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Description of development of an analytical method for measurement of PAA in tattoo ink and PMU

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Purpose

The purpose of this review is to give some background on azo pigments and primary aromatic amines (PAA) as well as to describe how to develop a robust method for analyzing these in tattoo ink.

Background

There is no well-established method for analyzing PAA in tattoo inks. The Danish EPA have experienced that the same batch of ink has given different analytical results when two labs have performed a chemical analysis of the PAA content. Further, different experiments with the tests of methods for reductive cleavage on the same sample have given different results. Further, none of the tested solvents or reagents tested for digesting the pigments has managed to produce more than a few percent of the theoretical amount of primary aromatic amine expected to be released (personal communication, Urs Hauri 2017). This is an important issue if the PAAs should be regulated in tattoo inks.

Most of the background in this review is taken from the extensive study, Safety of tattoos and permanent make-up. State of play and trends in tattoo practices by JRC 2015, primarily the report from work package 2 and the final report. JRC 2015 differentiate between tattoo ink and permanent make up (PMU). However, this distinction has not been made in this report.

Hypotheses

There are several possible explanations for the differences in the analytical results of PAAs in tattoo inks.

The inks are more dispersions than solutions. The colorants in the inks are pigment particles, which make the inks inhomogeneous. This can make it difficult to take out a representative sample from an inkbottle. Further, two bottles of inks can have different composition even though they are taken from the same batch.

Degradation of azo colorants can generate PAAs. Azo colorants can be degraded by irradiation: sunlight or laser (JRC 2015). Enzymatically degradation or bacterial degradation has also been shown (Sudha et al. 2014, Chacko and Subramaniam 2011). Degradation of the azo colorants may also lead to different result for the same sample. On the other hand, azo colorants are chemically very stable, which is why they are commonly used as dyes.

Investigations of the release of PAAs from azo colorants by different decomposition methods have shown very different results possibly due to the insolubility of pigments. Different solvents, conditions and perhaps reaction time seem to influence the release PAAs (personal communication, Urs Hauri 2017). Therefore, for an analytical method it would be relevant to investigate and decide if a specific decomposition method should be used.

Further, Azo colorants are produced from PAAs, and as a consequence of that PAAs can be present in the ink as impurities from the production and often the purity of the azo colorants is low (70-90%). However, it is not - in the first place - assumed that insufficiently homogeny in the production of the inks have resulted in inks frorm the same batch having different content of PAAs.

Azo-colorants and PAAs, definition and description

Azo pigments are defined by having an azo group, which is a double bonded nitrogen within the molecule (–N=N–). They have generally bright colors and are considered quite stable, which make them to one of the most common chemical groups of pigments used. Further, they are relatively cheap to produce. The chemical reactions do not require additional energy (such as heat) or catalysts.

In relation to tattoo ink and ink for permanent make up approximately 54% (67 in number) of the colorants are azo dyes. In a database for colorants (Artiscreation), approximately 630 colorants are described. Of these are 30% azo dyes (180 in number). The types of azo dyes applied for tattoo ink are mono azo and di azo colorants i.e. having one or two double bonded nitrogens, respectively. Mono azo colorants are most common of these two types (78%) regarding tattoo inks (JRC 2015). Figure 1 are examples of a mono azo and a di azo colorant used in tattoo ink.

a) b)

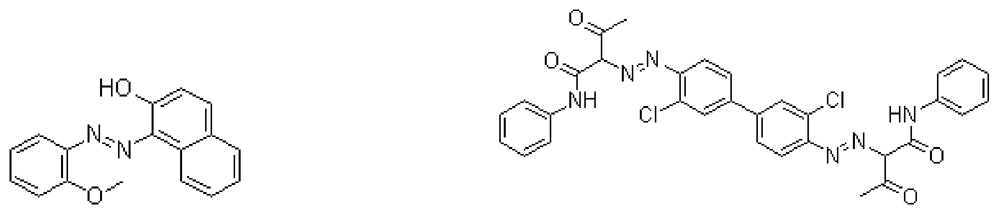
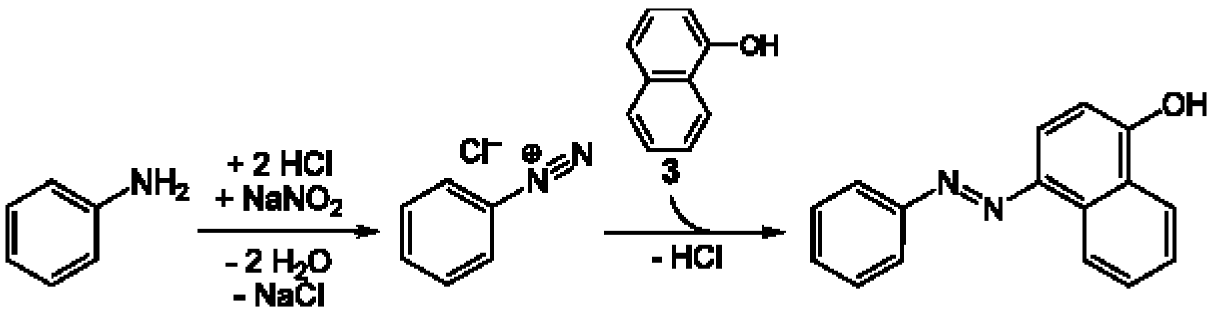


Figure 1. Examples of a mono azo colorant, a): pigment red 1 (PR 1) and a di azo colorant, b): pigment yellow 12 (PY 12).

Azo pigments are produced from a primary amine (1) which is treated with sodium nitrate under acidic condition to form an unstable diazonium salt (2). The reaction is called diazotization. The diazonium salt can react with a coupling agent such as an aromatic amine or a phenol (3) and form a stable azo dye (4) see figure 2 (Morrison and Boyd, 1987). The PAA used for producing azo pigments is normally a quit simple diazonium salt but the amine or phenol attached to the diazonium salt afterwards can be very complex molecules.



1 2 4

Figure 2. The chemical reaction for synthesis of an azo colorant.

Chemically a primary amine consist of nitrogen group (-NH2) attached to an aromatic backbone.

A number of PAAs have a harmonized classification as CMR (Carcinogenic, Mutagenic and Reprotoxic substances), as they are listed in Table 3.1 of the EC Regulation 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP regulation). Seven PAAs used for the production of tattoo colorants are classified as CMR, see also table 1.

Most azo dyes has normally one azo group (mono azo) but they can have two (di azo) or three (tri azo). In relation to tattoo inks, there are only pigments with one or two azo groups.

Azo dyes can provide almost all colors, but in tattoo inks it appears to be mainly red, yellow and orange colors that are applied (JRC 2015).

As primary aromatic amines (PAA) are used in the production of azo dyes impurities of PAA can be found in the dyes.

In JRC 2015, 67 azo colorants used for tattoo inks are described (see also Annex 3).

Even though the azo colorants used in tattoo inks are either pigments or lakes[1], both being considered insoluble in water and deposited in the derma as microcrystalline grains, equilibrium may exist between the solid phase and small amount of colorants dissolved in the fluid constantly circulating in the body. Therefore, it cannot be excluded that to a certain degree the azo compounds may be metabolically reductively cleaved into aromatic amines when situated in the viable skin layers – or in the liver upon release into the bloodstream (JRC 2015).

Pigments (or colorants) are the main component in ink (up to 60%). In addition, several additives are also present in the ink: surfactants (thixotropic agents to avoid sedimentation of pigments), preservatives (to avoid microbial growth), binding agents (polymers that bind the pigment particles together), solvents (water (primary), alcohol, glycerin) and fillers (disperablility of pigments, often silica and barium sulphate).

Colorants used for tattoo ink is not produced for this purpose, but normally by the chemical industry for outdoor applications in products like textiles, paints for cars and plastics, because they show good light fastness properties (resistant to fading when exposed to light). In addition the purity is often low (70-90%). The colorants are therefore not risk assessed for the use of injection and permanence into the human body. Further, because the colorants are produced for other purposes than tattoo ink there is probably no focus on removing impurities such as PAA if it is not altering the color of the product. In some cases, additional PAAs are added to a colorant (called free PAAs) for retrieving a specific nuance of a color (JRC 2015 and DEPA 2012). Another issue in respect to that is that the colorants are likely not to be produced sterile, so biological degradation can happen during storage of the colorants releasing PAAs.

Stability of Azo colorants

Dyes are designed to possess a high degree of chemical and photolytic stability. However, it is well documented that the azo colorants can degrade or decompose under different conditions, mainly by cleavage by the azo bond (reductive cleavage) which is the most labile portion of an azo colorant.

