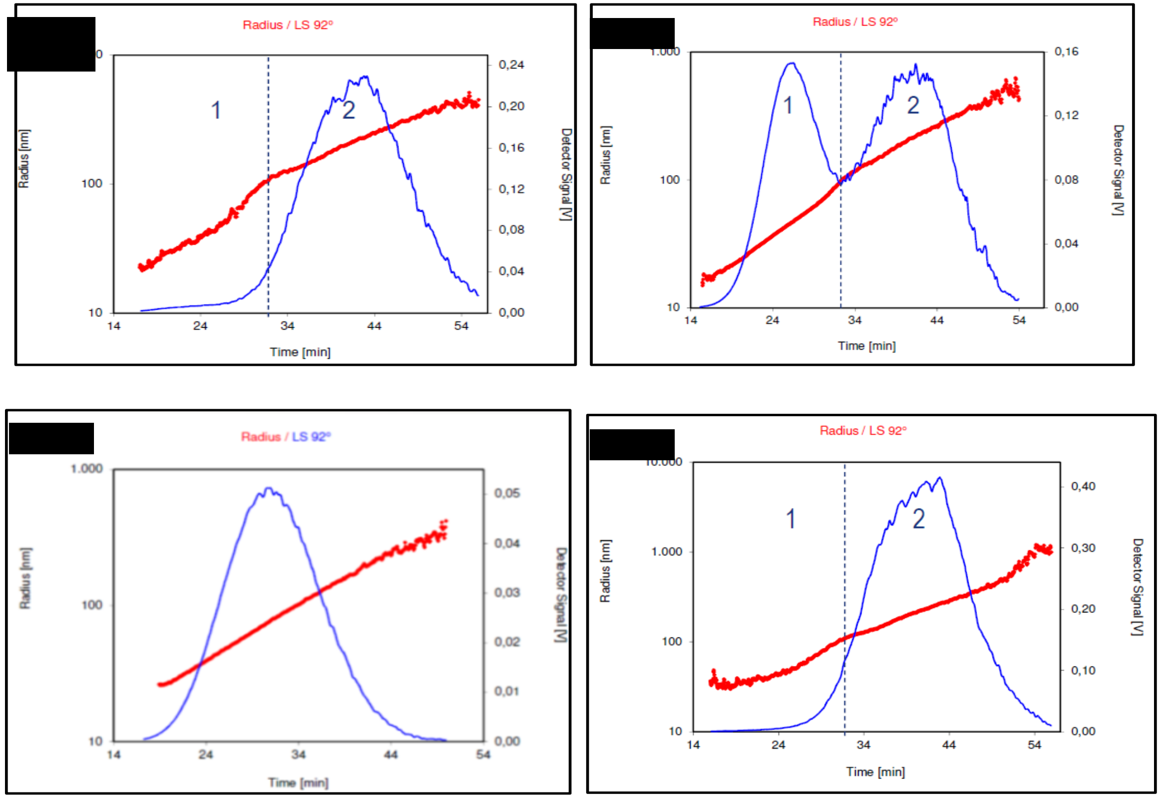
Principle: in the FFF separation, a laminar flow of the eluent carries the NPs toward a channel and a second flow field is applied ("cross-flow") orthogonal to the laminar flow. **Size separation is obtained using the interplay between cross-flow field and the different diffusivities of NPs determined by their sizes. Smaller particles diffuse higher and elute before larger particles**

Description: inks were diluted with ultrapure deionized water and sonicated for 10 min by ultrasound ice cold water bath. For the FFF fractionation ultrapure deionized water was used as mobile phase. Then the **MALS and ICP-MS detectors coupled on-line to FFF determined the particle size (radius of gyration, R_g) and the elemental composition and concentration in the separated monodispersed fractions of inks**

Validation: **Accuracy** on size (84% and 80%), **recovery** (> 92%), **repeatability** (RSD% < 10%) and **linearity** (R^2 > 0.99) in the range 0-170 µg/mL and 0-3.4 µg/mL were assessed by analyzing **certified reference NPs standards** of TiO_2 (<100 nm) and ZnO (<100 nm)

Bocca et al., 2017. Size and metal composition characterization of nano- and microparticles in tattoo inks by a combination of analytical techniques. JAAS 32:616-628

