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## Dyes in aquaculture and reference points for action

European Food Safety Authority (EFSA),  
André Penninks, Katleen Baert, Sara Levorato and Marco Binaglia

### Abstract

The European Commission requested EFSA to evaluate whether a series of dyes are covered by the 'Guidance on methodological principles and scientific methods to be taken into account when establishing Reference Points for Action (RPAs) for non-allowed pharmacologically active substances present in food of animal origin' and to which group they should be attributed according to this guidance. Although these substances are not registered for use in food-producing animals in the European Union, they may be used illegally in aquaculture for their antimicrobial properties. It was concluded that acriflavine, 3-aminoacridine, aminoacridine, basic blue 7, brilliant green, leucobrilliant green, C.I. basic blue 26, chloranil, crystal violet, leucocrystal violet, dichlone, ethyl violet, methylene blue, new methylene blue, Nile blue, pararosaniline base, proflavine, proflavine hydrochloride, rhodamine 6G and trypan red are covered by the guidance document and belong to group I. A toxicological screening value of 0.0025 µg/kg body weight per day is applicable. Azure blue and potassium permanganate were excluded from the evaluation due to their inorganic nature.

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**Keywords:** dyes, aquaculture, reference point for action, RPA, toxicological screening value, TSV

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**Correspondence:** [contam@efsa.europa.eu](mailto:contam@efsa.europa.eu)

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**Amendment:** An editorial correction was carried out that does not materially affect the contents or outcome of this scientific output. The title has been revised from 'Dyes used in aquaculture' to 'Dyes in aquaculture and reference points for action' to clarify the context in which this report should be considered. Furthermore, the illegal character of the use of these substances has been specified in the abstract and the wording "dyes used in aquaculture" has been replaced by "dyes" on page 9. To avoid confusion, the older version has been removed from the EFSA Journal, but is available on request, as is a version showing all the changes made.

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## 1. Introduction

### 1.1. Background and Terms of Reference as provided by the requestor

#### BACKGROUND

The EFSA CONTAM panel has adopted a scientific opinion entitled *Guidance on methodological principles and scientific methods to be taken into account when establishing Reference Points for Action (RPAs) for non-allowed pharmacologically active substances present in food of animal origin*.

In relation to the setting of reference points for action, the EFSA CONTAM Panel has subsequently delivered specific opinions related to the presence of chloramphenicol, nitrofurans, and malachite green and its metabolite leucomalachite green.

The already available opinions are used as a basis for discussions with Member States on an implementing act establishing reference points for action for non-allowed pharmacologically active substances present in food of animal origin.

#### TERMS OF REFERENCE

In the context of Article 31 of Regulation (EC) No 178/2002, the Commission requests EFSA's scientific and technical assistance on whether the dyes listed in Table 1 are covered by the above mentioned guidance document and, if so, to which group they should be attributed, or whether a specific scientific opinion would be needed.

**Table 1:** List of dyes covered by this Scientific report

Name	CAS number
Acriflavine	65589-70-0
3-Aminoacridine	581-29-3
Aminoacridine	90-45-9
Azure blue/ultramarine	57455-37-5
Basic blue 7/victoria pure blue BO	2390-60-5
Brilliant green/C.I. basic green 1	633-03-4
Leucobright green	82-90-6
C.I. basic blue 26	2580-56-5
Chloranil	118-75-2
Crystal violet/gentian violet	548-62-9
Leucocrystal violet/leucogentian violet	603-48-5
Dichlone	117-80-6
Ethyl violet	25275-06-3
Methylene blue	61-73-4
New methylene blue/victoria blue R	2185-86-6
Nile blue	2381-85-3
Pararosaniline base	25620-78-4
Potassium permanganate	7722-64-7
Proflavine	92-62-6
Proflavine hydrochloride	952-23-8
Rhodamine 6G	989-38-8
Trypan red	574-64-1

### 1.2. Interpretation of the Terms of Reference

The European Food Safety Authority (EFSA) concluded that this Scientific Report should comprise:

- the evaluation whether the substances listed in Table 1 are excluded from the *Guidance on methodological principles and scientific methods to be taken into account when establishing Reference Points for Action (RPAs) for non-allowed pharmacologically active substances present in food of animal origin (EFSA CONTAM Panel, 2013)*;
- the allocation of the substances that follow the above mentioned guidance document to the different groups of substances as defined in the guidance document.

### 1.3. Guidance on methodological principles and scientific methods to be taken into account when establishing Reference Points for Action

According<sup>1</sup> to Article 3 of Regulation (EC) No 470/2009 of the European Parliament and of the Council,<sup>2</sup> any pharmacologically active substance intended for use in the Union in veterinary medicinal products (VMPs), which are to be administered to food-producing animals, shall be subject to an opinion of the European Medicines Agency (EMA) on the maximum residue limit (MRL), formulated by the Committee for Veterinary Medicinal Products (CVMP). Pharmacologically active substances, for which the EMA opinion concludes that no MRL is needed or that a (provisional) MRL should be established, are subsequently classified in Table 1 'allowed substances' of Regulation (EU) 37/2010<sup>3</sup>. Use of other pharmacologically active substances in VMPs is not allowed.

Article 18 of Regulation (EC) No 470/2009 stipulates that, for substances which are not classified as 'allowed substances' in accordance with that Regulation, a RPA may be established in order to ensure the functioning of controls for food of animal origin. Food of animal origin containing residues of such substances at or above the RPA is considered not to comply with Union legislation.

The EFSA Scientific Opinion entitled 'Guidance on methodological principles and scientific methods to be taken into account when establishing Reference Points for Action (RPAs) for non-allowed pharmacologically active substances present in food of animal origin' (EFSA CONTAM Panel, 2013) identified a step-wise approach for establishing RPAs for various categories of non-allowed pharmacologically active substances. This Scientific Opinion is further referred in this Scientific Report to as 'guidance document'. In this approach, a toxicological screening value (TSV) is assigned to the substance under evaluation, following the use of a decision tree (Figure 1). A TSV of 0.0025 µg/kg body weight (bw) per day is assigned to non-allowed pharmacologically active substances for which there is direct evidence of genotoxicity, for which there is an alert for genotoxicity (from structural activity relationships or read across), or for which there is lack of information on genotoxicity. The substances that belong to this category are referred to as Group I substances.

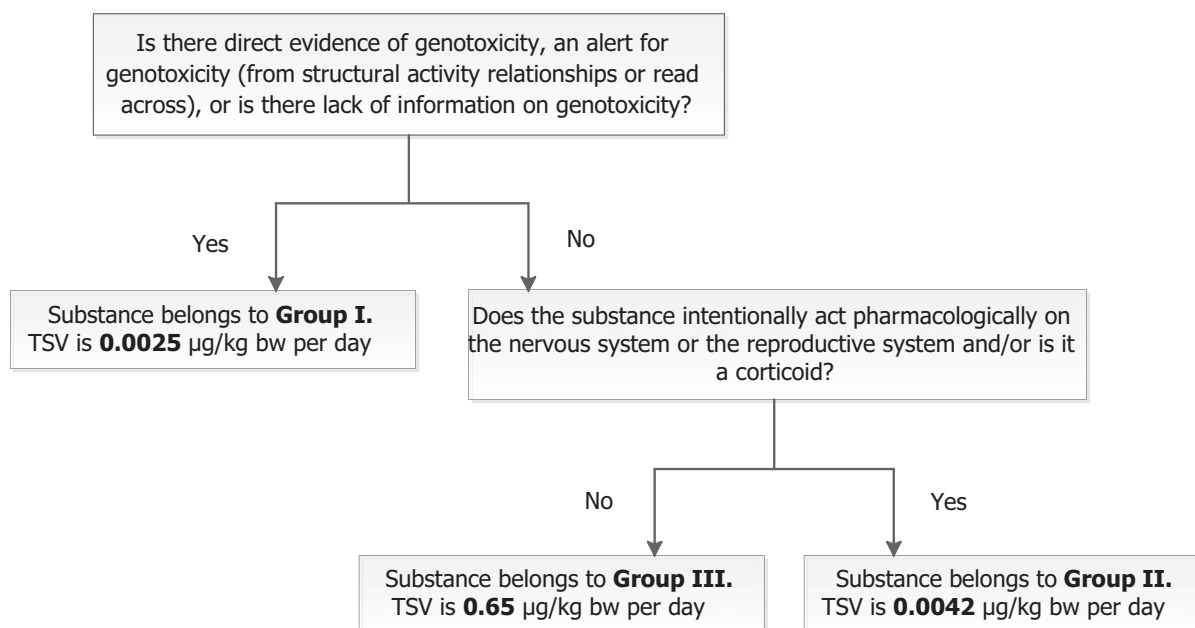
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<sup>1</sup> In this Scientific Report, where reference is made to European legislation (regulations, directives, decisions), the reference should be understood as relating to the most current amendment, unless otherwise stated.

<sup>2</sup> Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009, laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council Text with EEA relevance. OJ L 152, 16.6.2009, p. 11–22.

<sup>3</sup> Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1–72.





Substances causing blood dyscrasias (such as aplastic anaemia) or allergy, and high potency carcinogens are excluded.

**Figure 1:** Decision tree for assigning toxicological screening values (TSVs) for non-allowed pharmacologically active substances (based on EFSA CONTAM Panel, 2013)

Substances with intended pharmacological effects on the nervous system or the reproductive system, or that are corticoids, are referred to as Group II substances and a TSV of 0.0042 µg/kg bw per day is applied for this group.<sup>4</sup>

For the remaining classes of non-allowed pharmacologically active substances, referred to as Group III substances, a TSV of 0.65 µg/kg bw per day is applied.

The assigned TSV is then used to calculate a Toxicologically Based Limit of Quantification (TBLOQ), which is compared with the Reasonably Achievable Lowest Limit of Quantification (RALLOQ) using a decision tree to establish an appropriate RPA (EFSA CONTAM Panel, 2013).

However, it should be noted that the CONTAM Panel identified circumstances where the European Commission might consider it appropriate to conduct a substance-specific risk assessment. These might include '(i) where application of the proposed methodology results in a TBLOQ that is lower than the RALLOQ and there is little or no possibility of significant improvement in the analytical capability within a short to medium time frame, (ii) substances causing blood dyscrasias (such as aplastic anaemia) or allergy or that are high potency carcinogens,<sup>5</sup> which are outside the scope of this guidance document or (iii) where there is experimental or other evidence that the use of the TSV of 0.0025 µg/kg bw per day for Group I may not be adequately health protective'. Further details regarding the interpretation of these circumstances is given under Sections 2.1.2 and 2.2.1 of this Scientific Report.

## 2. Data and methodologies

The guidance document (EFSA CONTAM Panel, 2013) provides a simple and pragmatic approach to establish RPAs for non-allowed pharmacologically active substances present in food of animal origin. Following this approach, EFSA applied a screening methodology to search for information that allows answering the terms of reference. Further details are given below.

<sup>4</sup> The guidance document specifies that Group II substances are substances that are acting (or intend to act) pharmacologically on the nervous or reproductive system, or being a corticoid. The CONTAM Panel agreed at its 83rd meeting that only the intended use should be considered to allocate substances to Group II. <http://www.efsa.europa.eu/sites/default/files/event/170314-m.pdf>

<sup>5</sup> i.e. aflatoxin-like, azoxy- or N-nitroso-compounds, benzidines, hydrazines) (EFSA Scientific Committee, 2012).



Information regarding the identity of the substances, i.e. IUPAC name, EC number, chemical formula, smiles code and chemical structure was retrieved using Pubchem<sup>6</sup> and the ECHA website.<sup>7</sup>

## 2.1. Data and methodology to evaluate whether the substances are excluded from the guidance document

The following evaluations were carried out to evaluate whether the above mentioned guidance document (EFSA CONTAM Panel, 2013) is applicable to the substances listed in Table 1:

- evaluation whether the substance is a high potency carcinogen,
- evaluation whether the substance causes allergy,
- evaluation whether the substance causes blood dyscrasias.

