ABSTRACT

DOS SANTOS, TUANE CRISTINA. Natural Dyes: Structure Modification and Environmental Properties (Under the direction of Dr. Harold Stanley Freeman).

There has been increased interest in the use of natural dyes for textile coloration, as alternatives to synthetic dyes that are toxic to a variety of organisms. However, natural dyes have poor affinity for textiles, which can lead to high dye levels in the resultant wastewater and the need for treatment to remove color. Furthermore, their low fixation is one of the reasons why the textile industry hesitates to use these dyes. Conventional biological treatment is inefficient for color removal, and as a consequence dyes can remain in the environment. Chlorine treatment has proven to be efficient and economical for wastewater disinfection and decolorization, but for synthetic dyes, this process can lead to the formation of toxic products. However, there is a lack of information regarding the effect of chlorination treatment on natural dyes. Therefore, this dissertation is concerned with the study of the effect of chlorination on the ecotoxic, mutagenic and chemical properties of natural dyes, and with the design and synthesis of a natural dye analog capable of imparting color to textile fibers.

Chlorination of five natural dyes selected, madder (CI Natural Red 8), cochineal (CI Natural Red 4), weld (CI Natural Yellow 2), logwood grey (CI Natural Black 1) and fustic (CI Natural Yellow 11), led to decolorization of all dye solutions, as determined by UV-Vis spectroscopy. UV-Vis spectroscopy was also used to conduct the kinetics studies on the degradation of each dye. Cochineal had the highest initial degradation rate constant ($k = -1.119 \text{ min}^{-1}$) undergoing a nearly 100% of decolorization after 3 min of treatment, and weld had the lowest rate constant ($k = -0.006 \text{ min}^{-1}$), with about 6% of its color removed after 15 min. Although differences in degradation levels existed, a clear correlation between structure and degradation rates could not be established.
Chlorination increased the acute toxicity of cochineal, weld, logwood and fustic to *D. similis*. Chlorinated logwood solution was classified as harmful to aquatic life. Fustic, weld and cochineal had an EC$_{50}$ value between 10 and 100 mg/L and are classified as harmful to aquatic life (Acute III). Upon chlorination, weld and cochineal solutions were classified as Acute III and chlorinated fustic solution as toxic to aquatic life. Madder was classified as Acute II, with EC$_{50}$ = 4.4 mg/L acute toxicity to *D. similis*, and IC$_{50}$ = 8.9 mg/L toxicity to algae *R. subcapitata*. Chlorination decreased madder toxicity in both experiments, EC$_{50}$ increased to 45 mg/L for *D. similis* and non-inhibition of algae growth was observed in the limit of madder solution solubility. None of the five dye solutions showed mutagenicity to *S. typhimurium* strains TA98 and TA100, with or without metabolic activation (S9), before or after chlorination.

Further investigation of chlorinated madder by HPLC-DAD indicated the presence of six degradation products, detected at $\lambda$ = 254 nm. Two of those products were identified by ESI-MS as phthalic anhydride ($m/z$ 149.0229) and 2-hydroxynaphtalene-1,4-dione ($m/z$ 173.0250).

An alizarin analog, a vinyl sulfone reactive disperse dye (B120), $m/z$ 585.0496, with excellent light fastness on PET, was synthesized in a 2-step reaction. This novel dye was suitable for nylon, silk, wool and polyester, with the potential of being used for the coloration of polypropylene and modacrylic fabrics. Color strength was found to increase with the % B120 dye shade, as expected. In comparison with the parent compound, B120 gave a slightly better K/S value when applied to silk, using Irgasol FL as dispersing agent. The highest color strength for B120 was measured on dyed wool.
Natural Dyes: Structure Modification and Environmental Properties

by
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DEDICATION

This dissertation is dedicated to my mom, Clelia de Souza, who has raised me to be kind, caring and always pursue my dreams. She is my example of a strong leader, and a hard-working, independent woman who has always inspired me to be the best person I can be. I also dedicate this work to my husband, Zach Guilfoyle, who supported me in every possible way during my studies, understood my absence in many moments and encouraged me to never give up. All my love and gratitude for them.
BIOGRAPHY

Tuane Cristina dos Santos was born in November 22, 1986, in Araraquara, Brazil. She obtained her Bachelor of Science degree in Chemistry from Sao Paulo State University (UNESP) in 2007. After participating in an exchange student program in the US and working in the private sector for about 2 years, she enrolled in the Analytical Chemistry program also at UNESP and was awarded a Master of Sciences degree in 2013. She then worked as a researcher in analytical chemistry in the private sector until July 2014, when she began her doctorate studies at North Carolina State University towards the Doctor of Philosophy degree in Fiber and Polymer Science.
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CHAPTER 1. Introduction

1.1. Background

Natural dyes were the only source of color for products, such as leather, food and natural fibers (cotton, wool and silk) for many centuries. They can be extracted from plants, animals and mineral, with the majority of these dyes obtained from several parts of plants, such as bark, roots, leaves, and flowers. However, these compounds were not created by nature to be used as dyes for textiles. Therefore, they have poor fixation properties and, in general, mordants need to be used during the dyeing procedure to promote fixation. The most common mordants used are transition metals that can form complexes with dye molecules, allowing bonding with the fiber structure.

Due to their poor dyeing properties, natural dyes were gradually replaced after the discovery of synthetic dyes. These newly discovered dyes showed a much higher fixation level, reproducibility of shade, low production cost, and ease of application, when compared to natural dyes, making them much more commercially attractive. However, concerns with the safety of certain synthetic dyes to human health and the environment started to arise. Some synthetic dyes, were proved to cause DNA mutations, to be carcinogenic to humans and toxic to some aquatic organisms. Furthermore, although they improved fixation to the fiber, there is always a percentage of unfixed dye released into the wastewater, which in turn requires treatment at the wastewater treatment plants. In this environment, several of these dyes were degraded and their breakdown products exhibited increased toxicity/mutagenicity after exposure to oxidative methods, such as chlorination, used during the water treatment.

With the aforementioned points in mind, interest in using natural dyes was reborn, leading to an increased need for research regarding the safety of such dyes to humans and the environment. Although the literature presents data on the effects of certain natural dyes to human health, studies
concerning their effects to the environment and their transformations upon exposure to treatments in the wastewater treatment plants are very scarce and much needed.

In the present, work efforts to understand their environmental properties and toxicity and to improve their fixation to the fiber, reducing the amount of dye released to the environment, were undertaken. Chapter 1, briefly introduces the problem and motivation for this research, as well as the main questions to be answered. Chapter 2 describes the background information in more detail, presenting relevant data for understanding the methods used in this work. Chapter 3 involves a study of the behavior and toxicity of five natural dyes upon chlorination, and Chapter 4 presents the results obtained from the synthesis of a disperse dye analog of a natural dye, along with its dyeing behavior and fastness properties. Chapter 5 describes future work that would provide more information and complement the data and results presented herein.

1.2. Research objectives

The research questions to be answered in this work were:

1. What is the effect of chlorination on natural dyes, including the degradation products generated?
2. Are natural dyes and their chlorination products toxic to aquatic life such as the crustacean *Daphnia similis* and algae *Raphidocellis subcapitata*? Are they mutagenic to *S. typhimurium* strains TA98 and TA100?
3. Can the structure of natural dye Madder (CI Natural Red 8) be modified to improve dyeing properties?
4. Can the modified Madder dye be applied to natural fibers as well as synthetic fibers?

In order to answer these questions, experiments were undertaken to (1) evaluate the degradation level and rate during the chlorination of 5 natural dyes, madder, cochineal, logwood,
gray, fustic and weld; (2) evaluate the acute toxicity of these dyes to *D. similis* and algae; (3) establish the relationships between the degradation rate observed, toxicity and structure for the analyzed dyes; (4) chemically modify the structure of Alizarin (Madder) generating a reactive dye; (5) characterize the newly synthesized dye: chemically and toxicologically, and (6) assess the dyeing properties of the new dye to cotton and PET.

1.3. Dye selection

Five dyes, shown in [Figure 1.1](#), were selected as representative compounds for studying environmental properties of natural dyes as well as their dyeing behavior and toxicity/mutagenicity upon chlorination. These dyes were selected after considering: (1) the available information regarding their toxicity/mutagenicity, which is summarized in studies presented in [Table 2.8](#), (2) the lack of information on their ecotoxicity or the necessity for updated information, (3) predictions generated by ECOSAR, and (4) the relevancy of these dyes in terms of textile application and historical importance. Also, they were selected due to the lack of information regarding their behavior upon chlorination.
**Figure 1.1.** Structures of the reported main natural dye components: (A) Carminic acid in Cochineal, CI Natural Red 4, (B) Alizarin in Madder, CI Natural Red 8, (C) Hematoxylin in Logwood gray, CI Natural Black 1, (D) Luteolin in Weld, Natural Yellow 2 and (E) Morin in Fustic, Natural Yellow 11.

**Figure 1.2** (A-E) present the chemical properties for the 5 chosen dyes, as well as the ECOSAR predictions for aquatic toxicity obtained using the SMILE notation for the major constituent of each dye. End points reported are EC<sub>50</sub>, LC<sub>50</sub>, which will be discussed in Chapter 2, and ChV (Chronic Value) which represents the geometric mean of the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration). The results obtained include log K<sub>ow</sub> which is used in the calculation of the predicted effect level, and water solubility values (mg/L) are compared with predicted effect levels in order to identify effect levels that exceed the limit of water solubility. The ECOSAR program provides results for multiple classes, if the entered structure contains the defined base-structure from each of the classes identified in the ECOSAR. In that case, the most suitable class must be chosen for estimating toxicity, based on a knowledge of environmental toxicology, organic chemistry, and statistics. However, there is no one standard method for selecting the most representative predictions and, often, the best approach would be to select the most conservative effect level until measured data become available. 

\[^1\]
In these results, Alizarin, 0 B, was predicted to be at least harmful to aquatic life according to the GHS classification, which will be discussed in further detail in section 2.4.2.2, considering 3 trophic levels (algae, daphnids and fish). Carminic acid was included in two categories, phenols and hydroquinones, 0 A and considering the most conservative effect (the one associated with hydroquinone structures), this compound is also predicted to be harmful to aquatic life. The same is true for hematoxylin, Figure 1.2 C, Luteolin, Figure 1.2 D and Morin, Figure 1.2 E. All of these major components in the studied dyes were predicted to be acutely and or/ chronically harmful/toxic to aquatic organisms.
Figure 1.2. ECOSAR prediction data for the aquatic toxicity of (A) Carminic acid in Cochineal, CI Natural Red 4, (B) Alizarin in Madder, CI Natural Red 8, (C) Hematoxylin in Logwood gray, CI Natural Black 1, (D) Luteolin in Weld, CI Natural Yellow 2 and (E) Morin in Fustic, CI Natural Yellow 11, to daphnia, fish and algae.
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![Chemical Structure](image)

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### Structure

![Structure Image](image)

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Vinyl/Allyl/Propargyl Ethers
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Phenols, Poly
Class Results:

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<td>48h</td>
<td>LC50</td>
<td>63.05</td>
<td>5</td>
<td>• Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported</td>
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<tr>
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<td>96h</td>
<td>EC50</td>
<td>4.52</td>
<td>6.4</td>
<td></td>
</tr>
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<td>Fish</td>
<td></td>
<td>CC10</td>
<td>4.84</td>
<td>8</td>
<td></td>
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<tr>
<td>Daphnia</td>
<td></td>
<td>CC10</td>
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<td>8</td>
<td>• Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported</td>
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<tr>
<td>Green Algae</td>
<td></td>
<td>CC10</td>
<td>0.54</td>
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</tr>
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</table>

Structure:

\[
\text{\begin{leqy} \text{H} & \text{O} \\
\text{O} & \text{H} \\
\text{O} & \text{H} \\
\text{O} & \text{H}
\end{leqy}}
\]

Details:

<table>
<thead>
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<th>Property</th>
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<tbody>
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<tr>
<td>Selected Log Kow</td>
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</tr>
<tr>
<td>Selected Water Solubility (mg/L)</td>
<td>4121.75</td>
</tr>
<tr>
<td>Selected Melting Point (°C)</td>
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<tr>
<td>Estimated Log Kow</td>
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</tr>
<tr>
<td>Estimated Water Solubility (mg/L)</td>
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</tr>
<tr>
<td>Measured Log Kow</td>
<td></td>
</tr>
<tr>
<td>Measured Water Solubility (mg/L)</td>
<td></td>
</tr>
<tr>
<td>Measured Melting Point (°C)</td>
<td></td>
</tr>
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</table>
### Phenols, Poly

<table>
<thead>
<tr>
<th>Organism</th>
<th>Duration</th>
<th>End Point</th>
<th>Concentration (mg/L)</th>
<th>Max Log Kow</th>
<th>Flags</th>
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<tbody>
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<td>7.31</td>
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<td>Fish</td>
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<td>ChV</td>
<td>11.79</td>
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<td>Daphnids</td>
<td></td>
<td>ChV</td>
<td>66.42</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Green Algae</td>
<td></td>
<td>ChV</td>
<td>0.95</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
Alizarin, Luteolin and Morin have relatively high log K\textsubscript{ow} values, which can result in higher bioaccumulation factors\textsuperscript{2}, with carminic acid and hematoxylin having lower log K\textsubscript{ow} values. However, all of these dyes have sufficient hydrophilic character, dissolving partially or completely in water. The octanol/water partition coefficient (K\textsubscript{ow}) is defined as the ratio of concentration of a chemical in the octanol phase to its concentration in water. K\textsubscript{ow} values are usually expressed as log K\textsubscript{ow}, which has been shown to corelate well with bioconcentration factors in aquatic organisms and adsorption to soil or sediment\textsuperscript{3}. Chemicals with low K\textsubscript{ow} values (<10) can be considered to be hydrophilic, tending to be more water-soluble and have small soil/sediment adsorption coefficients as well as small bioconcentration factors for aquatic life. On the other hand, substances with high K\textsubscript{ow} values (>10\textsuperscript{4}), which correlates to log K\textsubscript{ow}>4.5, tend to be very hydrophobic, and absorb more rapidly to organic matter in soils or sediments.
CHAPTER 2. Literature review

2.1. Natural dyes

2.1.1. Historical background

Natural dyes are colorants derived from mineral, animal or plant sources. They have been used since pre-historic times for their application to leather and natural fibers like wool, silk and cotton\textsuperscript{4,5}. The practice of coloring materials by dyeing and printing techniques has played an important role in every civilization. The first western dyers were probably the Swiss Lake Dwellers who lived about 2000 BC. In China, craft and dye workshops originated some time before 3000 BC\textsuperscript{6}. Among other ancient civilizations, Egyptian mummies and documents from the Mughal periods in India are evidence of the early utilization of natural dyes\textsuperscript{7}. The Phoenicians were responsible for the first global dye, Tyrian purple, which was traded by them. The purple of Tyre was famous, as were the textiles dyed and produced by the Phoenicians. It is said that the Greeks named the Phoenicians after Phoinikes, the ancient Greek word for ‘red color’, probably because of their famous purple trade.

By the time of the founding of the Mediterranean civilizations, what was considered the classical palette for natural dyes had already been established, and the most valued colors were indigo for the blues, anthraquinone-based chromophores for the reds and dibromoindigo for purples. Yellow dyes, however, were obtained from a variety of sources. For dyeing, except for some browns, all other colors and shades, including green and orange, could be obtained with these blue, red, purple and yellow dyes. These color characteristics were preserved over centuries, with the first adjustment a consequence of the loss of Tyrian purple following the fall of Constantinople.
and the subsequent collapse of the Roman social and commercial influence. At that time, the Spanish brought cochineal red from the New World.

This classical palette was challenged from the mid-19th century when, in 1856, William H. Perkin, by chance, synthetized the basic dye Mauveine. He was studying the oxidation of phenylamine using potassium dichromate when he observed the formation of a reddish compound. This discovery created great excitement in England and France. For decades after aniline dyeing became the standard procedure, natural dyes continued to be used side by side with the manufactured dyes. However, because synthetic dyes had moderate to excellent color fastness and lower production costs, a decrease in the use of natural dyes was observed and by the end of the 19th century all but a few natural dyes such as logwood, indigo, catechu and cochineal had been replaced by the more dependable manufactured dyes.

A revival of interest in arts and crafts, which appeared during the first quarter of the 20th century, and the fact that certain synthetic dyes were found to be carcinogenic or considered potentially carcinogenic or mutagenic led to an increased interest in research pertaining to the presumed less hazardous and eco-friendly natural dyes. This increased interest is also due to the various natural dyes applications, Figure 2.1, and the extensive research in these areas.
There are more than 500 plant species identified as sources of natural dyes, but less than 200 species are usually referred to in the literature. The Color Index lists 92 natural dyes, for which 67 have disclosed structures. Many of these dyes have more than one component and some present duplicate structure\textsuperscript{12}. Plants are the main source of natural dyes, leading to the production of colors such as red, yellow, blue, black, brown and a combination of these\textsuperscript{13}. Almost all parts of the plants, root, bark, leaf, fruit, wood, seed, and flower produce dyes. It is interesting to note that over 2000 pigments are synthesized by various parts of plants, but only about 150 of them have been pursued commercially.
Only certain types of plants can produce dyes and these dye-yielding plants also benefit from this potential. The green pigment chlorophyll in leaves is important for the photosynthesis process, helping to capture sun’s energy and convert it to chemical energy, which is then stored and used by the plant. Colorful flowers are a survival adaptation, attracting insects and other pollinators, further aiding in their reproduction cycle. Although plants exhibit a wide range of colors, not all of the associated compounds can be used as dyes due to solubility limitations\textsuperscript{13}.

Red dyes, for example, are usually anthraquinone and naphthoquinone molecules present in the roots or bark of plants or in the body of insects. Madder, CI Natural Red 8, is extracted from the dried roots of the \textit{Rubia tinctorium} L plant and can produce shades varying from pink to black, depending on the mordant used\textsuperscript{14}. It can be used as food colorant or in the textile dyeing of natural fibers. The major component found in the color extract depends on the species from which extraction occurred. Extracts from \textit{Rubia cordifolia}, for example, have Purpurin as the major component\textsuperscript{7}.

Red dye Cochineal (CI Natural Red 4), on the other hand, is extracted from insects and it has Carminic acid, \textbf{Figure 1.1 A}, as its major component. It can be obtained from dried bodies of female insect \textit{Dactylopius coccus}. Carminic acid is soluble in water, alcohol, acid and alkali, but insoluble in petroleum ether, benzene and chloroform. This dye is currently used for cosmetics, plastic and textile applications\textsuperscript{15}.

The dihydropyran Logwood gray dye (CI Natural Black 1), a flavonoid derivative, also known as Campeachy wood, is extracted from \textit{Haematoxylon campechianum} L, a specie of flowering tree. Hematoxylin, \textbf{Figure 1.1 C}, (or its oxidized form haematein) is the strong component present in this dye. The dye itself is red but black shades can be achieved by using
copper and/or iron or chromium mordants. Gray (iron mordant), blue (alum and tin mordants) and purple (alum and tin mordants) shades can also be obtained, with good fastness properties\textsuperscript{14}.

Weld, CI Natural Yellow 2, has been used for 5,000 years\textsuperscript{16}. The dye is extracted from the Reseta luteola plant, with the flavonoid Luteolin, Figure 1.1 D, the major component\textsuperscript{17}. Weld is applied to the yarn, mostly wool or silk, using a mordant to produce pale flax to vivid lemon tints. Mordanting with copper sulfate or tin dichloride results in greenish yellows\textsuperscript{18}.

Fustic, CI Natural Yellow 11, also known as old Fustic, is extracted from the tropical hardwood Chlorophora tinctoria L. The main component in this dye is reported to be the flavanol morin\textsuperscript{14}, Figure 1.1 E.

2.1.3. Classification

Classification of dyes can be based on their structure, source, color or method of application\textsuperscript{19}. Examples of some natural dyes, their colors and sources are presented in Table 2.1. For textiles applications three types of natural dyes are used: (1) substantive or direct, which bond to a fiber without a mordant to fix color, improving light and wash fastness. Examples are turmeric, Figure 2.2 A, and pomegranate, Figure 2.2 B; (2) traditional or additive dyes that require the formation of a metal-fiber complex to be fixed, and (3) vat dyes, which are water insoluble and need to be reduced to the leuco form, using sodium hydrosulfite and sodium hydroxide to diffuse into the fiber. Examples of substantive dyes are extracts of tea and onion skin, traditional dyes are madder and lac, and indigo is an example of vat dye\textsuperscript{20}. Still considering the method of application, some natural dyes can be further classified as acid dyes, which have either sulfonic or carboxylic groups and are applied from an acidic bath, i.e. tannic acid, Figure 2.2 C; basic or cationic dyes, such as berberine, Figure 2.2 E, that form colored cations upon ionization and are applied from neutral to mildly acid medium; and disperse dyes, such as lawsone, Figure 2.2 D, which have a
relative low molecular weight and low solubility, being applied to hydrophobic fibers from neutral to mildly acid pH medium\textsuperscript{21}.

![Diagram of natural dyes](image)

**Figure 2.2.** (A) Curcumin, the key structure in turmeric, (B) Granattonine, the main compound in pomegranate, (C) Tannic acid, (D) Lawsone, and (E) Berberine.

If classification is done considering chemical structures, natural dyes are mainly divided into: indigoids, with indigo CI Natural Blue 1, **Figure 4.1**, the most important dye in this group; quinones, anthraquinones and naphthoquinones, characterized by good light and wash fastness. This group contains some of the most important red dyes used, such as Madder, cochineal and lac; flavones, most of the natural yellow dyes are hydroxyl and methoxy derivatives of flavones and isoflavones; anthocyanins that are water-soluble colorants found in most flowers and fruits and carotenoids in which color is due to the presence of conjugated double bonds\textsuperscript{19,22}. 
Table 2.1. Natural dye colors and sources\textsuperscript{20}.

<table>
<thead>
<tr>
<th>Color</th>
<th>Name</th>
<th>Botanical name</th>
<th>Parts used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Safflower</td>
<td>Carthamus tinctorius</td>
<td>Flower</td>
</tr>
<tr>
<td></td>
<td>Caesalpinia</td>
<td>Caesalpinia sappan</td>
<td>Wood chips</td>
</tr>
<tr>
<td></td>
<td>Madder</td>
<td>Rubia tinctorium</td>
<td>Root</td>
</tr>
<tr>
<td></td>
<td>Lac</td>
<td>Coccus lacca</td>
<td>Twigs inhabited by these insects</td>
</tr>
<tr>
<td></td>
<td>Cochineal</td>
<td>Insect on Cactuscoecinnillifer</td>
<td>-</td>
</tr>
<tr>
<td>Yellow</td>
<td>Parijata</td>
<td>Nyctanthesar bortristis</td>
<td>Flower</td>
</tr>
<tr>
<td></td>
<td>Marigold</td>
<td>Tagettes species</td>
<td>Flower</td>
</tr>
<tr>
<td></td>
<td>Teak</td>
<td>Tectona grandis</td>
<td>Leaves</td>
</tr>
<tr>
<td></td>
<td>Fustic</td>
<td>Morus tinctoria</td>
<td>Leaves</td>
</tr>
<tr>
<td></td>
<td>Weld</td>
<td>Resida luteola</td>
<td>Leaves</td>
</tr>
<tr>
<td>Blue</td>
<td>Indigo</td>
<td>Indigofera</td>
<td>Leaves</td>
</tr>
<tr>
<td></td>
<td>Woad</td>
<td>Isatis tinctoria</td>
<td>Leaves</td>
</tr>
<tr>
<td></td>
<td>Sunberry</td>
<td>Acacia nilotica</td>
<td>Seed pods</td>
</tr>
<tr>
<td>Orange/Peach</td>
<td>Dahlia</td>
<td>Dahlia species</td>
<td>Flower</td>
</tr>
<tr>
<td></td>
<td>Annatto</td>
<td>Bixa orellana</td>
<td>Seeds</td>
</tr>
<tr>
<td>Black</td>
<td>Alder</td>
<td>Alnus gultinosa</td>
<td>Bark</td>
</tr>
<tr>
<td></td>
<td>Rofblamala</td>
<td>Loranthus pentapetalus</td>
<td>Leaves</td>
</tr>
<tr>
<td></td>
<td>Custard apple</td>
<td>Amona reticulate</td>
<td>Fruit</td>
</tr>
<tr>
<td></td>
<td>Harda</td>
<td>Terminalia chebula</td>
<td>Fruit</td>
</tr>
<tr>
<td></td>
<td>Logwood</td>
<td>Hematoxylon</td>
<td>Wood chips</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Campeachianum</td>
<td></td>
</tr>
<tr>
<td>Brown</td>
<td>Caesalpinia</td>
<td>Caesalpinia sappan</td>
<td>Wood chips</td>
</tr>
<tr>
<td></td>
<td>Cutch</td>
<td>Carechu</td>
<td>Wood</td>
</tr>
<tr>
<td></td>
<td>Sumach</td>
<td>Rhus species</td>
<td>Berries</td>
</tr>
<tr>
<td></td>
<td>Marigold</td>
<td>Tagettes species</td>
<td>Flowers</td>
</tr>
<tr>
<td></td>
<td>Black berries</td>
<td>Rubus fructicosus</td>
<td>Berries</td>
</tr>
</tbody>
</table>

2.1.4. Application to textile fibers

Natural dyes are traditionally applied to natural fibers, mainly cotton, silk and wool. Figure 2.3 shows a diagram summarizing the classification of fibers according to their source and chemical composition.
Textile fibers are normally divided into two main classes: natural and man-made (or manufactured). All fibers coming from natural sources are classified as natural fibers. They include protein, cellulosic and mineral fibers. Manufactured fibers are those in which the repeat units have been formed by chemical synthesis followed by fiber formation or the polymers from natural sources have been dissolved and regenerated after passing through a spinneret.
2.1.4.1. Natural fibers

Although there are several natural fibers used for textiles, the next sections provide a discussion of the three most commercially relevant and mainly used in association with natural dyes.