4. Sizing of nano-pigments by FFF-MALS



A. Ice blue; **B**. Deep violet; **C**. Black outlining; **D**. Grasshopper green

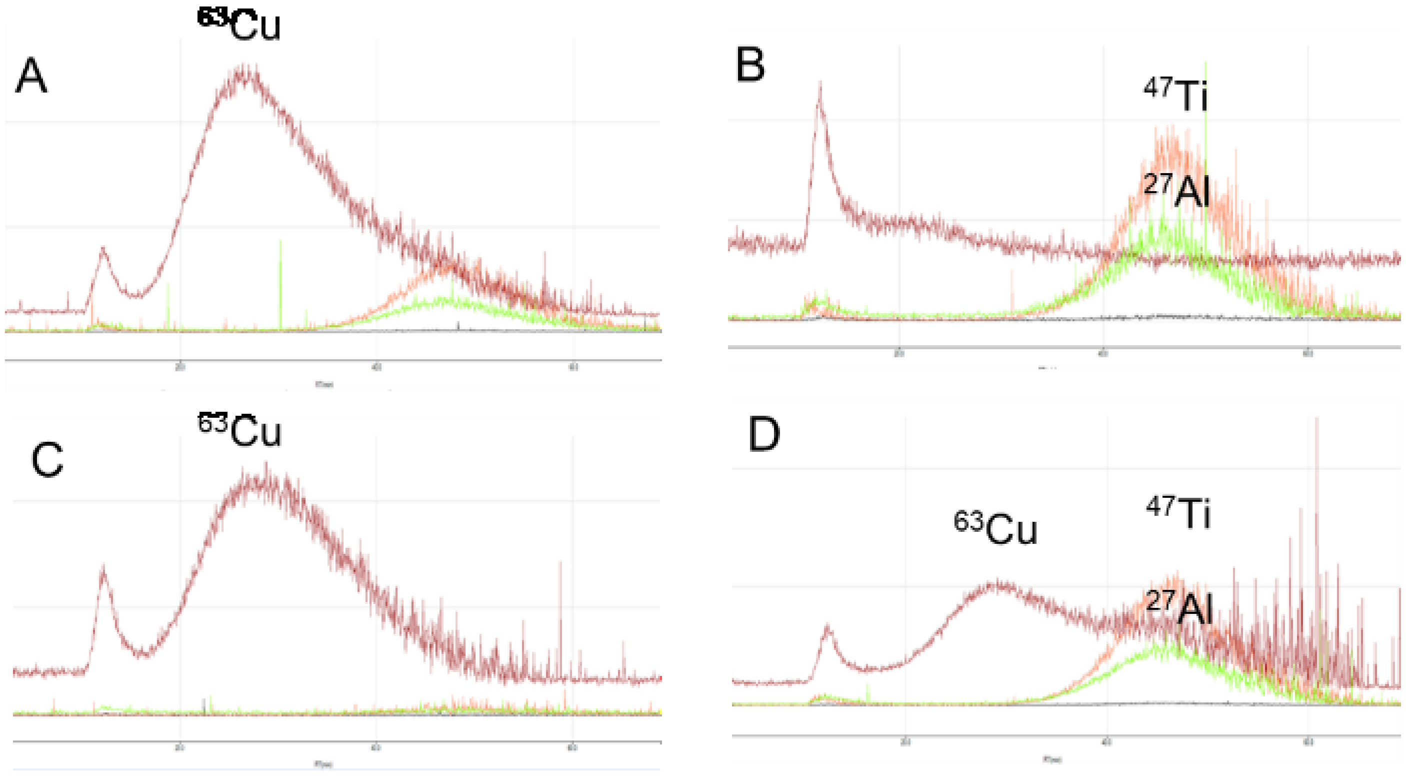
Ink	Peak	Rt (min)	Rg (nm)	< 100 nm
Ice blue	1	17-28	42 (16-65)	53%
	2	28-56	269 (60-490)	
Deep Violet	1	15-32	56 (11-100)	78%
	2	32-54	271 (100-490)	
Black Outlining	1	17-50	137 (21-330)	77%
Grasshopper Green	1	16-27	46 (18-56)	32%
	2	27-56	405 (50-1200)	

RT: retention time; Rg: Radius of gyration

Using FFF-MALS, **smaller particles from larger ones** were separated, revealing monomodal particle size distrbution in black ink, and bimodal distribution in blu, violet and green inks

By the **cumulant analysis** with FFF-MALS the inks (blue, violet and black) revealed to contain more than 50% of particles between 1-100 nm, thus classifiable as “**nanomaterials” according to EC definition**

4. Metals in nano-pigments by FFF-ICP-MS



A. Ice blue; B. Deep violet; C. Black outlining; D. Grasshopper green

Ink	Peak	Al (mg/mL)	Cu (mg/mL)	Ti (mg/mL)
Ice blue	1	nd	4.6±0.5	110±12
	2	0.4±0.1	nd	nd
Deep Violet	1	nd	nd	65±6
	2	0.3±0.05	nd	nd
Black Outlining	1	nd	1.8±0.2	nd
Grasshopper Green	1	nd	2.2±0.2	nd
	2	0.8±0.2	nd	160±15