The azo pigments may degrade, for example under solar or laser irradiation, into the original primary aromatic amines or other decomposition products (JRC 2015). Investigations have also shown that that the pigment can be degraded by enzymatic and microbial reaction by metabolic reductive cleavage releasing aromatic amines. This degradation might also occur in the skin due to the influence of skin bacteria. The primarily enzyme responsible for the azo cleavage is azo reductase which have been found in both bacteria

[1] An azo lake is an insoluble salt pigment formed by precipitation of a water-soluble anionic azo compound with a metal cation. The anionic part of the azo lake is typically carboxylic or sulfonic acids.

and in mammals, including humans. Bacterial degradation has also been found to be both aerobic and anaerobic (JRC 2015, Environment Canada 2012). Figure 3 and 4 shows the reaction of reductive cleavage, both enzymatically and chemically.

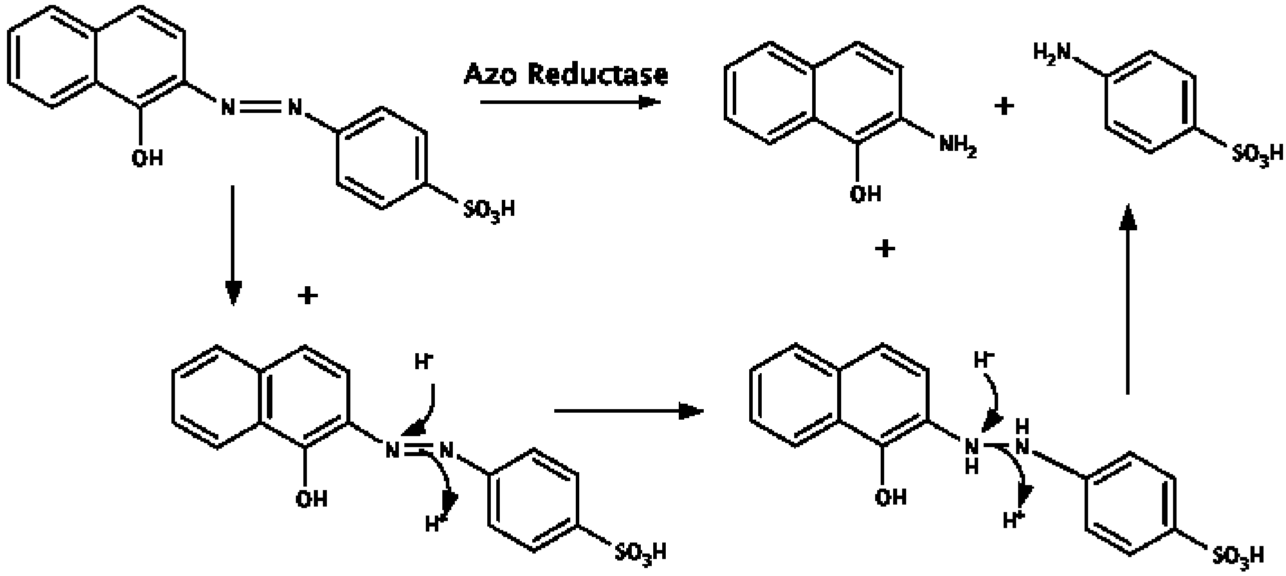
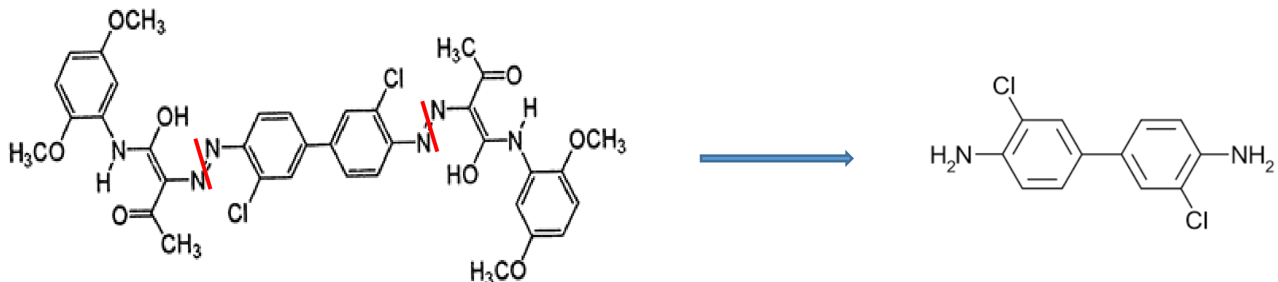


Figure 3. Azo bond cleavage by reductive cleavage of a mono azo bond, enzymatically (azo reductase) and chemically reactions, e.g. triggered by light.

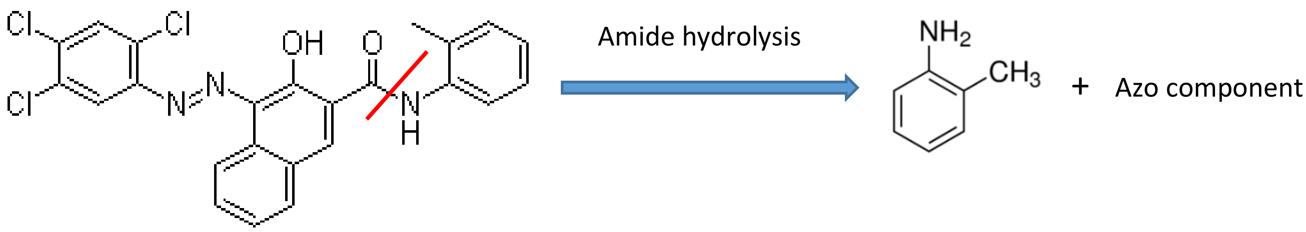


3,3'-dichlorobenzidine

Pigment yellow 87

Figure 4. Azo bond cleavage by reductive cleavage of a di azo bond

Another possible reaction that can generate PAAs is amide hydrolysis where a different type of bond (an amide bond) is cleavage, see figure 5.



O-Toludine

Pigment red 112

Figure 5. Bond cleavage by amide hydrolysis (JRC 2015).

It has not been possible to describe the stability quantitatively. However, some general remarks can be made based on the different investigation of decomposition of the azo colorants. In general, azo colorants with simple structures and low molecular weight exhibit higher rates of degradation and decomposition than high molecular weight compounds. Further, mono azo colorants are less stable than di azo colorants. Electron withdrawing groups such as SO_3H or SO_2NH_2 attached to the phenyl ring also increases the stability of the azo bond and azo colorants with hydroxyl groups is less stable compared to methyl, methoxy, sulpho or nitro groups attached to the phenyl ring (Environment Canada, 2012).

PAAs that might be found in azo-colorants in tattoo ink

In JRC 2015, 13 PAAs were found in different investigations mainly in Europe. In JRC 2015, aniline was included but there were no indication of findings. However, in a Danish survey aniline was found in tattoo inks (DEPA 2012) which brings the number of PAAs up to 14. These are listed in annex 5. In these investigations there were analysed 23 different PAAs in tattoo ink which except for aniline all were included in the negative list in CoE Resap (2008)1. Of these, 13, as mentioned, have been found in the inks, both by direct measurements of the inks and by different degradation studies. The compounds are listed in Annex 5 together with the number of analysis and the percentage of findings (JRC 2015).

In JRC 2015, Annex IX, the chemical structures of most of the pigments applied in tattoo inks, apart from the natural colors are shown. These structures were used in this study to predict the PAAs that might be found in the different azo colorants. The structures of the azo dyes are presented in Annex 3 in the present report together with CAS numbers and color index numbers.

JRC 2015 also investigated which colorants that might lead to unsafe amines by decomposition based on PAA negative list in CoE Resap (2008)1 based on the colorant structures.

This is followed up in the present study by including PAAs that are not present on the negative list of CoE Resap (2008)1. Further, in this study it was taken into account that purity for azo colorants is not high (70-90%), which means that it is possible that the colorant can contain PAAs from the production. Based on the structures of the single azo colorants presented in JRC 2015 the potential different PAAs either from the production or by degradation (azo cleavage) were identified. This list was compared and combined with the list of potential PAAs by decomposition, azo bond cleavage and amide hydrolysis from JRC 2015.

In table 1, the different possible PAAs are listed together with the azo colorants in which they may be found. For chemical structures and CAS no. of the PAAs, see Annex 1.

According to JRC 2015, 44 out of 67 azo colorants, corresponding to 66% of the azo colorants may potentially lead to the formation of one of the PAAs included in the negative list in CoE Resap (2008)1. All amines in the negative list in CoE Resap are classified as carcinogenic except for two: 6-amino-2-ethoxynaphthalene and 2,4-xylidine that are not classified as CMR according to Regulation (EC) No 1272/2008, Annex III.