2.1.4.1.1. Cellulosic fibers: cotton

Cotton is the most often used cellulosic fiber and is nearly pure cellulose. Cellulose is the main component of the cells of all plants\(^2\). It comprised of 1,4-\(\beta\) linkages of glucose units\(^2\), with each successive glucose unit rotated 180° around the axis of the polymer backbone chain, relatively to the last repeat unit, Figure 2.4.

Two different end groups are found on each cellulose chain end. At one end of each chain, a non-reducing group is present where a closed ring structure is found. A reducing group, with both an aliphatic structure and a carbonyl group, is found at the other end of the chain. The \(\beta\)-(1-4) linkage between glucose residues, in contrast to the \(\alpha\)-(1-4) linkage observed in starch, gives cellulose some unique structural features. Cellulose is a water-insoluble polymer with a rigid linear structure. Controlled cellulose biosynthesis allows arrangement of extensive linear chains which can be aligned side-by side, creating fibers of great mechanical strength. Consequently, the tensile strength of cellulose is comparable to that of steel\(^2\).
Both, intra- and intermolecular hydrogen bonding occur in cellulose. The presence of intramolecular H-bonds is of high relevance for the single-chain conformation and stiffness. It is likely that cellulose has a high melting temperature ($T_m$) because the $\Delta H_m$ is large due to the interchain H-bonds, facilitated by the long linear structure of cellulose chains, and $\Delta S_m$ (entropy) is small due to the limited number of conformations available. Therefore, although cellulose is difficult to dissolve, it can be swollen, which is very important for wet processing\textsuperscript{26}.

The experimental evidence available today leads to the understanding that cellulose has amorphous and high-order crystalline regions. Voids, spaces and irregularities occur in the amorphous region while the crystalline region is tightly packed. Penetration of dyes and auxiliary chemicals occurs more rapidly in the amorphous region. The degree of crystallization of cellulose is usually in the range of 40-60%\textsuperscript{27}.

Cotton (Figure 2.5) is the seed hair of the cotton plant, and the hairs on a single seed have different lengths. Each hair consists of a membranous cell, the wall which is formed of cellulose
and certain salts, nitrogenous combinations, and, in some cases, coloring matter. The longer fibers, average length of about 25 mm, are quite pure and are the ones used for textiles applications, whereas the shorter ones, about 2-3 mm in length, are employed for the manufacture of cellulose derivatives\(^{28,29}\).

![Longitudinal and cross-section views of cotton](image)

**Figure 2.5.** Longitudinal and cross-section views of cotton\(^{30}\).

The high hydrophilicity of cotton greatly impacts its tensile strength. Wet cotton is about 20% stronger than dry cotton\(^{31}\). Cotton is hydrolyzed by hot dilute or cold concentrated acids to form hydrocellulose but it has excellent resistance to alkalis. Cotton is slowly attacked by sunlight because most UV absorbing groups are not present in cellulose. However, over long periods, sunlight will degrade cotton causing strength loss. Cotton fabric has excellent hand and superior absorbency, making it a very comfortable fiber to wear\(^{24}\).

### 2.1.4.1 2. Protein fibers: silk and wool

Numerous protein fibers can be found in nature. They are the fine filaments seen in spider webs, silk filaments in cocoons, bird feathers, and human hair. Protein is a biopolymer formed by
linking several amino acid residues, -NH-CH(R)-CO-, and it achieves its diversity through the different sequences of 20 or more side groups (R) of varying nature. There are over 500 naturally occurring amino acids, 240 of which occur freely in nature or as intermediates in metabolism, and only 20, Figure 2.6, appear in the genetic code.$^{32,33}$

A protein structure is generally described by its primary (the amino acids sequence), secondary and tertiary structures. The secondary structure refers to the folding of protein chains, produced or maintained mainly by H-bonding. The α-helix, coiled crystals, and β-sheet, crystalline, configurations are possible secondary structures for polypeptides and proteins, Figure 2.7. The uniform L-configuration of the amino acids provides the regular twist of the helix, while the intramolecular H-bonds provide rigidity.$^{28}$ The tertiary structure indicates the arrangement of the secondary structure in space, i.e. folded or packed by side chain interactions and crosslinked by disulfide bonds. When the protein contains more than a single peptide chain, the organization resulting from the non-covalent bond of macromolecular subunits is called the quaternary structure.$^{34}$

Wool is the fiber sheared mainly from sheep, while silk comes from the cocoons of silk worms. Wool fibers are extremely complex, crosslinked keratin proteins made up of over 17 different amino acids. Keratin is the major protein in wool. In general, keratin fibers are crosslinked by disulfide bonds from cystine residues in the protein chain. These fibers tend to have helical portions periodically within the protein structure$^{31}$.

Wool contains a balanced distribution of amino acids, 30% of them being ionic. It can take up both, helical α-keratin and the sheet-like β-keratin, conformations. Because of its highly ionic nature, wool’s properties are largely dependent on the pH$^{28}$.
Silk is an extracellular continuous monofilament produced by a wide variety of animals in the phylum Arthropoda. Most silk produced by the classes *Insecta* and *Arachnida* are composed of fibroin. Fibroin protein is made up by 15 or 16 α- amino acids linked to form a biopolymer. The fibroin silk fibers are simpler in structure than keratin and are composed primarily of glycine, alanine, tyrosine and serine, but mainly glycine and alanine\(^{35}\).

Silk does not take up the α- helical conformation, it only appears as the β pleated-sheet form. It is constituted primarily of the four amino acids, glycine, alanine, serine and tyrosine, with glycine and alanine responsible for over 75% of the total composition. Serine and tyrosine are found only in the amorphous regions of the fiber.

For commercially available silk (formed by *Bombyx mori* moth larvae), little crosslinking is observed between fibroin protein chains due to the absence of cysteine. In the absence of crosslinking and with limited bulky side chains, fibroin molecules are aligned parallel to each other and undergo hydrogen bonding to form a highly crystalline and oriented β-sheet structure\(^{24,36}\).
The stress-strain behavior of commercial silk fibers is dependent on the moisture content and temperature. The amorphous regions become more mobile with the increase in water uptake and temperature, due to the H-bonds that hold the structure together. Acid, metal-complex, mordant, reactive and some vat dyes can be applied to wool. The fact that wool is obtained from animals, which have different breeds and hair thickness enables a variety of different types of fabric to be produced. However, this can cause problems regarding uniformity of dyeing as, for example, in a mixture containing fine and coarse wool, the fine fibers will appear lighter than the coarse fiber.
Dyes used for wool coloration are usually sodium salts of sulfonic acid based chromogens having one to three sulfonic acid groups per molecule, \([\text{chromophore-} (\text{SO}_3 \text{Na}^+)]_n\). Other water solubilizing groups, such as carboxyl and phenolic, can be present. During the dyeing process, dye-wool ionic interactions control the rate of dyeing, and hydrophobic interactions are responsible for the affinity of dyes for wool and their wet fastness properties on the substrate\(^{39}\).

As silk fibers are proteinaceous with a variety of functional groups, these fibers are relatively easy to dye. Most dye classes that are applicable to wool can be applied to silk as well: natural dyes and, among the synthetic ones, acid, basic, reactive and vat dyes have been used. Natural dyes can be applied to silk by using concentrated solutions of basic salts as mordants. After mordanting, the fiber is dyed in an acidic dyebath\(^{35}\).

2.1.4.2. *Synthetic fibers*

Certain characteristics differentiate natural and synthetic fibers. The latter are man-made by polymerization of monomers. These fibers are generally hydrophobic, and can have a variety of filament lengths, which are produced by melt, wet or dry spinning processes. Nylon, polyester,
acrylic, and polyolefin are some of the synthetic fibers used by the textile industry. However, nowadays nylon, acrylic (PAN) and polyester (PET) are the three major synthetic fibers in contrast to the three major natural fibers, wool, silk and cotton\textsuperscript{40}. Therefore, these will be discussed in more detail. Table 2.2 compares important parameters of the most relevant, synthetic and natural, textile fibers.

**Table 2.2.** Important parameters of the most relevant, synthetic and natural, textile fibers\textsuperscript{40}.

<table>
<thead>
<tr>
<th>Fibers</th>
<th>Tensile Strength (cN/dtex)</th>
<th>Moisture Regain (%)</th>
<th>Colorant used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry</td>
<td>Wet</td>
<td></td>
</tr>
<tr>
<td>Polyester (PET)</td>
<td>3.8-5.3</td>
<td>3.8-5.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Nylon 6</td>
<td>4.2-5.7</td>
<td>3.7-5.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Polyacrylonitrile (PAN, Acrylic)</td>
<td>2.2-4.4</td>
<td>1.8-4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>4.4-7.9</td>
<td>4.4-7.9</td>
<td>0</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>0.5-1.1</td>
<td>0.5-1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Viscose Rayon</td>
<td>1.5-2.0</td>
<td>0.7-1.1</td>
<td>11.0</td>
</tr>
<tr>
<td>Triacetate</td>
<td>1.1-1.2</td>
<td>0.6-0.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Silk</td>
<td>2.6-3.5</td>
<td>1.9-2.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Wool</td>
<td>0.9-1.5</td>
<td>0.7-1.4</td>
<td>15.0</td>
</tr>
<tr>
<td>Cotton</td>
<td>2.6-4.3</td>
<td>2.9-5.6</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Polyester is a general name given to polymers having an ester functional group in their main chain. Although the term “polyester” refers to a broad range of polymers, in a textiles context, it usually refers to polyethylene terephthalate (PET). PET is an aliphatic-aromatic polymer formed through the polymerization of terephthalic acid with ethylene glycol\textsuperscript{41,42}, Figure 2.8.
PET has a hydrophobic nature and high level of crystallinity, which explains its resistance to dye penetration. Therefore, when PET appeared on the market, it caused problems for traditional dyers as the usual dyes applied to natural fibers (wool, silk and cotton) had -SO$_3$H and -NR$_3^+$ solubilizing groups, which had no affinity for the hydrophobic PET. Later on, disperse dyes, which have very low water solubility, with higher molecular weight were developed, and were very suitable for PET applications$^{43}$.

PET fibers have a glass transition temperature (Tg) of about 80°C, so to achieve efficient dyeing rates, the procedure is usually done at temperatures at or above 100°C. Because of the low absorption rate, carriers are used to increase the amount of dye absorbed at 100°C. Carriers are low molecular weight organic molecules with high affinity for PET. These compounds swell the fiber promoting dye penetration. When carriers are not used, dyeing temperature must be raised, to around 130°C, to achieve satisfactory dyeing rates, increase dye penetration into the fiber and promote leveling$^{44}$.

**Figure 2.8.** Reaction for the synthesis of polyethylene terephthalate (PET).
Nylon, a polyamide, is a linear polymer whose structural units are linked by the amide group, \(-\text{NH-CO}\)-. Nylon polymers can be synthesized in many ways, but the condensation of diamines with diacids and the hydrolytic polymerization of lactams, which involves partial hydrolysis of lactam to an amino acid, are of major importance in the manufacture of fibers\(^{43}\).

Nylon’s structure is very similar to the protein polyamides in silk and wool natural fibers, but their properties are quite different. For example, nylon absorbs only a fraction of the moisture absorbed by wool, leading to its more hydrophobic nature\(^{45}\). Nylon is more durable, stronger and chemically inert, which affects its end-use properties. Synthetic polyamides derived from diacids and diamines are identified as nylon x,y where x is the number of carbon atoms in the diamine, and y the number of carbon atoms in the diacid. Nylon 6,6 is made from 1,6-diaminohexane and adipic acid, and it has excellent tensile strength, abrasion resistance and elasticity. Nylon 6 (Figure 2.9) is another polyamide of major commercial importance\(^{43}\).

Dyeing processes are dependent on the end-use. Hosiery ware, a major use for nylon, is dyed on preformed blanks or cards by a triad of disperse dyes, whereas nylon yarns are dyed in package form with selected acid and 1:2 premetallized dye complexes\(^{45}\).

\[
\begin{align*}
\text{Nylon 66} & : & \quad \begin{array}{cc}
\text{H} & \text{N} \\
\text{(CH}_2\text{)}_6 & \text{N} \\
\text{(CH}_2\text{)}_4 & \text{O} \\
\text{O} & \text{C} \\
\end{array} \\
\text{Nylon 6} & : & \quad \begin{array}{cc}
\text{H} & \text{N} \\
\text{(CH}_2\text{)}_5 & \text{C} \\
\end{array}
\end{align*}
\]

**Figure 2.9.** Molecular structures of nylon 6 and nylon 6,6\(^{46}\).
Commercial polyacrylonitrile fibers (PAN), Figure 2.10, are synthesized through free radical polymerization of acrylonitrile because it gives the combination of properties, such as molecular weight, whiteness, and the ability to incorporate desired comonomers and dyes sites (sulfonate groups). Acrylonitrile is usually copolymerized with at least one other monomer, which may contain sulfonic, carboxylic acid, amine or quaternary ammonium groups, resulting in a more open structure and glass transition of 85-95°C enabling better dye penetration. PAN fibers have excellent chemical and weathering resistance, which is the main reason for their industrial application.

![Chemical structures of acrylonitrile and polyacrylonitrile (PAN)](image)

Figure 2.10. Chemical structures of acrylonitrile and polyacrylonitrile (PAN).

Traditionally, synthetic fibers were only dyed by synthetic dyes. However, in recent years there has been a renewed interested in the application of natural dyes to man-made fibers, as a way to reduce the use of the less eco-friendly synthetic dyes. Henna (Natural Orange 6), also known as lawsone, was applied to PET without the use of any mordant. Dye absorption was found to increase with temperature, and the dyed fabric showed good wash, rubbing and perspiration fastness properties. Red kola dye, extracted from Kola nuts belonging to the Sterculiaceae family, showed good fastness properties when applied to PET. In this case, simultaneous mordanting using metal salts was employed. UV/ozone pretreated PET and nylon were premordanted and dyed with saffron and curcumin. This pretreatment causes surface roughening leading to an increase in the
dyeing rate as a consequence of increasing the fiber surface area. The dye extracted from the bark of mangrove tree (Rhizophora apiculata) showed affinity to nylon with and without the use of mordants. However, highest color strength and dye uniformity were achieved with post-mordanting using CuSO₄, which is known to form coordination complexes between dye molecules and the amino and carboxylic acid groups on the fiber. In another study, a Rhubarb extract was applied to alkaline treated PET without the presence of a mordant. This dye was found to behave as a disperse dye during the dyeing process, requiring a carrier (salicylic acid) to accelerate the rate of dyeing, and a non-ionic dispersing agent to provide better dye uniformity.

Nordmncanthal, Figure 2.11, an anthraquinone that can be present in madder dye, showed good affinity for nylon and PET, with dye uptake dependent on temperature. This study also pointed out the similarity of the anthraquinone compound with synthetic disperse dyes. However, the dye-fiber affinity observed was much lower than that obtained for other dyes, such as the mentioned lawsone.

![Nordmncanthal structure](image)

**Figure 2.11.** Nordmncanthal structure.

### 2.1.4.3. Mechanisms of dyeing

Depending on the structure of the dye, dyeing can be carried out in acidic, alkaline or neutral baths, using methods that may or may not involve the use of a mordant. Like synthetic
dyes, natural dyes can be classified as direct, reactive, disperse, basic, acid and vat. Each class has
different chemical characteristics and, therefore, a different mechanism of application.

In general, dyeing a fiber with a water-soluble dye involves three main processes:
adsorption, desorption and diffusion. Initially, dye molecules from the dyebath are adsorbed onto
the fiber surface. This process is dependent on dyebath concentration, dyeing temperature and
presence of auxiliaries, such as retarding agents, which influence dyebath exhaustion. Adsorption
is reversible and dye molecules are constantly going back and forth from fiber surface to dyebath,
reaching a dynamic equilibrium between adsorption and desorption, meaning there is no effective
gain or loss of dye by the fiber. Concomitantly, as the concentration of dye builds up on fiber
surface, dye molecules start to diffuse into the amorphous region of the fiber establishing a new
equilibrium between the dye on the fiber surface and the dye inside the fiber. Once the dye is inside
the fiber, it bounds to the fiber by various forces, such as coordinate bonds and Van der Waals
interactions. These bonds can hinder diffusion towards the center of the fiber. Therefore, there is
a need to break these initial interactions to promote further penetration, which can be achieved by
increasing temperature, for example\textsuperscript{12}.

Coordinate bonds are the ones existent in wool dyed with mordant dyes, such madder, and
metal complexes. Turmeric, annatto and marigold, for instance, are adsorbed onto cellulosic and
protein fibers through Van der Waals bonds, without any pretreatment. Silk, wool and nylon have
-NH\textsubscript{2} groups, which can be protonated under acidic pH, to be dyed with natural acid dyes, such as
lac, through the formation of ionic bonds. Vat dyes, such as indigo, are water insoluble and must
be reduced to their leuco form before being applied to the fiber\textsuperscript{12}.

As it can be seen in the examples above, each dye class will bond to a fiber through the
action of specific forces. The strength of these interactions will determine the need for auxiliaries
during the dyeing process. Below, is a more in-depth discussion on the traditional and modern dyeing methods regarding the application of natural dyes.

2.1.4.3.1. Direct dyeing

This method involves soaking/boiling the plant in water at a temperature determined by the fiber and dye used. Fibers are then immersed in this dyebath. This procedure can be used when dye molecules in the plants have polar functional groups, leading to the formation of ionic bonds between anionic dyes (curcumins from turmeric) or cationic dyes (berberine from many yellow dye-plants) and animal fibers. However, the nature of the interactions between many direct dyes, such as lichens, tannins and safflower, and vegetable fibers is not yet fully understood, and an explanation of why satisfactory results can be achieved without mordanting is still being searched\textsuperscript{56}.

2.1.4.3.2. Mordant dyeing

Mordanting is the treatment of the fabric with metallic salts or other complex forming agents generating a compound capable of fixation to the fiber\textsuperscript{57}. Historically, organic compounds, animal fat, excrements, and inorganic substances, such as salts and mud, were used as fixing agents\textsuperscript{58}. Mordanting can be achieved by one of three methods: pre-mordanting, simultaneously mordanting and post-mordanting.

Chemically, mordants are defined as compounds capable of forming coordinate and covalent complexes with certain dyes and fibers. In the actual dyeing process, mordant and dye combine to form an aggregated, insoluble lake of high molecular weight within the textile fiber, which interferes with the fastness properties by making the fiber resistant to external influences such as washing and finishing processes\textsuperscript{59}. They can be classified in three categories: (1) metallic
mordants such as aluminum, chromium, iron, tin and copper salts; (2) tannins that are usually used to increase the uptake of cationic dyes onto cotton wherein the fabric was first treated with tannins and then treated with metal salt solution prior dyeing; and (3) oil mordants which forms a complex with alum, used as the main mordant aiding to dye’s fixation. Figure 2.12 shows the formation of a chromium complex with the natural dye alizarin and Table 2.3 summarizes the conventional and newly discovered metallic mordants.

Before the 19th century, eco-toxic, transition metals such as Cu$^{2+}$ and Cr$^{6+}$ were the major mordants in use. However, some toxicity concerns related to these metals started to appear. Copper toxicity can occur through the ingestion of contaminated food or water. Occupational exposure to copper can be a result of working with fungicides, paints, mordants, among other materials, being hepatotoxicity, gastrointestinal toxicity and irritation of the mucous membranes, skin and eyes, the most common adverse effects associated with copper in humans.

Nowadays, due to the increasing concerns related to environmental issues, many studies have been published aiming the development and application of eco-friendly mordants. Shahid and co-workers used tamarind seed coat tannin by itself and in combination with copper sulfate to dye natural fabrics. The use of enzymes combined with tannic acid resulted in an enhancement in the dyeability. However, metallic mordants are still widely applied in the natural dyeing process, generating some environmental concerns.
Bonding between Cr\(^{3+}\) and alizarin\(^{19}\).

![Diagram of bonding between Cr\(^{3+}\) and alizarin](image)

**Figure 2.12.** Bonding between Cr\(^{3+}\) and alizarin\(^{19}\).

| Table 2.3. Mordants used in natural dye application to textiles\(^{11}\). |
|-------------------------------------|-------------------------------|
| **Mordants**                        | **Chemical Formula**          |
| **Conventional metallic mordants**  |                               |
| Alum                                | KAl(SO\(_4\))\(_2\).12H\(_2\)O |
| Ferrous sulphate                    | FeSO\(_4\).7H\(_2\)O          |
| Stannous chloride                   | SnCl\(_2\).2H\(_2\)O          |
| Copper sulphate                     | CuSO\(_4\).5H\(_2\)O         |
| Potassium dichromate                | K\(_2\)Cr\(_2\)O\(_7\)        |
| **Newner metallic mordants**        |                               |
| Magnesium sulphate                  | MgSO\(_4\).7H\(_2\)O         |
| Aluminium sulphate                  | Al\(_2\)(SO\(_4\))\(_3\)     |
| Zinc sulphate                       | ZnSO\(_4\).7H\(_2\)O         |
| Zinc tetrafluoroborate              | Zn(BF\(_4\))\(_2\)           |
| Zirconium oxy chloride              | ZrOCl\(_2\).8H\(_2\)O        |

2.1.4.3.3. Modern dyeing techniques

The major problems when dyeing with natural dyes are low exhaustion and general poor fastness properties. Solutions to overcome these problems are mostly focused on the use of mordants. However alternative methods have been developed to improve dyeing properties.

Enzymes have been used by the textile industry for several purposes: amylases are used in desizing, cellulases are employed in denim finishing and proteases for silk and wool processing.
Most of the enzymes used are derived from fungal sources, for example amylase comes from *Aspergillus niger* and protease from *Aspergillus oryzae*. Enzymatic pretreatment of wool fabrics resulted in increased pigment uptake when dyeing with chlorophyll, with the results similar to those obtained for synthetic dyes. Wool dyeing involved the formation of hydrophobic interactions, which were responsible for the better wash fastness observed. Pretreatment of cotton and silk with a tannic acid-enzyme complex promoted rapid dye adsorption, and a total higher adsorption in comparison with untreated fabrics. In this work, using a sonicator instead of conventional dyeing bath contributed to a more ecofriendly process.

Another approach is to use ultrasound power in the textile dyeing. Ultrasound can improve dyeing effectiveness by generating cavitation in liquid medium, which is the formation, growth and collapse of bubbles promoting molecular motion, in addition to other mechanical effects such as dispersion, degassing, diffusion, and intense agitation of liquid. When cavitation occurs at the fiber-dye bath interface, these implosions produce micro streaming towards the solid surface which greatly disrupts the diffusion interlayer and promotes mass transport in that direction. Moreover, ultrasonic energy causes decomposition of dye aggregates in solution further improving fixation. Sonication helped to increase dye fixation by 13% when dyeing cotton pretreated with enzymes (protease, α-amylase, lipase and diasterase), using catechin and tectoquinone (anthraquinone derived) natural dyes. Faster dye uptake and an improvement in light and wash fastness were observed. An ultrasonic method has also improved the dyeing properties of modified acrylic fiber dyed with indicaxanthin dye, by increasing dye uptake by 49.6% in comparison with conventional heating.

Another approach has involved the use of supercritical fluids as solvents for dyes in a waterless process. Carbon dioxide has been the most widely used supercritical fluid due to its...
relatively mild critical properties, availability and low cost. This process was patented in 1988 by Schollmeyer and further developed by Knittel, and it has been developed from laboratory to a semi-technical scale since.