### 2.1.1. Evaluation whether the substance is a high potency carcinogen

According to the EFSA Scientific Committee (2012), aflatoxin-like compounds, azoxy- or N-nitroso-compounds, benzidines and hydrazines are considered to be high potency carcinogens. Based on the chemical structure of the substance, it was determined whether the substance should be considered to be a high potency carcinogen.

### 2.1.2. Evaluation whether the substance causes allergy

As described in Section 1.3, a substance-specific risk assessment is required for non-allowed pharmacologically active substances that cause 'Allergy'. 'Allergy' can refer to substances that can cause food- or respiratory allergy (both Type I, immediate type hypersensitivity reactions, immunoglobulin E (IgE)-mediated) or can cause skin allergy (Type IV, delayed type hypersensitivity, T-cell-mediated hypersensitivity: ACD, allergic contact dermatitis).

Sometimes, systemic reactivation of ACD or systemic contact dermatitis (SCD) can occur when individuals with a contact allergy to a certain skin allergen are exposed systemically to the same allergen via exposure routes other than by cutaneous contact, like orally or by injection. In these situations, the systemically administered allergen may reach the skin through the circulatory system. However, only relatively few patients have been documented in literature as having had a significant contribution to their skin allergic state by allergen exposure through routes of exposure other than by cutaneous contact. SCD due to oral re-exposure has mainly been described (Lampel and Silvestri, 2014) for some antibiotics (e.g. neomycin and closely related antibiotics) and several metals (e.g. nickel, cobalt, chromium). A relatively high dose of the contact allergen is usually needed. From the results of a study by Jensen et al. (2006), it was shown that in case of oral nickel exposure about 1% of nickel-sensitive patients may react systemically to every day nickel exposure (0.22–0.35 mg Ni) and 10% may react to a diet composed of foods rich in nickel (0.55–1.33 mg Ni).

For certain dyes, skin sensitisation resulting in ACD can only be induced upon cutaneous contact. In individuals suffering already from contact allergy to a dye, reactivation of ACD will only occur upon renewed cutaneous contact, whereas after oral exposure to the dye present in food no reactivation of ACD or SCD has been reported in literature. Therefore, skin sensitisation by dyes is to be disregarded as exclusion criterion for establishment of RPAs for dyes when taking into account the irrelevance of the oral route of exposure in individuals already sensitised to certain dyes.

Overall, it is concluded that under the intended conditions for use of dyes in aquaculture, there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to the dye. The CONTAM Panel agreed with this conclusion at its 83rd plenary meeting.<sup>8</sup>

A stepwise approach was followed to collect information to evaluate whether the dyes cause allergy. First it was checked whether the dye had been evaluated by the European Chemicals Agency (ECHA), the EMA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) or one of the Scientific Committees advising the European Commission in relation to consumer safety, health and the environment. Also the following text books and databases were consulted to collect information regarding the potential of the substance to cause allergy.

<sup>6</sup> <https://pubchem.ncbi.nlm.nih.gov/#>

<sup>7</sup> <https://www.echa.europa.eu/>

<sup>8</sup> <http://www.efsa.europa.eu/sites/default/files/event/170314-m.pdf>

Rietschel RL and Fowler JF, 2008. Fisher's contact dermatitis 6. BC Decker Inc, Hamilton, 862 p.

Plakas SM, Doerge DR and Turnipseed SB, 1999. Disposition and metabolism of malachite green and other therapeutic dyes in fish. In Smith DJ, Gingerich WH, Beconi-Barker MG (Eds). *Xenobiotics in fish*. Springer, New York, 149–166.

Andersen KE and Maibach HI, 1983. Drugs used topically. In: de Weck AL and Bundgaard H (Eds), 1983. *Allergic reactions to drugs*. Springer-Verlag, Berlin, 313–377.

Martindale: The complete Drug Reference.<sup>9</sup>

In case no information was identified in the first step, scientific literature searches (Appendix A) were conducted and the outcome from computational toxicology was considered. For the latter, the following information sources were consulted:

- Alerts reported on the ECHA website<sup>7</sup>
- QSAR toolbox 3.3.5.17<sup>10</sup>
- Danish QSAR database.<sup>11</sup>

### 2.1.3. Evaluation whether the substance causes blood dyscrasias

A stepwise approach was followed to collect information for this evaluation.

First, it was checked whether the dyes had been evaluated by ECHA, EMA, JECFA or one of the Scientific Committees advising the European Commission in relation to consumer safety, health and the environment. Also, Martindale<sup>9</sup> was consulted to collect information regarding the potential of the substance to cause blood dyscrasias.

Secondly, a literature search was conducted (Appendix A) and the papers were retrieved for the relevant studies.

## 2.2. Data and methodology to allocate TSVs to the substances that are not excluded from the guidance document

### 2.2.1. Evaluation whether the substance should be regarded as genotoxic and whether the TSV of 0.0025 µg/kg bw per day is sufficiently protective

It was checked whether the dyes had been evaluated by ECHA, EMA, JECFA or one of the Scientific Committees advising the European Commission in relation to consumer safety, health and the environment. The available information was used in the evaluation.

In addition, a literature search was conducted to identify scientific papers in the open literature. Title and abstracts were screened to identify information to reply to this question. No evaluation of the full papers nor the quality of the study was conducted. The search strings are reported in Appendix A.

For the substances for which no or limited data were identified from the scientific literature and previous assessments, the outcome from computational toxicology was considered by consulting the following information sources:

- Alerts reported on the ECHA website<sup>7</sup>
- QSAR toolbox 3.3.5.17<sup>10</sup>
- Danish QSAR database.<sup>11</sup>

Since no evaluation of the quality of the identified information is conducted in the applied methodology, the classification of the substance regarding the genotoxicity based on the identified information from the literature and *in silico* methods should not be considered as conclusive. EFSA evaluated whether under the applied guidance document, the substance could be regarded as non-genotoxic and consequently be removed from group I. However, only a full evaluation of the available information, either by a previous assessment or (an) expert(s) in the area, would allow allocation of the substance to group II and III.

The CONTAM Panel noted at its 83rd plenary meeting<sup>8</sup> that high potency carcinogens are excluded from the guidance document and that the guidance document presents a simple and pragmatic approach for establishing RPAs. Therefore, the CONTAM Panel agreed that no specific evaluation of the

<sup>9</sup> <https://www.medicinescomplete.com/mc/martindale/current/login.htm?uri=https%3A%2F%2Fwww.medicinescomplete.com%2Fmc%2Fmartindale%2F2009%2F>

<sup>10</sup> <http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm>

<sup>11</sup> <http://qsar.food.dtu.dk/>

exclusion criterion (iii) (i.e. where there is experimental or other evidence that the use of the TSV of 0.0025 µg/kg bw per day for Group I may not be adequately health protective) was needed.

The TSV of 0.0025 µg/kg bw per day for substances for which there is direct evidence of genotoxicity or for which there is an alert for genotoxicity (from structural activity relationships or read across) is derived from the threshold of toxicological concern (TTC) concept. According to the RPA Opinion (EFSA CONTAM Panel, 2013), this also applies for substances for which there is insufficient information on genotoxicity. Inorganic substances are excluded from the TTC concept. The CONTAM Panel therefore concluded that inorganic substances should be excluded from the guidance document.<sup>8</sup>

### 2.2.2. Evaluation whether the substance has intended pharmacological effects on the nervous system or reproductive system and/or is a corticoid

The dyes evaluated in this Scientific Report are in principle used for antimicrobial purposes and not to act on the nervous or reproductive system and/or as a corticosteroid. Substances for which genotoxicity can be excluded are therefore classified in group III.

## 3. Answers to the terms of reference

### 3.1. Acriflavine (CAS 65589-70-0)

IUPAC name: acridine-3,6-diamine;10-methylacridin-10-ium-3,6-diamine;chloride

EC number: 617-132-5

Molecular formula: C<sub>27</sub>H<sub>25</sub>N<sub>6</sub>.Cl

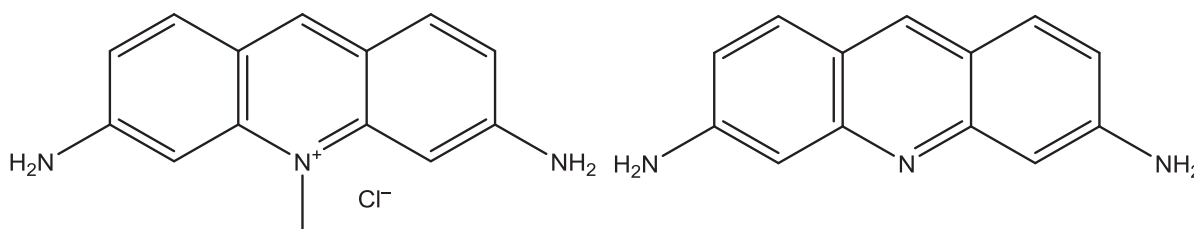
Smiles code:

C[N+]1=C2C=C(C=CC2=CC3=C1C=C(C=C3)N)N.C1=CC(=CC2=NC3=C(C=CC(=C3)N)C=C21)N.[Cl-]

#### 3.1.1. Inclusion/exclusion from the guidance document

##### 3.1.1.1. Q1: Is acriflavine a high potency carcinogen?

Acriflavine (Figure 2) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 2:** Chemical structure of acriflavine

##### 3.1.1.2. Q2: Is acriflavine causing allergy?

In several case reports, it has been described that upon occupational exposure, acriflavine can cause ACD (Plakas et al., 1999; Rietschel and Fowler, 2008), and was reported in a fishery worker after repeated application of acriflavine as an antiparasitic agent in aquarium fish (Goh, 1986). As no food or respiratory allergy was identified in the literature search for acriflavine, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of acriflavine.

##### 3.1.1.3. Q3: Is acriflavine causing blood dyscrasias?

No studies regarding blood dyscrasias caused by acriflavine were identified in the literature search.

##### 3.1.1.4. Conclusion

Based on the chemical structure of acriflavine and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for acriflavine.

### 3.1.2. Toxicological screening value

#### 3.1.2.1. Evaluation of genotoxicity

Several studies reported that acriflavine can induce mutations in different type of microorganisms (*i.e.* bacteria, yeast, moulds) (Witkin, 1947; Demerec et al., 1951; Marcovich, 1951; Arlett, 1957; Mejsel and Sokolova, 1960; Faulkner and Arlett, 1964; Morita and Mifuchi, 1965; Ball and Roper, 1966; Kot et al., 1975; Younis et al., 1975; Ali et al., 1978; Rosato and De Azevedo, 1978; Aoki et al., 2005). Acriflavine was also found to inhibit DNA-repair following UV-induced damage in bacteria (Doudney et al., 1964; Likhacheva, 1970; Setlow and Boling, 1970; Harm, 1973) and in human cells (Ben-Hur and Ben-Ishai, 1971; Kleijer et al., 1973).

The mutagenic potential of acriflavine was also reported in algae (Singh and Dikshit, 1976; Dorthu et al., 1992) and *Drosophila melanogaster* (Mitchell et al., 1981; Alba et al., 1983; Xamena et al., 1984).

Acriflavine exposure caused DNA damages in both cultured human lymphocytes and lung cancer cells (NCI-H460), as evaluated by the comet assay (Obstoy et al., 2015). Sudharsan Raj and Heddle (1980) reported increased frequencies of micronuclei and sister chromatid exchanges in Chinese hamster ovary cells exposed *in vitro* to acriflavine.

An *in vivo* study reported that acriflavine can induce chromosome alterations in C3H mice (Schle Iermacher, 1967).