Table 1: Potential PAAs witch either may origin from the production or generated by decomposition

Primary aromatic amine	**cas number**	**Azo colorants, cleavage of azo bond or originate from the production**	**Azo colorants, Amide hydrolysis** (JRC 2015)
3,3'-dichlorobenzidine#* [F]	91-94-1	PY 12, PY 14, PY 55, PY 83, PY 87, PO 13, PO 34	
Benzidine#*	92-87-5	DR 53	
3,3'-dimethoxybenzidine#* [F]	119-90-4	PB 25, PO 16	
4-aminobenzamide	2835-68-9	PR 120, PR 170, PR 210, PR 266	
4-chloro-o-toluidine#* [F]	95-69-2	PR 7	PR 7
5-nitro-o-toluidine#* [F]	99-55-8	PR, 12, PR 17, PR 22	
o-anisidine#* [F]	90-04-0	SR 1, PY 194,	PR 9, PR 15, PR 210, PO 74, PY 65, PY 74
o-toluidine#*[F]	95-53-4		PR 12, PR 14, PR 17, PR 112, PY 14
p-toluidine*	106-49-0		PY 55
2,5-dichloroaniline	95-82-9	PR 2, PO 22, PBr 25	
2-amino-4-nitroanisole	99-59-2	PR 23, PY 65, PY 74	
2,4,5-trichloroaniline	636-30-6	PR 112	
Aniline#* [F]	62-53-3		PR 2, PR 22, PR 146, PR 269, PO 16, PY 1, PY 12, PY 97
o-amino-benzoic acid (Anthralinic acid)	118-92-3	PR 60, PY 151	
3-chloro-p-toluidine	95-74-9	PR 48:1	
2-(Triflouromethyl)aniline	88-17-5	PY 154	
2-chloro-5-(trifluoromethyl)aniline	121-50-6	PR 242	
4-chloro-2-nitroaniline	89-63-4	PR 9, PR 14, PR 15, PY 1, PY 3, PO 36	
1-amino-2-naphthol	2834-92-6	PR 3, PR 4, PR 49, PR 49:2, PR 51, PR 53:1, PO 5	
4-Aminotoluene-3-sulfonic acid	88-44-8	PR 57:1, PR 57:2	
2-Amino-1-naphthalenesulfonic acid	81-16-3	PR 63:1	
4-Amino-1-naphthalenesulfonic acid	84-86-6	AR 14, AR 18	
4-Aminobenzenesulfonic acid*	121-57-3	AY 23, AY 104, FY 3, PY 100	
5-amino-6-hydroxy-2-naphthalenesulfonic acid	7248-98-8	FR 17:1	

Explanations to the table: #: compounds listed on the negative list in CoE Resap (2008)1*: Compounds classified as CMR. [F]: Found in tattoo inks JRC 2015 either by direct measurements or by decomposition. Colorants: First letter: P: pigment, A: acid, F: food, S: solvent, D: direct. Second letter: R: red, Y: yellow, B: blue, O: orange and Br: brown.

Three azo colorant has not been possible to identify: AY 9, arylide yellow and diarylide yellow. The last two are types of colorants, which arenot specific. 8 colorants did not generate simple PAAs by azo cleavage but followed other reaction patterns that lead to more complex molecules and are therefore not included. These are: PR 5, PR 146, PR 222, PR 269, PY 93, PY 97, PY 155 and PY 180.

24 PAAs can be identified based on the chemical structures and possible decomposition of the azo colorants from this study and from JRC 2015. The findings from different surveys shown in table 1 indicate that also amide hydrolysis may be a relevant and important decomposition route for the azo colorants, which should be considered in connection to developing an analytical method.

PAAs classified as CRM in table 1 are all, except for one, classified as carcinogenic. 4-Aminobenzenesulfonic acid is only classified as sensitizing, not carcinogenic.

PAAs that might be found in azo-colorants on the negative list in CoE ResAP (2008)1

In CoE ResAP (2008)1 35 colorants are listed that should not be used in tattoo ink. CoE ResAP (2008)1 states that these colorant are selected particularly with regard to their carcinogenic, mutagenic, reprotoxic and/or sensitizing properties. The list is non-exhaustive. 30 of the colorants can be found in Regulation (EC) No 1272/2008, Annex III. 17 of the colorants are azo colorants. The chemical structures of the azo colorants are shown in Annex 4. One of the 17 azo colorants is presently used in tattoo ink: Pigment Orange 5.

By using the same principle as above, a list of potential PAAs (13 in number) witch either can origin from the production or be generated by decomposition is shown in table 2. According the chemical structures of the 17 azo pigments none of these can generate PAAs by amide hydrolysis.

The chemical structures from table 2 can be found in Annex 2.

Table 2: Potential PAAs that either may origin from the production or be generated by decomposition from the negative list of azo colorants in CoE ResAP (2008)1

Primary aromatic amine	**CAS no.**	**Azo colorants, cleavage of azo bond or originate from the production**
2,4-dimethylaniline#	95-68-1	AR 26, SO 7
4-nitroaniline	100-01-6	DiR 1, DiR 17, DiO 3
3-amino-2-naphthol*	5417-63-0	PR 53, SR 24
2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9	PR 53
3-Aminobenzenesulfonic acid	121-47-1	AY 36
2-hydroxy-5-methylaniline, 2-Amino-4-methylphenol*	95-84-1	DiY 3
p-Phenylenediamine, 1,4-Benzenediamine#[F]	106-50-3	SY 1, DiO 3
2,5-Diaminotoluene, 2-Methyl-benzene-1,4-diamine	95-70-5	SY 3
2,6-Dichloro-4-nitroaniline*	99-30-9	DiO 37
2,4-Dinitroaniline	97-02-9	PO 5
o-toludine#*[F] (from table 1)	95-53-4	SR 24, SY 3, DiB 106, DiB 124
Aniline#*[F] (from table 1)	62-53-3	SY 1, SY 2
1-amino-2-naphthol (from table 1)	2834-92-6	PO 5, PO 7

Explanations to the table: #: Compounds listed on the negative list in CoE Resap (2008)1*: Compounds classified as CMR. [F]: Found in tattoo inks JRC 2015- Colorants: First letter: P: pigment, A: acid, S: solvent, Di: Disperse. Second letter: R: red, Y: yellow, B: blue, O: orange.

The last three PAAs are also present in table 1.

10 additional PAAs can be identified based on the chemical structures and possible decomposition of the azo colorants. The PAAs in the table 2, classified as CRM, are all carcinogenic.

There are further 15 PAAs from the negative list in CoE Resap (2008)1 which are not present in the table 1 and 2. See table 3.

Table 3. List of amines from the negative list in CoE Resap (2008)1, which are not included in the table 1 and 2.

Primary aromatic amine	cas number
6-amino-2-ethoxynaphthalene	293733-21-8
4-aminoazobenzene*	60-09-3
3,3'-dimethylbenzidine* [F]	119-93-7
6-methoxy-m-toluidine*	120-71-8
4-methoxy-m-phenylenediamine*	615-05-4
4,4'-methylenebis(2-chloroaniline)*	101-14-4
4,4'-methylenedianiline*	101-77-9
4,4'-methylenedi-o-toluidine*	838-88-0
4-methyl-m-phenylenediamine* [F]	95-80-7
2-naphtylamine* [F]	91-59-8
4,4'-oxydianiline*	101-80-4
4,4'-thiodianiline* [F]	139-65-1
2,4,5-trimethylaniline*	137-17-7
2,6-xylidine*	87-62-7

Explanations to the table: *: Compounds classified as CMR. [F]: Found in tattoo inks JRC 2015.

Other degradation patterns

Apart from the three lists of PAAs (table 1, 2 and 3), additional PAAs were found in different chemical investigations (JRC 2015). These are listed in table 4.

Table 4. PAAs found in different chemical investigations (JRC 2015) that are not listed in table 1-3.

Primary aromatic amine	cas no.
4-Chloroaniline*	106-47-8
3,3'-Dimethylbenzidine#*	119-93-7
4-Methyl-m-phenylendiamine#*	95-80-7
2-Naphthylamine#*	91-59-8
4,4'-Thiodianiline#*	139-65-1

Explanations: #: Compounds listed on the negative list in CoE Resap (2008)1. *: Compounds classified as CMR.

Table 4 shows that the use of theoretical prediction of PAAs from the colorants by azo cleavage and amide hydrolysis cannot completely explain all PAAs found in colorants. It is possible that other biological and chemical decomposition pattern can appear. As an example, 4-chloroaniline has been found in investigations for decomposition under irradiation with light (JRC 2015). The reaction is likely to be due to a radical reaction.