Gases under high temperature, and pressure that exceeds the critical point are known as supercritical fluids. A liquid can be converted to a supercritical fluid by increasing its temperature (T), and consequently its vapor pressure, and simultaneously increasing the pressure (p). Under these conditions, a closed system reaches critical values where it cannot be seen any boundaries between the liquid and gaseous state, this is the supercritical state. Further increases in pressure will result in a great increase of the dielectric constant of the system, thus imparting dissolving powers to the fluid. A supercritical fluid can be best characterized by the phase diagram in Figure 2.13. The critical point (CP) of carbon dioxide is at 7.3 MPa and 31°C, making it a suitable solvent for less polar and non-polar molecules with molecular weights up to 400 g/mol.

This technique has been applied in several studies. CI Disperse Yellow 23 was modified by introducing the reactive groups 1,3,5-trichloro-2,4,6-triazine and 2-bromoacrylic acid and applied to natural (cellulose and protein) as well as man-made fibers, in supercritical carbon dioxide (SCO$_2$). Results showed it was possible to dye natural fibers in supercritical CO$_2$ without any pretreatment. High color yields and excellent fastness were obtained with the 2-bromoacrylic acid dye applied to protein fibers (wool and silk) due to the easier activation of the amino groups. Polyester and polyamide fibers were also successfully dyed using this method. Modification of cellulose by reaction with benzoyl chloride allowed cotton to be dyed with CI Disperse Yellow 82 from a SCO$_2$ solution. Fixation values between 85-100% and good washfastness were obtained in comparison with polyester (PET) dyed with the same dye. Dyeing was achieved at lower temperatures than that required for PET dyeing.
As natural dyes are similar to the sparingly soluble, non-ionic low molecular weight coloring compounds, the same principles of dyeing with disperse dyes on synthetic or natural substrate can be applied. The knowledge of dye solubility in \( \text{SCO}_2 \) is essential for the design and optimization of the dyeing process. In general, dye solubility in \( \text{SCO}_2 \) is low, so the use of a co-solvent is required to enhance the solubility to an appropriate level. Solubility of natural dyes in \( \text{SCO}_2 \) depends on the temperature, molecular weight and polarity, which are a function of chain length and functional groups. Carotenoids are more soluble at 55°C than at 35°C, over a pressure range of 300-600 bar. Solubility of bixin doubled, at 350 bar, by increasing the temperature 20°C. Furthermore, the higher the polarity or molecular weight, the lower is the solubility in \( \text{SCO}_2 \) at a given condition\(^1\). Guzel and co-workers\(^7\) analyzed the solubility of three mordant dyes (CI Mordant Yellow 12, CI Mordant Red 11 and CI Mordant Brown), at 323-343 K and pressures in the range 132-195 bar. Results showed the higher solubility of CI Mordant Brown due to its smaller size and the formation of an oxime structure through tautomerization (equilibrium between enol and carbonyl forms), which promoted the formation of strong intra-molecular hydrogen bonds, fixing the size of the molecule. The same three dyes were dissolved in \( \text{SCO}_2 \), and five different

\( \text{Figure 2.13.} \) Phase diagram for carbon dioxide: \( P_c \) and \( T_c \) are the critical values of pressure and temperature; \( \text{TP} \) is the triple point and \( \text{CP} \) the critical point\(^8\).
mordanting metal ions (Cr$^{3+}$, Al$^{3+}$, Fe$^{2+}$, Cu$^{2+}$ and Sn$^{2+}$) and applied to pre-mordanted wool at 333-353 K and 150-230 atm. CI Mordant Brown provided excellent color strength and washfastness$^{66}$.

### 2.1.5. Fastness properties

The fastness of dyes refers to their capacity to resist fading when exposed to light, crocking, bleach, perspiration, water and other end use treatments$^{20}$. In the next two sections, we discuss two of these categories, light and wash fastness, in more details.

#### 2.1.5 1. Lightfastness

In general, natural dyes have poor light fastness specially when compared to synthetic dyes. Most dyes fade rapidly when tested using different types of lightfastness standards. A few exceptions are cochineal, madder, and others that are brightly colored and present adequate fading ratings. This better behavior is usually exhibited by anthraquinone or anthraquinone derivative dyes. While most of the yellow dyes fade to a duller-browner color, weld is an exception as it fades without a hue change$^{72}$.

Several factors influence the lightfastness of a dye: the characteristics of the fabric used, the chemical structure of the dye molecule, the physical state of the dye, the type of mordant used and wavelength of the light source of exposure$^{72}$. Dye structure and the type of mordant are the main responsible for causing color change. The resistance of a dye to chemical or photochemical degradation is an inherent property of the dye chromophore, but the auxochromes can also alter the fastness for the better or worse. For hydroxyanthraquinones, lightfastness was observed to decrease with the increase in the number of hydroxyl groups, being the extend of this decrease dependent on the position of the substituents present. For example, luteolin (Figure 2.14), a flavone extracted from weld, has higher light fastness on wool than its 3-hydroxy derivative,
quercetin, due to the high susceptibility to photodegradation exhibited by the hydroxyl at position two \textsuperscript{12}.

![Chemical structures of flavonoid derivatives (A) Luteolin and (B) Quercetin.](image)

Figure 2.14. Chemical structures of flavonoid derivatives (A) Luteolin and (B) Quercetin.

Regarding the nature of the incident light, absorbed wavelengths in the ultraviolet and visible regions are not equally effective in initiating the fading process. Visible radiation is the main responsible for the fading of fugitive dyes whereas UV radiation plays the most important role on the fading of dyes with higher lightfastness\textsuperscript{12}.

When studying the impact of several mordants on yellow dyes’ color change, it was observed that the greatest amount of color change occurred with alum and tin, while the least amount occurred with chrome, copper and iron\textsuperscript{73}. For two Madder components, Purpurin and Munjistin, applied to nylon, post-mordanting with copper and ferrous sulfate provided higher resistance to photofading than stannous chloride and alum, whereas pre-mordanting showed the opposite result\textsuperscript{74}.

Because lightfastness is one limitation of natural dyes in comparison with the more light stable synthetic ones, studies have been performed attempting to overcome this disadvantage. Using additives like UV absorbers and antioxidants, such as vitamin C and gallic acid, improved the lightfastness of madder, weld and wood applied to cotton yarn\textsuperscript{75}. Lee et al\textsuperscript{76} analyzed the effect of adding UV absorbers to mordant-dyed wool and silk fabrics and found out that this after-
treatment led to an improved lightfastness of wool mordant-dyed with goldthread, amur cork tree and redwood dyes with no changes in color or shade.

2.1.5 2. Washfastness

As observed for lightfastness, generally, textiles dyed with natural dyes (without using mordants) do not have good wash fastness properties relative to synthetic dyes. Direct natural dyes, such as turmeric, annatto and safflower are attached to the fiber by hydrogen bonds or weak Van der Waals forces, which are easily broken even when washing is done under mild conditions fiber

For some dyes, better results were also obtained when using mordants. When dyeing cotton, pre- mordanting improved the wash fastness for dyes that would otherwise show low to moderate results, such as cochineal, logwood and safflower. Samples dyed with madder, sandalwood and indigo showed better wash fastness even without using mordants. For those samples, pre-mordanting caused an increase in dye adsorption on cotton, but the excess of dye was only superficially adsorbed being easily washed off.

Another example of how several factors play a role when studying the fastness properties of natural dyes is the use of saffron to dye cotton and wool. The use of mordant was advantageous for cotton fabrics but its presence resulted in a reduction in washfastness for wool.
2.1.5 3. **Fastness assessment**

Different organizations have developed standard test methods to evaluate fastness properties. Among these organizations is America Association of Textile Chemists and Colourists (AATCC) and the International Organization for Standardization (ISO), but some manufacturers have their own standards for testing and grading the fastness properties of dyes (natural and synthetic)\(^{20}\).

AATCC Test Method 16-2014\(^{78}\) is a commonly used standard method that determines the general principles and procedures currently used for determining the colorfastness of textiles materials to light. The fabric is exposed to a Xenon arc lamp under set conditions. AATCC Test Method 61-2013\(^{79}\) is commonly used to evaluate the colorfastness to laundering of textiles which are expected to withstand frequent laundering. The fabric color loss and surface changes resulting from detergent solution and abrasive action of five typical hand or home launderings are roughly approximated by one 45 min test. A typical test method to determine the amount of color transferred from the surface of colored textile materials to other surfaces by rubbing is the AATCC Test Method 8-2016\(^{80}\).

The effects of washing and light exposures can be determined by colorimetric analysis of the color strength (K/S) and L*a*b values. The effect of crocking, can be rated by using a Chromatic Transference Scale or the Gray Scale for color change.

2.1.5 4. **Color evaluation**

Color strength is usually measured as K/S, which is a function of color depth and calculated by through the Kubelka-Munk equation,

\[
\frac{K}{S} = \frac{(1 - R)^2}{2R}
\]
where $R$ is the reflectance of the dyed fabric, $K$ is the absorption coefficient, which is dependent on dye concentration, and $S$ is the scattering coefficient of the dyed substrate$^{81}$.

The amount of fading or color alteration with environmental exposure or washing can also be evaluated using the Gray Change (AATCC EP1-2012)$^{82}$, Figure 2.15, and Gray Staining (AATCC EP2-2012)$^{83}$ scales. Both are based on a gray set of a visual paint chips and are rated on a scale of 1 (most difference) to 5 (no difference)$^{84}$.

Another evaluation procedure is through the use of the Chromatic Transference Scale, AATCC EP8-2017$^{85}$. This scale is also used to visually evaluate color transfer or staining. Five hues, red, yellow, green, blue and purple, are used, which may facilitate evaluations.

The Commission International d’Eclairage (CIE) established some principles for the measurement of color of surfaces with results given in terms of CIE 1976 L* a* b* (CIELAB) color space parameters, Figure 2.16.

Figure 2.15. Gray Scale for color change$^{84}$.
The perceived color of an object is dependent on the nature of the illuminating light, its modification by interaction with the object, and the observer response. The CIELAB is an opponent color system that considers these parameters and uses the tristimulus values to calculate the L* a* b* color space values. The central vertical axis (L*) represents lightness and measures the changes along the gray scale from 0 (black) to 100 (white). The color axes are based on the fact that red and green, and blue and yellow are opposite colors and, therefore, a color cannot be both (red and green, for example) at the same time. On the “a” axis, positive values indicate amounts of red whereas negative values indicate amounts of green. On the “b” axis, yellow is positive and blue is negative. For both axes, zero is neutral gray.

2.2. Synthetic dyes

2.2.1. Historical background

The earliest synthetic dyes prepared were picric acid, by Peter Woulfe in 1771, and aurine (rosolic acid), by Runge in 1834, Figure 2.17. However, these dyes were not prepared commercially due to the high cost of raw materials at the time. For that reason, William H Perkin
is considered the founder of synthetic dyes, for his discovery of Mauveine and later, the synthesis of a dye called safranine by oxidation of mauve using lead dioxide\(^\text{88}\).

![Structures of picric acid (on the left) and rosolic acid (on the right)\(^\text{88}\).](image)

**Figure 2.17.** Structures of picric acid (on the left) and rosolic acid (on the right)\(^\text{88}\).

A key milestone for the dye industry was the discovery of the diazotization reaction by Peter Gries in 1858. The coupling reaction combined diazotized aniline with \(\alpha\)-naphthyl amine. This discovery allowed the synthesis of the first bisazo dye, Bismarck brown, five years later\(^\text{89}\).

Following this discovery, further research allowed the synthesis of dyes that had only occurred naturally before, such as indigo and alizarin, as well as the creation of new dye classes, i.e. triarylmethane. A series of indanthrene vat dyes were prepared between 1901-1911, and in the same period, many functionalization processes were discovered: sulfonation, nitration, alkali fusion, among other, which were used in the production of important dyes intermediates, like H and J-acids and anthraquinone derivatives\(^\text{88,89}\).

The commercial value, availability, better fastness properties, reproducibility obtained in the dyeing processes and broader gamut of colors offered by synthetic dyes led to the collapse of the natural dye industry in Europe at the end of the nineteenth century, and soon the situation extensively affected dye markets worldwide, including the Chinese dye market\(^\text{90}\).
2.2.2. Classification

Generally, synthetic dyes are aromatic organic compounds that can be categorized in three main groups: non-ionic, anionic and cationic. There are about 2100 anionic dyes listed in the Color Index. With a few exceptions, anionic dyes are manufactured as metal salts, with the majority sodium salts of sulfonic acids. However, they can also be sodium salts of carboxylic acids. On the other hand, cationic dyes, usually have chloride as the counter ion\textsuperscript{91}.

Like natural dyes, synthetic ones can be classified according to their chemical structure and mode of application to the fiber. When classification is done based on the mode of application, first they are divided in two groups: the ready-made and the Ingrain dyes (those synthetized inside the fiber). The main class of ingrain dyes is the Azoic dyes. Ready-made dyes can be further divided into water soluble (Direct, Acidic, Basic, Reactive dyes) and water insoluble (Vat, Sulphur and Disperse dyes). \textbf{Figure 2.18}\textsuperscript{49}.

In the water soluble anionic class are the Direct, Acid and Reactive dyes. Acid dyes generally contain one or more -SO$_3$Na or -SO$_3$H groups. They have affinity for protein fibers, nylon, modified acrylic fibers, leather and they do not dye cotton. They are applied to fibers from an acidic bath (containing dilute sulfuric or acetic acid), which is responsible for protonating the amino groups on the fabric making them attractive to the anionic dye, and to the formation of the ionic bond\textsuperscript{92,93}.

Direct dyes, also known as substantive dyes, are closely related to acid dyes with their substantivity to natural or regenerated cellulose the distinguishing property. For a dye to be classified in this group it has to exhaust from an aqueous salt-containing solution onto cellulosic substrates. They generally contain sulfonic acid groups and are bound to cotton through H-bonds
in the presence of salt (Na₂SO₄ or NaCl). Despite their low light and washfastness, they are widely used due to their low cost and easiness of application⁹²,⁹⁴.

Reactive dyes consist of a dye molecule attached to a fiber reactive system. They contain -Cl or -O-SO₃Na as leaving groups, enabling the formation of covalent bonds with fibers (mainly cellulosic fibers). The dyeing process is carried out under heat and mild alkaline conditions and after fixation, the fabric is washed with soap to remove the unfixed hydrolyzed reactive dye molecules⁹³,⁹⁴.

The only water soluble cationic dyes are the basic dyes. These dyes can be applied to wool, silk, leather, under slightly acid conditions, but are mainly applied to acrylic fibers. They contain basic groups (i.e. NH₂, NR₂) and form ionic bonds with negatively charged polymer chains⁹³,⁹⁵.

Regarding the water insoluble dyes, the disperse class contains dyes that form hydrophobic interactions with hydrophobic fibers, such as cellulose acetate, polyester and polyamide. They are applied from a water dispersion containing a dispersing agent. Application requires the use of
carriers or high temperature, which allows the diffusion of the fine dye particles into the fiber. These dyes must have a relatively low molecular weight\textsuperscript{88}.

Vat dyes cannot be directly applied to the fiber due to their water insolubility. They must be reduced first, into their leuco water soluble form, by using sodium hydrosulfite in an alkaline medium, then they exhaust to the fiber similarly to a direct dye. After diffusion, the dye molecule is oxidized, using air or hydrogen peroxide, to its original form becoming trapped in the fiber. Anthraquinoids constitute the great majority of dye structures within this group but there are a few indigoids, like indigo, and thioindigoids.

Sulfur dyes have complex structures composed by disulfide linkages. They also need to be reduced prior application to textiles and, upon reduction, have their S-S bond broken, generating a simpler water-soluble dye, which can be applied to cellulosic fibers. After diffusion, the insoluble dye is regenerated by oxidation\textsuperscript{49,93}.

Azoic dyes are made inside the fiber. During the dyeing process, the cellulosic fiber is first impregnated with the coupling component, a naphtholate salt, followed by treatment with the solution of diazonium salt. The coupling reaction takes place at low temperatures forming the water insoluble azoic dye.

Another approach is the classification based on dyes chemical structures, which is primarily based on characteristic structural units. A summary of the main dyes classes is presented in Table 2.4.
Table 2.4. Classification of dyes based on their chemical structure\textsuperscript{49,95}.

<table>
<thead>
<tr>
<th>Dye class</th>
<th>Characteristic structural unit</th>
<th>Details</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitro dyes</td>
<td>NO$_2$</td>
<td>Generally acid dyes.</td>
<td>Naphthol Yellow S</td>
</tr>
<tr>
<td>Nitroso dyes</td>
<td>-NO or =N-OH</td>
<td>Used as complexes with iron generating green colored compounds.</td>
<td>Naphthol Green G</td>
</tr>
<tr>
<td>Azo dyes</td>
<td>Ar-N=N-Ar’(1)</td>
<td>Can be further classified according to the number of azo groups.</td>
<td>Aniline Yellow</td>
</tr>
<tr>
<td>Triphenylmethane dyes</td>
<td>Ar-C=Ar</td>
<td>Have quinonoid group as chromophores.</td>
<td>Malachite Green</td>
</tr>
<tr>
<td>Xanthene dyes</td>
<td>Derivative of xanthene</td>
<td>Have quinonoid group as chromophores.</td>
<td>Fluorescein</td>
</tr>
</tbody>
</table>
### Table 2.4. continued.

<table>
<thead>
<tr>
<th>Anthraquinone dyes</th>
<th>Anthraquinone or anthrone</th>
<th>Good fastness properties.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duranol Blue B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cyanines</th>
<th>Heterocyclic rings united by -CH(-CH=CH)_n- or =CH-</th>
<th>Used as photographic sensitizers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinacyanol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1)Ar and Ar’= aromatic groups
2.2.2.1. Reactive dyes

Reactive dye molecules contain (1) a chromogen that is responsible for its color, (2) a reactive system that enables fixation to the fiber, (3) a bridging group that connects chromogen, and (4) one or more solubilizing groups. The reactive groups are responsible for the formation of covalent bonds with the nucleophilic groups on the polymer chains within the fiber.\textsuperscript{96}

The first covalently bound combination of a dye with cellulose was reported in 1895 by Cross and Bevan, who described the synthesis of a colored polymer by benzylation of alkali-treated cellulose, nitration of the benzoate ester, reduction to the aminobenzoate and finally diazotization and coupling.\textsuperscript{97} The first commercially available dye capable of covalently bond with a textile fiber is believed to be Supramine Orange R (CI Acid Orange 30), Figure 2.19, which was introduced by I G Farbenindustrie in 1930 for the dyeing of wool. It was a chloroacetylamino substituted dye that had a chlorine atom readily replaced to form a dye-fiber bond, under weakly acidic dyeing.\textsuperscript{97}

![Figure 2.19. CI Acid Orange 30 structure.](image)

Reactive dyes can be classified according to the number and nature of the reactive groups existent in the molecule. Another useful classification method for these reactive systems is based on the parameters that control the dyeing process. Both are discussed below.
2.2.2.1.1. Classification based on the reactive groups

Reactive dyes can be classified as monofunctional, bifunctional or polyfunctional dyes and Table 2.5 shows some important reactive systems belonging to the mono and bifunctional classes\textsuperscript{96}.

Monofunctional systems contain dyes that have only one reactive center. Examples are halotriazine and vinylsulphone systems. Regarding the dichlorotriazine, difluoropyrimidine and dichloroquinoxaline heterocyclic ring systems, there are two equivalents replaceable halogeno substituents. However, when one of these halogen atoms is displaced by reaction with functional groups in the fiber or with alkali in the dyebath, the remaining halogeno substituent is no longer reactive. The s-triazine, Figure 2.20, has a unique behavior amongst the six-membered nitrogen heterocycles due to its three electronegative atoms, which provide the necessary activation of the halogen atoms attached to the adjacent carbons. Polarization of the carbon-chlorine bonds makes the chloro substituents susceptible to nucleophilic substitution. S-triazine has highest reactivity on the carbon atoms. The electronegativity of the nitrogen atoms in these heterocycles activates the system for nucleophilic substitution\textsuperscript{98,99}.

Table 2.5. Monofunctional and bifunctional reactive systems\textsuperscript{96}.

<table>
<thead>
<tr>
<th>System</th>
<th>Commercial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monofunctional</td>
<td></td>
</tr>
<tr>
<td>Dichlorotriazine</td>
<td>Procion MX</td>
</tr>
<tr>
<td>Monochlorotriazine</td>
<td>Procion H</td>
</tr>
<tr>
<td>Monofluorotriazine</td>
<td>Cibacron F</td>
</tr>
<tr>
<td>Trichloropyrimidine</td>
<td>Drimarene X</td>
</tr>
<tr>
<td>Difluorochloropyrimidine</td>
<td>Drimarene K</td>
</tr>
<tr>
<td>Dichloroquinoxaline</td>
<td>Levafix E</td>
</tr>
<tr>
<td>Sulfatoethul sulfone</td>
<td>Remazol</td>
</tr>
<tr>
<td>Sulfatoethyl sulfonamide</td>
<td>Remazol D</td>
</tr>
<tr>
<td>Bifunctional</td>
<td></td>
</tr>
<tr>
<td>Bis (monochlorotriazine)</td>
<td>Procion HE</td>
</tr>
<tr>
<td>Bis (mononicotinotriazine)</td>
<td>Kayacelon React</td>
</tr>
<tr>
<td>Monochlorotriazine-sulfatoethyl sulfone</td>
<td>Sumifix Supra</td>
</tr>
</tbody>
</table>
Table 2.5 continued

<table>
<thead>
<tr>
<th>Bifunctional</th>
<th>Monofluorotriazine-sulfatoethylsulfone</th>
<th>Cibacron C</th>
</tr>
</thead>
</table>

Bifunctional systems are the ones containing two reactive centers, being more likely to combine with a hydroxyl group in the cellulose molecule. A covalent bond can be formed with -OH groups in the glucose units of the cellulose chain. If other factors are equal, using a bifunctional dye rather than its monofunctional analog increases dye fixation from a typical 60% to around 96% in exhaust dyeing\textsuperscript{100-102}.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Pyridazine & Pyrazine & Pyridine & Pyrimidine & s-Triazine \\
-0.083 & 0.071 & -0.109 & 0.064 & -0.144 & 0.042 & -0.031 & 0.101 & 0.133 & -0.178 & 0.202 & 0.23 \\
\hline
\end{tabular}
\caption{Charge distribution values (levels of reactivity) of various heterocyclic rings\textsuperscript{99}.}
\end{table}

Bifunctional dyes can be further characterized as homo-bifunctional, having two of the same reactive groups, or hetero-bifunctional, having two different reactive groups. Examples of dyes belonging to these two group are shown in Figure 2.21.
2.2.2.1.2. Parameters controlling the dyeing process of cellulose

Success of dye fixation to cellulose depends on many parameters, such as the liquor ratio, temperature and pH of the dyeing bath, the affinity between dye and fiber, and consequently, on the dye and fiber structures, the diffusion coefficient of the dyes in the fiber, and the fiber surface area available to adsorb the dye\textsuperscript{96,103}. Liquor ratio is dependent on the dyeing machine used. However, using lower liquor ratios enables the application of a wide range of reactive dyes, specially the low-affinity ones, leading to higher fixation due to reduction of wasteful hydrolysis in the dyebath. A greater surface area per unit weight is observed on linear low density fibers, which results in a more efficient dyeing. The substantivity ratio, which is the relationship between the concentration of dye absorbed into the fiber and remaining in the dyebath, remains constant in the pH 7-11 range, but it significantly decreases above pH 11 (especially with highly sulfonated dyes) revealing a decrease in dye fixation related to pH.

In all known dyeing systems, the exhaustion of a dyebath increases with decreasing temperature due to the reduction in the substantivity ratio and acceleration of dye’s hydrolysis rate.
Temperature of application also varies with the reactive group present, **Figure 2.22**. In dichlorotriazine systems, the two chlorine groups tend to polarize the C-Cl bond, contributing to the buildup of the positive charge on the carbon atom. Therefore, nucleophilic attach can take place at the carbocation. Two chlorines have a higher activation power on the carbon for nucleophilic substitution than a single chlorine, and that is why the dichlorotriazine system is more reactive and dyed at lower temperatures than the monochlorotriazine system\(^96,103\).

<table>
<thead>
<tr>
<th>Warm (60°C)</th>
<th>Colder (40°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichlorotriazine</td>
<td></td>
</tr>
<tr>
<td>Difluorochloropyrimidine</td>
<td></td>
</tr>
<tr>
<td>Monofluorotriazine</td>
<td></td>
</tr>
<tr>
<td>Dichloroquinoxaline</td>
<td></td>
</tr>
<tr>
<td>Vinyl sulfone</td>
<td></td>
</tr>
<tr>
<td>Monochlorotriazine</td>
<td></td>
</tr>
<tr>
<td>Trichloropyrimidine</td>
<td></td>
</tr>
<tr>
<td><strong>Reactivity</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.22.** Reactivity and application temperature for reactive dyes\(^{104}\).