Based on this information, EFSA concluded that under the applied approach, acriflavine should be regarded as genotoxic. This substance consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.2. 3-Aminoacridine (CAS 581-29-3) and aminoacridine (CAS 90-45-9)

#### 3-Aminoacridine

IUPAC name: acridin-3-amine

EC number: 209-461-4

Molecular formula: C<sub>13</sub>H<sub>10</sub>N<sub>2</sub>

Smiles code: C1=CC=C2C(=C1)C=C3C=CC(=CC3=N2)N

#### Aminoacridine

IUPAC name: acridin-9-amine

EC number: 201-995-6

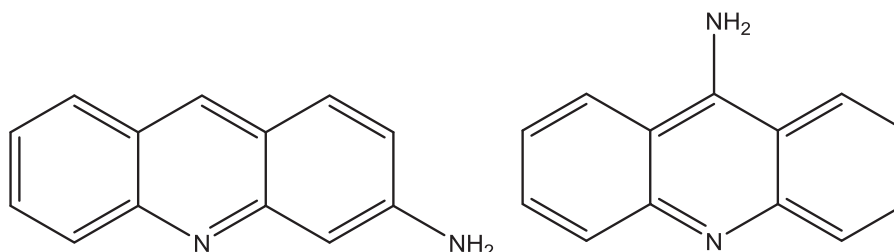
Molecular formula: C<sub>13</sub>H<sub>10</sub>N<sub>2</sub>

Smiles code: C1=CC=C2C(=C1)C(=C3C=CC=CC3=N2)N

#### 3.2.1. Inclusion/exclusion from the guidance document

##### 3.2.1.1. Q1: Are 3-aminoacridine and aminoacridine high potency carcinogens?

3-Aminoacridine and aminoacridine (Figure 3) do not belong to the chemical classes defined as high potency carcinogens.



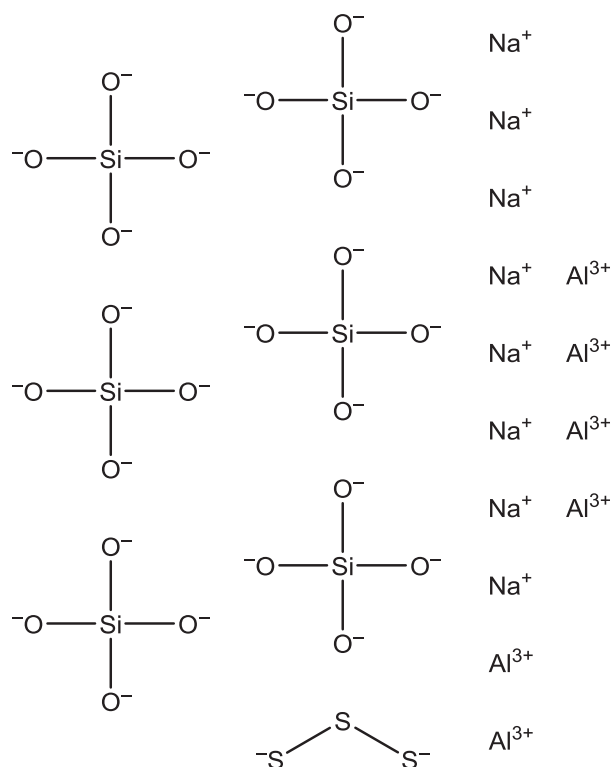
**Figure 3:** Chemical structure of 3-aminoacridine (left) and aminoacridine (right)



### 3.3.1. Inclusion/exclusion from the guidance document

As shown in Figure 4, azure blue is a mixture of different inorganic compounds, of which thiozonide  $S_3^-$  has an intense blue colour. The other compounds are not known as dyes or as pharmacologically active substances. EFSA noted that besides thiozonide, azure blue contains sodium aluminium silicate which is used as an anticaking agent in foods (E 554).

The CONTAM Panel agreed at the 83rd Panel meeting<sup>8</sup> that inorganic substances should be excluded from the guidance document (see Section 2.2.1). Therefore, the guidance document cannot be applied to azure blue and no TSV can be assigned to this compound.



**Figure 4:** Chemical structure of azure blue

### 3.4. Basic blue 7/victoria pure blue BO (CAS 2390-60-5)

IUPAC name: [4-[bis[4-(diethylamino)phenyl]methylidene]naphthalen-1-ylidene]-ethylazanium;chloride  
EC number: 219-232-0

Molecular formula:  $C_{33}H_{40}ClN_3$

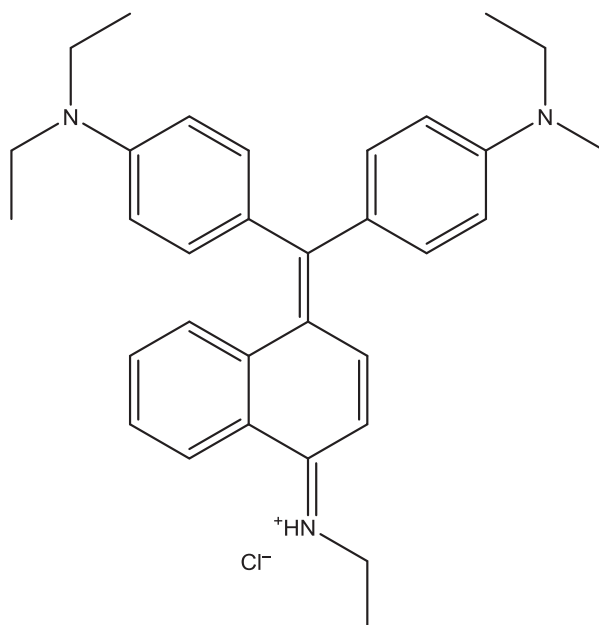
Smiles code: CC[NH+]=C1C=CC(=C(C2=CC=C(C=C2)N(CC)CC)C3=CC=C(C=C3)N(CC)CC)C4=CC=CC=C14.[Cl-]

#### 3.4.1. Inclusion/exclusion from the guidance document

##### 3.4.1.1. Q1: Is basic blue 7 a high potency carcinogen?

Basic blue 7 (Figure 5) does not belong to the chemical classes defined as high potency carcinogens.





**Figure 5:** Chemical structure of basic blue 7

#### 3.4.1.2. Q2: Is basic blue 7 causing allergy?

In 2000, the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers (SCCNFP) evaluated basic blue 7 as a hair tinting product and concluded that 'on the basis of the data provided, it cannot be excluded that this substance is a contact allergen. Cosmetic products containing this substance shall carry a label warning of a risk of sensitisation'.<sup>14</sup>

As no food or respiratory allergy was identified in the literature search for basic blue 7, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of basic blue 7.

#### 3.4.1.3. Q3: Is basic blue 7 causing blood dyscrasias?

No studies regarding blood dyscrasias caused by basic blue 7 were identified in the literature search.

#### 3.4.1.4. Conclusion

Based on the chemical structure of basic blue 7 and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for basic blue 7.

### 3.4.2. Toxicological screening value

#### 3.4.2.1. Evaluation of genotoxicity

Basic blue 7 was found to efficiently bind to DNA and to mediate its photochemical destruction in tumour cells (Lewis and Indig, 2001, 2002). No other relevant information was identified in the literature search.

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. As indicated on the ECHA website, basic blue 7 is a suspected mutagen as shown in different models. The CAESAR Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q)SAR platform predicts with moderate reliability that basic blue 7 is a mutagen.<sup>15</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for *in vitro* mutagenicity (Ames), *in vivo* mutagenicity (micronucleus)<sup>16</sup> and structural

<sup>14</sup> [http://ec.europa.eu/health/scientific\\_committees/consumer\\_safety/opinions/sccnfp\\_opinions\\_97\\_04/sccp\\_out118\\_en.htm](http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out118_en.htm)

<sup>15</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.017.485>

<sup>16</sup> EFSA noted that micronuclei are due to clastogenic effects.



alerts for genotoxic carcinogenicity by ISS. Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, Ames test in *Salmonella* Typhimurium (*in vitro*), Syrian hamster embryo cell transformation, sex-linked recessive lethal test in *Drosophila* and sister chromatid exchange in mouse bone marrow cells (only predictions inside the applicability domain are reported in this Scientific Report).

Based on this information, EFSA concluded that under the applied approach, basic blue 7 should be regarded as genotoxic. Basic blue 7 consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.5. Brilliant green/C.I. basic green 1 (CAS 633-03-4) and leucobrilliant green (CAS 82-90-6)

#### Brilliant green

IUPAC name: [4-[[4-(diethylamino)phenyl]-phenylmethylidene]cyclohexa-2,5-dien-1-ylidene]-diethylazanium;hydrogen sulfate

EC number: 211-190-1

Molecular formula: C<sub>27</sub>H<sub>34</sub>N<sub>2</sub>O<sub>4</sub>S

Smiles code: CCN(CC)C1=CC=C(C=C1)C(=C2C=CC(=[N+](CC)CC)C=C2)C3=CC=CC=C3.OS(=O)(=O)[O-]

#### Leucobrilliant green

IUPAC name: 4-[[4-(diethylamino)phenyl]-phenylmethyl]-*N,N*-diethylaniline

EC number: -

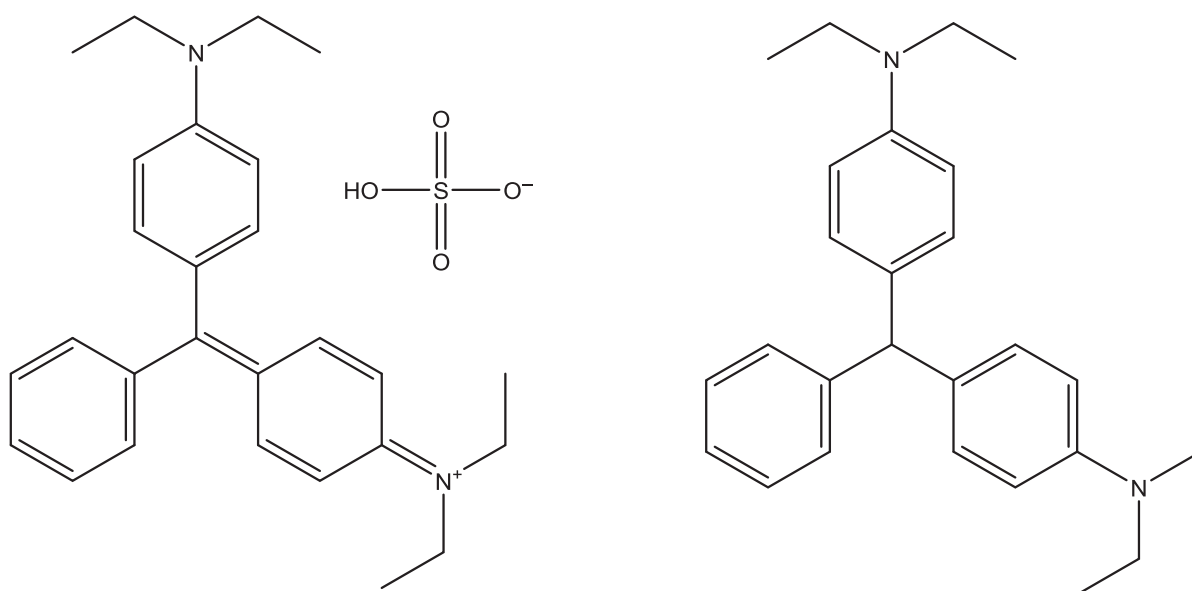
Molecular formula: C<sub>27</sub>H<sub>34</sub>N<sub>2</sub>

Smiles code: CCN(CC)C1=CC=C(C=C1)C(C2=CC=CC=C2)C3=CC=C(C=C3)N(CC)CC

#### 3.5.1. Inclusion/exclusion from the guidance document

##### 3.5.1.1. Q1: Are brilliant green and leucobrilliant green high potency carcinogens?

Brilliant green and leucobrilliant green (Figure 6) do not belong to the chemical classes defined as high potency carcinogens.



**Figure 6:** Chemical structure of brilliant green (left) and leucobrilliant green (right)

### 3.5.1.2. Q2: Are brilliant green and leucobrilliant green causing allergy?

Information in the REACH registration dossier of brilliant green on structural analogues indicates that brilliant green is suspected to be a dermal contact sensitiser.<sup>17</sup> It is classified as skin sensitiser category 1,<sup>18,19</sup> by the registrant.<sup>20</sup>

In literature, triphenylmethane dyes such as brilliant green and leucobrilliant green are indicated as weak sensitisers (Andersen and Maibach, 1983; Rietschel and Fowler, 2008). Bielicky and Novak (1969) described 11 patients with contact allergy to brilliant green, with eight patients being sensitive also to crystal violet and 6 of them were also sensitive to malachite green.