Summary on the number of PAAs for chemical analysis

Table 1, 2, 3 and 4 can be regarded as a list of possible PAAs that might be included in an analytical method. There is 49 PAAs from these four lists, of these 29 PAAs are classified as CRM. All classified PAAs are carcinogenic, except for one: 4-aminobenzenesulfonic acid, which is only classified as sensitizing. The four lists are combined in Annex 6, where they are ranked with the CRM classified compound first. It must be emphasized that the list is not necessarily complete as it only can account for the knowledge present on the use of tattoo ink and the azo colorants listed in the CoE Resap (2008)1.

Development of an analytical method

The aim of developing a new analytical method is to get more robust and comparable results.

There are several methods for analyzing PAAs from colorants. However, as mentioned earlier the amount of PAAs found in the colorants can vary significantly, possibly due to impurities but decomposition of the azo colorants is also an issue.

Taking out a representative sample is also an issue that possibly can be one of the main reasons for the variation of analytical results. A Danish survey described that the composition and texture of the various inks were very different: some very fluent and other thick and in many cases inhomogeneous. According to this survey, it was a challenge to take out a representative sample. All samples in this survey were carefully shaken and samples taken out by weight instead of volume in order to take the differences in viscosity of the different inks into account (DEPA 2012). Test should be done with different shaking method and temperatures in order to find a standardize method.

Surfactants are added in tattoo inks to avoid sedimentation of the pigments. Whether adding more surfactants to the sample would increase the homogeneity could be considered. However, this could also lead to analytical difficulties as surfactants have a tendency to foam in extractions. Nevertheless, it would could be relatively easy teste.

Another possibility is to normalize the analytical results to dry matter instead of wet weight. That is a common approach used for other environmental sample such as sediment and sludge and might give more uniform results.

CoE Resap (2008)1 recommend two analytical methods for analyzing PAAs in tattoo ink which are based on two international standards. None of these methods was developed for ink as a matrice.

Analytical method should be developed from the two mentioned standard methods, as there is experience with these two methods. However, standardized methods are not necessary the best available technology and they need to be further developed.

Method EN 14362 has a method for analyzing PAAs in textile products by GC-MS and EN 71-7: 2002 has a method for analyzing for PAAs in finger-paint by LC-MS. An updated version of EN 71-7 was published in 2014 and has an improved analytical method for analyzing PAAs It would be more relevant to use the updated version in the development of an analytical method. Further, there has been improvements by adding dimethylformamide (DMF) to the reaction solutions (personal communication, Urs Hauri 2017).

Both methods use reductive cleavage in the methods by sodium dithionite, which give the total amount of PAAs in the ink not just the free PAAs. An analytical method should be able to measure both the total PAAs as well as the free PAAs. However, different analytical methods and conditions have shown to very different measureable amounts of total PAAs likely based on the chemical decomposition conditions and only a few percent of the theoretical amount of PAAs found (personal communication, Urs Hauri 2017). One of the reasons for the low measured amounts can be that the colorants can be present as insoluble pigments, which might reduce the contact to the solvents and reagents.

Different method should be tested for the release of PAAs. Reductive cleavage is well known but from the result from Urs Hauri, it seems possible to optimize further on that reaction. From the findings from different surveys, some PAAs are generated by amide hydrolysis so it could be relevant to test that as well. Finally, enzymatic cleavage by azo reductases could also be a possibility. Enzymatic reactions are often more efficient than chemical. However, it is not expected to achieve a 100% recovery of PAAs from the colorants.

Instrumentation: GC-MS and HPLC-MS are used in above-mentioned standards and these methods are well proven if the PAAs to be included in the method are approximately the same as those already found in the different surveys it might not be necessary to make significant changes. However, there have been good results using LC-MS-MS together with derivatization of the PAAs (Mortensen et al. 2005). Derivatization can, in some cases, improve the detection limits as well as increase the number of compounds that can be analyzed with the same method. Derivatization could therefore be relevant to test in relation to GC-MS and LC-MS-MS. Further, several companies have developed specific columns for LC and GC for analyzing PAAs. A survey of what is available on the market is relevant.

Extraction: Different solvents have been used for the extraction of PAAs: t-butylmethylether, methanol, MBTE, acetonitrile. However, some of these solvents are not commonly used today and further the solubility of the pigment can be influenced of different solvents.

During the extraction there should be focus on how much the samples are exposed to light as this might alter the results. Further exposure to heat, ultrasonic etc. during extraction is also relevant to investigate. The extractions should be tested by standard addition with pure standard.

A literature survey showed that there are no scientific papers addressing analysis of tattoo ink. There are papers that address analysis of PAAs from azo colorants, but they have analyzed pure pigments, not ink (e.g. Sudha et al. 2014, Chacko and Subramaniam 2011). Further, there are also several papers describing the analysis of PAAs. In some cases these papers focus on the matrices; papers used for food or food, which are completely different matrices than ink. Input from these can be seen above.

As mentioned previously 49 potentially and measured PAAs can be identified from tattoo colorants and the two negative lists in CoE Resap (2008)1 (see Annex 6). It should be possible to develop methods for these; however, it will probably not be possible to develop just one analytical method for all of them. To prioritize one should start the PAAs found in different surveys (Annex 5). Most analytical methods have measured PAAs from the negative list of PAAs in CoE Resap (2008)1 together with aniline but not all of these have been found in tattoo ink. This can be combined with the potential CRM classified PAAs in Annex 6 which not previously been analysed. As several of the other PAAs (Annex 1 and 2) have similar structures they can easily be included when a robust method exist (e.g. derivatives of aniline and toluidine). However, some of the PAAs chemical structures are quite different and might need to have their own analytical methods.

When a method has been developed, it will be necessary to test the robustness.

Further, it should be tested how much light and microbial degradation can influence the results by leaving samples for a longer period in their original bottles and then reanalyze them. Several bacteria have been identified for being able to perform azo cleavage, and it could be relevant to add some of these bacteria strains to the inks if the resources for the method development allow it. These results can also give guidelines for how long and under which conditions the sample can be stored, as well as the handling during extraction in the laboratory.

Finally, it would be a relevant when the method is developed to perform a parallel testing in several laboratories for comparison and control of robustness.

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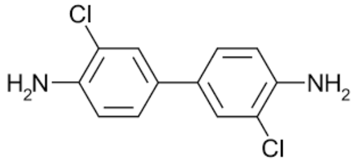
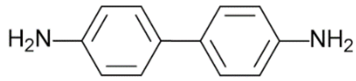
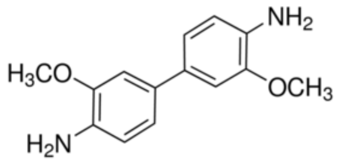
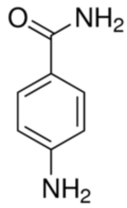
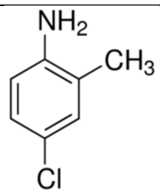
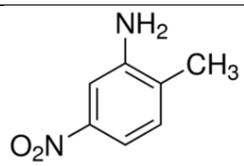
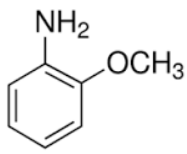
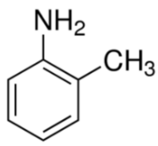
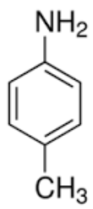
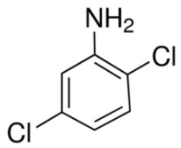
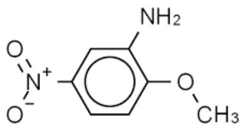
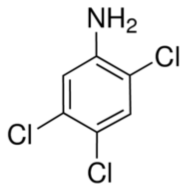
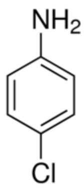
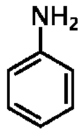
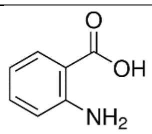
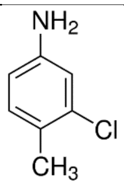
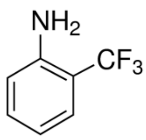
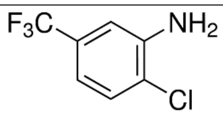
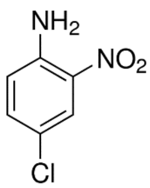
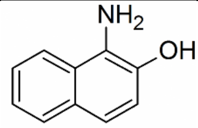
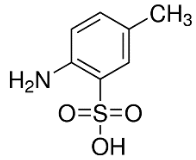
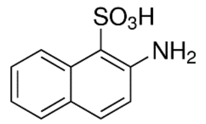
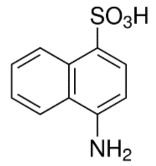
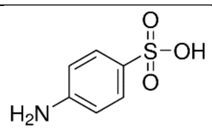
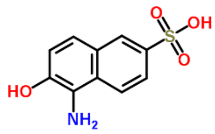
Annex 1. Chemical structures of possible PAAs from azo colorants in tattoo ink

Chemical structures of possible PAAs in tattoo ink based on the structures of the azo colorants.