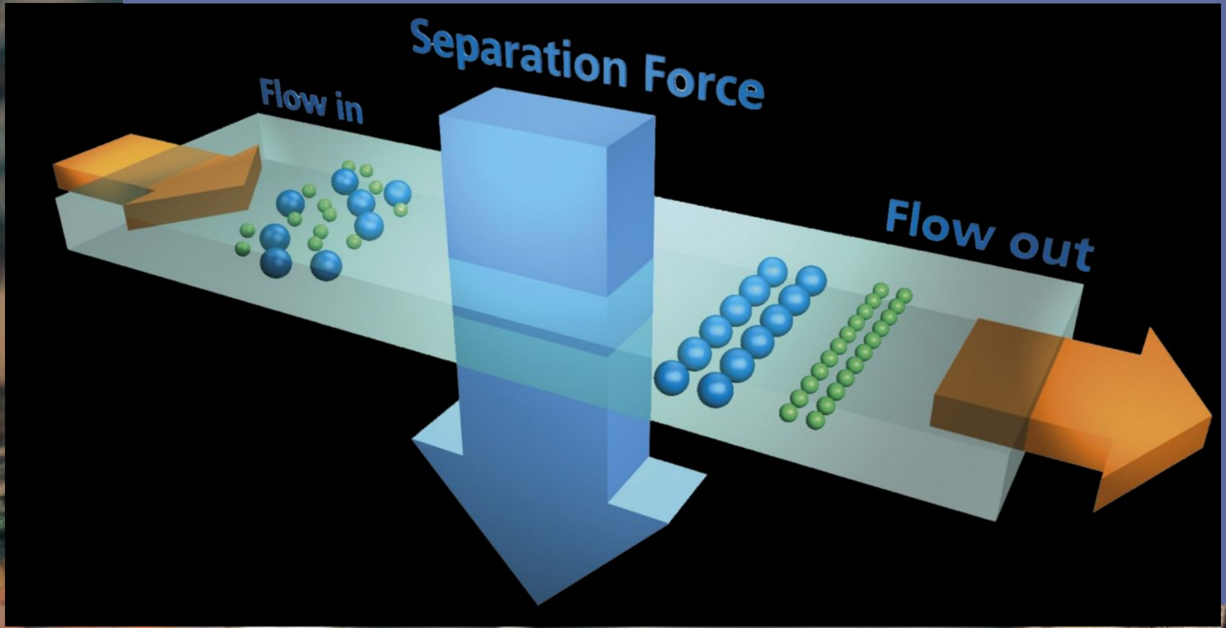
Al (0.3-0.8 mg/mL) at low content was associated to particles above 100 nm in blue, violet and green

Cu (1.8-4.6 mg/mL) was associated to NPs of size between 16-65 nm in black, blue and green inks

Ti was found at the highest concentration **(65-160 mg/mL)** associated to particles above 100 nm in blue, violet and green

4. Asymmetric Flow Field Fractionation and Multi-Angle Light Scattering (FFF-MALS)-ICP-MS: DISCUSSION

The analysis by FFF-MALS-ICP-MS was beneficial for the separation of complex and polydispersed tattoo inks, providing the elemental distributions of various metals as a function of size



The cumulant analysis by FFF-MALS permitted to classify tattoo inks as ‘nanomaterials’ under the terms of the EC definition

In some tattoo inks more than 25% weight of particle between 1-100 nm were found, hence exceeding the safe level for TiO_2 and ZnO permitted in the Cosmetic Directive for dermal use

Conclusion and health impact

ICP-MS based methods have been specifically developed and validated under a quality assurance system for the analysis of tattoo inks, with the aim to fill the gap of non-existent or not standardized analytical methods

It is clear that tattoo inks can and do contain metals classified with regard to carcinogenicity (Cr(VI)) and skin sensitization (Co, Cr(VI) and Ni) and so adverse effects of these substances in tattooed individuals cannot be excluded

Moreover, a mixture of several metals mainly as oxides and at different particle sizes (from nano to submicron-level) are present simultaneously in tattoo inks, and these combinations may generate cumulative effects of hazard substances

Due to the evidence of NPs in tattoo inks that may not have the same behavior of the particles at a micro-level, it is mandatory to perform a risk assessment and regulation system for intradermal exposure to NPs which is inadequately explored so far

The present project:

Assessment of skin and systemic toxicity in patients undergoing laseR tatTOO removal (ARTOO)

Research financially supported by the Italian Ministry of Health, involves the collaboration of Istituto Superiore di Sanità (ISS)-Istituto Dermopatico dell'Immacolata (IDI) of Rome

Background: the potential risk of pigment fragmentation or degradation into more toxic chemicals upon **tattoo-laser removal is a serious health-related issue**

Objective: the **new knowledge about the assessment of chemicals** generated by laser-treatment of tattoos using **in-vitro, ex-vivo and in-vivo (human tissues, blood and urine) experiments,** and the evaluation of **dermal and systemic toxic effects** during a **3-years follow-up survey**

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Thanks for the attention

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