Exhaustion of the dyebath is also affected by addition of electrolytes. Increasing electrolyte concentration improves substantivity, with this effect more evident when a high number of sulfonated groups are present in the molecule. The function of salt, usually Na\(_2\)SO\(_4\) and/or NaCl, during the dyeing process is to negate the negative charge on the fiber surface and decrease the polarity of the dyebath (which reduces dye solubility in the bath), just so the negatively charged dye can adsorb on the fiber\(^96,103\).

Considering the variables described above, reactive dyes can be further aggregated into three groups. The first group, alkali-controllable reactive dyes, includes dyes that show low
exhaustion values in neutral salt solution before the addition of alkali. They are fixed at an optimum temperature between 40 and 60°C. Alkali addition should be slow to control dyeing levelness. Vinylsulfone and dichlorotriazine systems belong to this group. Group 2, salt-controlled reactive dyes, includes low-reactivity systems, such as trichloropyrimidine, which exhibit somewhat high exhaustion at neutral pH, so the addition of salt must be controlled and careful to ensure good levelness. Optimal fixation temperature for this dye class is between 80 and 100°C. For dyes in the third group, temperature-controlled reactive dyes, the rate of temperature rise to the fixation temperature must be controlled. Some dyes react with cellulose at temperatures above 100°C in the absence of alkali. There are not many dyes in this group but one example is the Kayacelon React (KYK) range of bis(aminonicotinotriazine)\textsuperscript{101,103}.

2.2.2.1.3. Fixation to cellulosic fibers

Based on the reactive group present, dye fixation, through the formation of a covalent bond, can happen through nucleophilic substitution or nucleophilic addition reactions. Reactive dyes containing a nitrogen heterocyclic ring with halogeno groups undergo nucleophilic substitution. The electronegative heteroatoms activate the aryl ring for attack by nucleophiles. The electron pair on the nucleophile bonds to the positively charged, or partially positively charged, electrophile as the labile group leaves with an electron pair. This reaction is illustrated for a dichlorotriazine dye in Figure 2.23. The nucleophile, X\textsuperscript{−}, can either be a cellulosate anion or a hydroxyl group. In the first case (X=O-Cellulose), dye and fiber bond and fixation is successful, in the latter case (X=OH), dye is hydrolyzed and is no longer capable of being bound to cellulose\textsuperscript{101}.
Sulfatoethylsulfone dyes, the precursors for the vinylsulfone reactive group, undergo nucleophilic addition during fixation. In this case, an alkaline 1,2-elimination of the bisulfonate group in the precursor molecule is necessary before addition. The presence of the electron withdrawing sulfone group polarizes the carbon-carbon double bond, which gives the terminal carbon a partial positive charge enabling nucleophilic addition of an electron rich specie. In this case too, the electrophile can either be a cellulosate anion or a hydroxyl group leading to dye fixation and hydrolysis, respectively. This reaction mechanism is shown on Figure 2.24\textsuperscript{101,103}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure23.png}
\caption{Nucleophilic substitution reactions of a dichlorotriazine dye\textsuperscript{101}.
}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure24.png}
\caption{Fixation mechanism of vinylsulfone reactive dyes to cellulose\textsuperscript{105}.
}
\end{figure}
Understanding the mechanism of fixation of dyes to the fiber enhances knowledge about the dyeing process. When the dye is applied from a neutral solution, some molecules are adsorbed on the cellulose surface while others diffuse into the fiber, until an equilibrium is reached. At this point, interaction is mainly physical because the concentration of hydroxyl and cellulosate ions is very low. This is the exhaustion phase. The reversible equilibrium reaction between cellulose and alkali generates the nucleophile that will react with the dye, Figure 2.25. Equilibrium is reached instantaneously when alkali is added to the system. Upon the addition of alkali, the fixation phase starts, and adsorbed dye bonds to cellulose at a significant rate\textsuperscript{101,106}.

![Figure 2.25](image)

**Figure 2.25.** Reaction between cellulose and alkali to generate the cellulosate anion.

A typical procedure for exhaust dyeing at high temperature is described as follows\textsuperscript{107}: (1) fill machine with water; (2) load fabric; (3) heat to 50 °C; (4) add pre-dissolved reactive dyes to the bath; (5) raise the temperature 1 °C/ min until the bath reaches a temperature of 80 °C; (6) add salt to the bath in parts; (7) run for an additional 15 min; (8) add alkali over 15 min in parts; (9) run for an additional 45-75 min; (10) empty dyebath; (11) rinse substrate with warm water and then soapy warm water; (12) rinse final time with cold water.

### 2.2.2.1.4. Vinylsulfone systems

Although vinylsulfone (VS) reactive dyes first appeared commercially in 1952 and were applied to wool, the commercial exploitation of VS dyes only started in 1958, with the introduction of the Remazol range of dyes for cellulosic fibers\textsuperscript{108}. Vinylsulfone groups, -SO\textsubscript{2}-CH=CH\textsubscript{2}, are not
typically found in a reactive dye product. What is found is generally the functional group -SO₂-CH₂-CH₂-OSO₃⁻ (H⁺ or Na⁺), the sulfatoethylsulfone form. The presence of the hydrogen ion or the sodium ion depends on the pH. The sulfone, -SO₂-, stabilizes the intermediate carbanion that forms in the presence of alkali, as shown in Figure 2.24

As previously mentioned, hydrolysis of the vinyl sulfone moiety in lieu of fixation is one of the fundamental problems associated with reactive dye technology. For most of vinyl sulfone reactive dyes, the degree of fixation on cotton ranges from 75 to 80%.109

2.2.2.2. Disperse dyes

The first disperse dyes were synthesized in the early 1920s for dyeing secondary cellulose acetate, and the discovery that aqueous dispersions of almost water-insoluble dyes were highly suitable for the dyeing of such fibers was a major technological breakthrough. Disperse dyes are defined by The Society of Dyers and Colourists as substantially water-insoluble dyes having substantivity for hydrophobic fibers, such as polyester, nylon and acetate, and usually applied from a fine aqueous dispersion. They are non-ionic, crystalline materials having melting point higher than 150°C, and relatively low molecular weight substances, mostly based on azo, anthraquinone and diphenylamine structures.110

In the 1970s, disperse dyes were classified, according to their sublimation fastness and dyeing properties, in classes A to D. Class A contains dyes with low molecular weight, which results in poor sublimation fastness, and fast dye uptake properties, whereas class D contains dyes with relatively high molecular weight (good sublimation fastness), but slow dye uptake properties. Classes B and C contain dyes with properties in between. Nowadays, the Disperse Dye Committee of the Society of Dyers and Colourists classifies these dyes as high and low energy disperse dyes. The first group contains level dyeing dyes, which are small dye molecules with low polarity, and
rapid dyeing properties with poor heat resistance. On the other hand, high energy disperse dyes are more polar, higher molecular weight molecules that have low dyeing rates, poor migration during dyeing and good heat and sublimation fastness. Like the other dye classes mentioned before, disperse dyes can be further classified according to their chromophore structure.

The growth of disperse dyes is associated with the development of the new man-made hydrophobic fibers, cellulose acetate. However, nowadays, these dyes play a dominant role in dyeing polyester fibers. They constitute the second largest sector in the dyeing industry, with 125 thousand tons of dye produced in 2006, with a value of $900 million. The discover and commercial introduction of PET in 1948 increased the interest in disperse dyes. With this discovery, new methods of dye application had to be developed as PET, a highly crystalline and hydrophobic fiber, would only slightly swell in water. Therefore, higher temperatures had to be used to open the fiber structure allowing dye penetration, and disperse dyes behaved very well under such conditions.

2.2.2.2.1. Dyeing polyester with disperse dyes

Essential auxiliaries in dyeing PET fibers with disperse dyes include dispersing agents and agents to control the pH. Although disperse dyes are considered to be substantially water insoluble, some degree of solubility is needed for dyeing to take place from an aqueous medium, which is achieved by using dispersing agents. In water, dispersing agents work by forming micelles with the hydrophobic tail on the inside of the micelle, surrounding the dye molecule, and it the hydrophilic portion on the outside in contact with water. During the dyeing process, dye molecules dissolved in dyebath are transferred to the fiber surface, and the solution in the dyebath is restored by the dissolution of more solid material from the dispersion. The absorbed dye diffuses into the fiber. This process is illustrated in Figure 2.26.
Parameters to consider when dyeing PET with disperse dyes will now be illustrated in detail. The solubility of disperse dyes generally increases with temperature and dispersing agent concentration, but these effects vary enormously with the dye and dispersing agent’s structures\textsuperscript{112}. In general, dispersing agents are anionic, ligninsulfonates which facilitate milling by preventing agglomeration of the dye particles. Hence primary dispersing agents usually do not enhance levelling, levelling agents can be used to promote dyeing uniformity. Levelness can be improved by controlling the exhaustion rate, which can be done by adding non-ionic surfactants selected for their ability to form surfactant micelles attractive to the dye. They compete with the fiber for the dye, leading to the decreased in the rate at which the dye becomes more available to the fiber. Ideally, dyebath pH should be in the 4.5–5.5 range to minimize the possibility of dye hydrolysis. This can be achieved by adding acetic acid when needed. Temperature is another crucial factor to consider, as dyeing begins to occur more rapidly above PET’s glass transition temperature. When dyeing temperature is reduced, a carrier is needed to promote dye levelling and exhaustion. At temperatures much below 100°C, PET fibers dye very slowly, but when extended time is allowed dyeing can be successful\textsuperscript{111-113}. 
2.2.2.2. Fastness properties

The relationship between dye structure and fastness properties is quite complex. As mentioned before, disperse dyes general structures are small, non-ionic, planar, with no water solubilizing groups, which, in general, leads to good wash fastness when these dyes are applied to PET. Sublimation fastness is related to molecular size and polarity, and is important due to exposures to heat during outdoor applications. It tends to increase with molecular size and polarity. However, a balance needs to be achieved between sublimation fastness and dyeing properties, as an increase in molecular size and polarity also leads to a reduction in the rate of diffusion.41

There is a close relationship between the chemical structure of a dye and its ability to resist photodegradation. However, as previously mentioned for natural dyes, other factors play an important role in lightfastness levels. The chromophore is the most important element in determining the lightfastness of a dye, but substituents can significantly alter the dye’s properties. In general, fastness increases with increasing dye concentrations within the fiber. The fiber also

![Figure 2.26. Disperse dyeing mechanism](image)
influences the lightfastness, as different fibers contain different chemical groups that react differently upon exposure to light. The moisture content in the atmosphere can greatly affect the fading rates of certain dyes\textsuperscript{111}.

For example, for dyes derived from phenylindole, N-alkylaminobenzene, carbazole and pyridone the tendency to undergo photodegradation was strongly dependent on the electron donating ability of the coupling component. The dye, whose coupling component was phenylindole, possessed the excellent dyeing properties and the high degree of lightfastness\textsuperscript{114}. A study with anthraquinone and benzanthrone disperse dyes showed that the substitution of a hydroxy group \textit{ortho} to a carbonyl group gave dyes of much higher lightfastness than if the same group had been amino or alkyl amino\textsuperscript{115}.

### 2.3. Environmental fate of dyes

Many factors need to be considered when attempting to determine what happens to a compound and its breakdown products in the environment:

1. The mechanisms that break down the chemical need to be considered. These mechanisms include photolysis, metabolism and bioaccumulation in plants and animals, biodegradation, hydrolysis, dissociation, and sorption in all environment compartments (air, water, soil, plants and animals).

2. The half-life ($t_{1/2}$) or dissipation rate of the chemical in the environment. The lost of 50% of the chemical can be in the form of generation of a breakdown product. Dissipation can be real, generating the breakdown of the parent chemical, or not real, due to absorption or movement of the chemical. Therefore, it is important to study the breakdown products in the same way as the parent chemical.
(3) The natural movement of the chemical in the environment, which can occur by volatilization, leaching, runoff, and food-chain contamination.

(4) The effect of absorption or adsorption, in soil, plants or animals, on the chemical structure.

(5) Food-chain contamination, which occurs when one environment is contaminated by a chemical, and the chemical is released into another environment compartment, with the end result the ingestion of this chemical by animals.

(6) The accumulation and bioaccumulation factors, referring to the build up of the chemical instead of its dissipation\textsuperscript{116}.

**Figure 2.27** illustrates the ways that chemicals can reach the environment. Considering all the variables presented, the study of all the aspects influencing the fate of a dye in the environment is rather complex.

Dyes, synthetic and natural, are present in several household products. They can be discharged from houses, several types of industries (those that produce, extract, and/or apply the dyes), or undergo treatment in a wastewater treatment plant before being released in the environment. Because the interest of this work is on textiles dyes, the focus is on the dyes reaching the environment directly, without going through any treatment, and those released from wastewater treatment plants.
The next section will cover the steps and procedures a dye can undergo once it reaches the wastewater treatment plant, highlighting the oxidation process, chlorination, which is the focus of this work.

2.3.1. Wastewater treatment

Uncontrolled discharge of untreated, domestic and industrial, wastewater contaminates surface and underground water by releasing wastewater with high BOD (biochemical oxygen demand) and COD (chemical oxygen demand). Textile wastewater, for instance, is known to contain strong color, large amounts of suspended solids, highly fluctuating pH and high temperature. The strong color of the wastewater effluent, if not removed can cause disturbance
to the ecological system of the receiving water\textsuperscript{120}, affecting algae photosynthesis for example. Therefore, wastewater treatment is extremely important and is the subject of studies that aim to develop more efficient, cleaner technologies.

According to the Code of Federal Regulations 40 CRF Part 403, regulations were established in the late 1970s and early 1980s to help publicly owned treatment works control industrial discharges to sewers. These regulations were designed to ensure pollutants were properly treated\textsuperscript{121}. Treatment must occur before a municipal or industrial facility can discharge its wastewater into the receiving environment. A scheme for the unit processes for a typical wastewater treatment plant is shown on Figure 2.28.

Conventional wastewater treatment plants provide primary, secondary, and tertiary (or advanced) treatment, depending on their purity level capacity. They employ physical, chemical and biological methods. In primary treatment plants, screening and sedimentation (physical processes) remove a portion of pollutants that will settle or float. Large pollutants that pass-through screening devices are also removed, followed by disinfection. Removal of about 35% of the BOD and 60% of the suspended solids is achieved by primary treatments.

Secondary treatment plants use the same processes employed by primary treatment, with the addition of microbial oxidation of wastes, removing about 90% of the BOD and suspended solids. Advanced treatment processes can be used following primary and secondary treatments or replace these conventional processes completely. They are very specialized and their action depends on the pollutant structure and characteristics. Table 2.6 summarizes the purpose of each step\textsuperscript{122}.  

70
Figure 2.28. Unit processes for wastewater treatment\textsuperscript{122}. 
### Table 2.6. Wastewater treatment processes.

<table>
<thead>
<tr>
<th>Process</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection</td>
<td>Conveys wastewater to treatment plant.</td>
</tr>
<tr>
<td>Screening</td>
<td>Removes debris that could damage equipment.</td>
</tr>
<tr>
<td>Shredding</td>
<td>Alternative to screening; generates smaller solids that can be handled by plant equipment.</td>
</tr>
<tr>
<td><strong>Primary treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Grit removal</td>
<td>Removes gravel, sand and other gritty materials.</td>
</tr>
<tr>
<td>Flow measurement</td>
<td>Provides data for hydraulic and organic loading calculations.</td>
</tr>
<tr>
<td>Preaeration</td>
<td>Reduces odors and corrosion and improves solids separation and settling.</td>
</tr>
<tr>
<td>Primary sedimentation</td>
<td>Removes settleable organic and floatable solids from wastewater.</td>
</tr>
<tr>
<td><strong>Secondary treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Biological treatment</td>
<td>Biological processes that converts dissolved and colloidal organic wastes to more stabled solids leading to further BOD removal.</td>
</tr>
<tr>
<td><strong>Tertiary treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Effluent polishing</td>
<td>Filtration to remove additional BOD.</td>
</tr>
<tr>
<td>Nitrogen removal</td>
<td>Removes nutrients controlling algal blooms in the receiving body.</td>
</tr>
<tr>
<td>Phosphorus removal</td>
<td>Removes limiting nutrients that could affect the receiving body.</td>
</tr>
<tr>
<td>Land application</td>
<td>Used as an alternative to tertiary treatments; reduces BOD, phosphorus and nitrogen compounds.</td>
</tr>
<tr>
<td>Disinfection</td>
<td>Destroys pathogens that might have survived the treatment.</td>
</tr>
<tr>
<td>Dechlorination</td>
<td>Protects aquatic life from high chlorine concentrations.</td>
</tr>
<tr>
<td>Discharge</td>
<td>Treated effluent is released back to the environment.</td>
</tr>
<tr>
<td>Solids treatment</td>
<td>Transforms sludges to biosolids for use as soil conditioners.</td>
</tr>
</tbody>
</table>
2.3.1.1. Oxidation and disinfection

In an oxidation reaction, electrons are transferred from the oxidized species to the oxidizing agent. Oxidation is defined as a loss of electrons, and the higher the oxidation potential of a chemical, the higher is its oxidizing capacity. Fluorine is a stronger oxidant than permanganate, because its oxidation potential is 3.03 V which is higher than the value for permanganate (1.68 V). Besides these two compounds, typical oxidizing agents include ozone, hydrogen peroxide, chlorine gas and Fenton’s reagent. Oxidation processes are applied to wastewater and water treatments to destroy organic matter responsible for color and odor, to reduce COD, destroy ammonia and pesticides, and other applications mainly focused on degradation of organic matter\textsuperscript{123}.

Photochemical methods have been widely researched and employed for the destruction of organic pollutants in wastewater. Hydroxyl radical, the active species, attacks and destroys the undesired compounds. This radical can be produced by homogeneous or heterogeneous processes, such as $\text{H}_2\text{O}_2 + \text{Fe}^0$/Fe (nano-zero valent iron). Homogenous processes, such as UV-$\text{H}_2\text{O}_2$ and UV-$\text{O}_3$, are widely known and in practical use. Another homogeneous reaction, the Fenton reaction, produces hydroxyl radicals by interaction of hydrogen peroxide with ferrous salts:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-$$

In the dark, the reaction is retarded after complete conversion of $\text{Fe}^{2+}$ to $\text{Fe}^{3+}$. However, illumination of the $\text{Fe}^{2+}/\text{Fe}^{3+}/\text{H}_2\text{O}_2$ system increases the degradation rate of many organic substances, i.e. nitrophenols, nitrobenzene, chlorobenzene\textsuperscript{124} and indigo carmine\textsuperscript{125}.

Oxidation usually takes place alongside disinfection. Bacteria are ubiquitous organisms that can be a public health problem regarding potable water treatment, as well as other industrial processes, because bacteria can cause fouling of heat exchange surfaces and blockage of pipework.
Also, there is a public health issue of legionellosis and similar diseases in cooling towers and air conditioning systems.

Disinfection processes are not only applicable to bacteria. In fact, they inactivate virtually all recognized pathogens, but not necessarily all microbial life. It works by killing *Giardia* cysts, bacteria and viruses (primary disinfection), and maintaining a disinfectant residual preventing regrowth of microorganisms.

The first temporary use of chlorination for disinfection purposes dates from 1896, and its first reported continuous use for water supply disinfection occurred in England and Chicago, in 1905. This is the best-known and most commonly used method in the US. Chlorination is also the most suitable and effective method for decolorizing and reducing the COD of waste dyebaths containing azo dyes\textsuperscript{126}.

Chlorine can be applied as a gas (elemental chlorine, Cl\textsubscript{2}), solid calcium hypochlorite or liquid sodium hypochlorite. They all work, in bacteria, by breaking the cell wall, disrupting the cell. Among these methods, gaseous chlorine is the most cost-effective and efficient in terms of available chlorine. Treatment in large scale commonly works by generating chlorine gas from liquid chlorine stored under pressure. Although this method is the most efficient, gaseous chlorine is also the most inherently dangerous method and its use requires extensive safety measures\textsuperscript{123,127}.

All-vacuum chlorinators, Figure 2.29, are probably the most used and safe chlorine feed device. They are installed directly on the chlorine cylinder, making sure chlorine is continuously fed to the influent.

Other than the safety risks associated with the use of chlorine, several studies concerning the formation of chlorination by-products have been reported and will be discussed in further detail.
on in the next section. Because of the safety issues associated with this method and formation of hazardous by-products, in some cases, alternative disinfection methods have been developed\textsuperscript{123}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig29.png}
\caption{All-vacuum chlorinator\textsuperscript{127}.}
\end{figure}

\section*{2.4. Toxicity}

An area of growing concern for the public and scientists is the presence of dyes in the environment and the potential adverse effects these may have. Although significant of work has already been done aiming to understand the adverse environmental effects of, mainly, synthetic azo dyes through either direct measurement, or accurate prediction, of their toxicological effects on diverse organisms, there is still a lack of information regarding natural dyes environmental toxicity. Below is a discussion of a prediction model used by EPA and some studies regarding the toxicity of natural and synthetic dyes.
2.4.1. ECOTOX and ECOSAR

ECOTOX and ECOSAR are tools that were created and are maintained by the US Environmental Protection Agency (EPA). Structure activity relationships (SARs) have been used, since 1981, by EPA to predict the aquatic toxicity of chemicals based on the structural similarity of a compound to chemicals for which the aquatic toxicity has been previously measured\textsuperscript{128}. ECOTOX database is the source for locating single chemical toxicity data for aquatic life, terrestrial plants and wildlife\textsuperscript{129}. The acute toxicity of a chemical to fish (both fresh and saltwater), aquatic invertebrates (daphnids), and green algae has been the focus of the development of SARs. These organisms are group model-organisms and thus not specific species. The SARs are all based on algorithms reflecting a linear regression relationship between the decadic logarithm of the aquatic toxicity of the chemicals used to develop the SAR for the specific chemical class (training set) and the decadic logarithm of their octanol/water-partition coefficients (log $K_{\text{OW}}$)\textsuperscript{130}.

ECOSAR (short for Ecological Structure Activity Relationship) is a computerized predictive system that estimates aquatic toxicity, acute and chronic, to organisms such as fish, aquatic invertebrates and aquatic plants\textsuperscript{131}. It has been developed within the regulatory constrain of the Toxic Substances Control Act (TSCA) and is a pragmatic approach to SAR as opposed to a theoretical approach\textsuperscript{128}. The only input data needed is the SMILES notation of the substance and the related log $K_{\text{OW}}$ value. If the measured log $K_{\text{OW}}$ is not available, the value is calculated by ECOSAR. Based on the SMILES notation, each substance is allocated to a chemical class as defined by ECOSAR. It might happen, however, that due to its molecular structure a substance is assigned to more than one chemical class\textsuperscript{130}.

SMILES (Simplified Molecular Input Line Entry System) is a chemical notation that allows the representation of a chemical structure in a way that can be used by the computer. More
detailed information can be found in Anderson et al.\textsuperscript{132}, but succinctly, SMILES has five basic syntax rules regarding the notation of atoms and bonds, simple chains, branches, rings and charged atoms\textsuperscript{133}.

2.4.2. Aquatic toxicity assessment

A wide variety of dyes has been proved to pose toxicity, mutagenicity and carcinogenicity to aquatic and terrestrial organisms. Bioassays and chemical analysis can be used to determine the degree of pollution in water bodies, being bioassays able to show the total impact of the pollutants\textsuperscript{134}.

2.4.2.1. Dose-response and concentration-response curves

Dose-response (concentration-response) relationships represent the association between dose (concentration) and the incidence of a defined biological effect in an exposed population, which is usually expressed as percentage. The classic dose-response relationship is shown in Figure 2.30. It is important to say that this Gaussian curve is theoretical and practical results can differ from this ideal. From the graphic, LD\textsubscript{50}, the statistically derived single dose of a chemical that can be expected to cause death in 50% of the exposed population, can be obtained. Furthermore, the minimum dose (or concentration) required to produce a detectable response in the test population (the threshold) can be derived, but because this value cannot be obtained with absolute certainty, the LOEL (lowest observed effect level) or the NOEL (no observed effect level) values have been used instead when deriving regulatory standards\textsuperscript{135}. 
2.4.2.2. **Acute toxicity testing**

In toxicology, the word “acute” is used in combination with exposure, toxicity and effect. Acute exposure is a single or very short-lasting dosing by any route\textsuperscript{136}. Acute toxicity studies evaluate the adverse biological effects resulting from a short-term (less than 24 h) exposure to a compound. However, some effects considered to be acute can occur up to as long as 96 hours after exposure. Although this definition is widely used, differentiating between acute and chronic toxicity is a more complex matter because it’s related to the time of sexual maturity and life spans of the studied organisms, which varies from hours to tens of years. From Figure 2.31, it is clear that acute and chronic effects are very different for marine mammals, long-lived fish, crustaceans with long and short generation times, unicellular algae, protozoans, and aquatic bacteria\textsuperscript{137}.