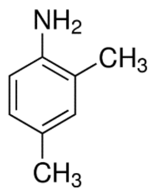
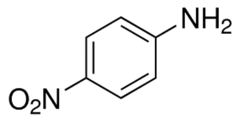
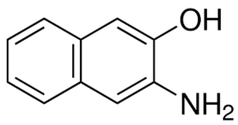
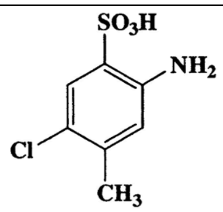
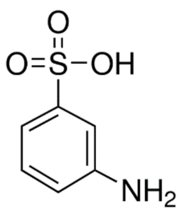
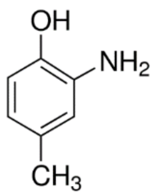
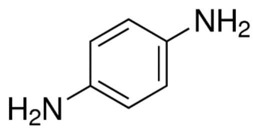
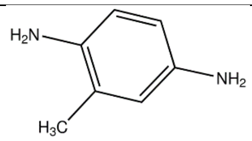
Name	CAS no.	Structural formula
3,3'-dichlorobenzidine	91-94-1	
Benzidine (DB 53)	92-87-5	
3,3'-dimethoxybenzidine	119-90-4	
4-aminobenzamide	2835-68-9	
4-chloro-o-toluidine	95-69-2	
5-nitro-o-toluidine	99-55-8	
o-anisidine	90-04-0	
o-toluidine	95-53-4	
p-toludine	106-49-0	

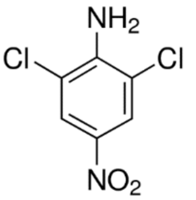
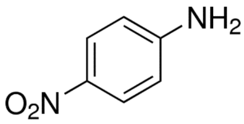
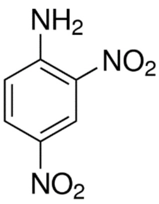
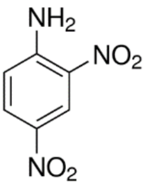
2,5-dichloroaniline	95-82-9	
2-amino-4-nitroanisole	99-59-2	
2,4,5-trichloroaniline	636-30-6	
4-chloroaniline	106-47-8	
aniline	62-53-3	
o-amino-benzoic acid (Anthralinic acid)	118-92-3	
3-chloro-p-toluidine	95-74-9	
2-(Triflouromethyl)aniline	88-17-5	
2-chloro-5-(trifluoromethyl)aniline	121-50-6	
4-chloro-2-nitroaniline	89-63-4	

1-amino-2-naphthol	2834-92-6	
4-Aminotoluene-3-sulfonic acid or 4-Methylaniline-2-sulfonic acid	88-44-8	
2-Amino-1-naphthalenesulfonic acid	81-16-3	
4-Amino-1-naphthalenesulfonic acid	84-86-6	
4-Aminobenzenesulfonic acid, Aniline-4-sulfonic acid, Sulfanilic acid	121-57-3	
5-amino-6-hydroxy-2-naphthalenesulfonic acid	7248-98-8	

Annex 2. Chemical structures of possible PAAs from the azo colorants from the negative list in CoE ResAP (2008)1.

Chemical structures of possible PAAs based on the structures of the azo colorants from the negative list, table 2, in CoE ResAP (2008)1.

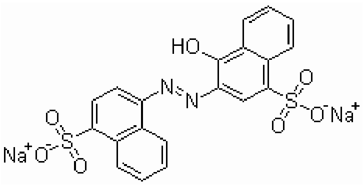
Name	CAS no.	Structural formula
2,4-dimethylaniline	95-68-1	
4-nitroaniline	100-01-6	
3-amino-2-naphthol	5417-63-0	
2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9	
3-Aminobenzenesulfonic acid	121-47-1	
2-hydroxy-5-methylaniline, 2-Amino-4-methylphenol	95-84-1	
p-Phenylenediamine, 1,4-Benzenediamine	106-50-3	
2,5-Diaminotoluene, 2-Methyl-benzene-1,4-diamine	95-70-5	

2,6-Dichloro-4-nitroaniline	99-30-9	
4-Nitroaniline	100-01-6	
2,4-Dinitroaniline	97-02-999-30-9	
2,4-Dinitroaniline	97-02-9	

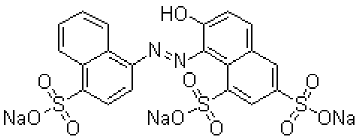
Annex 3. Chemical structures of azo colorants used in tattoo ink

Structures of azo colorants used in ink according to JRC 2015. The pictures of the structures have mainly been copied from Chemblink.

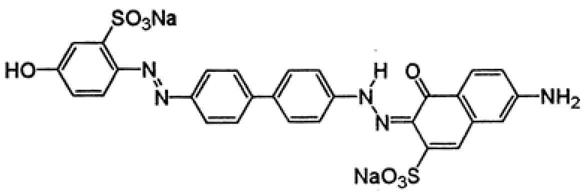
Under each structure is a table including: CI name: the common name used for the colorant according to the colour index, SH: short name sued by CI, cas no., CI no. : colour index number, chemical class: either mono azo colorants or di azo compounds. Comments concerning the compounds is placed under the table.



CI Name	SH	cas no.	CI no.	Chemical class
Acid red 14	AR 14	3567-69-9	14720	monoazo

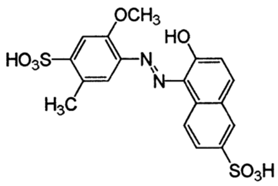


CI Name	SH	cas no.	CI no.	Chemical class
Acid red 18	AR 18	2611-82-7	16255	monoazo

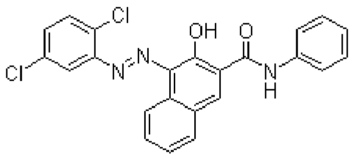


CI Name	SH	cas no.	CI no.	Chemical class
Direct Red 53	DR 53	6375-58-2	22405	diazo

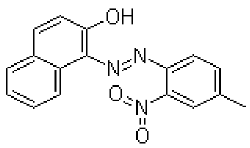
The structure is not correct. There should be at double bond between N-N not N-biphenyl.



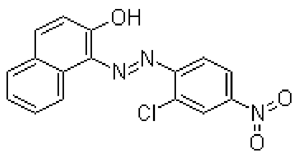
CI Name	SH	cas no.	CI no.	Chemical class
Food red 17:1	FR 17:1	68583-95-9	16035:1	monoazo



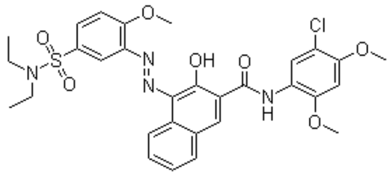
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 2	PR 2	6041-94-7	12310	monoazo



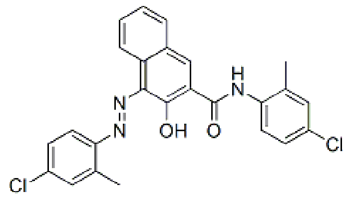
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 3	PR 3	2425-85-6	12120	monoazo



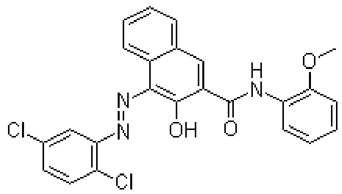
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 4	PR 4	2814-77-9	12085	monoazo



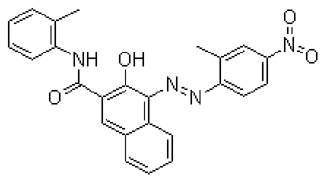
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 5	PR 5	6410-41-9	12490	monoazo



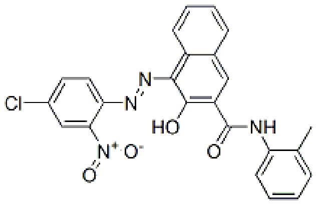
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 7	PR 7	6471-51-8	12420	monoazo



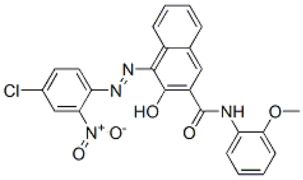
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 9	PR 9	6410-38-4	12460	monoazo



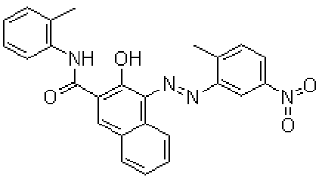
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 12	PR 12	6410-32-8	12385	monoazo



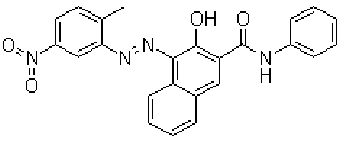
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 14	PR 14	6471-50-7	12380	monoazo