As no food or respiratory allergy was identified in the literature search for brilliant green and leucobrilliant green, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of brilliant green and leucobrilliant green.

### 3.5.1.3. Q3: Are brilliant green and leucobrilliant green causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.5.1.4. Conclusion

Based on the chemical structures of brilliant green and leucobrilliant green and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for these substances.

## 3.5.2. Toxicological screening value

### 3.5.2.1. Evaluation of genotoxicity

Brilliant green increased the frequency of mutations in bacteria (*Salmonella Pullorum*; Smith, 1962) and yeast (*S. cerevisiae*; Zimina and Pavlenko, 1990).

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. As indicated on the ECHA website, brilliant green is a suspected mutagen as shown in different models. The CAESAR Mutagenicity model, the ISS Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q) SAR platform predicts that brilliant green is a mutagen (experimental value available or prediction with moderate reliability).<sup>21</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as an alert for 'in vivo mutagenicity (micronucleus)' by ISS.

Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, mutations in thymidine kinase locus in mouse lymphoma cells, Syrian hamster embryo cell transformation, sex-linked recessive lethal test in *Drosophila* and sister chromatid exchange in mouse bone marrow cells (only predictions inside the applicability domain are reported in this Scientific Report).

No information was retrieved regarding leucobrilliant green in the scientific literature. Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for *in vitro* mutagenicity (Ames), 'in vivo mutagenicity (micronucleus)' and structural alerts for genotoxic carcinogenicity by ISS.

Based on this information, EFSA concluded that under the applied approach, brilliant green and leucobrilliant green should be regarded as genotoxic. Both substances consequently belong to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

<sup>17</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/17567/7/5/2>

<sup>18</sup> According to regulation (EC) No 1272/2008 substances shall be classified as skin sensitisers (Category 1) (a) if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons; or (b) if there are positive results from an appropriate animal test.

<sup>19</sup> Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 (Text with EEA relevance) (OJ L 353, 31.12.2008, p. 1)

<sup>20</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/17567/2/1>

<sup>21</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.010.174>

### 3.6. Basic blue 26 (CAS 2580-56-5)

IUPAC name: (4-((4-Anilino-1-naphthyl)(4-(dimethylamino)phenyl)methylene)cyclohexa-2,5-dien-1-ylidene)dimethylammonium chloride

EC number: 219-943-6

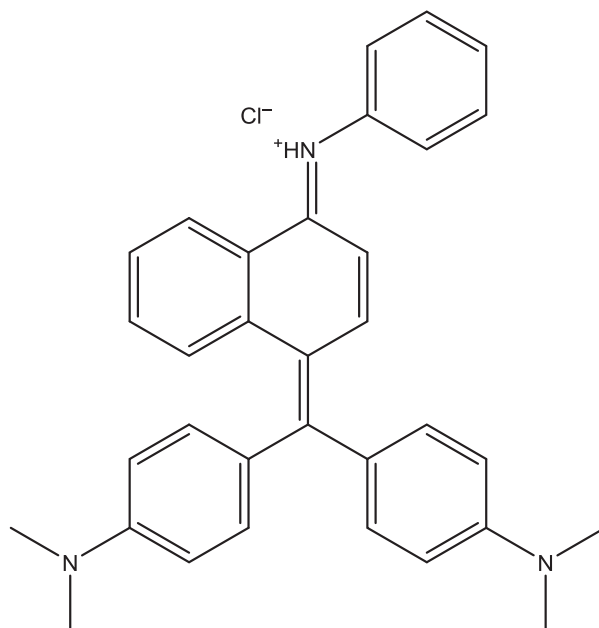
Molecular formula: C<sub>33</sub>H<sub>32</sub>N<sub>3</sub>.Cl

Smiles code: CN(C)C1=CC=C(C=C1)C(=C2C=CC(=[NH<sup>+</sup>]C3=CC=CC=C3)C4=CC=CC=C24)C5=CC=C(C=C5)N(C)C.[Cl<sup>-</sup>]

#### 3.6.1. Inclusion/exclusion from the guidance document

##### 3.6.1.1. Q1: Is basic blue 26 a high potency carcinogen?

Basic blue 26 (Figure 7) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 7:** Chemical structure of basic blue 26

##### 3.6.1.2. Q2: Is basic blue 26 causing allergy?

Basic blue 26 is a triarylmethane dye. Based on a read across with basic blue 7 (see Section 3.4.1.2), it is suspected to be a skin sensitiser.

As no food or respiratory allergy was identified in the literature search for basic blue 26, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of basic blue 26.

##### 3.6.1.3. Q3: Is basic blue 26 causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

##### 3.6.1.4. Conclusion

Based on the chemical structure of basic blue 26 and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for basic blue 26.

### 3.6.2. Toxicological screening value

#### 3.6.2.1. Evaluation of genotoxicity

In the study conducted by Janik-Spiechowicz et al. (1997), basic blue 26 failed to induce mutagenicity in *S. Typhimurium* strains (Ames test), and did not cause the formation of micronuclei and sister chromatid exchanges in mice bone marrow cells *in vivo*.

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. As indicated on the ECHA website, basic blue 26 is a suspected mutagen as shown in different models. The Toolbox profiler Protein binding alerts for Chromosomal aberration by OASIS v1.1 gives an alert for mutagenicity. The CAESAR Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q)SAR platform predicts with moderate reliability that basic blue 26 is a mutagen.<sup>22</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for *in vitro* mutagenicity (Ames), '*in vivo* mutagenicity (micronucleus)' and structural alerts for genotoxic carcinogenicity by ISS.

Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, Ames test in *S. Typhimurium* (*in vitro*), Syrian hamster embryo cell transformation and sister chromatid exchange in mouse bone marrow cells and the comet assay in mouse (only predictions inside the applicability domain are reported in this Scientific Report).

One study on the genotoxic properties of basic blue 26 was identified which showed negative results. On the other hand, positive alerts were identified in several models. Since no evaluation of the quality of the studies is conducted in the applied methodology (see Section 2.2.1), EFSA followed a conservative approach and concluded that in the context of this evaluation, basic blue 26 should be regarded as genotoxic. This substance consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.7. Chloranil (CAS 118-75-2)

IUPAC name: 2,3,5,6-tetrachloro-1,4-benzoquinone

EC number: 204-274-4

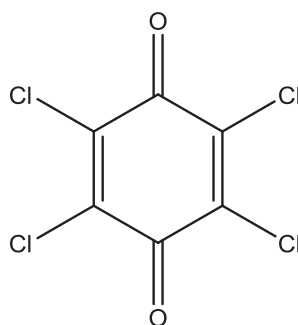
Molecular formula: C<sub>6</sub>Cl<sub>4</sub>O<sub>2</sub>

Smiles code: Cl1(=C(C(=O)C(=C(C1=O)Cl)Cl)Cl)Cl

#### 3.7.1. Inclusion/exclusion from the guidance document

##### 3.7.1.1. Q1: Is chloranil a high potency carcinogen?

Chloranil (Figure 8) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 8:** Chemical structure of chloranil

<sup>22</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.018.131>

### 3.7.1.2. Q2: Is chloranil causing allergy?

Chloranil was found to be a skin sensitiser in a mouse local lymph node assay reported in the REACH registration dossier available on the ECHA website.<sup>23</sup> It is classified as skin sensitiser category 1 by the registrant.<sup>24</sup>

As no food or respiratory allergy was identified in the literature search for chloranil, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of chloranil.

### 3.7.1.3. Q3: Is chloranil causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.7.1.4. Conclusion

Based on the chemical structure of chloranil and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for chloranil.

## 3.7.2. Toxicological screening value

### 3.7.2.1. Evaluation of genotoxicity

Chloranil was indicated as one of the metabolites responsible for the genotoxicity and carcinogenicity of pentachlorophenol (PCP) (Dahlhaus et al., 1996; Zhu and Shan, 2009; Zhu et al., 2011).

In the study conducted by Dong et al. (2014), chloranil induced a significant increase of DNA and chromosomal damage (comet assay and micronucleus test) as well as histone H2AX phosphorylation (enzyme-linked immunosorbent assay (ELISA) and western blot analyses) in human hepatoma cells (HepG2). The authors concluded that the genotoxic effects probably occur through the induction of oxidative stress. Also other studies reported the ability of chloranil to cause oxidative base damage (Dahlhaus et al., 1996; Liu et al., 2011).

Chloranil was found to cause DNA and protein adducts both *in vitro* (Lin et al., 2001; Jia et al., 2010) and *in vivo*, following the administration of its parent compound (PCP) (Waidyanatha et al., 1994; Waidyanatha et al., 1996; Lin et al., 1997, 1999, 2002; Vaidyanathan et al., 2007).

Chloranil was considered to be non-mutagenic in *S. Typhimurium* and *E. coli* (Ames test) as reported in the REACH registration dossier available on the ECHA website.<sup>25</sup> In the same dossier, a negative result was reported for an *in vivo* mammalian erythrocyte micronucleus test (OECD 474).<sup>26</sup> The available data are insufficient for classification.<sup>27</sup>

Based on this information, EFSA concluded that under the applied approach, chloranil should be regarded as genotoxic. Chloranil consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

## 3.8. Crystal violet/gentian violet (CAS 548-62-9) and leucocrystal violet/leucogentian violet (CAS 603-48-5)

### Crystal violet

IUPAC name: [4-[bis[4-(dimethylamino)phenyl]methylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium;chloride

EC number: 208-953-6

Molecular formula: C<sub>25</sub>H<sub>30</sub>ClN<sub>3</sub>

Smiles code: CN(C)C1=CC=C(C=C1)C(=C2C=CC(=[N+](C)C)C=C2)C3=CC=C(C=C3)N(C)C.[Cl-]

<sup>23</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/2069/7/5/2>

<sup>24</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/2069/2/1>

<sup>25</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/2069/7/7/2>

<sup>26</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/2069/7/7/3>

<sup>27</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/2069/2/2>

**Leucocrystal violet**IUPAC name: 4-[bis[4-(dimethylamino)phenyl]methyl]-*N,N*-dimethylaniline

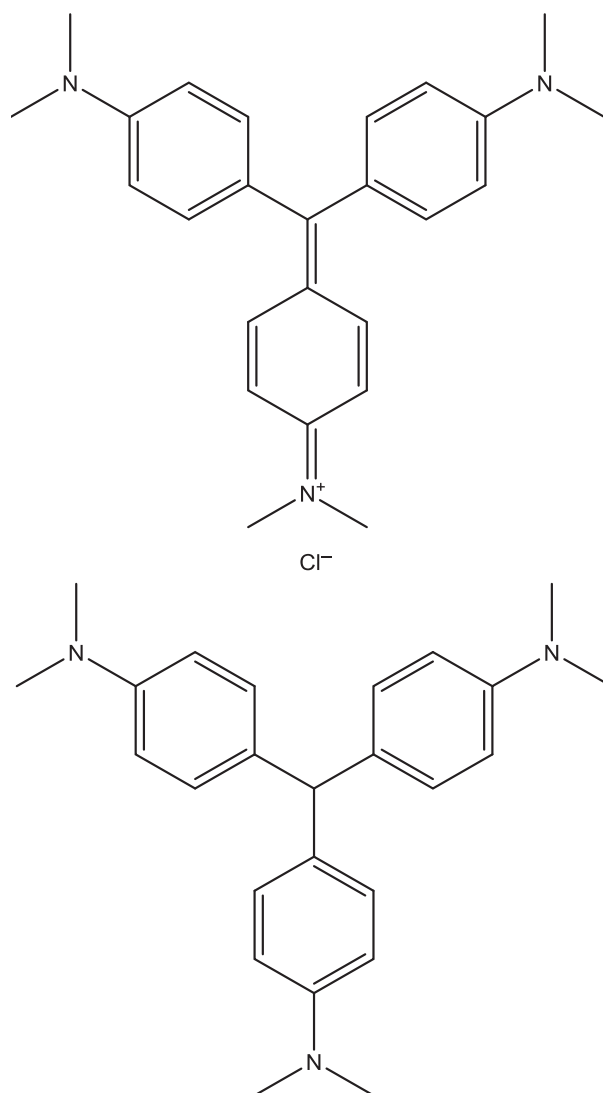
EC number: 210-043-9

Molecular formula: C<sub>25</sub>H<sub>31</sub>N<sub>3</sub>

Smiles code: CN(C)C1=CC=C(C=C1)C(C2=CC=C(C=C2)N(C)C)C3=CC=C(C=C3)N(C)C

**3.8.1. Inclusion/exclusion from the guidance document****3.8.1.1. Q1: Are crystal violet and leucocrystal violet high potency carcinogens?**

Crystal violet and leucocrystal violet (Figure 9) do not belong to the chemical classes defined as high potency carcinogens.