Acute toxicity assessments are the oldest and most common of the toxicity or safety evaluations. They have a prehistoric origin (man determining what was safe to eat by testing plants) and are also the oldest formalized tests. They were the first tests performed to begin the evaluation
of potential hazard and have been perceived as being cheap (in comparison with chronic toxicity tests).\textsuperscript{138}

![Diagram showing generation times and life lengths of organisms](image)

**Figure 2.31.** The minimum generation time (indicated in blue) in comparison with the average life length (shown in pink) of several organisms.\textsuperscript{137}

Most of the regulatory agencies and research organizations use the well-established conventional acute toxicity test with fish and Daphnia, a genus of small planktonic crustaceans, in which survival/death/immobility is the most frequently monitored end point\textsuperscript{134,139}. Daphnids, commonly known as water fleas, are routinely used as standard test organism in aquatic toxicity bioassays (acute and chronic) because of their rapid reproduction, sensitivity to chemical environment, and key ecological role in the aquatic food chain by serving as an intermediate between primary producers and fish. Although Daphnia bioassays results are reproducible, it is difficult to extrapolate the results to other species, community structures and ecosystem functions as all species do not respond identically to the same pollutants showing varying sensitivity.\textsuperscript{134}

Daphnia belongs to the Phyllopoda group (sometimes called Branchiopoda), which are characterized by flattened leaf-like legs. Within the branchiopods, daphnia belongs to the
Cladocera group, whose bodies are enclosed by the carapace, which is an uncalcified shell, Figure 2.32. The carapace is largely made of the polysaccharide chitin. At the end of the abdomen is a pair of claws. The body length of Cladocera ranges from less than 0.5 mm to more than 6 mm and males are distinguished from females by their smaller size, larger antennules, modified post-abdomen, and first legs, which are armed with a hook used in clasping. The genus Daphnia includes more than 100 known species of freshwater plankton organisms found around the world, i.e. *D. similis*, *D. magna*, *D. longispina* and *D. cucullata*. They inhabit most types of standing freshwater except for extreme habitats, such as hot springs. All age classes are good swimmers and are mostly found in the open water. They live as filter feeders, but some species may frequently be seen clinging to substrates such as water plants or even browsing over the bottom sediments of shallow ponds. Adults range from less than 1 mm to 5 mm in size, with the smaller species typically found in ponds or lakes with fish predation\textsuperscript{140}.

OECD 202\textsuperscript{139} describes the acute immobilization test. In this test, the toxic effects on the swimming capability of daphnids is investigated for a range of concentrations of the substance analyzed. Certain concentrations result in certain percentages of Daphnia being no longer capable of swimming at 48 h. *Daphnia magna* is the preferred test species but other suitable Daphnia species can be used as long as the animals are less than 24 h old, in the beginning of the test. Evaluation of *D. similis* as test organisms in ecotoxicological assays has been performed by several researchers, showing satisfying results\textsuperscript{141-143}. The percentage immobility at 48 h is plotted against test concentrations. Data is analyzed by statistical methods to calculate the slope of the curve and the EC\textsubscript{50} with 95% confidence.
Effects associated with acute toxicity commonly consist of mortality or morbidity. From a quantitative point of view, these effects are measured as the LC$_{50}$, EC$_{50}$, LD$_{50}$, or ED$_{50}$. EC$_{50}$ is defined as experimentally derived concentration of test substance in dilution water that is calculated to cause an effect (other than kill) 50% of a test population during continuous exposure over a specified period. Likewise, LC$_{50}$ represents the concentration of the material to which the organisms were exposed that causes mortality in 50% of an exposed population. The LD$_{50}$ and ED$_{50}$ are normalized to the weight of the animal (mg chemical/kg body weight); whereas, LC$_{50}$
and EC$_{50}$ are normalized to the environment in which the organisms were exposed (mg chemical/L water), being that the difference being dose and concentration$^{144}$.

LC$_{50}$ values are used as indicator of relative acute toxicity and are not, by any means, an indicative of an acceptable level of the chemical in the environment. LC$_{50}$ and EC$_{50}$ values are interpreted according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)$^{145}$, as given in Table 2.7. This table also provides the classification of chronic toxicity results, which will be discussed in detail on the next section. Chronic category IV is considered a “safety net” classification used when the available data does not allow classification under the formal criteria, but there are some reasons for concern$^{146}$.

Table 2.7. Relationship between LC$_{50}$ and EC$_{50}$ and toxicity rating$^{145,146}$.

<table>
<thead>
<tr>
<th>Category</th>
<th>Acute Toxicity</th>
<th>Chronic Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category I</td>
<td>96 h LC$_{50}$(fish) ≤ 1 mg/L</td>
<td>Acute toxicity ≤ 1.00 mg/L and lack of rapid degradability and log K$_{ow}$ ≥ 4 unless *BCF &lt; 500.</td>
</tr>
<tr>
<td>Very toxic to aquatic life</td>
<td>48 h EC$<em>{50}$(crustacea) ≤ 1 mg/L 72/96 h ErC$</em>{50}$(aquatic plants) ≤ 1 mg/L</td>
<td></td>
</tr>
<tr>
<td>Category II</td>
<td>96 h LC$_{50}$(fish)c &gt; 1 ≤ 10 mg/L</td>
<td>Acute toxicity &gt; 1 ≤ 10 mg/L and lack of rapid degradability and log K$_{ow}$ ≥ 4 unless BCF &lt; 500 and unless chronic toxicity &gt; 1 mg/L.</td>
</tr>
<tr>
<td>Toxic to aquatic life</td>
<td>48 h EC$<em>{50}$(crustacea) &gt;1 ≤ 10 mg/L 72/96 h ErC$</em>{50}$(aquatic plants) &gt;1 ≤ 1 mg/L</td>
<td></td>
</tr>
<tr>
<td>Category III</td>
<td>96 h LC$_{50}$(fish) &gt;10 ≤ 100 mg/L</td>
<td>Acute toxicity &gt; 10 ≤ 100 mg/L and lack of rapid degradability and log K$_{ow}$ ≥ 4 unless BCF &lt; 500 and unless chronic toxicity &gt; 1 mg/L.</td>
</tr>
<tr>
<td>Harmful to aquatic life</td>
<td>48 h EC$<em>{50}$(crustacea) &gt;10 ≤ 100 mg/L 72/96 h ErC$</em>{50}$(aquatic plants) &gt;10≤ 100 mg/L</td>
<td></td>
</tr>
<tr>
<td>Category IV</td>
<td>-</td>
<td>Acute toxicity &gt; 100 mg/L and lack of rapid degradability and log K$_{ow}$ ≥ 4 unless BCF &lt; 500 and unless chronic toxicity &gt; 1 mg/L.</td>
</tr>
<tr>
<td>May cause long lasting harmful effects to aquatic life</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*BCF=Bioconcentration Factor
2.4.2.3. Chronic toxicity testing

Chronic toxicity usually occurs after repeated or prolonged exposures. However, it can also be observed after single exposures if they develop slowly or are long-lasting. It usually results in a progressive loss of organ function and are often irreversible\textsuperscript{136}.

Several methods have been used to determine the chronic effects of a wide range of compounds to aquatic organisms. The effect of the eight veterinary antibiotics on the reproductive output was assessed in a semi-static test according to the standard protocol for \textit{D. magna} reproduction test, in which the organisms are exposed for a period of 21 days to about six different concentrations of the chemical. Reproductive effects were observed for 4 of those antibiotics, in the range of 5 to 50 mg/L. These antibiotics caused mortality in the parent generation during the long-term (3-week) exposure\textsuperscript{147}. The chronic toxicity of two xanthene dyes, fluorescein sodium salt and phloxine B, to the aquatic cladoceran \textit{D. pulex}, under static conditions, was evaluated in a 10-day study. Day 10 was chosen as the time to census because it was the minimum necessary to guarantee a minimum of two broods for individuals in the control population. Fluorescein elicited greater sublethal effects on \textit{D. pulex} neonates. Following 10-day exposure concentrations of fluorescein at 250 mg/L and below did not show large declines in survivorship. However, exposure at these concentrations did result in substantial declines in the reproductive potential of surviving individuals\textsuperscript{148}.

All chemicals elicit acute toxicity at a sufficiently high dose, but all chemicals do not elicit chronic toxicity. Paracelsus famous phrase “all things are poison; the dose determines a poison” is clearly about acute toxicity. Even the most benign substances will elicit acute toxicity if administered at a sufficiently high dose. However, raising the dose of a chemical does not ensure
that chronic toxicity will ultimately occur. Chronic toxicity typically occurs at dosages below those that elicit acute toxicity\textsuperscript{144}.

One of the standardized tests used is the OECD 201 test\textsuperscript{149}, which can be used to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance over a period of normally 71 h. Despite the relatively brief test duration, effects over several generations can be assessed. Test endpoint is the inhibition of growth, expressed as the logarithmic increase in algal biomass during the exposure time.

\textbf{2.4.3. Natural dye toxicity/mutagenicity}

Although natural dyes are obtained from natural sources, some of them and their dyeing procedures may not be safe, potentially causing health hazard and environmental issues. Metallic mordants used for fixation of natural dyes to the fibers are not always eco-friendly as discussed previously. Furthermore, pesticides may be used in large quantities during the growing of crops, causing further harm\textsuperscript{12}. Toxicity/mutagenicity assessment of natural dyes has been done to provide information regarding adverse effects to human health when used in foods. Most studies present in the literature deals with the mutagenic and carcinogenic effects that natural food dyes have in the human body but, to the author’s best knowledge, very little has been done to understand the effect of these dyes to water organisms and to the environment. Furthermore, there is a need to understand the toxicity of these dyes’ breakdown products. \textbf{Table 2.8} summarizes some results of studies using natural dyes.
Table 2.8. Toxicity/mutagenicity information on some of the most used natural dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Dye class</th>
<th>CAS*</th>
<th>Results</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysophanol CI Natural Yellow 23 (2)</td>
<td>Anthraquinone</td>
<td>481-74-3</td>
<td>Moderately anti-inflammatory\textsuperscript{150}</td>
<td>\textit{in vitro} COX-1 and COX-2 and \textit{in vivo} assay using the carrageenan induced rat paw edema method mouse leukemia L1210 and human leukemia HL-60 cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytotoxic\textsuperscript{151}</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antifungal activity\textsuperscript{152}</td>
<td>\textit{Blumeria graminis} f. sp. hordei</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No mutagenic activity \textsuperscript{153}</td>
<td>TA100 and TA2638 with or without S9 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No mutagenic activity \textsuperscript{153}</td>
<td>TFT-resistance in mouse L5178Y cells, eukaryotic cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity \textsuperscript{154}</td>
<td>Strain TA2637 +S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity \textsuperscript{155}</td>
<td>Strain TA1537 + S9</td>
</tr>
<tr>
<td>Emodin CI Natural Yellow 23</td>
<td>Anthraquinone</td>
<td>518-82-1</td>
<td>Mutagenic activity \textsuperscript{154}</td>
<td>Strains TA2637 +S9 and TA100 +S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Induces apoptosis \textsuperscript{156}</td>
<td>Mouse blastocysts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytotoxic\textsuperscript{157}</td>
<td>Cultured human liver cells (L-02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antiviral activity \textsuperscript{158}</td>
<td>Herpes simplex viruses (HSV-1 and -2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anti-tumor activity \textsuperscript{159}</td>
<td>Chronic myeloid leukemia K562 cell line</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity \textsuperscript{160}</td>
<td>TA 1537 + S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity \textsuperscript{161}</td>
<td>TA1537 + S9</td>
</tr>
<tr>
<td>Substance</td>
<td>Type</td>
<td>Formula</td>
<td>Activity (synthetic compound)</td>
<td>Mutagenic Activity</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------------------------</td>
<td>---------</td>
<td>--------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Lawsone CI Natural Orange 6</td>
<td>Naphthoquinone</td>
<td>83-72-7</td>
<td>Weak mutagenic activity&lt;sup&gt;162&lt;/sup&gt;</td>
<td>TA98 – S9 (S9 prevents mutagenicity) TA2637+ S9, Slightly mutagenic to TA 98+ S9 TA1535, 1537, 98, 100, E.coli WP2 uvrA mouse lymphoma L5178Y tk mutation assay catalase-deficient E. coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity (synthetic compound)&lt;sup&gt;163&lt;/sup&gt;</td>
<td>TA98, TA 98 + S9, TA100, TA100 + S9, TA102+ S9 L-arabinose resistance test of S.typhimurium (BA13, (AuvrB, pKM1O1) glioma cell growth in vivo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No mutagenic activity&lt;sup&gt;164&lt;/sup&gt;</td>
<td>TA 1537+S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slight mutagenic activity&lt;sup&gt;164&lt;/sup&gt;</td>
<td>TA 2637 + S9 TA100 + S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytotoxic&lt;sup&gt;165&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slightly toxic&lt;sup&gt;166&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Islandicin Funiculosin (pigment)</td>
<td>Anthraquinone</td>
<td>476-56-2</td>
<td>Slight mutagenic activity&lt;sup&gt;155&lt;/sup&gt;</td>
<td>TA98, TA 98 + S9, TA100, TA100 + S9, TA102+ S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic activity&lt;sup&gt;154&lt;/sup&gt;</td>
<td>TA98, TA100, TA100 + S9, TA102 + S9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Flavonoid</td>
<td>117-39-5</td>
<td>Increased brain tumor volume&lt;sup&gt;169&lt;/sup&gt;</td>
<td>a bone marrow micronucleus assay and an unscheduled DNA synthesis (UDS)-in vivo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Not genotoxic&lt;sup&gt;170&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Type</td>
<td>Reference</td>
<td>Mutagenic activity</td>
<td>TA98</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>--------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Kaempferol Indigo yellow CI 75640</td>
<td>Flavonol</td>
<td>171, 172</td>
<td>Mutagenic activity</td>
<td>TA98 + S9, TA 100 + S9, TA 102+ S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L-arabinose resistance test of <em>S. typhimurium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dreissena polymorpha zebra mussels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V79 Chinese hamster cells + S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TA98</td>
</tr>
<tr>
<td>Weld Natural Yellow 2 (main component is Luteolin)</td>
<td>Flavonoid</td>
<td>171</td>
<td>Signs of mutagenic activity</td>
<td>TA102+ S9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protective effect (can inhibit oxidative stress and apoptosis induced by Ang II)</td>
<td>Mice peritoneal macrophages</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mutagenic upon nitrosation</td>
<td>TA100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No mutagenic activity</td>
<td><em>S. typhimurium</em> TA1535, TA1538, TA97, TA98, TA100 and TA102</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>S. typhimurium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TA100, TA98 and TA1537 with and without S9</td>
</tr>
<tr>
<td>Rutin CI 75730</td>
<td>Flavonoid</td>
<td>171</td>
<td>Does not prevent reattachment of zebra mussels</td>
<td>Dreissena polymorpha zebra mussels</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weak mutagenic activity; S9 not obligatory for mutagenicity</td>
<td>L-arabinose resistance test of <em>S. typhimurium</em></td>
</tr>
<tr>
<td>Compound</td>
<td>Type</td>
<td>Activity</td>
<td>Additional Information</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------------------</td>
<td>----------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Carminic acid</td>
<td>Anthraquinone</td>
<td>Non-mutagenic activity(^{180})</td>
<td>comet assay and micronucleus test \textit{S. typhimurium} TA98</td>
<td></td>
</tr>
<tr>
<td>Natural Red 4 CI 75410</td>
<td></td>
<td></td>
<td>TA1535, TA1537, TA98 and TA100 + and - S9, chromosome aberrations and sister chromatid exchanges \textit{in vitro} on Chinese hamster ovary cells</td>
<td></td>
</tr>
<tr>
<td>(major component in Cochineal)</td>
<td></td>
<td>Non-genotoxic(^{181})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cochineal</td>
<td>Anthraquinone</td>
<td>Non-genotoxic(^{182})</td>
<td>Mammalian tissue human recombinant cytochrome P450 (CYP) isozymes</td>
<td></td>
</tr>
<tr>
<td>Natural Red 4 CI 75470</td>
<td></td>
<td></td>
<td>SMART of \textit{Drosophila} melanogaster</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toxic(^{183})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Did not suppress the activities of any CYPs examined(^{184})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-genotoxic(^{185})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Madder</td>
<td>Anthraquinone</td>
<td>Non-carcinogenic(^{186})</td>
<td>B6C3F1 mice rats in utero \textit{Drosophila in vivo} DNA repair assay</td>
<td></td>
</tr>
<tr>
<td>Natural Red 8 CI 75470</td>
<td></td>
<td>Non-carcinogenic(^{187})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-genotoxic activity(^{188})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carcinogenic(^{189})</td>
<td>Rat medium-term multi-organ assay \textit{S. typhimurium} TA98 and TA 100 + and - S9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutagenic activity(^{190})</td>
<td>\textit{S. typhimurium} TA98 and TA 100 + and - S9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutagenic activity(^{191})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.8. continued.

<table>
<thead>
<tr>
<th></th>
<th>Mutagenic activity[^192]</th>
<th>UDS assay in primary rat hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fustic Natural Yellow 11 CI 75240</td>
<td>Prevents acute liver damage[^193]</td>
<td>Rat’s liver</td>
</tr>
<tr>
<td></td>
<td>Flavonol 90-34-6</td>
<td>Increased liver weight in males; no mortality[^194]</td>
</tr>
</tbody>
</table>

[^192]: CAS for the major component identified.
2.4.4. Synthetic dyes toxicity

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, and leather industries. Among the several classes of synthetic dyes, the azo class is the largest, accounting for 60-70% of the total annual production. Approximately, 40,000 different dyes and pigments are used industrially, and more than 2,000 different azo dyes are currently used, with a total production worldwide of $7 \times 10^5$ tons. A large amount of azo dyes is used in the textile industry, with about 10% of the dyes used in the dyeing processes released to the environment.

Due to its wide application, the azo class is the most studied regarding toxicity evaluation. As an example, the acute toxicity of copper containing azo dye CI Direct Blue 218, Figure 2.33, A, to *D. magna* was evaluated. LC$_{50}$ was determined to be 3.6-6 mg/L, showing the toxicity of the dye to the daphniids, and suggesting potential damage to every receptor ecosystem. This toxicity is probably due to the copper present in the dye structure. Some heavy metals, including copper, are essential for many organisms, but they can also be very toxic when in present in excess.

Furthermore, sludge from wastewater treatment plants are frequently applied to agricultural fields, which may result in significant concentrations of dyes in agricultural soils, reducing the its fertility. Generally, ionic azo dyes released into surface waters or wastewater are expected to bind primarily to suspended organic matter due to electrostatic interactions and ultimately sequester to sediments or wastewater sludge. However, a proportion of ionic dyes will likely remain dissolved in the water column due to their relatively high water solubility, presenting, therefore, a risk to aquatic animals that may be exposed to azo-contaminated sediment and/or water. In a series of acute toxicity tests, involving 46 azo and other organic dyes, to fathead minnows (*Pimephales promelas*), the most toxic azo dyes were CI Acid Blue 113 (LD$_{50}$, 4 mg/L), CI Basic Brown 4 (5.6 mg/L), and CI
Mordant Black 11 (6 mg/L), Figures 2.33 B-D. CI Acid Red 97, a benzidine-based azo dye, Figure 2.33 E, and Bismarck Brown Y (BBY), Figure 2.33 F, were not lethal to *Silurana tropicalis* embryos, which were exposed to sediment spiked with these dyes. However, BBY significantly induced malformations in 14-16% of frog larvae.

![Structures of synthetic dyes](image)

**Figure 2.33.** Structures of synthetic dyes (A) CI Direct Blue 218, (B) CI Acid Blue 113, (C) CI Basic Brown 4, (D) CI Mordant Black 11, (E) CI Acid Red 97 and (F) Bismarck Brown Y.

Studies concerning azo dyes toxicity may be the most abundant in literature, but they are certainly not the only class of synthetic dyes examined by researchers. Although some studies have indicated that most textile dyes do not exhibit significant toxicity to microbial populations, triphenylmethane dyes like CI Basic Violet 1 (Figure 2.34 A) were toxic to the fresh microbiota organisms tested, *Bacillus cereus* and *E. coli*. The dye toxicity was evaluated by determining the percent survival of microbiota when the dye was incorporated into plating media. CI Basic Violet 3 (Figure 2.34 B) had a mean survival rate of 20.7± 6.57% at a dye concentration of 5 mg/L.
Furthermore, the dye had a high sorption value (measure by the partition coefficient for live and killed cells, $K_d$) for viable cells, which suggests that a metabolically active process may be involved in the sorption\textsuperscript{198}.

CI Disperse Red 1 and CI Disperse Red 13, Figure 2.34 C-D, both azo dyes, were acutely toxic to *D. similis*, with EC$_{50}$ values of 127 µg/L and 18.7 µg/L, respectively\textsuperscript{142}. Remazol Blue RR, an anthraquinone reactive dye, showed highly toxicity to *D. magna*\textsuperscript{199}. Remazol Brilliant Blue R and CI Disperse Blue 3, Figure 2.34 E-F, two anthraquinone dyes, inhibited luminescence for the bacteria *V. fischeri*, after 30 min of exposure. This “flash” bioluminescence test is a kinetic application of luminescent bacteria test especially tailored for measuring the toxicity of solid and colored samples. CI Disperse Blue 3 was also toxic to the microalgae *S. capricornutum*\textsuperscript{200}.

![Figure 2.34. Structures of synthetic dyes (A) CI Basic Violet 1, (B) CI Basic Violet 3, (C) CI Disperse Red 1, (D) CI Direct Red 13, (E) Remazol Brilliant Blue R, (F) CI Disperse Blue 3.](image)

Furthermore, substances reaching sewage and drinking water treatment plants may undergo different reactions, resulting in partial or complete transformation and/or degradation of the parent compound. Sometimes degradation is not complete and intermediates can even be more
stable and have different toxic, mutagenic and accumulation properties than the parent compounds\textsuperscript{201}.

Traditionally, chlorination is one step used worldwide in the wastewater treatment plant for water disinfection purposes, as previously mentioned. On the other hand, the main disadvantages are related to the formation of disinfection by-products and to chlorine phytotoxicity\textsuperscript{202}. The presence of chlorinated mutagenic by-products in the Cristais River Drinking Water Treatment Plant was detected and evaluated by Oliveira et.al (2006)\textsuperscript{203} through the study of the black dye commercial product composed of CI. Disperse Blue 373, CI. Disperse Orange 37, CI. Disperse Violet 93, Figure 2.35. Authors also observed mutagenic activity in the \textit{Salmonella/microsome} assay. In another study, chlorination of CI. Disperse Red 1, known to exist as a complex mixture, generated four aromatic degradation products, which were also ecotoxic\textsuperscript{204}.

Figure 2.35. Structure of synthetic dyes (A) CI Disperse Blue 373, (B) CI Disperse Orange 37, (C) CI Disperse Violet 93.
CHAPTER 3. The effect of chlorination on natural dyes

3.1. Environmental properties of natural dye madder (CI Natural Red 8): Effect of chlorine-based decolorization

3.1.1. Abstract

There has been increased interest in the use of natural dyes for textile coloration as alternatives to synthetic ones that are toxic to a variety of organisms. However, natural dyes have poor affinity for textiles, which can lead to a high dye amount in the resultant wastewater and the need for treatment to remove color. Conventional biological treatment is inefficient for color removal, and as a consequence dyes can remain in the environment. Chlorine treatment has proven to be efficient and economical for wastewater disinfection and decolorization. For synthetic dyes, this process can lead to the formation of toxic products. However, little information is available concerning the effects of natural dyes on the environment, as well as the effect of chlorination of wastewater containing natural dyes. The aim of this study was to assess the ecotoxicity and mutagenicity of the well-known natural dye Madder (used as a prototype dye), and to evaluate the effect of chlorine treatment on dye properties, as a step towards understanding natural dyes environmental fate. Madder was analyzed by LC-ESI/MS and the results showed the presence of Alizarin as the main component (89.8%). An aqueous solution of the dye was treated with Cl₂(g) until total decolorization, which was followed by UV-Vis, HPLC-DAD, and ESI-MS. Madder was verified as toxic to aquatic life but non-mutagenic. Chlorination decreased toxicity to *D. similis* but the solution was still considered harmful to aquatic life (Acute II). Furthermore, there was no evidence from the *Salmonella/microsome* that shows any mutagenic activity from the chlorinated
products. Two of the degradation products generated after chlorination were identified as phthalic anhydride and 2-hydroxynaphtalene-1,4-dione by using high-resolution mass spectrometry.