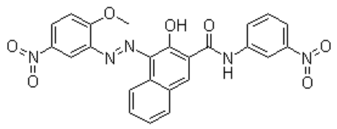
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 15	PR 15	6410-39-5	12465	monoazo



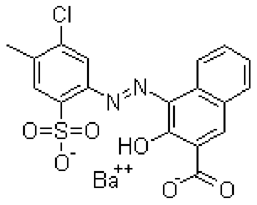
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 17	PR 17	6655-84-1	12390	monoazo



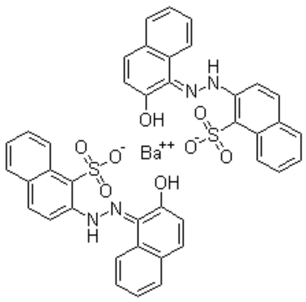
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 22	PR 22	6448-95-9	12315	monoazo



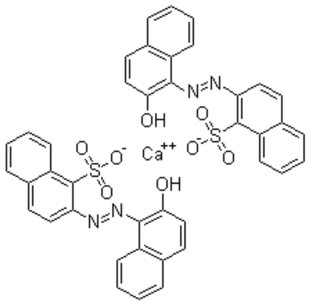
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 23	PR 23	6471-49-4	12355	monoazo



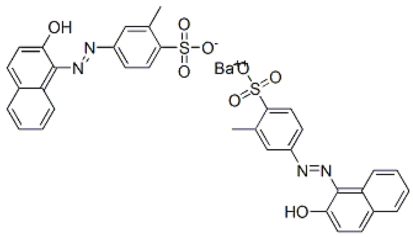
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 48:1	PR 48:1	7585-41-3	15865:1	monoazo



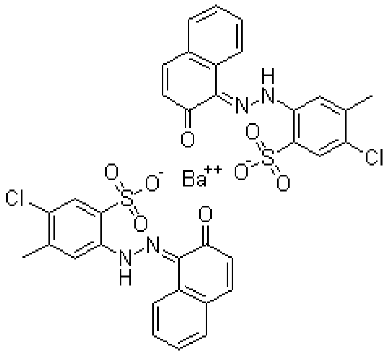
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 49	PR 49	1103-38-4	15630	monoazo



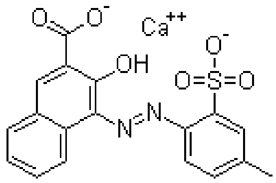
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 49:2	PR 49:2	1103-39-5	15630:2	monoazo



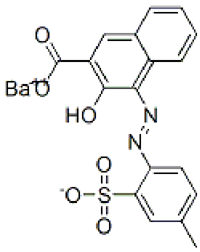
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 51	PR 51	5850-87-3	15580	monoazo



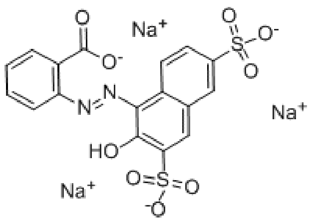
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 53:1	PR 53:1	5160-02-1	15585	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 57:1	PR 57:1	5281-04-9	15850:1	monoazo

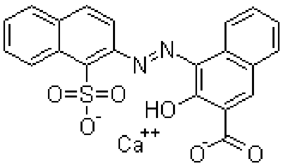


CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 57:2	PR 57:2	17852-98-1	15850:2	monoazo

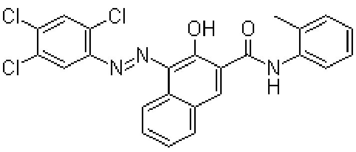


CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 60	PR 60	1836-22-2	16105	monoazo

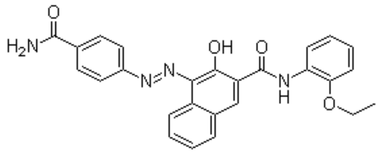
PR 60 is complexed with Ba not Na



CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 63:1	PR 63.1	6417-83-0	15880	monoazo

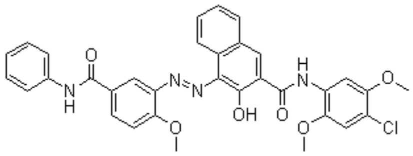


CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 112	PR 112	6535-46-2	12370	monoazo

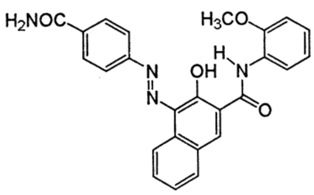


CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 120 &170	PR 120, PR 170	2786-76-7	12475	monoazo

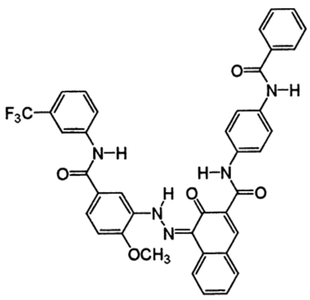
Pigment red 120 and 170 is the same colorant



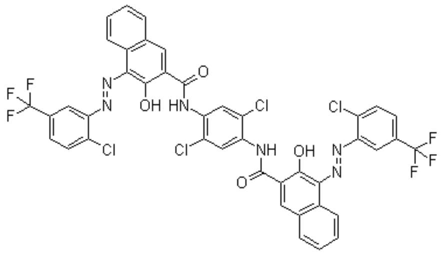
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 146	PR 146	5280-68-2	12485	monoazo



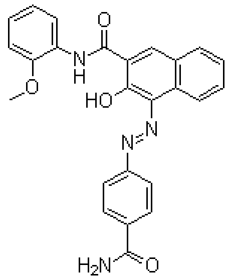
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 210	PR 210	61932-63-6	12477	monoazo



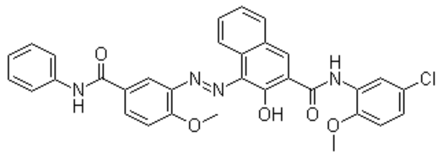
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 222	PR 222	71872-63-4	123665	monoazo



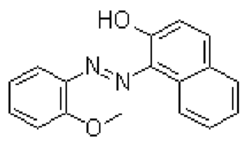
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 242	PR 242	52238-92-3	20067	diazo



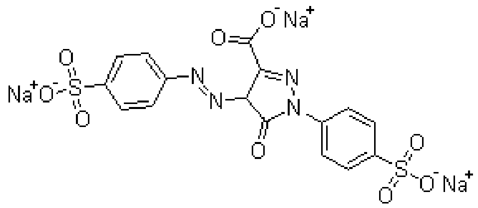
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 266	PR 266	36968-27-1	12474	monoazo



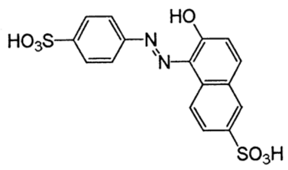
CI Name	SH	cas no.	CI no.	Chemical class
Pigment red 269	PR 269	67990-05-0	12466	monoazo



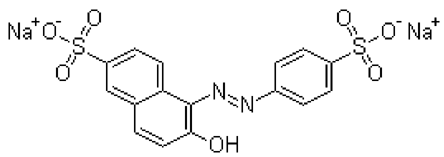
CI Name	SH	cas no.	CI no.	Chemical class
Solvent red 1	SR 1	1229-55-6	12150	monoazo



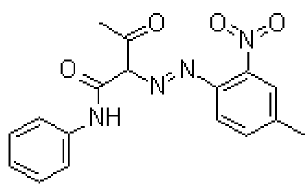
CI Name	SH	cas no.	CI no.	Chemical class
Acid yellow 23	AY 23	1934-21-0	19140	monoazo



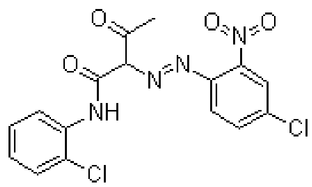
CI Name	SH	cas no.	CI no.	Chemical class
Acid yellow 104	AY 104	12227-60-0	15985:1	monoazo



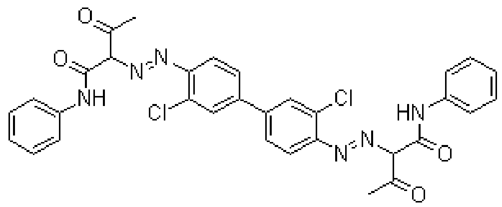
CI Name	SH	cas no.	CI no.	Chemical class
Food yellow 3	FY 3	2783-94-0	15985	monoazo



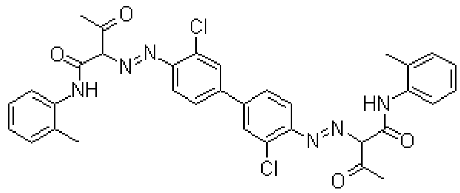
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 1	PY 1	2512-29-0	11680	monoazo