**Figure 9:** Chemical structure of crystal violet (up) and leucocrystal violet (bottom)

**3.8.1.2. Q2: Are crystal violet and leucocrystal violet causing allergy?**

The JECFA evaluated crystal violet at its 78th meeting in 2013 and noted that crystal violet is shown to cause dermal irritation/sensitisation (FAO/WHO, 2014).

Inconclusive results regarding skin sensitisation are reported in the dossier of crystal violet available on the ECHA website and no self-classification regarding skin sensitisation has been proposed by the registrant.<sup>28</sup>

<sup>28</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/10024/7/5/2>

Triphenylmethane dyes such as crystal violet and leucocrystal violet are weak sensitizers and are capable of producing phototoxic dermatitis (Andersen and Maibach, 1983; Rietschel and Fowler, 2008). Cross-reactivity with some triphenylmethane dyes was described by Bielicky and Novak (1969) in 11 patients with contact allergy to the triphenylmethane dye brilliant green, of which 8 patients showed cross-reactivity with crystal violet, and 6 of these patients also showed cross-reactivity with malachite green.

As no food or respiratory allergy was identified in the literature search for crystal violet and leucocrystal violet, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of crystal violet and leucocrystal violet.

### 3.8.1.3. Q3: Are crystal violet and leucocrystal violet causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.8.1.4. Conclusion

Based on the chemical structures of crystal violet and leucocrystal violet and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for these substances.

## 3.8.2. Toxicological screening value

### 3.8.2.1. Evaluation of genotoxicity

Several studies reported that crystal violet is mutagenic towards bacteria (Wild and Hinshelwood, 1956; Smith, 1962; Takahashi, 1972; Fujita et al., 1976; Bonin et al., 1981; Malachová et al., 2006). Moreover, the compound was found to enhance the mutagenic potency of UV (Witkin, 1961; Hill and Feiner, 1964; Levin et al., 1982).

In the onion root tip, crystal violet did not induce any chromosome mutations (Battaglia, 1950).

In the study by Au et al. (1979), crystal violet was negative in the Ames test, as well as in cytogenetic assays (Chinese hamster ovary cells *in vitro*, the chicken-embryo and mouse-bone-marrow cells *in vivo*), but caused repairable DNA damage in the Rosenkranz bacterial assay. However, no further information regarding this test was identified. In another study, crystal violet showed clastogenic property in different mammalian cell lines *in vitro* (Au et al., 1978). Positive genotoxic effects were also observed in the comet assay performed on leukocytes from rat blood exposed *in vitro* to crystal violet (Diaz Gomez and Castro, 2013).

In the review published by Docampo and Moreno (1990), crystal violet was considered as mutagenic and clastogenic.

Negative results were reported by NTP<sup>29</sup> for *in vitro* cytogenetics (sister chromatid exchange and chromosomal aberrations) and for mutagenicity in *Drosophila*. Equivocal results were reported for the Ames test.

No toxicological information was retrieved in the scientific literature regarding leucocrystal violet. Therefore, the outcome from computational toxicology was taken into consideration. As indicated on the ECHA website, leucocrystal violet is a suspected mutagen as shown in different models. The KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q)SAR platform predict with moderate reliability that leucocrystal violet is a mutagen.<sup>30</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox, as well as alerts for *in vitro* mutagenicity (Ames), *in vivo* mutagenicity (micronucleus) and structural alerts for genotoxic carcinogenicity by ISS. Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ames test in *S. Typhimurium* (*in vitro*), mutations in thymidine kinase locus in mouse lymphoma cells, Syrian Hamster embryo cell transformation, sister chromatid exchange in mouse bone marrow cells and the comet assay in mouse (only predictions inside the applicability domain are reported in this Scientific Report).

Based on this information, EFSA concluded that under the applied approach, crystal violet and leucocrystal violet should be regarded as genotoxic. Both substances consequently belong to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

<sup>29</sup> <https://ntp.niehs.nih.gov/testing/status/agents/ts-10008-r.html>

<sup>30</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.009.131>



### 3.9. Dichlone (CAS 117-80-6)

IUPAC name: 2,3-dichloronaphthalene-1,4-dione

EC number: 204-210-5

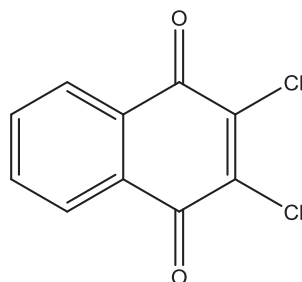
Molecular formula: C<sub>10</sub>H<sub>4</sub>Cl<sub>2</sub>O<sub>2</sub>

Smiles code: C1=CC=C2C(=C1)C(=O)C(=C(C2=O)Cl)Cl

#### 3.9.1. Inclusion/exclusion from the guidance document

##### 3.9.1.1. Q1: Is dichlone a high potency carcinogen?

Dichlone (Figure 10) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 10:** Chemical structure of dichlone

##### 3.9.1.2. Q2: Is dichlone causing allergy?

Two studies are reported in the REACH registration dossier available on the ECHA website. From an *in vitro* keratinocyte activation assay (LuSens), it was concluded that dichlone has a keratinocyte activating potential.<sup>31</sup> From a direct peptide reactivity assay (DPRA), it was concluded that dichlone shows a high chemical reactivity.<sup>32</sup> Dichlone is classified as skin sensitisation category 1 by the registrant.<sup>33</sup>

As no food or respiratory allergy was identified in the literature search for dichlone, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of dichlone.

##### 3.9.1.3. Q3: Is dichlone causing blood dyscrasias?

Haematology was investigated in a subacute toxicity study in rats (REACH registration dossier), however no conclusion regarding blood dyscrasias could be drawn from this study.<sup>34</sup>

##### 3.9.1.4. Conclusion

Based on the chemical structure of dichlone and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for dichlone.

#### 3.9.2. Toxicological screening value

##### 3.9.2.1. Evaluation of genotoxicity

Dichlone showed high mutagenicity in *S. Typhimurium* strains (Ames test) as reported by Hakura et al. (1994), whereas it was negative in the study by Onodera et al. (1982).

<sup>31</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/7/5/2/?documentUUID=2bb75381-66a4-4d4d-89a5-f1047dd0e026>

<sup>32</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/7/5/2>

<sup>33</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/2/1>

<sup>34</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/7/6/2>

Dichlone was not mutagenic in *S. Typhimurium/E. coli* (Ames test) in the absence and the presence of metabolic activation as reported in the REACH registration dossier available on the ECHA website.<sup>35</sup> The available data are insufficient for classification.<sup>36</sup>

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. Alerts for DNA binding by OECD and by OASIS v.1.3 were identified in the QSAR toolbox as well as DNA alerts for Ames test, micronucleus and chromosomal aberrations by OASIS v. 1.3 and alerts for *in vitro* mutagenicity (Ames), '*in vivo* mutagenicity (micronucleus)' and structural alerts for genotoxic carcinogenicity by ISS.

Based on this information, EFSA concluded that under the applied approach, dichlone should be regarded as genotoxic. This substance consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.10. Ethyl violet (CAS 25275-06-3)

IUPAC name: [4-[bis[4-(diethylamino)phenyl]methylidene]cyclohexa-2,5-dien-1-ylidene]-diethylazanium

EC number: 246-781-3

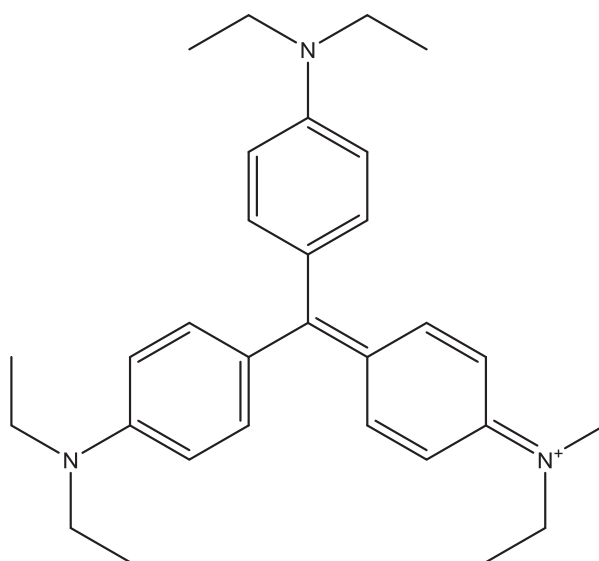
Molecular formula: C<sub>31</sub>H<sub>42</sub>N<sub>3</sub><sup>+</sup>

Smiles code: CCN(CC)C1=CC=C(C=C1)C(=C2C=CC(=[N+](CC)CC)C=C2)C3=CC=C(C=C3)N(CC)CC

#### 3.10.1. Inclusion/exclusion from the guidance document

##### 3.10.1.1. Q1: Is ethyl violet a high potency carcinogen?

Ethyl violet (Figure 11) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 11:** Chemical structure of ethyl violet

##### 3.10.1.2. Q2: Is ethyl violet causing allergy?

Ethyl violet is a triphenylmethane dye and therefore suspected to be a skin sensitiser (see Section 3.5.1.2).

As no food or respiratory allergy was identified in the literature search for ethyl violet, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of ethyl violet.

<sup>35</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/7/7/2>

<sup>36</sup> <https://www.echa.europa.eu/web/guest/registration-dossier/-/registered-dossier/16993/2/2>

### 3.10.1.3. Q3: Is ethyl violet causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.10.1.4. Conclusion

Based on the chemical structure of ethyl violet and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for ethyl violet.

## 3.10.2. Toxicological screening value

### 3.10.2.1. Evaluation of genotoxicity

Lewis and Indig (2001) reported that ethyl violet efficiently binds to DNA, mediating its photochemical destruction.

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. As indicated on the ECHA website, ethyl violet is a suspected mutagen as shown in different models. The CAESAR Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in VEGA (Q)SAR platform predicts on the basis of experimental values that ethyl violet is a mutagen.<sup>37</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for *in vitro* mutagenicity (Ames), '*in vivo* mutagenicity (micronucleus)' and structural alerts for genotoxic carcinogenicity by ISS.

Based on this information, EFSA concluded that under the applied approach, ethyl violet should be regarded as genotoxic. This substance consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

## 3.11. Methylene blue (CAS 61-73-4)

IUPAC name: [7-(dimethylamino)phenothiazin-3-ylidene]-dimethylazanium;chloride

EC number: 200-515-2

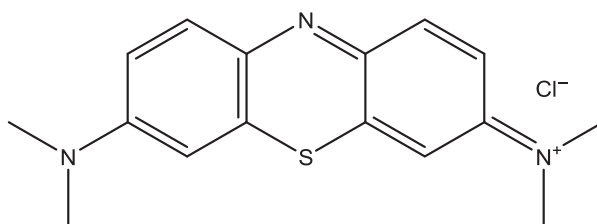
Molecular formula: C<sub>16</sub>H<sub>18</sub>ClN<sub>3</sub>S

Smiles code: CN(C)C1=CC2=C(C=C1)N=C3C=CC(=[N+](C)C)C=C3S2.[Cl-]

### 3.11.1. Inclusion/exclusion from the guidance document

#### 3.11.1.1. Q1: Is methylene blue a high potency carcinogen?

Methylene blue (Figure 12) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 12:** Chemical structure of methylene blue

#### 3.11.1.2. Q2: Is methylene blue causing allergy?

No alerts for skin sensitisation are reported on the ECHA website.<sup>38</sup> Also in the QSAR toolbox no alerts were reported for skin sensitisation.