3.1.2. Introduction

Natural dyes were the only source of color to several subtracts for centuries. They were widely used in the textile dyeing until the accidental discovery of the Mauveine dye by William Perkins, in 1856\textsuperscript{205}. With this discovery, synthetic dyes started to be accepted through the world, and by 1880 most natural dyes were replaced by synthetic dyes on an industrial scale\textsuperscript{21,205}. Natural dyes had the disadvantage of needing a mordant for their application to textile fibers, due to their general poor affinity, whereas synthetic dyes showed higher reproducibility of the shade, wider range of colors and overall easiness of application.

In the early days of synthetic dyes, the discussions about their use were mainly related to performance and costs, as the industrial age was not yet concerned with environmental pollution and the potential adverse effects to human health\textsuperscript{206}. Later on, the connection between certain types of cancer and exposure to early aniline-based dyes became clear. In fact, bladder cancer was observed in workers involved in the manufacture of those dyes. At that time, an epidemiological examination of human male bladder cancer deaths during a 30-year period, among a group of azo dye workers and a control group, provided evidence for such correlation\textsuperscript{207}.

Additionally, concerns about the effects of synthetic dyes on aquatic organisms, and consequently to the environment, started to arise. Although their fixation to the fiber is generally higher than that of natural dyes, there is still a percentage of unfixed dye released to the environment. As an example of the dimension of the problem, approximately 900,000 metric tons of dye are produced annually. It is known that 17-20\% of the industrial waste is comprised of dye
effluents and up to 50% of the total annual dye production reaches the environment directly, as effluent, or indirectly, as losses occurring during dyeing processes\textsuperscript{208}.

Studies showing the toxicity of anthraquinone synthetic dyes to different aquatic organisms have been previously reported\textsuperscript{199,200}. Concerns with synthetic dye toxicity/mutagenicity are accentuated by the additional processes the discharged dye can undergo. Substances reaching sewage and drinking water treatment plants can undergo different reactions, which may result in partial or complete transformation and/or degradation of the parent compound. When degradation is incomplete, intermediates generated can be more stable and have different toxic, mutagenic and accumulation properties than the parent compound\textsuperscript{196,201,209}. One of these reactions is chlorination, which is used worldwide in wastewater treatment plants for water disinfection purposes. This is the best-known and most common disinfection method used in the USA. Chlorine can be applied as a gas (elemental chlorine, Cl\textsubscript{2}), which is cost-effective and efficient in terms of available chlorine\textsuperscript{123,127}. However, this procedure has been associated with the formation of toxic degradation products. The presence of mutagenic by-products resulting from the chlorination of the commercial black dye, composed of C.I. Disperse Blue 373, C.I. Disperse Orange 37, and C.I. Disperse Violet 93, was detected in water from the Cristais River Drinking Water Treatment Plant, in Brazil\textsuperscript{203}. In another study, chlorination of C.I. Disperse Red 1 generated four aromatic degradation products, which were also ecotoxic\textsuperscript{204}.

The increase of data regarding the toxic and mutagenic effects of several synthetic dyes to a variety of organisms\textsuperscript{8-10}, led to a revival of interest in the use of natural dyes as alternatives to synthetic ones. Among these natural dyes is the orange-reddish Madder (CI Natural Red 8), extracted from dried roots of the \textit{Rubia tinctorium L}, that has been used since ancient times to color silk, wool and cotton, producing shades from pinks to blacks, depending on the mordant
used14. Madder is composed of a mixture of thirty-six identified anthraquinone derivatives7,205. However, the major component found in the dye depends on the substance from which extraction occurred7.

Although there are several studies reported in the literature on the potential toxicity of madder to human health189,190,192,210, little information is available concerning its environmental fate, ecotoxicity, and behavior upon chlorination treatment. Therefore, in this study, evaluation of the ecotoxic/ mutagenic potential of madder and its chlorinated by-products was evaluated, as a step towards understanding the environmental fate of this anthraquinone natural dye.

3.1.3. Experimental

3.1.3.1. Chemicals

Madder (CI Natural Red 8), was obtained from the Pilot Plant at North Carolina State University. Solvents used for HPLC-DAD and LC-MS analysis were all HPLC grade, bought from Fisher Scientific®.

3.1.3.2. Methods

3.1.3.2.1. Madder chlorination procedure

A 100 mg/L aqueous Madder solution was treated with chlorine gas generated from the dropwise addition of 20 mL of 12 M HCl to 3 g of potassium permanganate (KMnO4). Chlorine gas was bubbled into 250 mL of the stirred dye solution. The apparatus used is shown in Figure 3.1. Color removal was monitored by UV-Vis spectrophotometry204. After 60 min of chlorination, the amount of residual free chlorine was determined using the N,N’-diethyl-para-phenylenediamine (DPD) method211.
The redox reaction leading to the generation of chlorine gas is shown below:

$$2 \text{KMnO}_4(s) + 16 \text{HCl}(l) \rightarrow 2 \text{KCl}(aq) + 2 \text{MnCl}_2(s) + 5 \text{Cl}_2(g) + 8 \text{H}_2\text{O}(l)$$

3.1.3.2.2. Ecotoxicity tests

The effect of Madder and its chlorinated solution at different trophic levels, toxicity to *Daphnia similis* and algae *Raphidocellis subcapitata* was evaluated. Chlorinated solutions were tested after no residual free chlorine was detected.

The acute toxicity to *D. similis* was evaluated according to OECD 202 guidelines\textsuperscript{139}. The madder stock solution was prepared in water containing 0.1% DMSO to aid dissolution. Chlorinated dye concentrations were expressed in percentages. In each replicate (4 replicates total), five neonate organisms, 6-24h old, were exposed to the test solutions for 48h, at 20 °C ± 2 °C, with a light intensity of 1000 lux under photoperiod (16:8 light/dark), without feeding. After the 48h exposure period, the number of immobilized organisms was counted. The tests were
considered valid if the immobilization rate was less than 10% in the negative control group. Results presented in Figure 3.17 A were statistically analyzed using the trimmed Spearman–Karber method for estimating the median immobilization concentration, EC$_{50}^{212}$.

For the algae *Raphidocellis subcapitata* toxicity test, three replicates of each concentration of madder and chlorinated Madder solutions were analyzed. The amount of algal biomass added was calculated according to the OECD 201 guidelines$^{149}$. To each test solution, 45 mL of growth medium was added to the needed amount of dye solution and sterile water to achieve the desired concentration for a final volume of 50 mL. After the 72-hour exposure period, at 22 °C ± 2 °C, with a light intensity of 1000 lux, the number of cells was counted. The test was considered valid if cellular growth in the control solution was at least 16 times. The endpoint IC$_{50}$ (median inhibition concentration) was determined statistically using analysis of variance and the results are presented in Figure 3.17 B.

### 3.1.3.2.3. The *Salmonella*/microsome assay

The mutagenicity of the natural dye and its chlorinated solution was evaluated using the *Salmonella*/microsome assay according to the ISO 16240:2005 guidelines$^{213}$. *S.typhimurium* strains TA98 (*hisD3052, rfa, Abio, ΔuvrB*, and pKM101) and TA100 (*hisG46, rfa, Abio, ΔuvrB*, pKM101), with and without exogenous metabolic activation (S9), were used. Metabolic activation was provided by Arochlor-1254- induced rat liver S9 fraction (Sprague Dawley), which was prepared at a concentration of 4% (v/v). For both strains, positive controls were 4-nitroquinoline-1-oxide (4NQO) (Sigma–Aldrich) at 0.5 μg/plate and 2-aminoanthracene (2AA) (Sigma–Aldrich) at 2.5 μg/plate, both dissolved in dimethylsulfoxide. Sterile water was used as the negative control. The test was performed using a single dose for each treatment. For each plate, 0.5 mL of sodium phosphate buffer or S9 mix and 1 mL of sample solution were added.
Sample solutions were sterilized by filtration, using a 0.45 μm filter. A single dose was tested, according to the maximum solubility of the dye, and using three replica plates. After 66 h incubation at 37°C, colonies were automatically counted. Results are expressed as the number of revertants/plate. Samples were considered positive when significant ANOVA (p<0.05).

3.1.3.2.4. Chemical characterization of Madder and its chlorinated solution

HPLC-DAD aided the assessment of Madder degradation by chlorination. Separation of products was performed using a C18 Waters column (150 x 3.9 mm), flow rate was 1 mL/min and the volume of sample injected was 10 μL. Solvents 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile (ACN) were used in a gradient elution: 0-3 min: 15% ACN, 3-10 min: 15-45% ACN and 10-15 min: 45% ACN.

Further characterization was done using mass spectrometry. Analyzes were carried out using two instruments. For Madder characterization, a high-resolution mass spectrometer (Thermo Fisher Scientific Exactive Plus MS, a benchtop full-scan Orbitrap™) using Heated Electrospray Ionization (HESI). Samples were analyzed via LC injection into the mass spectrometer at a flow rate of 250 μL/min. The mobile phase B was acetonitrile with 0.1% formic acid and mobile phase A was water with 0.1% formic acid. The mass spectrometer was operated in negative ion mode. Gradient elution was employed as follows: 0-3 min: 15% B, 3-10 min: 15-45% B, 10-15 min: 15% B and 15-20 min: 15% B. HESI source parameters were: spray voltage 3.5 kV, capillary temperature 350°C, heater temperature 300°C, and S lens RF level 70 V, sheath gas flow rate 60, resolution 70,000 and scan range 50-750 m/z. The chlorinated solution was analyzed in the Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer using Electrospray Ionization (ESI) in positive and negative mode. Instrument parameters were: gas temperature 350°C, drying gas 10 L/min, nebulizer 30 psi, fragmentor voltage 175 V, capillary voltage 3500V.
Madder chlorinated products were isolated by flash column chromatography as follows. In a glass column of 2.5cm diameter x 60 cm length, glass wool, sand (quartz, SiO₂, sea washed catalog 525500 Fisher, CAS 14808-60-7) was applied in the bottom and then the column was filled with silica gel (Selecto Scientific, Georgia, USA, particle size 32-63mm, catalog 132824, lots 306273301; 306082201) and then sand was again added. Sample was prepared by mixing 0.5128 g of the chlorinated dye with 10 mL of methanol and 1 g of silica gel. After drying, the mixture was applied to the top of the column and sand was added again. The solvent mixture, chloroform/methanol/water (65:30:5) was used to carry the elution under pressure.

Since the silica gel used to prepare the column did not have the fluorescent UV indicator, separation could not be observed under UV light unlike for the case for the TLC. Therefore, 9 fractions, were collected and analyzed by HPLC-DAD, Figure 3.11

3.1.4. Results and Discussion

3.1.4.1. Madder characterization

LC/MS analysis of madder revealed Alizarin (m/z 239.03482, mass error of -1.59 ppm) and Purpurin (m/z 255.02980, mass error of -1.76 ppm) as the major components (Figure 3.2), with 89.8% of the sample comprised of Alizarin.
3.1.4.2. Chlorination of natural dye Madder

The degradation of the anthraquinone chromophore was evidenced by the disappearance of the absorption band at $\lambda_{\text{max}} = 430$ nm (Figure 3.3 A). After 60 min of treatment, the solution was colorless. HPLC-DAD, Figure 3.3 B, also confirmed this observation, as the peak correspondent to Alizarin, retention time ($r_t$) 11.85 minutes, virtually disappeared.
Chlorine decolorization of Madder solution was evident from disappearance of the absorption band at $\lambda_{\text{max}} = 430$ nm in the UV-Vis spectrum and drastic reduction in the intensity of the alizarin peak in the HPLC-DAD chromatogram.

Similarly, the MS spectrum confirmed the loss of the characteristic peak ($m/z$ 239.0334) for Alizarin, Figure 3.4, and Figure 3.4 B shows the appearance of two peaks, $m/z$ 178.9903 and $m/z$ 212.8924, that could not be identified. However, phthalic anhydride ($m/z$ 149.0229), and 2-hydroxynaphthalene-1,4-dione ($m/z$ 173.0250), Figure 3.5, were identified as two of the degradation products.
Figure 3.4. (-)ESI/MS spectra comparing (A) Madder and (B) its chlorinated solution.
Figure 3.5. ESI/MS spectra showing the two identified degradation products (A) Phthalic anhydride (B) 2-hydroxynaphthalene-1,4-dione, obtained from Madder exposure to chlorine gas.
3.1.4.3. Further analysis for the identification of Madder chlorinated products

Further analyzes were performed in attempts to identify the compounds corresponding to the peaks observed in the chlorinated Madder spectrum, Figure 3.4 B.

3.1.4.3.1. Isolation of Madder chlorinated compounds by Thin Layer Chromatography (TLC) and column chromatography

Because Madder chlorinated products could not be identified when analyzing the final product mixture, TLC and column chromatography were used to isolate each compound prior to HPLC-DAD and LC-MS analysis, in an effort to facilitate the identification. This section contains results obtained following isolation of the products using thin layer chromatography and flash column chromatography.

3.1.4.3.1.1. Optimization of TLC elution system

TLC eluent optimization was performed, Table 3.1, to determine the best conditions to separate the Madder components and the degradation products in the chlorinated solution. Silica gel plates (TLC Silica Gel 60 F254, EMD Chemicals, Germany) were used. Madder samples were solubilized in methanol and spotted on the plates.

**Table 3.1.** Solvent systems tested for the analysis of Madder.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Madder</td>
<td>Chloroform/ Methanol (95:5)(^{214})</td>
</tr>
<tr>
<td></td>
<td>Hexane/Ethyl acetate (1:1)</td>
</tr>
<tr>
<td></td>
<td>Acetic acid/Methanol/Water (3:3:4)(^{215})</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate/THF/water (6:35:47)(^{74})</td>
</tr>
<tr>
<td></td>
<td>Methanol/ 10% aqueous Acetic acid (80:20)(^{216})</td>
</tr>
<tr>
<td></td>
<td>Butanol/DMF/ NH(_4)OH (8:4:7)</td>
</tr>
<tr>
<td></td>
<td>Chloroform/ Acetic acid (9:1)</td>
</tr>
</tbody>
</table>
The best separation of chlorinated Madder components was obtained using chloroform/methanol/water (65:30:5) as the mobile phase. Preparative TLC was also performed using the same system. Good separation of the chlorination products was also achieved using reverse phase silica plates (R18) as the stationary phase and 15% Acetonitrile/85% (aqueous 0.1% Formic acid) as the mobile phase, however this was not the preferred method due to the high cost of the R18 TLC plates.

### 3.1.4.3.1.2. TLC and ESI/MS analysis of Madder chlorinated degradation products

A Madder solution was chlorinated following the procedure described in section 3.2.3.2.1. The water in the resulting solution obtained was evaporated, the solid was resuspended in methanol and applied to a TLC plate. Five main fractions can be observed under UV light (254 nm), Figure 3.6, after developing the plate. Preparative TLC (not shown) was performed for the isolation of these 5 fractions, R<sub>f</sub> (retention factors) 0, 0.27, 0.53, 0.55 and 0.93.

Individual fractions were analyzed by ESI/MS, in both negative, Figure 3.8, and positive (not shown) modes. The mass spectra for fractions 1, 2, 3, and 4, with R<sub>f</sub> 0, 0.27, 0.53, and 0.55, respectively, in both ionization modes, positive and negative, did not show any of the expected ions, the ones not identified in Figure 3.4 B as well as other ions presented in Table 3.2. For
fraction 5, R_t=0.93, a peak with m/z 257.0445 was detected, with the proposed molecular formula C_{14}H_{10}O_{5} and possible chemical structures shown (ppm error 1.95) in Figure 3.7.

Figure 3.6. Chlorinated Madder solution (circled) separated on a silica gel plate and chloroform/methanol/water (65:30:5) mobile phase, detection under UV light (254 nm).

Figure 3.7. Proposed chemical structures for the chlorinated compound with m/z 257.0445.
Figure 3.8. (-) ESI/MS of fractions Rf 0 (B), 0.27 (C), 0.53 (D), 0.55 (E) and 0.93 (F), obtained by TLC for a 100 mg/L aqueous chlorinated Madder solution. A is the background spectra.
Considering the degradation compounds shown in Figure 3.5 and Figure 3.8 F, and based on the results obtained and literature observations for the decomposition of two synthetic anthraquinone dyes, Figure 3.9\textsuperscript{217-219}, a projected pathway of madder degradation, based on its major component Alizarin, upon chlorination was proposed, Figure 3.10. The obtained results suggest that chlorine promotes discoloration by cleaving Alizarin bonds and not by addition or substitution reactions.

![Anthraquinone dyes](image)

**Figure 3.9.** Anthraquinone dyes, Reactive Blue 19 (on the left) and Acid Blue 62 (on the right), used in decolorization studies.

<table>
<thead>
<tr>
<th>Expected product</th>
<th>MW (g/mol)</th>
<th>Expected Ions observed in first order MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{14}H\textsubscript{8}O\textsubscript{4} (Alizarin)</td>
<td>240.21</td>
<td>[M-H]\textsuperscript{−} = 239.04</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{8}O\textsubscript{5} (Purpurin)</td>
<td>256.21</td>
<td>[M-H]\textsuperscript{−} = 256.04</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{7}ClO\textsubscript{4}</td>
<td>274.66</td>
<td>[M-H]\textsuperscript{−} = 273.00</td>
</tr>
<tr>
<td>C\textsubscript{13}H\textsubscript{8}O\textsubscript{4}</td>
<td>228.20</td>
<td>[M-H]\textsuperscript{−} = 227.03</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{4}Cl\textsubscript{4}O\textsubscript{5}</td>
<td>393.98</td>
<td>[M-H]\textsuperscript{−} = 390.88</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{6}O\textsubscript{5}</td>
<td>254.20</td>
<td>[M-H]\textsuperscript{−} = 253.02</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{10}O\textsubscript{7}</td>
<td>290.23</td>
<td>[M-H]\textsuperscript{−} = 289.04</td>
</tr>
<tr>
<td>C\textsubscript{14}H\textsubscript{22}O\textsubscript{7}</td>
<td>206.33</td>
<td>[M-H]\textsuperscript{−} = 205.17</td>
</tr>
<tr>
<td>C\textsubscript{8}H\textsubscript{4}O\textsubscript{3}</td>
<td>148.12</td>
<td>[M-H]\textsuperscript{−} = 147.02</td>
</tr>
<tr>
<td>C\textsubscript{10}H\textsubscript{10}O\textsubscript{4}</td>
<td>194.19</td>
<td>[M-H]\textsuperscript{−} = 193.06</td>
</tr>
<tr>
<td>C\textsubscript{7}H\textsubscript{6}O\textsubscript{2}</td>
<td>122.12</td>
<td>[M-H]\textsuperscript{−} = 121.04</td>
</tr>
</tbody>
</table>

**Table 3.2.** Molecular weight and expected ions observed in (-) ESI, first order MS for major components in Madder, Alizarin and Purpurin, and for expected madder chlorination products.
The chromatograms obtained for the analysis of the fractions collected from column chromatography showed the disappearance of the Alizarin peak (retention time 11.66 min) over time and the appearance of a new peak, identified at $\lambda=254$ nm, with retention time 10.38 minutes, Figures 3.11 (B-I). Fractions B, F and I were also analyzed by LC-ESI/MS and the results did not present any relevant information towards the compound identification.
Figure 3.11. Chromatograms, at 254 nm, of fractions A-I (first to last eluted, respectively) of a 100 mg/L chlorinated Madder solution, separated by column chromatography.
3.1.4.3.2. Comparison between chlorinated Madder and chlorinated Alizarin solutions

As mentioned in the background information section, depending on the source, madder can contain a variety of substituted anthraquinones\textsuperscript{220}. Therefore, identifying the degradation pathway following chlorination of the dye solution is a complex process. The strategy was to narrow down the possibilities for the final breakdown products, by comparing the chromatograms, Figure 3.12, and MS full scan spectra obtained for chlorinated madder to the one obtained for chlorinated alizarin, Figure 3.13. Chlorination process for alizarin was the same used for madder.

![Figure 3.12](image)

**Figure 3.12.** Comparison between the chromatograms obtained for (a) Madder, (b) chlorinated Madder, (c) Alizarin and (d) chlorinated Alizarin, at 254 nm.

Chromatogram profiles are the same for chlorinated madder and chlorinated alizarin, Figure 3.12 (b) and (d), suggesting that the degradation products observed for madder resulted from the degradation of Alizarin. Both MS spectra have the same profile, Figure 3.13, except by a few ions, such as the ones with \textit{m/z} 212.8924 and \textit{m/z} 214.8883 present in chlorinated madder. Analysis of the fragmentation pattern of ion with \textit{m/z} 212.8924, Figure 3.14 A, showed the loss
of a chlorine, peak \( m/z 177.9215 \) \([M-\text{Cl}]^-\), and a carbonyl, peak \( m/z 149.9271 \) \([M-35-\text{CO}]^-\). Samples were also run in the positive mode (spectra not shown) and the same trend was observed. Both samples had the same spectra except for the ion \( m/z 196.1092 \) present in chlorinated madder, suggesting these compounds originated from the degradation of another compound present in madder.

![ESI spectra for chlorinated Alizarin (A) and Madder (B)](image)

**Figure 3.13.** ESI spectra obtained for A (chlorinated Alizarin) and B (chlorinated Madder), in the negative mode.

It is important to mention that some degradation products containing one chlorine atom were detected in Alizarin and Madder chlorinated solutions. **Figure 3.14** shows the fragmentation pattern for two of those ions, and **Figure 3.15** presents other spectra showing the presence of the peaks with the 3:1 isotopic ratio characteristic for a monochlorinated compound\(^{221}\).
ESI-MS/MS for ions with (A) \( m/z \) 212.8924 and (B) \( m/z \) 214.8883 detected in chlorinated Madder solution.

Figure 3.14. (-) ESI-MS spectra showing the presence of two monochlorinated compounds, one with \( m/z \) 144.9698 in chlorinated Madder (A), and the other with \( m/z \) 145.0260 in chlorinated Alizarin (B).

Figure 3.15. (-) ESI/MS spectra showing the presence of two monochlorinated compounds, one with \( m/z \) 144.9698 in chlorinated Madder (A), and the other with \( m/z \) 145.0260 in chlorinated Alizarin (B).

Figure 3.15 also shows that the monochlorinated compound generated (\( m/z \) 144.9698) is not due to the chlorination of Alizarin, as the same peak is not observed in the Alizarin spectrum.

Figure 3.15 B. Table 3.3 lists other monochlorinated compounds detected in Alizarin and Madder.
chlorinated solutions. Some of the compounds observed in Madder chlorinated solution arise from the chlorination of Alizarin.

Although present, some of these peaks have low relative intensity. Furthermore, they may represent products involving the minor anthraquinone components present in the parent dye Madder. Isolation of each compound and further characterization, by LC-MS/MS and NMR for example, would be necessary to elucidate the chemical structure of each compound.

Table 3.3. Monochlorinated ions detected in Madder and Alizarin chlorinated solutions.

<table>
<thead>
<tr>
<th>Ions observed in first order ESI-MS</th>
<th>Chlorinated Madder</th>
<th>Chlorinated Alizarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M-H]^− = 144.9698</td>
<td>[M-H]^− = 145.0260</td>
<td></td>
</tr>
<tr>
<td>[M+H]^+ = 345.0138</td>
<td>[M+H]^+ = 345.0138</td>
<td></td>
</tr>
</tbody>
</table>

3.1.4.3.3. Chlorination of Madder under different conditions

Chlorination was performed by varying the time in which the dye was exposed to chlorine gas generated, to help characterize products formed during the early stages of the chlorination process. The hypothesis was that madder was being degraded quickly and its degradation products were also being degraded, making it difficult to detect and identify the compounds generated. Controlling the level of degradation could allow the detection and identification of the initial degradation products.
In the first experiment, 250 mL of a chlorinated Madder solution was obtained by treating 100 mg/L aqueous Madder solution with chlorine gas generated from the dropwise addition of 4.5 mL of 12 M HCl (around 0.5 mL/min) to 3 g of potassium permanganate (KMnO₄), for 10 min. Sample was stirred for 48 h to remove the unreacted chlorine and concentrated. This solution was labeled (B 103).