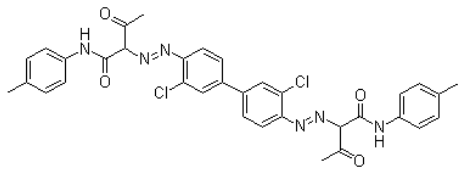
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 3	PY 3	6486-23-3	11710	monoazo



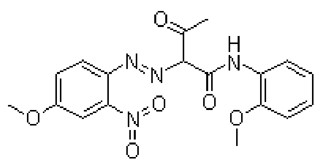
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 12	PY 12	6358-85-6	21090	diazo



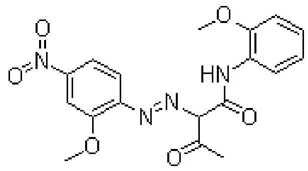
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 14	PY 14	5468-75-7	21095	diazo



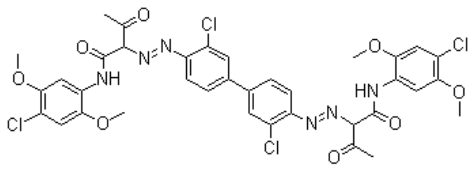
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 55	PY 55	6358-37-8	21096	diazo



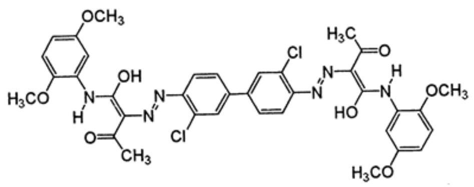
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 65	PY 65	6528-34-3	11740	monoazo



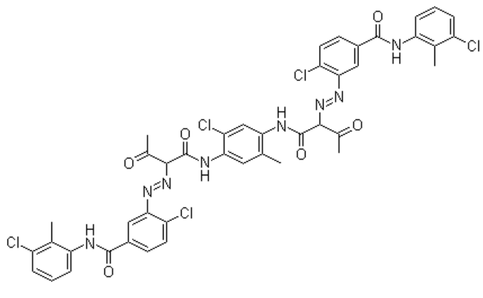
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 74	PY 74	6358-31-2	11741	monoazo



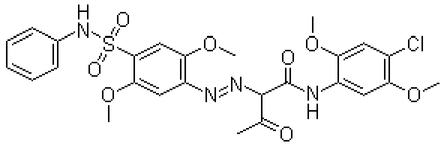
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 83	PY 83	5567-15-7	21108	diazo



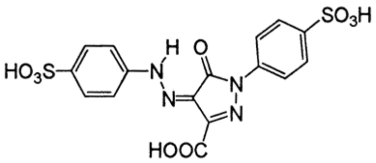
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 87	PY 87	15110-84-6	21107:1	diazo



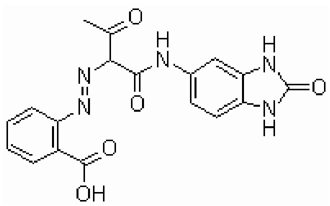
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 93	PY 93	5580-57-4	20710	diazo



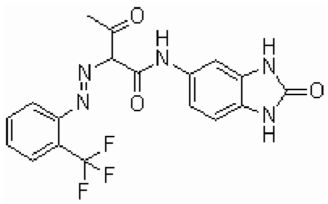
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 97	PY 97	12225-18-2	11767	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 100	PY 100	12225-21-7	19140:1	monoazo

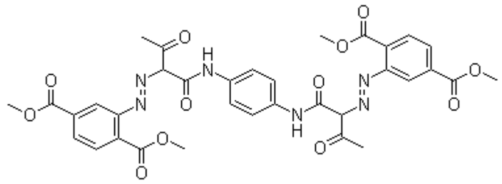


CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 151	PY 151	31837-42-0	13980	monoazo



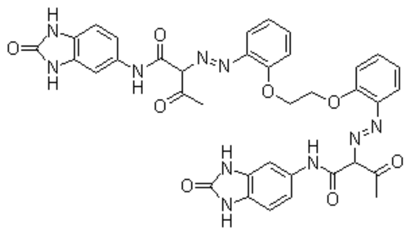
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 154	PY 154	68134-22-5	11781	monoazo
Pigment yellow 154	PY 154	68134-22-5	11781	monoazo

PY 154 has two cas numbers.

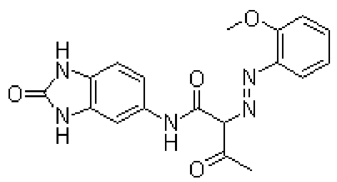


CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 155	PY 155	68516-73-4	200310	diazo
Pigment yellow 155	PY 155	77465-46-4	200310	diazo

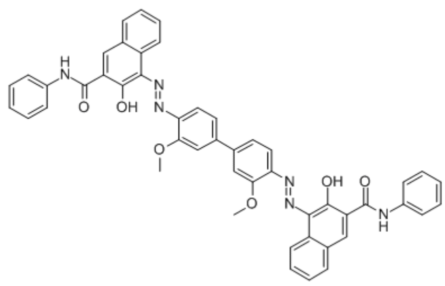
PY 155 has two cas numbers.



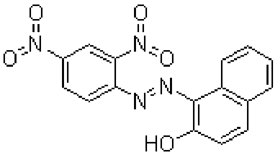
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 180	PY 180	77804-81-0	21290	diazo



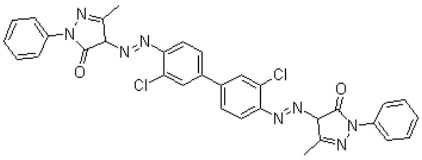
CI Name	SH	cas no.	CI no.	Chemical class
Pigment yellow 194	PY 194	82199-12-0	11785	monoazo



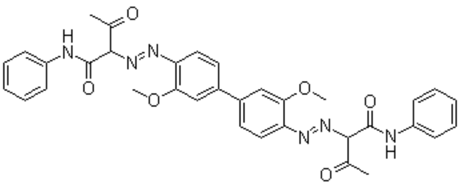
CI Name	SH	cas no.	CI no.	Chemical class
Pigment blue 25	PB 25	10127-03-4	21180	diazo



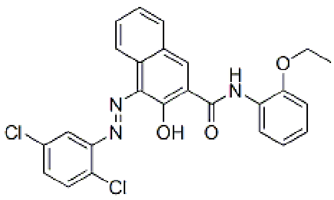
CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 5	PO 5	3468-63-1	12075	monoazo



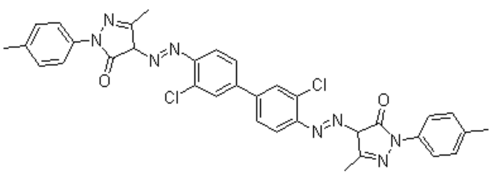
CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 13	PO 13	3520-72-7	21110	diazo



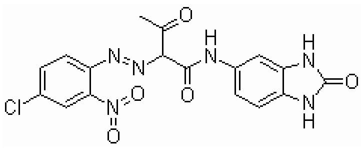
CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 16	PO 16	6505-28-8	21160	diazo



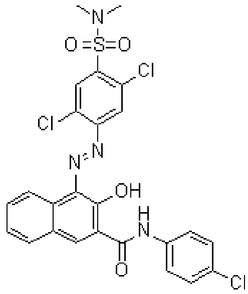
CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 22	PO 22	6358-48-1	12470	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 34	PO 34	15793-73-4	21115	diazo

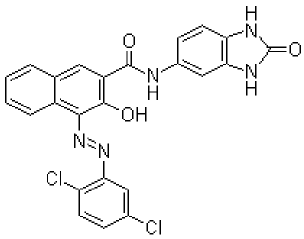


CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 36	PO 36	12236-62-3	11780	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Pigment orange 74	PO 74	85776-14-3	n.a.	monoazo

NB se kommentar - mangler flere ting og er I uoverensstemmelse med Paolas struktur



CI Name	SH	cas no.	CI no.	Chemical class
Pigment brown 25	PBr 25	6992-11-6	12510	monoazo

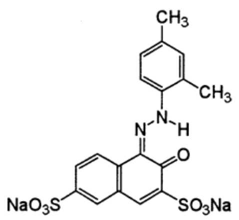
Three azo colorant has not been possible to identify: Acid yellow 9, arylide yellow and diarylide yellow the last two are type of colorants not specific colorants.