A few very rare case reports regarding methylene blue-induced anaphylactic reactions upon injection in surgery patients were identified in the scientific literature (Dewachter et al., 2005, 2011; Jangjoo et al., 2010). These reactions were IgE mediated and probably caused by a conjugation of methylene blue as hapten to a protein.

<sup>37</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.042.514>

<sup>38</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.000.469>

As no food or respiratory allergy nor skin allergy was identified in the literature search for methylene blue, and concern for anaphylactic reactions upon oral exposure is low, there are no safety concerns with respect to allergenicity of methylene blue.

### 3.11.1.3. Q3: Is methylene blue causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.11.1.4. Conclusion

Based on the chemical structure of methylene blue and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for methylene blue.

## 3.11.2. Toxicological screening value

### 3.11.2.1. Evaluation of genotoxicity

Methylene blue was found to induce mutations in different bacteria strains (Smith, 1962; Webb and Hass, 1984). In one study, methylene blue was efficient in producing yeast mutants (Mitchell and Bevan, 1973), while in others it was not (Nagai, 1963; Morita and Mifuchi, 1965). Morita and Mifuchi (1965) reported a low increase in the mutation rate (1.2–4.5%) in yeast at pH 9.0, but not at pH values from 5.0 to 7.0.

Many studies reported that, in the presence of light, methylene blue can generate oxidative DNA damage in bacteria (Czeczot et al., 1991), yeast (Meniel and Waters, 1999) and mammalian cells (Zhang et al., 2000; Slamenova et al., 2002, 2003; Lazarova et al., 2006).

DNA damage and chromosome aberrations were also found in plants exposed to methylene blue (Sushil and Natarajan, 1965; Li et al., 2002).

Genotoxic effects were detected in mammalian cells using the comet assay (Masannat et al., 2009) and the mouse lymphoma TK assay (Wagner et al., 1995). *In vivo*, methylene blue gave negative results in the sister chromatid exchange test (Speit, 1982) and in the micronucleus assay (Sychyova et al., 2007; Masannat et al., 2009).

Based on this information, EFSA concluded that under the applied approach, methylene blue should be regarded as genotoxic. This substance consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

## 3.12. New methylene blue/victoria blue R (CAS 2185-86-6)

IUPAC name: [4-[bis[4-(dimethylamino)phenyl]methylidene]naphthalen-1-ylidene]-ethylazanium;chloride  
EC number: 218-572-7

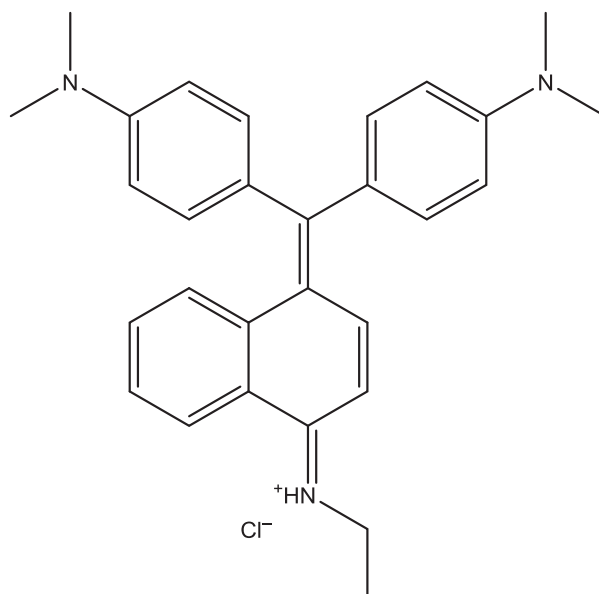
Molecular formula: C<sub>29</sub>H<sub>32</sub>ClN<sub>3</sub>

Smiles code: CC[NH+]=C1C=CC(=C(C2=CC=C(C=C2)N(C)C)C3=CC=C(C=C3)N(C)C)C4=CC=CC=C14.[Cl-]

### 3.12.1. Inclusion/exclusion from the guidance document

#### 3.12.1.1. Q1: Is victoria blue R a high potency carcinogen?

Victoria blue R (Figure 13) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 13:** Chemical structure of Victoria blue R

### 3.12.1.2. Q2: Is victoria blue R causing allergy?

Victoria blue R is a triarylmethane dye. Based on a read across with basic blue 7 (see Section 3.4.1.2), victoria blue R is suspected to be a skin sensitiser.

As no food or respiratory allergy was identified in the literature search for victoria blue R, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of victoria blue R.

### 3.12.1.3. Q3: Is victoria blue R causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.12.1.4. Conclusion

Based on the chemical structure of victoria blue R and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for victoria blue R.

## 3.12.2. Toxicological screening value

### 3.12.2.1. Evaluation of genotoxicity

Victoria blue R significantly increased the frequency of both cytoplasmic and nuclear mutants in yeast (*S. cerevisiae*) (Pavlenko and Zimina, 1983; Zimina and Pavlenko, 1990).

Lewis and Indig (2001) reported that victoria blue R mediates the photochemical destruction of DNA in tumour cells.

In one *in vivo* study, oral administration of victoria blue R induced chromosome aberrations in the bone marrow of male rats (Chesnokov et al., 1979).

Based on this information, EFSA concluded that under the applied approach, victoria blue R should be regarded as genotoxic. Victoria blue R consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

## 3.13. Nile blue (CAS 2381-85-3)

IUPAC name: [9-(diethylamino)benzo[a]phenoxazin-5-ylidene]azanium;chloride

EC number: 219-181-4

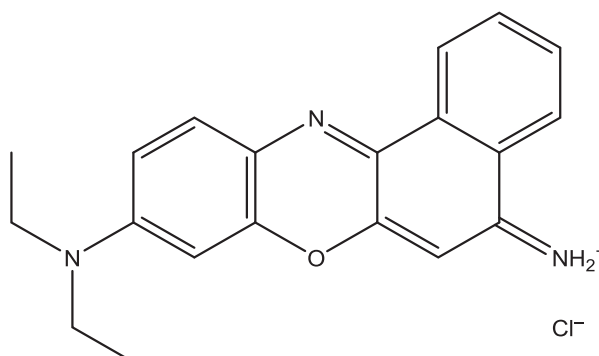
Molecular formula: C<sub>20</sub>H<sub>20</sub>ClN<sub>3</sub>O

Smiles code: CCN(CC)C1=CC2=C(C=C1)N=C3C4=CC=CC=C4C(=O)C=C3O2.[Cl-]

### 3.13.1. Inclusion/exclusion from the guidance document

#### 3.13.1.1. Q1: Is Nile blue a high potency carcinogen?

Nile blue (Figure 14) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 14:** Chemical structure of Nile blue

#### 3.13.1.2. Q2: Is Nile blue causing allergy?

As no food or respiratory allergy nor skin allergy was identified in the literature search for Nile blue, there are no safety concerns with respect to allergenicity of Nile blue.

#### 3.13.1.3. Q3: Is Nile blue causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

#### 3.13.1.4. Conclusion

Based on the chemical structure of Nile blue and the lack of evidence that Nile blue is causing allergy or blood dyscrasias, the guidance document can be applied to establish an RPA for Nile blue.

### 3.13.2. Toxicological screening value

#### 3.13.2.1. Evaluation of genotoxicity

Nagai (1962) reported mutagenicity of Nile blue in yeast and several authors reported DNA binding (e.g. Zhao et al., 2002; Mitra et al., 2009; Gattuso et al., 2016).

Given the limited information identified in the scientific literature, EFSA took into consideration the outcome from computational toxicology. As indicated on the ECHA website, Nile blue is a suspected mutagen as shown in different models. The Toolbox profilers 'DNA alerts for AMES, MN and CA by OASIS v.1.3' and 'in vitro mutagenicity (Ames test) alerts by ISS' give alerts for mutagenicity. The CAESAR Mutagenicity model, the ISS Mutagenicity model and the KNN Mutagenicity model in the VEGA (Q)SAR platform predicts with good to moderate reliability that Nile blue is a mutagen.<sup>39</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for in vitro mutagenicity (Ames), in vivo mutagenicity (micronucleus) and structural alerts for genotoxic carcinogenicity by ISS.

Based on this information EFSA concluded that under the applied approach, Nile blue should be regarded as genotoxic. Nile blue consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.14. Pararosaniline base (CAS 25620-78-4<sup>40</sup>)

IUPAC name: tris(4-aminophenyl)methanol

EC number: 207-395-0

Molecular formula: C<sub>19</sub>H<sub>19</sub>N<sub>3</sub>O

Smiles code: C1=CC(=CC=C1C(C=C(C=C2)N)(C3=CC=C(C=C3)N)O)N

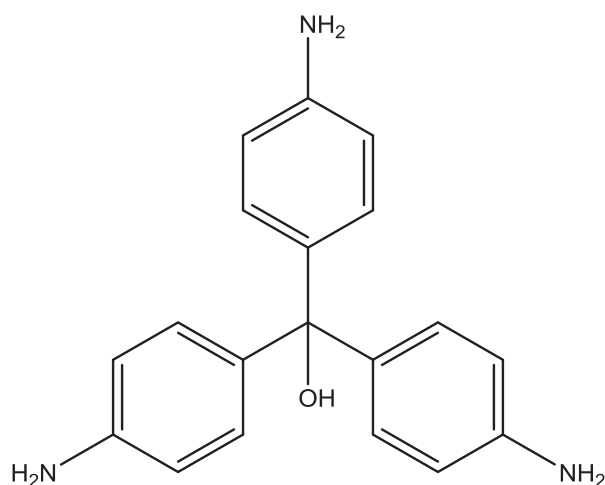
<sup>39</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.017.439>

<sup>40</sup> CAS number as provided by EC; however EFSA noted that the CAS number 467-62-9 is more often used for this compound

### 3.14.1. Inclusion/exclusion from the guidance document

#### 3.14.1.1. Q1: Is pararosaniline base a high potency carcinogen?

Pararosaniline base (Figure 15) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 15:** Chemical structure of pararosaniline base

#### 3.14.1.2. Q2: Is pararosaniline base causing allergy?

As indicated on the ECHA website, pararosaniline base is a suspected skin sensitiser as predicted with moderate reliability with the CAESAR skin sensitisation model in the VEGA (Q)SAR platform.<sup>41</sup> Moreover, a positive prediction was obtained for 'Allergic Contact Dermatitis in Guinea Pig and Human' in the Danish QSAR database. However, no alerts were reported for skin sensitisation in the QSAR toolbox.

As no food or respiratory allergy was identified in the literature search for pararosaniline base, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of pararosaniline base.

#### 3.14.1.3. Q3: Is pararosaniline base causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

#### 3.14.1.4. Conclusion

Based on the chemical structure of pararosaniline base and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for pararosaniline base.

### 3.14.2. Toxicological screening value

#### 3.14.2.1. Evaluation of genotoxicity

No papers were identified in the scientific literature on the genotoxicity of pararosaniline base.

As indicated on the ECHA website, pararosaniline base is a suspected mutagen as shown in different models. The Toolbox profiler *in vitro* mutagenicity (Ames test) alerts by ISS gives an alert for mutagenicity. The ISS Mutagenicity model, the KNN Mutagenicity model and the SARPYP Mutagenicity model in the VEGA (Q)SAR platform predicts with moderate reliability that pararosaniline base is a mutagen.<sup>41</sup> Alerts for DNA binding by OECD were identified in the QSAR toolbox as well as alerts for *in vitro* mutagenicity (Ames), *in vivo* mutagenicity (micronucleus) and structural alerts for genotoxic carcinogenicity by ISS. Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, Ames test in *S. Typhimurium* (*in vitro*), chromosome

<sup>41</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.006.724>



aberrations in chinese hamster lung cells, unscheduled DNA synthesis in rat hepatocytes, sister chromatid exchange in mouse bone marrow cells and the comet assay in Mouse (only predictions inside the applicability domain are reported in this Scientific Report).