Next, solution B103a was generated by reacting 100 mg/L aqueous Madder solution with chlorine gas generated from the dropwise addition of 10 mL of 12 M HCl (0.5 mL/min) to 0.6 g of potassium permanganate (KMnO₄). Chlorine gas was bubbled into 250 mL of the dye solution, for 20 min. Solution B105 was obtained by treating 100 mg/L aqueous Madder solution with chlorine gas generated from the dropwise addition of 20 mL of 12 M HCl (0.5 mL/min) to 1.5 g of potassium permanganate (KMnO₄). Chlorine gas was bubbled into 250 mL of the dye solution, for 40 min. Both samples were stirred for 48 h to remove the unreacted chlorine and concentrated. The three samples were analyzed by ESI-MS, Figure 3.16 (A-C).
Figure 3.16. (-) ESI/MS spectra for chlorinated Madder solutions (A) B103, (B) B103a and (C) B105.
In the redox reaction used for the production of chlorine gas, shown below, chlorine ion \((\text{Cl}^-)\) is oxidized to \(\text{Cl}_2\) (oxidation number 0), and manganese is reduced, from \(\text{Mn}^{7+}\) to \(\text{Mn}^{2+}\). In this process, 2 mols of \(\text{KMnO}_4\) react with 16 mols of \(\text{HCl}\) generating 5 mols of chlorine gas. Therefore, considering the amount of \(\text{KMnO}_4\) used, the amount of chlorine gas generated in each reaction for the production of solutions B103, B103a and B105 was 3.36g, 0.6736g and 1.6822g, respectively.

\[
2 \text{KMnO}_4(s) + 16 \text{HCl}(l) \rightarrow 2 \text{KCl}(aq) + 2 \text{MnCl}_2(s) + 5 \text{Cl}_2(g) + 8 \text{H}_2\text{O}(l)
\]

In general, the peaks observed in Figure 3.16, were the same observed for chlorinated samples B103, B103a and B105, \(m/z\) 276.9967, \(m/z\) 241.0193 and \(m/z\) 156.9725, for example. However, some differences are worth mentioning. Sample B103a still had not degraded alizarin \((m/z\ 239.0379)\), Figure 3.16 B, and B105 showed the presence of a new peak \(m/z\ 173.0250\), identified as 2-hydroxynaphtalene-1,4-dione, \(\text{C}_{10}\text{H}_6\text{O}_3\), Figure 3.16 C.

Alizarin was not completely degraded when the amount of gas involved a nearly 5-fold decrease (3.36g for B103 to 0.6736g for B103a) but the reaction time was doubled. Furthermore, when sufficient \(\text{Cl}_2\) gas was present and enough reaction time was allowed (B105), dye degradation was efficient (disappearance of the alizarin peak), and additional degradation compounds could be identified.

3.1.4.4. Ecotoxicity assessment

Madder and its chlorinated solution were tested for acute toxicity to \(\text{D. similis}\), giving \(\text{EC}_{50}\) values of 4.4 (4.1-4.7, 95% confidence interval) and 45 (39-53, 95% confidence interval) \(\text{mg L}^{-1}\), respectively (Figure 3.17 A). \(\text{EC}_{50}\) for the chlorinated dye solution is expressed as equivalent concentration. According to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), Madder is classified as toxic to aquatic life (Acute II), and although there
was a nearly 10-fold decrease in acute toxicity upon chlorination, the generated product mixture is classified as harmful to aquatic life (Acute III). Tests with the synthetic dyes CI Disperse Red 1, carried out by Vacchi et al (2013) showed the same behavior and the chlorinated dye was less toxic than the original dye in the *D. similis* acute test.

Both solutions were also tested for toxicity to algae *Raphidocellis subcapitata*, Figure 3.17. The inhibition of growth for 50% of algae population occurred when Madder concentration was 8.9 ± 0.4 mg L⁻¹, showing the dye is also toxic to this species. Chlorination decreased dye toxicity to algae, as no inhibition in cells growth was observed, even when the assay was performed at the limit of dye solution solubility (200 mg L⁻¹).

![Figure 3.17](image)

**Figure 3.17.** The effect of chlorination of Madder on (A) Acute toxicity to *D. similis* and (B) Toxicity to algae *Raphidocellis subcapitata*.

### 3.1.4.5 Mutagenicity assessment

Madder and its chlorinated solution did not show mutagenic responses to either of the tested strains (TA98 and TA100), with or without metabolic activation, Table 3.4. Therefore, chlorine treatment led to the formation of products that were not mutagenic to tested strains and
produced fewer revertants than parent dye madder, unlike synthetic dyes, such as CI Disperse Red 1, CI Disperse Red 13 and CI Disperse Orange 1.

Table 3.4. Number of revertants/plate for Madder and its chlorinated solution in the mutagenicity assay to *S. typhimurium* strains TA98 and TA100, with and without metabolic activation (S9).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of revertants/plate (TA 98)</th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without S9</td>
<td>With S9</td>
<td>Without S9</td>
<td>With S9</td>
<td>Without S9</td>
<td>With S9</td>
<td>Without S9</td>
<td>With S9</td>
<td>Without S9</td>
<td>With S9</td>
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<tr>
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<td>17</td>
<td>18</td>
<td>18</td>
<td>21</td>
<td>19</td>
<td>23</td>
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<tr>
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<td>17</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<td>Number of revertants/plate (TA 100)</td>
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<tr>
<td>Chlorinated Madder</td>
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<td>3333</td>
<td>3338</td>
<td>2753</td>
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<td></td>
</tr>
</tbody>
</table>

3.1.5. Conclusions

This study was designed to evaluate the effects of anthraquinone natural dye Madder to aquatic organisms at different trophic levels and to assess the influence of chlorination on the dye degradation products. (--) ESI/MS analysis of Madder showed the presence of Alizarin (*m/z* 239.03482) as the major component. Chlorination led to complete decolorization after 60 min, which was confirmed by UV-Vis spectrophotometry, HPLC-DAD and LC-MS. Absorption band, at \( \lambda_{max} = 430 \) nm, disappeared as did the alizarin peak (\( r_t = 11.85 \) min) on the chromatogram and in the MS spectrum. Liquid chromatography also showed the appearance of peaks, identified by ESI-MS as phthalic anhydride (*m/z* 149.0229) and 2-hydroxynaphthalene-1,4-dione (*m/z* 173.0250).
Madder was classified as toxic to aquatic life (Acute II), with EC$_{50}$ = 4.4 mg L$^{-1}$ for acute toxicity to *D. similis*, and IC$_{50}$ = 8.9 mg L$^{-1}$ for toxicity to algae *R. subcapitata*. Chlorination decreased toxicity in both experiments, with EC$_{50}$ increasing to 45 mg L$^{-1}$ for *D. similis* and non-inhibition of algae growth was observed at the limit of Madder solubility. However, the chlorinated dye was classified as harmful to aquatic life (Acute III). Madder and its chlorinated solution did not show any mutagenicity to *S. typhimurium* strains TA98 and TA100, with or without metabolic activation (S9). Therefore, although it is natural, madder is not fully benign in the aquatic environment.

### 3.2. Effect of chlorine treatment on the degradation and toxicity of natural dyes

#### 3.2.1. Introduction

Synthetic dyes are extensively used by different industries including textile, cosmetic, paper, leather, pharmaceutical and nutrition industries. Over 70,000 tons of synthetic dyes are produced annually, and for the textile industry, about 15% is lost during the dyeing process$^{223}$ When discharged into the wastewater, even trace concentrations of these dyes can be visible, generating an aesthetic impact. Furthermore, direct discharge into a water body or improper treatment can lead to adverse effects on aquatic and human life$^{223,224}$. After the azo class, anthraquinones constitute the most important group of dyes. They are widely used in commercial dyeing formulations for cellulose, wool and polyamide fibers$^{225}$. They can be found in a wide range of colors, but are mostly used for violet, blue and green$^{219}$. However, there is some concern about these dyes as literature shows that some of them are mutagenic and carcinogenic, posing a risk to human health and the environment$^{226}$. For example, Reactive Blue 4 was found to be phytotoxic, cytotoxic and significantly induced DNA damage on lymphocytes.
Disperse Blue 3 was proven to be toxic to bacteria, algae, and protozoan, exhibiting mutagenic effects upon metabolic activation in all *S. typhimurium* strains used. Remazol Brilliant Blue R was phytotoxic to rye grass and presented acute toxicity to *D. magna*. Supplementary to the toxicity concerns about the dyes themselves, there is the added problem generated by the production of degradation products by wastewater treatment. With a history of generating disinfection by-products, chlorination is a relevant treatment for consideration. Chlorine is used as a chemical disinfectant, which is effective for killing harmful microorganisms in water. It has an oxidation potential of 1.36 V being able to oxidize the organic matter, and anthropogenic contaminants present in the water. The effects of chlorination on waste water containing azo dyes, have been extensively reported in the literature. However, despite of their great importance and high applicability worldwide, biological and physical–chemical investigations on the effects of wastewater treatments on the degradation and toxicity of anthraquinone dyes are not nearly as common as those carried out with azo dyes.

Among the limited examples is the study on Reactive Blue 19, which showed the generation of a toxic benzofuran, and other intermediate transformation products upon ozonation, another disinfection method used in wastewater treatment plants.

This growing concern regarding the effect of direct and indirect (treated wastewater coming from treatment plants) release of synthetic dyes into water bodies led to a growth in the interest of using natural dyes as alternatives to problematic synthetic colorants. However, information about natural dye ecotoxicity and transformation upon chlorine treatment is scarce. In previous studies, section 3.2, the natural dye Madder was found to be acutely toxic to *D. similis*, and degraded upon chlorination generating products, such as phthalic anhydride (*m/z* 149.0229) and 2-hydroxynaphtalene-1,4-dione (*m/z* 173.0250). The chlorinated Madder solution, although
less toxic than the initial dye, was still classified as harmful to the environment. To augment these results four other natural dyes were examined to compare the effects of dye structure on degradation rate, acute toxicity and mutagenicity.

The four dyes studied in this work were Cochineal, Fustic, Logwood and Weld. Cochineal (CI Natural Red 4), an anthraquinone dyes, was one of the most important red dyes until the latter half of the 19th century. Carminic acid, the major component in Cochineal, is extracted from dried bodies of the insect *Dactylopius coccus* 233. This dye is currently used for cosmetics, plastic and textile applications15. The dihydropyran logwood grey dye (CI Natural Black 1), a flavonoid derivative, also known as Campeachy Wood, is extracted from *Haematoxylon campechianum L*, a species of flowering tree. Haematein is the strong chromophore present in this dye. The dye itself is red but black shades can be achieved by using copper and/or iron or chrome mordants. Gray (iron mordant), blue (alum and tin mordants) and purple (alum and tin mordants) shades can also be obtained, with good fastness properties14. Weld, CI Natural Yellow 2, has been used for 5,000 years16. The dye is extracted from the *Reseta luteola* plant, with the flavonoid Luteolin the major component17. Weld is applied to the yarn, mostly wool or silk, using a mordant to produce pale flax to vivid lemon tints. Mordanting with cooper sulfate or tin dichloride results in greenish yellows 18. Old fustic, CI Natural Yellow 11, is extracted from the tropical hardwood *Chlorophora tinctoria L*14.
3.2.2. Materials and Methods

3.2.2.1. Dye solutions

Natural dyes had no specification of purity and where used without further purification. Logwood Grey and cochineal were obtained from Jeffrey D. Krauss, Pilot Plant manager at North Carolina State University. Fustic extract and weld were purchased from Botanical Colors.

For the toxicological assessment, solutions to be chlorinated were prepared with the following concentrations: 100 mg/L of Cochineal, 500 mg/L of Logwood, 130 mg/L of Weld, and 150 mg/L of Fustic. Dyes were diluted in ultra-pure water, and the pH of each solution was 5.11, 5.48, 6.14 and 6.22, respectively. There was an addition of 0.1% (V/V) DMSO to the Weld and Fustic solutions to improve solubility. These concentrations where chosen due to solubility concerns, for weld and fustic, and according to pre-screening data obtained for acute toxicity to *D. similis* assessment.

For the kinetic assessment, all solutions were prepared at the concentration of 100 mg/L prior to the chlorination treatment. Cochineal and Logwood solutions were prepared by solubilizing the dye in in ultra-pure water. Madder and Fustic solutions were prepared in methanol/water (1:1), and Weld solutions were prepared in DMSO/water (1:20).

3.2.2.2. Chlorination procedure

Chlorination of natural dye solutions was performed according to Vacchi et al (2013). Dye solutions (250 mL) were treated with chlorine gas generated from the dropwise addition of 20 mL of 12 M HCl to 3 g of potassium permanganate (KMnO₄). The duration of treatment varied for each dye: 60 minutes for Weld, 30 minutes for Cochineal, 20 min for Fustic and 50 min for Logwood.
For the kinetic analysis, solutions were treated with chlorine gas generated from the dropwise addition of 7.5 mL of 12 M HCl (at a 0.5mL/min rate) to 1.5 g of potassium permanganate (KMnO₄). Chlorination was performed during 15 min.

Color removal was monitored by UV-Vis spectrophotometry and the amount of free chlorine was determined using the N,N'-diethyl-para-phenylenediamine (DPD) method.

3.2.2.3. Analytical procedures

UV-Vis spectra were collected on an Agilent Cary Series spectrophotometer, between 200 and 800 nm using a quartz cuvette (path length 1 cm). Dyes calibration curves were prepared between 0.1 and 200 mg/L. Cochineal and Logwood solutions were prepared by solubilizing the dye in ultra-pure water. Madder and Fustic solutions were prepared in methanol/water (1:1), and weld solutions were prepared in DMSO/water (1:20).

Natural dyes solutions were analyzed in the Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer using Electrospray Ionization (ESI) in negative mode. Instrument parameters were: gas temperature 350°C, drying gas 10 L/min, nebulizer 30 psi, fragmentor voltage 175 V, capillary voltage 3500V.

3.2.2.4. Toxicity and mutagenicity assessment

The acute toxicity of the dye solutions (before and after chlorination) was evaluated. Chlorinated solutions were tested after no residual free chlorine was detected.

Toxicity to D. similis was tested according to OECD 202 guidelines. Chlorinated dye solutions concentrations were expressed in percentages. The five neonates (6-24 h old) used in each replicate (4 replicates total) were exposed to the dye for 48h, at 20 °C ± 2 °C, with a light intensity of 1000 lux under photoperiod (16:8 light/dark). Organisms were not fed. The number of
immobilized organisms was counted after the 48-h exposure time. Results presented in Figure 3.27, were statistically analyzed using the trimmed Spearman–Karber method for estimating the median immobilization concentration, EC$_{50}$

The mutagenicity of the natural dyes before and after chlorination was evaluated using the Salmonella/microsome assay according to the ISO 16240:2005 guidelines. Salmonella enterica serovar Typhimurium strains TA98 (hisD3052, rfa, Abio, ΔuvrB, and pKM101) and TA100 (hisG46, rfa, Abio, ΔuvrB, pKM101), with and without exogenous metabolic activation (S9), were tested. The metabolic activation was provided by Arochlor 1254-induced Sprague Dawley rat liver S9 fraction (Moltox Inc, NC, USA), which was prepared at a concentration of 4% (v/v). For both strains, positive controls were 4-nitroquinoline-1-oxide (4NQO) (Sigma–Aldrich) at 0.125 μg/plate and 2-aminoanthracene (2AA) (Sigma–Aldrich) at 0.625 μg/plate, both dissolved in dimethyl sulfoxide. Sterile water was used as negative control. The test was performed using a single dose for each treatment. For each plate, 0.5 mL of 0.015 M sodium phosphate buffer or S9 mix and 1 mL of sample solution were added. Sample solutions were sterilized by filtration, using a 0.45 μm filter. A single dose was tested, according to the maximum solubility of the dye, and using two replicates. After 66 h incubation at 37°C, colonies were automatically counted. Results were expressed as the number of revertants and mutagenetic ratio (MR), which is the average number of revertants in the sample test divided by the average number of revertants of negative control. Data was analyzed using the Salanal computer program (Integrated Laboratory Systems, Research Triangle Park, NC). Samples were considered positive when ANOVA provided significant responses (p<0.05).
3.2.3. Results and Discussion

3.2.3.1. Characterization of natural dyes

Natural dyes need to be extracted from their sources to be applied to the substrate. Various techniques, solvents and parameters are reported for extraction of natural dyes in the literature\textsuperscript{21}. The source of extraction and extraction method used can affect the final dye composition. Therefore, identification of the major components in each dye is important.

Luteolin ($m/z$ 285.0432), Figure 3.18 A, and Carminic acid ($m/z$ 491.0840), Figure 3.18 C, were identified as the major components in Weld and Cochineal, respectively. Hematoxylin, Figure 3.19, is the naturally occurring chemical found in the heartwood of the Logwood tree\textsuperscript{234}, however its oxidation product Hematein ($m/z$ 299.0566), Figure 3.18 B, was detected as the main component in logwood.
Figure 3.18. (-) ESI/MS spectra for natural dyes (A) Weld, (B) Logwood, (C) Cochineal and (D) Fustic.
Oxidation of Hematoxylin to Hematein.

Old fustic was introduced into Europe and widely used, in the 16th century, as a yellow mordant dye. The main chromophores in this source are reported to be the flavanol Morin, small amounts of Kaempferol and the benzophenone Maclurin. Maclurin (m/z 261.0413), Figure 3.18 D, and a compound with m/z 303.0518 (proposed chemical formula C₁₅H₁₂O₇), with the proposed structure in Figure 3.20, were the main components in the old fustic extract. Morin (m/z 301.0359) was present but was not one of the main components, and Kaempferol (MW= 286.23 g/mol) was not detected.

Figure 3.19. Oxidation of Hematoxylin to Hematein.

Figure 3.20. Zoomed in (-) ESI/MS spectra for natural dye Fustic.
3.2.3.2.  **Effect of chlorination on color removal**

Chlorination promoted discoloration of the 4 dye solutions as the absorption bands at the maximum wavelength decreased, Figure 3.21. Due to its higher starting concentration, logwood starting and chlorinated solutions, Figure 3.21 B, were diluted with ultra-pure water to a 1:10 ratio prior to UV-Vis analysis.

![UV-Visible spectra](image)

**Figure 3.21.** UV-Visible spectra showing the discoloration of (A) Cochineal, (B) Logwood, (C) Weld and (D) Fustic after exposure to chlorine gas.

Analysis of the discoloration reaction kinetics was performed, using 100 mg/L dye solutions, Figure 3.22. Although all solutions were at the same concentration, Weld and Fustic
solutions are a lot lighter, suggesting they have a small molar extinction coefficient in the visible range.

![Figure 3.22. Dye solutions, 100 mg/L, used for the chlorination kinetics studies: (A) Cochineal, (B) Logwood, (C) Madder, (D) Fustic and (E) Weld.]

Table 3.5 shows the equations obtained for the calibration curves constructed, at the maximum wavelength ($\lambda$) for the major component identified in each dye (section 3.2.3.1.).

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Calibration curve equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
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<td>$A = 0.00898 \ C + 0.02428$</td>
<td>0.99250</td>
</tr>
<tr>
<td>Fustic</td>
<td>280</td>
<td>$A = 0.01527 \ C - 0.06881$</td>
<td>0.99614</td>
</tr>
<tr>
<td>Logwood</td>
<td>438</td>
<td>$A = 0.00660 \ C + 0.01030$</td>
<td>0.99940</td>
</tr>
<tr>
<td>Madder</td>
<td>430</td>
<td>$A = 0.00812 \ C + 0.01893$</td>
<td>0.99685</td>
</tr>
<tr>
<td>Weld</td>
<td>330</td>
<td>$A = 0.00792 \ C - 0.03621$</td>
<td>0.99747</td>
</tr>
</tbody>
</table>

Concentration (C) in mg/L.
Changes in the dye absorbances, which are related to their concentration, as a function of time were observed for Logwood, Weld, Cochineal, Fustic and Madder, **Figure 3.23 A**. The decrease in dye absorbances (concentrations) as a function of time was plotted, **Figure 3.23 B**. A linear relationship was obtained, being its slope correspondent to the degradation rate for each dye, at the maximum wavelength observed (mentioned on Table 3.5), **Table 3.6**. These equations are extremely useful in the experimental determination of rate constants and reaction order.

**Figure 3.23.** (A) Changes in the dye absorbances as a function of time, and (B) initial degradation rate curves for the natural dyes, from the chlorination process.

For dyes except weld, absorbance, and consequently dye concentration, decreased as the chlorination time increased, **Figure 3.23 A**. The initial linear relationship observed, **Figure 3.23 B**, is characteristic of a first-order reaction in dye consumption\(^{235}\). This behavior has been previously observed for the degradation of some synthetic dyes\(^{236}\). The rate equation for a first order reaction can be expressed as:

\[
rate = \frac{d[A]}{dt} = -k[A]
\]
Integration of the above equation for the boundary conditions \( A=A_o \) at \( t=0 \) and \( A=A_t \) yields the rate equation for a first order reaction, where, \( k_t \) is the rate constant, which is a concentration-independent measure of the velocity of a reaction, and \( A \) is the dye concentration (absorbance).

\[
\ln \left( \frac{A_t}{A_0} \right) = -kt
\]

**Table 3.6.** Initial degradation rate equations and degradation rate constants for the natural dyes at their maximum wavelength.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Degradation rate constant (min(^{-1}))</th>
<th>Degradation rate equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cochineal</td>
<td>-1.119</td>
<td>( \ln (\text{Abs}<em>{t}/\text{Abs}</em>{0}) = 0.459-1.119t )</td>
<td>-0.97935</td>
</tr>
<tr>
<td>Fustic</td>
<td>-0.088</td>
<td>( \ln (\text{Abs}<em>{t}/\text{Abs}</em>{0}) = -0.216-0.088t )</td>
<td>-0.95564</td>
</tr>
<tr>
<td>Logwood</td>
<td>-0.282</td>
<td>( \ln (\text{Abs}<em>{t}/\text{Abs}</em>{0}) = 0.326-0.282t )</td>
<td>-0.96260</td>
</tr>
<tr>
<td>Madder</td>
<td>-0.400</td>
<td>( \ln (\text{Abs}<em>{t}/\text{Abs}</em>{0}) = 0.208-0.400t )</td>
<td>-0.95661</td>
</tr>
<tr>
<td>Weld</td>
<td>-0.006</td>
<td>( \ln (\text{Abs}<em>{t}/\text{Abs}</em>{0}) = 0.034-0.006t )</td>
<td>-0.97151</td>
</tr>
</tbody>
</table>

For Weld, at \( \lambda=330 \) nm, absorbance did not decrease as fast as it did for the other dyes, which can be observed by comparing the degradation rate constants, suggesting its initial degradation does not follow a first-order reaction. At least initially, this observation disagrees with the information reported by Wang et al (2016)\(^{237} \) that the degradation of flavonoids follows a first-order reaction. Cochineal had the highest \( k \) value, suggesting the chromophore in its major component, Carminic acid, is quickly degraded, whereas weld had the lowest value, suggesting the chromophore in luteolin is more slowly degraded upon chlorination. The negative sign means the compound is consumed (degraded during the reaction).

Using the calibration equations presented in **Table 3.5**, the final concentrations of the 5 dye solutions were measured in order to determine the color removal efficiency, expressed by

\[
[CR(\%) = \left( 1 - \frac{C_t}{C_0} \right) \times 100]^{224} \text{ Figure 3.24.}
\]

Cochineal, logwood and madder solutions were 100% decolorized within the 15 min reaction time. Cochineal has the highest degradation rate constant,
which led to nearly 100% discoloration in 3 min of chlorination. Weld reached a maximum of 6% discoloration within 15 min of chlorination.

![Graph showing color removal percentage over time for different dyes.](image)

**Figure 3.24.** Percentage of discoloration promoted by chlorination of the five natural dye solutions.

### 3.2.3.3. Relation with structure

**Table 3.7** lists the dyes’ degradation rate constants from the highest to the lowest negative value as well as significant structural features of the dyes. It is clear from the data that one single structural feature does not control the rate of color removal and that it was rather a combination of features that determined this rate.

Several studies regarding the relationship between enzymatic, microbial and biological treatments and chemical structures regarding dye degradation. Costa et al (2012) investigated the effects of dye structure and redox mediators on the biological degradation of anthraquinone dyes CI Reactive Blue 4 and Remazol Brilliant Blue R. **Figure 3.25.** The results showed that redox mediators did not catalyze the reduction reaction, which the authors attributed to the dye structural stability, high molecular volume and steric hindrance effects. CI Reactive Blue 4 was the most
recalcitrant dye for anaerobic decolorization, presenting the lowest kinetic constant, authors related with the presence of the triazine group and the anthraquinone chromophore. On the other hand, the kinetic degradation constant of Remazol Brilliant Blue R was much higher probably due to its lower molecular volume and the absence of the triazine group. However, not much specific information could be found in the literature regarding the influence of dye structure, especially anthraquinone and flavonoid derivatives, on the efficacy of degradation promoted by oxidative treatments, such as chlorination.