Annex 4. Chemical structures of azo colorants used in the negative list of colorants in CoE ResAP (2008)1

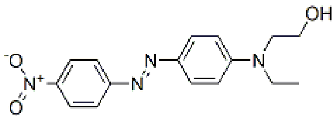
Structures of azo colorants Listed in table 2 in CoE ResAP(2008)1: The colorants are listed with particularly regard to carcinogenic, mutagenic, reprotoxic and/or sensitizing properties, which tattoo and PMU products should not contain (BC/CEN/97/29.11). The colorants are grouped according to their color.

The pictures of the structures have mainly been copied from Chemblink.

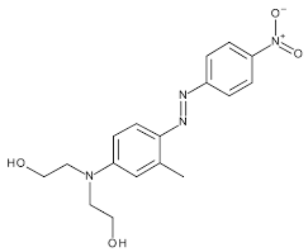
Under each structure is a table including: CI name: the common name used for the colorant according to the color index, SH: short name issued by CI, cas no., CI no.: color index number, chemical class: either mono azo colorants or di azo compounds.



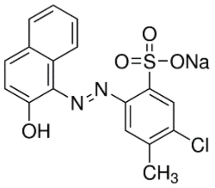
CI Name	SH	cas no.	CI no.	Chemical class
Acid Red 26	AR 26	3761-53-3	16150	monoazo



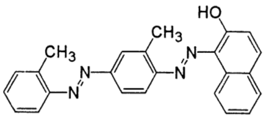
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Red 1	DiR 1	2872-52-8	11110	monoazo



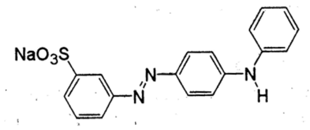
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Red 17	DiR 17	3179-89-3	11210	monoazo



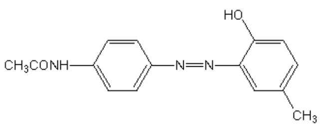
CI Name	SH	cas no.	CI no.	Chemical class
Pigment Red 53	PR 53	2092-56-0	15585	monoazo



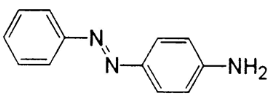
CI Name	SH	cas no.	CI no.	Chemical class
Solvent Red 24	SR 24	85-83-6	26105	diazo



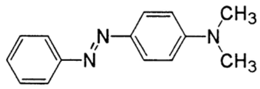
CI Name	SH	cas no.	CI no.	Chemical class
Acid Yellow 36	AY 36	587-98-4	13065	monoazo



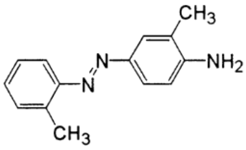
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Yellow 3	DiY 3	2832-40-8	11855	monoazo



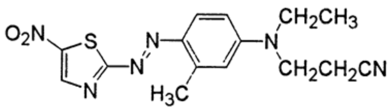
CI Name	SH	cas no.	CI no.	Chemical class
Solvent Yellow 1	SY 1	60-09-3	11000	monoazo



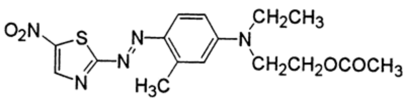
CI Name	SH	cas no.	CI no.	Chemical class
Solvent Yellow 2	SY 2	60-11-7	11020	monoazo



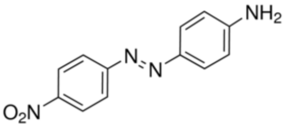
CI Name	SH	cas no.	CI no.	Chemical class
Solvent Yellow 3	SY 3	97-56-3	11160	monoazo



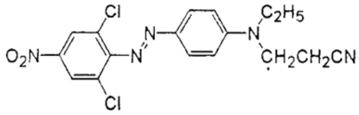
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Blue 106	DiB 106	12223-01-7	111935	monoazo



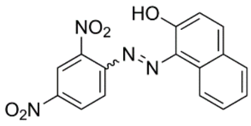
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Blue 124	DiB 124	61951-51-7	111938	monoazo



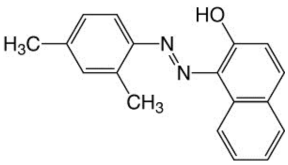
CI Name	SH	cas no.	CI no.	Chemical class
Disperse Orange 3	DiO 3	730-40-5	11005	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Disperse Orange 37	DiO 37	12223-33-5	11132	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Pigment Orange 5	PO 5	3468-63-1	12075	monoazo



CI Name	SH	cas no.	CI no.	Chemical class
Solvent Orange 7	SO 7	3118-97-6	12140	monoazo

Annex 5. PAAs found in surveillance campaign all over Europe

From JRC 2015 and DEPA 2012

cas nr	PAA	no. Of analysis	% non-compliant samples
90-04-0	o-Anisidine	3655	10
106-47-8	4-Chloroaniline	2958	2
95-69-2	4-Chloro-o-toluidine	43	1
91-94-1	3,3'-Dichlorobenzidine	3647	2,4
119-90-4	3,3'-Dimethoxybenzidine	827	0,5
119-93-7	3,3'-Dimethylbenzidine	829	0,1
95-80-7	4-Methyl-m-phenylendiamine	3516	2,5
91-59-8	2-Naphthylamine	19	1,2
99-55-8	5-Nitro-o-toluidine	2129	1,2
106-50-3	p-Phenylenediamine	29	3
139-65-1	4,4'-Thiodianiline	100	1
95-53-4	o-Toludine	3675	5
95-68-1	2,4-xylidine	120	1
62-53-3	Aniline[1]	24	58

[1] The data for aniline was taken from a Danish survey (DEPA 2012)

Annex 6. Potential PAAs for an analytical method

List of PAAs found in this study that could be relevant for developing an analytical method

Primary aromatic amine	cas number
4,4'-methylenebis(2-chloroaniline)#*	101-14-4
4,4'-methylenedianiline#*	101-77-9
4,4'-oxydianiline#*	101-80-4
4-Chloroaniline*[F]	106-47-8
p-toluidine*	106-49-0
p-Phenylenediamine, 1,4-Benzenediamine#*[F]	106-50-3
3,3'-dimethoxybenzidine#* [F]	119-90-4
3,3'-Dimethylbenzidine#*[F]	119-93-7
6-methoxy-m-toluidine#*	120-71-8
4-Aminobenzenesulfonic acid*	121-57-3
2,4,5-trimethylaniline#*	137-17-7
4,4'-Thiodianiline#*[F]	139-65-1
3-amino-2-naphthol*	5417-63-0
4-aminoazobenzene#*	60-09-3
4-methoxy-m-phenylenediamine#*	615-05-4
Aniline#* [F]	62-53-3
4,4'-methylenedi-o-toluidine#*	838-88-0
2,6-xylidine#*	87-62-7
o-anisidine#* [F]	90-04-0
2-Naphthylamine#*[F]	91-59-8
3,3'-dichlorobenzidine#* [F]	91-94-1
Benzidine#*	92-87-5
o-toluidine#*[F]	95-53-4
2,4-dimethylaniline#*	95-68-1
4-chloro-o-toluidine#* [F]	95-69-2
4-Methyl-m-phenylendiamine#*[F]	95-80-7
2,6-Dichloro-4-nitroaniline*	99-30-9
5-nitro-o-toluidine#* [F]	99-55-8
2-hydroxy-5-methylaniline, 2-Amino-4-methylphenol*	95-84-1

Explanations to the table: #: compounds listed on the negative list in CoE Resap (2008)1*: Compounds classified as CMR. F: Found in tattoo inks JRC 2015

Primary aromatic amine	**cas number**
6-amino-2-ethoxynaphthalene#	293733-21-8
4-nitroaniline	100-01-6
o-amino-benzoic acid (Anthralinic acid)	118-92-3
3-Aminobenzenesulfonic acid	121-47-1
2-chloro-5-(trifluoromethyl)aniline	121-50-6
1-amino-2-naphthol	2834-92-6
4-aminobenzamide	2835-68-9
2,4,5-trichloroaniline	636-30-6
5-amino-6-hydroxy-2-naphthalenesulfonic acid	7248-98-8
2-Amino-1-naphthalenesulfonic acid	81-16-3
4-Amino-1-naphthalenesulfonic acid	84-86-6
2-(Triflouromethyl)aniline	88-17-5
4-Aminotoluene-3-sulfonic acid	88-44-8
2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9
4-chloro-2-nitroaniline	89-63-4
2,5-Diaminotoluene, 2-Methyl-benzene-1,4-diamine	95-70-5
3-chloro-p-toluidine	95-74-9
2,5-dichloroaniline	95-82-9
2,4-Dinitroaniline	97-02-9
2-amino-4-nitroanisole	99-59-2

Description of development of an analytical method for measurement of PAA in tattoo ink and PMU

This review gives a background on azo pigments and primary aromatic amines (PAA) and describe how to develop a robust method for analyzing these in tattoo ink.

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