Based on this information, EFSA concluded that under the applied approach, pararosaniline should be regarded as genotoxic. Pararosaniline base consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.15. Potassium permanganate (CAS 7722-64-7)

IUPAC name: potassium oxido(trioxo)manganese

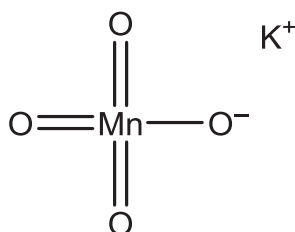
EC number: 231-760-3

Molecular formula:  $\text{KMnO}_4$

Smiles code: [O-][Mn](=O)(=O)=O.[K+]

#### 3.15.1. Inclusion/exclusion from the guidance document

Potassium permanganate (Figure 16) is an inorganic compound. The CONTAM Panel agreed at the 83rd Panel meeting<sup>8</sup> that inorganic substances should be excluded from the guidance document (see Section 2.2.1). Therefore, the guidance document cannot be applied to potassium permanganate and no TSV can be assigned to this compound.



**Figure 16:** Chemical structure of potassium permanganate

### 3.16. Proflavine (CAS 92-62-6) and proflavine hydrochloride (CAS 952-23-8)

#### Proflavine

IUPAC name: acridine-3,6-diamine

EC number: 202-172-4

Molecular formula:  $\text{C}_{13}\text{H}_{11}\text{N}_3$

Smiles code: C1=CC(=CC2=NC3=C(C=CC(=C3)N)C=C21)N

#### Proflavine hydrochloride

IUPAC name: acridine-3,6-diamine;hydrochloride

EC number: 213-459-9

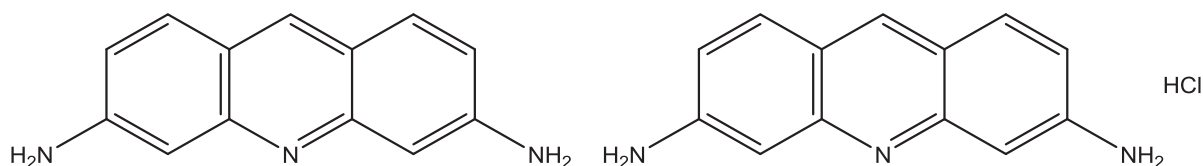
Molecular formula:  $\text{C}_{13}\text{H}_{12}\text{ClN}_3$

Smiles code: C1=CC(=CC2=NC3=C(C=CC(=C3)N)C=C21)N.Cl

#### 3.16.1. Inclusion/exclusion from the guidance document

##### 3.16.1.1. Q1: Are proflavine and proflavine hydrochloride high potency carcinogens?

Proflavine and proflavine hydrochloride (Figure 17) do not belong to the chemical classes defined as high potency carcinogens.



**Figure 17:** Chemical structure of proflavine (left) and proflavine hydrochloride (right)

### 3.16.1.2. Q2: Are proflavine and proflavine hydrochloride causing allergy?

Proflavine-induced human contact sensitivity is well documented and allergic skin reactions may be severe (Plakas et al., 1999).

No information was identified for proflavine hydrochloride, but proflavine dihydrochloride has been reported to cause ACD (Rietschel and Fowler, 2008).

As no food or respiratory allergy was identified in the literature search for proflavine and proflavine hydrochloride, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of proflavine and proflavine hydrochloride.

### 3.16.1.3. Q3: Are proflavine and proflavine hydrochloride causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.16.1.4. Conclusion

Based on the chemical structures of proflavine and proflavine hydrochloride and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for proflavine and proflavine hydrochloride.

## 3.16.2. Toxicological screening value

### 3.16.2.1. Evaluation of genotoxicity

Proflavine induced significant mutagenicity in bacteriophages (Demars, 1953; Orgel and Brenner, 1961; Drake, 1964; Hessler, 1965; Ritchie, 1965), bacteria (Dean and Hinshelwood, 1951; Randall et al., 1964; Ferguson et al., 1991; Sun and Stahr, 1993) and yeast (Marcovich, 1953). Mutant RNA viruses were observed after co-exposure to proflavine and light (Gendon, 1963).

Proflavine induced chromosome breakages in *Vicia faba* (Ockey, 1957) and increases the frequency of sister chromatid exchanges in both human lymphocytes and Chinese hamster ovary cells (Morgan and Crossen, 1982). In human fibroblasts, proflavine affected DNA synthesis, but did not induce any unscheduled DNA synthesis (Benigni et al., 1990).

No studies were identified in the scientific literature on the genotoxicity of proflavine hydrochloride. Therefore, the outcome from computational toxicology was taken into consideration. As indicated on the ECHA website, proflavine hydrochloride is a suspected mutagen as shown in different models. The CAESAR Mutagenicity model, the ISS Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q)SAR platform predicts based on experimental values that proflavine hydrochloride is a mutagen. It is also a mutagen according to ISSSTY database.<sup>42</sup>

Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, Ames test in *S. Typhimurium* (*in vitro*), chromosome aberrations in Chinese hamster lung cells, unscheduled DNA synthesis in rat hepatocytes, Syrian hamster embryo cell transformation, sister chromatid exchange in mouse bone marrow cells and the comet assay in Mouse (only predictions inside the applicability domain are reported in this Scientific Report).

Based on this information, EFSA concluded that under the applied approach, proflavine and proflavine hydrochloride should be regarded as genotoxic. Both substances consequently belong to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

<sup>42</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.012.236>

### 3.17. Rhodamine 6G (CAS 989-38-8)

IUPAC name: ethyl 2-[3-(ethylamino)-6-ethylimino-2,7-dimethylxanthen-9-yl]benzoate hydrochloride

EC number: 213-584-9

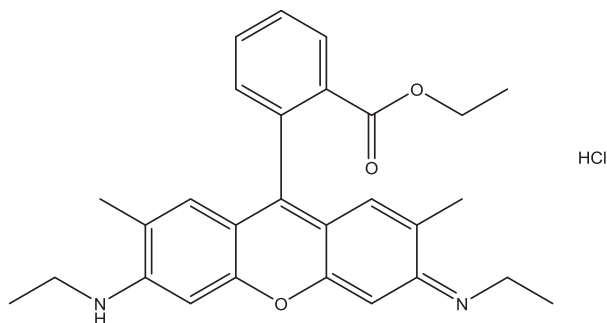
Molecular formula: C<sub>28</sub>H<sub>31</sub>ClN<sub>2</sub>O<sub>3</sub>

Smiles code: CCNC1=C(C=C2C(=C1)OC3=CC(=NCC)C(=CC3=C2C4=CC=CC(=O)OCC)C)C.Cl

#### 3.17.1. Inclusion/exclusion from the guidance document

##### 3.17.1.1. Q1: Is rhodamine 6G a high potency carcinogen?

Rhodamine 6G (Figure 18) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 18:** Chemical structure of rhodamine 6G

##### 3.17.1.2. Q2: Is rhodamine 6G causing allergy?

Although no alerts for skin sensitisation are reported on the ECHA website,<sup>43</sup> and no alerts for skin sensitisation were found in the QSAR toolbox, rhodamine 6G has been reported to cause contact sensitisation (Albert, 1997).

As no food or respiratory allergy was identified in the literature search for rhodamine 6G, and there is no concern of reactivation of ACD or SCD upon oral exposure of individuals with an existing contact allergy to a dye (see Section 2.1.2), there are no safety concerns with respect to allergenicity of rhodamine 6G.

##### 3.17.1.3. Q3: Is rhodamine 6G causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

##### 3.17.1.4. Conclusion

Based on the chemical structure of rhodamine 6G and the absence of safety concerns upon oral exposure with respect to allergenicity or blood dyscrasias, the guidance document can be applied to establish an RPA for rhodamine 6G.

#### 3.17.2. Toxicological screening value

##### 3.17.2.1. Evaluation of genotoxicity

Rhodamine 6G was found to induce mutations in bacteria (Nestmann et al., 1979), yeast (Carignani et al., 1977) and DNA damage (single-strand breaks) in Chinese hamster ovary cells, as detected by alkaline sucrose sedimentation (Nestmann et al., 1979).

In the NTP study of rhodamine 6G (NTP, 1989), there was equivocal evidence of genotoxicity activity. In particular, the compound gave negative results in different *S. Typhimurium* strains (Ames test). In the mouse lymphoma assay, rhodamine 6G was negative in the presence of exogenous metabolic activation (S9), whereas positive results were reported in the absence of S9. Sister chromatid exchanges and chromosomal aberrations were induced in Chinese hamster ovary cells in the presence, but not the absence, of S9.

<sup>43</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.012.350>

Based on this information EFSA concluded that under the applied approach, rhodamine 6G should be regarded as genotoxic. Rhodamine 6G consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

### 3.18. Trypan red (CAS 574-64-1)

IUPAC name: pentasodium;3-amino-4-[[4-[4-[(2-amino-3,6-disulfonatophthalen-1-yl)diazenyl]-3-sulfonatophenyl]phenyl]diazenyl]naphthalene-2,7-disulfonate

EC number: 209-372-0

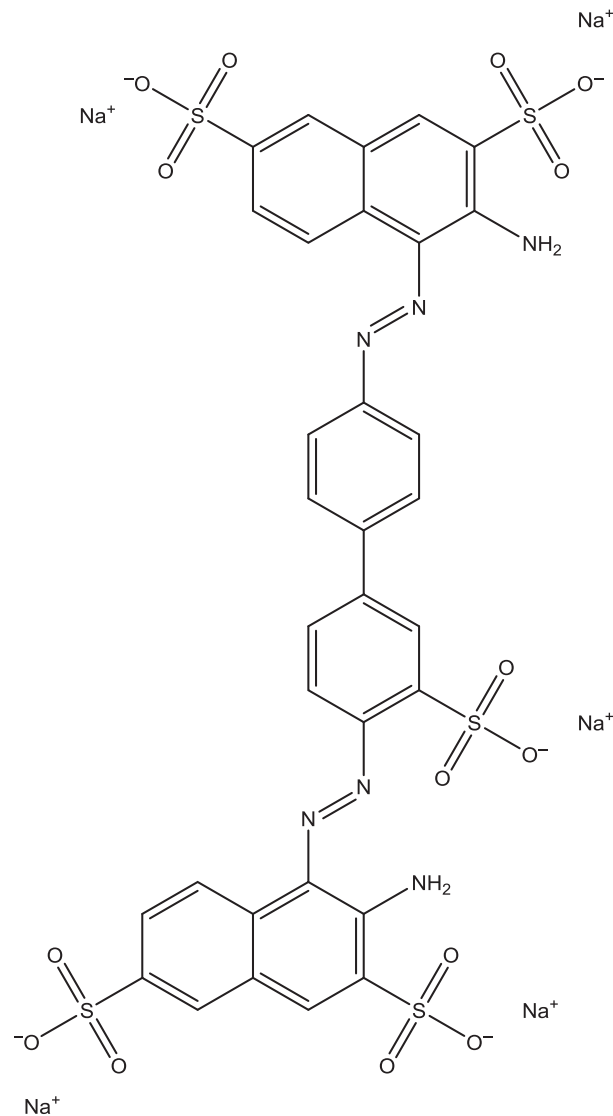
Molecular formula: C<sub>32</sub>H<sub>19</sub>N<sub>6</sub>Na<sub>5</sub>O<sub>15</sub>S<sub>5</sub>

Smiles code: C1=CC(=CC=C1C2=CC(=C(C=C2)N=NC3=C4C=CC(=CC4=CC(=C3N)S(=O)(=O)[O-])S(=O)(=O)[O-])S(=O)(=O)[O-])N=NC5=C6C=CC(=CC6=CC(=C5N)S(=O)(=O)[O-])S(=O)(=O)[O-].[Na+].[Na+].[Na+].[Na+].[Na+]

#### 3.18.1. Inclusion/exclusion from the guidance document

##### 3.18.1.1. Q1: Is trypan red a high potency carcinogen?

Trypan red (Figure 19) does not belong to the chemical classes defined as high potency carcinogens.



**Figure 19:** Chemical structure of trypan red

### 3.18.1.2. Q2: Is trypan red causing allergy?

No alerts for skin sensitisation are reported on the ECHA website<sup>44</sup> and no alerts were found in the QSAR toolbox.

As no food or respiratory allergy nor skin allergy was identified in the literature search for trypan red, there are no safety concerns with respect to allergenicity of trypan red.

### 3.18.1.3. Q3: Is trypan red causing blood dyscrasias?

No studies regarding blood dyscrasias were identified in the literature search.

### 3.18.1.4. Conclusion

Based on the chemical structure of trypan red and the lack of evidence that trypan red is causing allergy or blood dyscrasias, the guidance document can be applied to establish an RPA for trypan red.

## 3.18.2. Toxicological screening value

### 3.18.2.1. Evaluation of genotoxicity

No toxicological information was retrieved in the scientific literature regarding trypan red following the approach described in Section 2.2.1. Therefore, the outcome from computational toxicology was taken into consideration. As indicated on the ECHA website, trypan red is a suspected mutagen as shown in different models. The CAESAR Mutagenicity model, the ISS Mutagenicity model, the KNN Mutagenicity model and the SARPY Mutagenicity model in the VEGA (Q)SAR platform predicts that trypan red is a mutagen with moderate or good reliability.<sup>44</sup> Alerts for DNA binding by OASIS v1.3 and OECD were identified in the QSAR toolbox as well as an alert for 'in vivo mutagenicity (micronucleus)' by ISS. Positive alerts were also reported in the Danish QSAR database using the battery algorithm for Ashby structural alerts, Ames test in *S. Typhimurium* (*in vitro*), chromosome aberrations in Chinese hamster lung cells, unscheduled DNA synthesis in rat hepatocytes, Syrian hamster embryo cell transformation, sister chromatid exchange in mouse bone marrow cells and the comet assay in mouse (only predictions inside the applicability domain are reported in this Scientific Report).

Based on this information, EFSA concluded that under the applied approach, trypan red should be regarded as genotoxic. Trypan red consequently belongs to Group I as defined in the guidance document and a TSV of 0.0025 µg/kg bw per day should be used in case an RPA were to be established.

## 3.19. Summary (Table 2)

**Table 2:** Overview of the evaluation for each dye

Substance	Part I				Part II		
	Q1 <sup>(a)</sup>	Q2 <sup>(a)</sup>	Q3 <sup>(a)</sup>	Excluded from guidance document?	Q4 <sup>(a)</sup>	Q5 <sup>(a)</sup>	TSV (µg/kg bw per day)
Acriflavine	No	No	No	No	Yes	–	0.0025
3-Aminoacridine	No	No	No	No	Yes	–	0.0025
Aminoacridine	No	No	No	No	Yes	–	0.0025
Azure blue/ultramarine	– <sup>(b)</sup>	–	–	Yes	–	–	–
Basic blue 7/victoria pure blue BO	No	No	No	No	Yes	–	0.0025
Brilliant green/C.I. basic green 1	No	No	No	No	Yes	–	0.0025
Leucobrilliant green	No	No	No	No	Yes	–	0.0025
C.I. basic blue 26	No	No	No	No	Yes	–	0.0025
Chloranil	No	No	No	No	Yes	–	0.0025

<sup>44</sup> <https://www.echa.europa.eu/web/guest/information-on-chemicals/annex-iii-inventory/-/dislist/details/AIII-100.008.521>

Substance	Part I				Part II		
	Q1 <sup>(a)</sup>	Q2 <sup>(a)</sup>	Q3 <sup>(a)</sup>	Excluded from guidance document?	Q4 <sup>(a)</sup>	Q5 <sup>(a)</sup>	TSV ( $\mu\text{g}/\text{kg}$ bw per day)
Crystal violet/gentian violet	No	No	No	No	Yes	–	0.0025
Leucocrystal violet/leucogentian violet	No	No	No	No	Yes	–	0.0025
Dichlone	No	No	No	No	Yes	–	0.0025
Ethyl violet	No	No	No	No	Yes	–	0.0025
Methylene blue	No	No	No	No	Yes	–	0.0025
New methylene blue/victoria blue R	No	No	No	No	Yes	–	0.0025
Nile blue	No	No	No	No	Yes	–	0.0025
Pararosaniline base	No	No	No	No	Yes	–	0.0025
Potassium permanganate	– <sup>(a)</sup>	–	–	Yes	–	–	–
Proflavine	No	No	No	No	Yes	–	0.0025
Proflavine hydrochloride	No	No	No	No	Yes	–	0.0025
Rhodamine 6G	No	No	No	No	Yes	–	0.0025
Trypan red	No	No	No	No	Yes	–	0.0025

bw: body weight; TSV: toxicological screening value.

(a): Q1: is the substance a high potency carcinogen?; Q2: are there safety concerns upon oral exposure with respect to allergenicity?; Q3: is the substance causing blood dyscrasias?; Q4: is there direct evidence of genotoxicity, an alert for genotoxicity (from structural activity relationships or read across), or is there lack of information on genotoxicity?; Q5: Does the substance intentionally act pharmacologically on the nervous system or the reproductive system and/or is it a corticoid

(b): Excluded from the guidance document since it is an inorganic compound.

## 4. Conclusions

EFSA evaluated whether the guidance document is applicable to the dyes listed in Table 1 of this Scientific Report and concluded the following:

- Acriflavine, 3-aminoacridine, aminoacridine, basic blue 7, brilliant green, leucobrilliant green, C.I. basic blue 26, chloranil, crystal violet, leucocrystal violet, dichlone, ethyl violet, methylene blue, new methylene blue, Nile blue, pararosaniline base, proflavine, proflavine hydrochloride, rhodamine 6G and trypan red are covered by the guidance document and belong to group I. A TSV of 0.0025  $\mu\text{g}/\text{kg}$  bw per day is applicable.
- Azure blue and potassium permanganate are excluded from the guidance document since they are inorganic compounds.

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## Abbreviations

ACD	allergic contact dermatitis
Bw	body weight
CVMP	Committee for Veterinary Medicinal Products
DPPRA	Direct Peptide Reactivity Assay
EC	Enzyme Commission
ECHA	European Chemicals Agency
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
HepG2	human hepatoma cells
IgE	immunoglobulin E
IUPAC	International Union of Pure and Applied Chemistry

JECFA	Joint FAO/WHO Expert Committee on Food Additives
MRL	maximum residue limit
Ni	nickel
OECD	Organisation for Economic Co-operation and Development
PCP	pentachlorophenol
(Q)SAR	quantitative structure–activity relationship
RALLOQ	reasonably achievable lowest limit of quantification
RPA	reference point for action
SCCNFP	Scientific Committee on cosmetic products and non-food products intended for consumers
SCD	systemic contact dermatitis
TBLOQ	toxicologically based limit of quantification
TSV	toxicological screening value
TTC	threshold of toxicological concern
UV	ultraviolet
VMP	veterinary medicinal product



## Appendix A – Literature search

### A.1. Identification and selection of evidence related to allergy

#### 1) WEB OF SCIENCE

**TOPIC:** (substance OR synonym) AND **TOPIC:** (allergy or dermatitis); *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=All years*

#### 2) PUBMED

(substance OR synonym) AND (allergy or dermatitis)

### A.2. Identification and selection of evidence related to blood dyscrasias

#### 1) WEB OF SCIENCE

**TOPIC:** (substance OR synonym) AND **TOPIC:** (anaemia or 'blood dyscrasias'); *Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-EXPANDED, IC Timespan=All years*

#### 2) PUBMED

(substance OR synonym) AND (anaemia or 'blood dyscrasias')

### A.3. Identification and selection of evidence related to genotoxicity

#### A.3.1. Nile blue and trypan red

##### 1) EMBASE

(substance OR synonym) AND ('toxicity'/exp OR 'toxic substance'/exp OR 'toxicity assay'/exp OR 'toxicity testing'/exp OR 'mutagenesis'/exp OR 'mutagen testing'/exp OR 'carcinogenesis'/exp OR 'carcinogenic activity'/exp OR 'carcinogen dna interaction'/exp OR 'dna adduct'/exp OR 'carcinogen testing'/exp OR 'genetic damage'/exp OR 'chromosome aberration'/exp OR 'chromatid aberration'/exp OR toxic\*:ab,ti OR genotox\*:ab,ti OR carcinogen\*:ab,ti OR mutagen\*:ab,ti OR teratogen\*:ab,ti OR aneugen\*:ab,ti OR clastogen\*:ab,ti OR promutagen\*:ab,ti OR tum\*rigen\*:ab,ti OR 'dna adduct\*':ab,ti OR chromosom\*:ab,ti OR chromatid\*:ab,ti OR 'dna damage':ab,ti)

##### 2) PUBMED

(substance OR synonym) AND (genotox\* OR muta\* OR DNA OR damage OR repair OR clastogen\* OR aneugen\* OR chromosom\* OR cancer\* OR carcino\* OR tumor\* OR tumour\* OR rat OR rats OR mouse OR mice)

##### 3) SCIFINDER

- toxicity of CAS number
- mutagenicity of CAS number
- genotoxicity of CAS number
- carcinogenity of CAS number

##### 4) WEB OF SCIENCE

TOPIC: (substance OR synonym) AND TOPIC: (genotox\* OR muta\* OR DNA OR damage OR repair OR clastogen\* OR aneugen\* OR chromosom\* OR cancer\* OR carcino\* OR tumor\* OR tumour\* OR rat OR rats OR mouse OR mice)

#### A.3.2. Other substances

##### 1) EMBASE

(substance OR synonym) AND ('genotoxicity'/exp OR 'mutagenic agent'/exp OR 'genetic damage'/exp OR 'dna adduct'/exp OR 'dna binding'/exp OR 'dna repair'/exp OR 'chromosome aberration'/exp OR 'chromatid aberration'/exp OR 'mutation'/exp OR 'genotoxicity assay'/exp OR 'mutagen testing'/exp OR genotox\*:ab,ti OR mutation\* OR mutagen\*:ab,ti OR aneugen\*:ab,ti OR clastogen\*:ab,ti OR (dna

OR chromosom\* OR chromatid\* OR genetic\*) NEAR/3 (damage OR alteration\* OR break\* OR mutation\* OR aberration\* OR interaction\* OR adduct\* OR repair)) AND ([article]/lim OR [article in press]/lim OR [review]/lim)

## 2) **PUBMED**

(substance OR synonym) AND ("Mutagens"[Mesh] OR "DNA Damage"[Mesh] OR "DNA repair"[Mesh] OR "Mutagenesis"[Mesh] OR "Mutation"[Mesh] OR "Mutagenicity Tests"[Mesh] OR genotox\*[tiab] OR mutation\*[tiab] mutagen\*[tiab] OR aneugen\*[tiab] OR clastogen\*[tiab] OR ((DNA [tiab] OR chromosom\*[tiab] OR chromatid\*[tiab] OR genetic\*[tiab]) AND (damage[tiab] OR alteration\* [tiab] OR break\*[tiab] OR mutation\*[tiab] OR aberration\*[tiab] OR interaction\*[tiab] OR adduct\*[tiab] OR repair[tiab])))Filters: Journal Article; Review

## 3) **SCIFINDER**

Mutagenicity or genotoxicity of CAS number

## 4) **WEB OF SCIENCE**

TOPIC: (substance OR synonym) AND TOPIC: (genotox\* OR mutation\* OR mutagen\* OR aneugen\* OR clatogen\* OR (DNA OR chromosom\* OR chromatid\* OR genetic\*) NEAR/3 (damage OR alteration\* OR break\* OR mutation\* OR aberration\* OR interaction\* OR adduct\* OR repair)); Refined by: DOCUMENT TYPES: (ARTICLE OR REVIEW); Timespan: All years; Search language=Auto