![Structures of the anthraquinone dyes](image)

**Figure 3.25.** Structures of the anthraquinone dyes (A) CI Reactive Blue 4 and (B) Remazol Brilliant Blue R.

Anthraquinones have a resonance effect on their conjugated structure, which stabilizes the dye structure and theoretically makes the degradation reaction is less favorable. Of the 5 dyes studied, Cochineal, the one with highest molecular weight, was the one degraded more quickly. This is probably due to the -OH groups present with add electron density to the aromatic system, activating it for electrophilic aromatic attack by the chlorine ions.
Flavonoids in aqueous solutions show instability, resulting in degradation. According to Wang et al. (2016)\textsuperscript{237}, the degradation of flavonoids involves oxidation and ring-cleavage. Furthermore, more hydroxyl groups in a flavonoid molecule leads to lower stability. For example, Fisetin and Quercetin, Figure 3.26, have 4 and 5 hydroxyl groups, respectively. Thus, it is reasonable that Quercetin was more liable than Fisetin. A similar comparison can be made between the natural dyes Fustic and Weld. Considering that fustic has the flavonoid Morin, which contains 5 hydroxyl groups, in its composition and weld is composed by the flavonoid Luteolin (with 4 -OH groups), this may explain the higher stability observed for weld (slower initial degradation).

![Figure 3.26. Structures of flavonoid derivatives (A) Fisetin and (B) Quercetin.](image)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Degradation rate constant (min(^{-1}))</th>
<th>Number of Aromatic rings</th>
<th>Number of Hydroxyl groups</th>
<th>Number of Carbonyl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cochineal</td>
<td>-1.119</td>
<td>2</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Madder</td>
<td>-0.400</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Logwood</td>
<td>-0.282</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Fustic</td>
<td>-0.088</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Weld</td>
<td>-0.006</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

The simple structural analysis provided by the parameters described in Table 3.7 does not consider the full complexity of the tested molecules. Therefore, a more detailed analysis using
molecular descriptors (molecular modelling) would help to better understand the relationship between structure and degradation promoted by chlorination\textsuperscript{224}.

3.2.3.4. *Toxicological and mutagenicity tests*

$EC_{50}$ values for natural dyes and their chlorinated solutions are presented in Figure 3.27. $EC_{50}$ for the chlorinated dye solutions are expressed as equivalent concentration. Figure 3.28 shows Daphnia test results before and after exposure to logwood, indicating the dye presence in the organism digestive tract.

According to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)\textsuperscript{146}, Logwood is not classified as toxic or harmful to aquatic life as its $EC_{50}$ value is higher than 100 mg/L, considering the 95% confidence interval. Fustic, weld and cochineal have $EC_{50}$ that in between 10 and 100 mg/L and are classified as harmful to aquatic life (Acute III).

Chlorination produced a nearly 7-fold increase in acute toxicity of logwood, generating a solution classified as harmful to aquatic life (Acute III). The same behavior upon chlorination was observed for the other natural dyes: a 3.5-fold increase in acute toxicity for Cochineal and Weld, and a 10-fold increase for fustic. Chlorinated weld and cochineal solutions are classified as Acute III and chlorinated fustic solution as toxic to aquatic life (Acute II).

These results differ from the ones observed for the natural dye Madder, which showed a nearly 10-fold decrease in acute toxicity upon chlorination. $EC_{50}$ values for madder and its chlorinated solution were 4.4 (4.1-4.7, 95% confidence interval) and 45 (39-53, 95% confidence interval) mg L$^{-1}$, respectively. However, this trend agrees with the observations reported in the literature. An increase in acute toxicity to *D. similis* of textile effluents upon chlorination using sodium hypochlorite was also reported by Chen at al. (2001)\textsuperscript{241}.
**Figure 3.27.** EC$_{50}$ values obtained in the acute toxicity to *D. similis* test for Logwood (L) and its chlorinated solution (L-Cl), Fustic (F) and its chlorinated solution (F-Cl), Weld and its chlorinated solution (W-Cl) and Cochineal (C) and its chlorinated solution (C-Cl).

**Figure 3.28.** Daphnia before (A) and after (B) exposure to logwood dye solution.

All dyes and their chlorinated solutions were non-mutagenic in strains TA98 and TA100, with and without metabolic activation, as the mutagenic ratios (MR) obtained were below 2 for all tested conditions. **Tables 3.8-3.11** show the complete data. The number of revertants/plate was not significantly higher than the number produced by the negative control. Therefore, chlorine treatment led to products that were not mutagenic to tested strains, unlike azo dyes such as the azo dyes CI Disperse Red 1, CI Disperse Red 13 and CI Disperse Orange 1$^{222}$. 
Table 3.8. Mutagenic activity for natural dyes to TA 98 with metabolic activation (S9), before and after chlorination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg/plate)</th>
<th>Number of revertant</th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th>SD</th>
<th>MR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>Plate2</td>
<td>Plate3</td>
<td>Plate4</td>
<td>Plate5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control (H₂O)</td>
<td>0</td>
<td>26</td>
<td>26</td>
<td>28</td>
<td>34</td>
<td>21</td>
<td>27.0</td>
<td>4.7</td>
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<td>-</td>
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<td>3.5</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>5.0</td>
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<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>9.2</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>10.6</td>
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<tr>
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<td>27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30.5</td>
<td>5.0</td>
</tr>
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<td>28</td>
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<td>-</td>
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<td>27.0</td>
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<td>1109</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1109.5</td>
<td>0.7</td>
</tr>
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</table>

*equivalent dose; MR=mutagenic ratio.
Table 3.9. Mutagenic activity for natural dyes to TA 98 without metabolic activation (S9), before and after chlorination

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg/plate)</th>
<th>Number of revertants</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th>SD</th>
<th>MR</th>
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<td>Plate4</td>
<td>Plate5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control (H₂O)</td>
<td>0</td>
<td>34</td>
<td>27</td>
<td>26</td>
<td>26</td>
<td>24</td>
<td>27.4</td>
<td>3.8</td>
<td>-</td>
</tr>
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<td>26</td>
<td>-</td>
<td>-</td>
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<td>32.0</td>
<td>8.5</td>
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<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.5</td>
<td>2.1</td>
<td>0.9</td>
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<td>1.2</td>
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<td>32</td>
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<td>-</td>
<td>32.5</td>
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<td>30</td>
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<td>34.0</td>
<td>7.1</td>
<td>1.2</td>
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<td>36</td>
<td>32</td>
<td>-</td>
<td>-</td>
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<td>34.0</td>
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<td>1.2</td>
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<td>583</td>
<td>563</td>
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<td>-</td>
<td>573.0</td>
<td>14.1</td>
<td>20.9</td>
</tr>
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</table>

*equivalent dose; MR=mutagenic ratio.
Table 3.10. Mutagenic activity for natural dyes to TA 100 with metabolic activation (S9), before and after chlorination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg/plate)</th>
<th>Number of revertants</th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th>SD</th>
<th>MR</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
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<td>Negative Control (H₂O)</td>
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<td>216</td>
<td>204</td>
<td>188</td>
<td>204</td>
<td>179</td>
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<td>-</td>
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<td>235</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>31.8</td>
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<td>228</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>218.0</td>
<td>14.1</td>
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<td>-</td>
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<td>237.0</td>
<td>17.0</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>3314.0</td>
<td>59.4</td>
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</table>

*equivalent dose; MR=mutagenic ratio.
Table 3.11. Mutagenic activity for natural dyes to TA 100 without metabolic activation (S9), before and after chlorination

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg/plate)</th>
<th>Number of revertants</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th>SD</th>
<th>MR</th>
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<tr>
<td>Negative Control (H₂O)</td>
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<td>203</td>
<td>175</td>
<td>189</td>
<td>203</td>
<td>194.4</td>
<td>12.4</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>210.0</td>
<td>2.8</td>
<td>1.1</td>
</tr>
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<td>176</td>
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<td>-</td>
<td>-</td>
<td>195.0</td>
<td>26.9</td>
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<td>-</td>
<td>-</td>
<td>221.5</td>
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</tr>
<tr>
<td>Fustic</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>228.5</td>
<td>17.7</td>
<td>1.2</td>
</tr>
<tr>
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<td>233</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>225.0</td>
<td>11.3</td>
<td>1.2</td>
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<tr>
<td>Weld</td>
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<td>211</td>
<td>229</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>220.0</td>
<td>12.7</td>
<td>1.1</td>
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<tr>
<td>Chlorinated Weld</td>
<td>130*</td>
<td>231</td>
<td>195</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>213.0</td>
<td>25.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Positive Control (4NDQO)</td>
<td>0.125</td>
<td>3310</td>
<td>3566</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3438.0</td>
<td>181.0</td>
<td>17.7</td>
</tr>
</tbody>
</table>

*equivalent dose; MR=mutagenic ratio.
The Ames test (Salmonella typhimurium reverse mutation assay) is a bacterial in vitro short-term test for identification of carcinogens using mutagenicity in bacteria as an endpoint. A high but not complete correlation has been found between carcinogenicity in animals and mutagenicity in this test, which detects mutations in a gene of a histidine-requiring bacterial strain that produces a histidine-independent strain\textsuperscript{242}. Therefore, the negative results obtained for the studied dyes and their chlorination solutions suggests these compounds are not mutagenic.

3.2.4. Conclusions

The major components in the four natural dyes studied, Weld, Cochineal, Logwood and Fustic were identified by (-) ESI/MS analysis as Luteolin (m/z 285.0432), Carminic acid (m/z 491.0840), Hematein (m/z 299.0566) and Maclurin (m/z 261.0361), respectively. Chlorination led to decolorization of all dye solutions, which was confirmed by UV-Vis spectrophotometry. UV-Vis analysis also allowed the kinetic studies of the degradation rate for these natural dyes as well as for the anthraquinone natural dye Madder. Cochineal showed the highest initial degradation rate constant (k= -1.119 min\textsuperscript{-1}) achieving a nearly 100\% of decolorization after 3 min of treatment. On the other hand, Weld has the lowest rate constant (k= -0.006 min\textsuperscript{-1}), having about 6\% of its color removed after 15 min of treatment.

Chlorination increased the acute toxicity of the 4 dye solutions to D. similis. Logwood was classified as toxic or harmful to aquatic life, and upon chlorination, had a 7-fold increase in acute toxicity generating a solution classified as harmful to aquatic life. Fustic, Weld and Cochineal had EC\textsubscript{50} in between 10 and 100 mg/L and are classified as harmful to aquatic life (Acute III). Upon chlorination, Weld and Cochineal solutions were classified as Acute III and chlorinated Fustic solution as toxic to aquatic life. The four dye solutions, in the solubility limit, did not show any
mutagenicity to *S. typhimurium* strains TA98 and TA100, with or without metabolic activation (S9), before and after chlorination.

From the degradation rate results obtained it is clear that the structure of the molecules affects the degradation behavior of dyes. However, a clear association between structure and degradation could not be made and further examination should be performed, perhaps using molecular descriptors and molecular modelling to better understand these relations.

Therefore, attention should be paid when using these natural dyes, as once discharged into wastewater and submitted to oxidation treatments, such as chlorination, they can be degraded to compound that pose potential harm to aquatic organisms, and consequently, the environment.
CHAPTER 4. Synthesis of a reactive dye analog of a natural dye

Despite the increasing interest for natural dyes, textile industries hesitate to use these dyes due to their low color strength and poor fixation properties\textsuperscript{243}, which can lead to a high volume of dyes in the wastewater. Furthermore, an introduction of natural dyeing into a full-scale technical dyeing process is rather difficult due to the requirements of the dyehouse, such as the water and energy consumption\textsuperscript{244}. Therefore, modifying natural dye structures to increase fixation to textile fibers, while keeping or improving the genotoxic characteristics, is important to establish these dyes as safe and economical appealing. The different approaches adopted, using different starting materials are listed below.

4.1. Synthesis of an Indigo carmine analog

4.1.1. Introduction and synthesis approach A

Indigo and Indigo carmine are well known dyes that have been used for centuries and can be extracted from natural sources or be synthesized. Due to the high amount of information available and the dyes’ applicability, these dyes were first chosen as models to produce dye reactive analogs. The goal was to apply the developed methodology to the generation of anthraquinone and flavonoid modified natural dyes.

Indigo, CI Natural Blue 1, is extracted from the plants \textit{Indigofera tinctoria} and \textit{Isatis tinctoria}, which have been cultivated for at least 4000 years for coloring textiles. The economic importance of indigo grew in the 19th century, which led to the commercialization of synthetic indigo in 1887. Indigo is the leading textile dye mainly employed in the dyeing of cotton for blue jeans. It has an extremely low water solubility and a high melting point (390–392°C)\textsuperscript{245}. 
Indigo carmine (IC), CI Natural Blue 2, can be extracted from a natural source or synthesized from Indigo, Figure 4.1. It is mainly used as a pH indicator and in the food and textile industries\textsuperscript{246}. Chemically, IC is Indigo disulfonic acid, and the consequence of this chemical modification is that the dye is water-soluble, which facilitates the dyeing process in contrast with the vat dyeing process for indigo. The main disadvantage is the decrease in washfastness. Historically, indigo carmine was mainly used in smaller plants for dyeing silk, wool and leather, in printing and coloring soap\textsuperscript{247}.

Figure 4.1. Scheme of the typical reaction for the synthesis of Indigo carmine from Indigo.

Due to its higher solubility in comparison with Indigo, Indigo carmine was chosen as the starting material for the reaction described next. Furthermore, synthetic IC was used in order to determine the reaction parameters, due to its higher purity in comparison with the dyes obtained from natural sources. The goal was to develop the reaction method and then apply it using natural IC. A 2-step approach to incorporating a reactive group into the IC system has been adopted in this work. The first step involved chlorination of the -SO\textsubscript{3}Na group generating a sulfonyl chloride compound to be reacted with vinylsulfone para ester, forming the bireactive indigo dye shown in Figure 4.2.
The bireactive dye, Figure 4.2 C, has a higher molecular weight but the same chromogen as the starting material IC, so fixation to the fiber would be improved without a change in color. Another goal was to change the reactants, vinylsulfone para ester and chlorinated dye, Figure 4.2 B, molar ratio in order to synthetize a monoreactive dye, aiming to improve color strength and water solubility.

4.1.1.1. Experimental

4.1.1.1.1. Reaction conditions

Several attempts to synthesize the intermediate dye, Figure 4.2 B, and the target dye, Figure 4.2 C, were made. Table 4.1 summarizes the reaction conditions used. Further reaction details can be found in the references included. Indigo carmine 85% was purchased from Sigma Aldrich and used without further purification. Vinyl sulfone para ester (VSPE) 96.5% was obtained from Hoechst Celanese. All solvents used were ACS grade, purchased either from VWR International or Sigma Aldrich.
Indigo carmine in its acidic form (IC-free) indicated on Table 4.1, was obtained by solubilizing 0.5 g of IC in 100 mL of pure water and adding 3mL of HCl (36%) until pH 1.47. Water was removed from the solution and 150 mL of DMF was added. Solution was filtered and DMF evaporated.
Table 4.1.  Experimental conditions tested for the synthesis of the sulfonyl chloride derivative of IC and the bireactive dye.

<table>
<thead>
<tr>
<th>Starting Compound</th>
<th>Conditions</th>
<th>Samples obtained</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.5 g) IC</td>
<td>(34 mL) chlorobenzene + (0.5 mL) DMF + (1 mL) thionyl chloride (SOCl₂) + heated at 95°C for 1.5 h.</td>
<td>P59</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
<tr>
<td>(1.5 g) IC</td>
<td>(50 mL) chlorobenzene + (1 mL) DMF + (2 mL) SOCl₂ + heated at 95°C for 2 h.</td>
<td>P61</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
<tr>
<td>(1.5 g) IC</td>
<td>(34 mL) chlorobenzene + (0.5 mL) DMF + (1 mL) SOCl₂ + heated at 95°C for 4 h.</td>
<td>P62</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
<tr>
<td>(1 g) IC</td>
<td>(24 mL) chlorobenzene + (0.5 mL) DMF + (1 mL) SOCl₂ + heated at 95°C for 15 h.</td>
<td>P63</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
<tr>
<td>(1.3 g) IC</td>
<td>(30 mL) chlorobenzene + (0.5 mL) DMF + (1 mL) SOCl₂ + heated at 60°C for 17 h, under N₂ atmosphere.</td>
<td>P64</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
<tr>
<td>(0.2 g) P64</td>
<td>P64 in 17 mL pyridine + (0.3 g) vinyl sulfone para ester (VSPE).</td>
<td>PA</td>
<td>-</td>
</tr>
<tr>
<td>(0.6 g) IC</td>
<td>(15 mL) chlorobenzene + (0.5 mL) DMF + (0.5 mL) SOCl₂ + heated at 60°C for 1 h + (20 mL) pyridine + (0.8 g) VSPE + heated at 120°C for 16 h.</td>
<td>P67</td>
<td>-</td>
</tr>
<tr>
<td>(0.4 g) IC</td>
<td>(5 mL) SOCl₂ + heated at 110°C for 4 hours+ (10 mL) pyridine + (0.8 g) VSPE+ heated at 120°C for 4 hours.</td>
<td>P69</td>
<td>Haring (1960)²⁴⁹</td>
</tr>
<tr>
<td>(0.5 g) IC</td>
<td>(8 mL) POC₁₃ + heated at 40°C for 50 min + (0.4 g) PCl₅ + heated at 95°C for 10 min + cooled overnight and filtered.</td>
<td>P70</td>
<td>Haring (1960)²⁴⁹</td>
</tr>
<tr>
<td>P70 solid</td>
<td>Washed with toluene + (15 mL) pyridine+ (0.5 g) VSPE + heated at 120°C for 25 h.</td>
<td>P70 (2)</td>
<td>-</td>
</tr>
<tr>
<td>(0.5 g) IC</td>
<td>(31 mL) dichloromethane (CH₂Cl₂) + chlorosulfonic acid (HSO₃Cl) + heated at 40°C for 6 h.</td>
<td>P74</td>
<td>Feng et al. (2002)²⁵⁰</td>
</tr>
<tr>
<td>(0.5 g) IC</td>
<td>(3 mL) triethylamine + (2.5 g) cyanuric acid + heated at 55°C for 24 h.</td>
<td>P75</td>
<td>Blotny (2003)²⁵¹</td>
</tr>
<tr>
<td>(0.5 g) IC</td>
<td>(5 g) AlCl₃ + (2.5 g) NaCl + heated at 180°C for 1 h.</td>
<td>P77</td>
<td>Singh (2005)²⁵²</td>
</tr>
<tr>
<td>(0.5 g) IC-free</td>
<td>(80 mL) DMF+(5 mL) SOCl₂ + heated at 110°C</td>
<td>B1</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
</tbody>
</table>

IC: indigo carmine sodium salt; IC-free: indigo carmine in its acidic form
4.1.1.1.2. Analytical procedures

Reaction progress was followed by TLC, using silica gel plates (TLC Silica Gel 60 F254, EMD Chemicals, Germany) and butanol/ethanol/NH$_3$/pyridine (4:1:3:2) as the mobile phase. Indigo carmine characterization was performed by running ESI-MS and HPLC-analysis. An Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer using Electrospray Ionization (ESI) was used. Equipment conditions were: gas temperature 300°C, drying gas 5 L/min, nebulizer 30 psig, Vcap voltage 3500V, fragmentor voltage 90V, skimmer 65V, oct1 RF Vpp 750V. Samples were directly injected into MS at an infusing rate of 1 µL/min.

An Alliance Waters HPLC-DAD was used, with an Atlantis C$_{18}$ (150 x 4.6 mm, 5 µm), flow rate 1.0 mL/min and gradient elution as follows: A (10 mM ammonium phosphate monobasic, NH$_4$H$_2$PO$_4$), B (ACN), 0-10 min (5-35 %B), 10-15 (35%B), at room temperature.

4.1.1.2. Results and discussion

Indigo carmine disodium salt was found to be 95.6% pure, and its chemical structure was confirmed to be the one with molecular weight 466.35 g/mol, as its characteristic doubly charged [M-2Na]$^{2-}$ (m/z 209.9874) and singly charged [M-Na]$^-$(m/z 442.9634) anions were identified by ESI-MS, Figure 4.3 B. The characteristic absorption spectrum for the peak with rt= 6.179, dye’s major component, Figure 4.3 A.

For all reactions listed on Table 4.1, TLC plates did not show the presence of new components, observed with the naked eye, which would be an indicative of the formation of a new dye, as both product B and C on Figure 4.2 were expected to have different retention times from the starting compound IC. This indicated that the reactions were not successful.
Figure 4.3. (A) Chromatogram at 608 nm and (B) (-) ESI/MS spectrum of Indigo carmine.

Table 4.2 shows the ions expected to be detected assuming synthesis’ success. Although the presence of a new compound was not detected by TLC, reaction mixture P59 was analyzed by ESI/MS, which showed the presence of the ion $m/z$ 456.9118, Figure A1, in negative mode, which matched the expected exact mass and peak ratio (9:6:1) for the presence of 2 chlorine atoms expected for product B, Figure 4.2 B. However, trying to replicate this synthesis did not lead to the same results, suggesting the compound was probably an impurity. As the results could not be replicated, further structure investigation was not performed for this compound. Figure A1 spectrum shows the sample was not pure, as a major unidentified peak ($m/z$ 496.8594) was detected.
Table 4.2. Expected reaction products and their ions from MS.

<table>
<thead>
<tr>
<th>Possible reaction product</th>
<th>MW (g/mol)</th>
<th>Expected Ions observed in MS$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{16}$H$_8$ClN$_2$NaO$_7$S$_2$</td>
<td>462.81</td>
<td>[M-Na]$^+$ = 439.82</td>
</tr>
<tr>
<td>C$_{16}$H$_8$Cl$_2$N$_2$O$_6$S$_2$</td>
<td>459.28</td>
<td>[M-H]$^-$ = 456.91</td>
</tr>
<tr>
<td>C$<em>{24}$H$</em>{18}$ClN$<em>3$O$</em>{12}$S$_4$</td>
<td>704.12</td>
<td>[M-H]$^-$ = 701.94</td>
</tr>
<tr>
<td>C$<em>{32}$H$</em>{28}$N$<em>4$O$</em>{18}$S$_6$</td>
<td>948.97</td>
<td>[M-H]$^-$ = 946.97, [M-2H]$^{2-}$ = 472.49</td>
</tr>
</tbody>
</table>

4.1.2. Synthesis approach B

Approach B was the esterification of the sulfonic groups in Indigo carmine, generating the intermediary product in Figure 4.4 B, prior to the reaction presented in approach A (section 4.1.1.) to obtain the sulfonyl chloride product, Figure 4.2 B.

![Figure 4.4. Scheme for the proposed reaction to obtain the esterification (B) of the acid form of Indigo carmine (A).](image)

4.1.2.1. Experimental

4.1.2.1.1. Reaction conditions

Table 4.3 summarizes the reaction conditions tested, using Indigo carmine acidic form as starting material.
Table 4.3. Reaction conditions tested for the esterification of Indigo Carmine.

<table>
<thead>
<tr>
<th>Starting compound</th>
<th>Conditions</th>
<th>Samples obtained</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.5 g) IC-free</td>
<td>(60 mL) trimethyl orthoformate/MeOH (1:1) + heated at 120°C for 30 min under N₂ atmosphere.</td>
<td>IC1</td>
<td>Padmarpriya et al. (1986)²⁵³</td>
</tr>
<tr>
<td>(0.5 g) IC-free</td>
<td>(60 mL) triethyl orthoformate/MeOH (1:1) + heated at 120°C for 21 h under N₂ atmosphere.</td>
<td>B20</td>
<td>Rossback et al. (2000)²⁴⁸</td>
</tr>
</tbody>
</table>

4.1.2.1.2. Analytical procedures

Reaction progress was followed by TLC, using silica gel plates (TLC Silica Gel 60 F254, EMD Chemicals, Germany) and butanol/ethanol/NH₃/pyridine (4:1:3:2) as the mobile phase. HPLC-DAD analysis was performed under the same conditions stated in section 4.1.1.1.2.

4.1.2.2. Results and discussion

TLC did not show new spots for reaction mixtures B20 and IC1, suggesting no products were generated from the reactions. Further HPLC-DAD analysis confirmed this observation, Figure B1, as no new chromatographic peaks were observed.

As the reaction conditions tested did not generate the desired products, another dye was used as the starting material, having the same end result in mind. These experiments are described in the next section.

4.2. Synthesis of Alizarin analogs

4.2.1. Synthesis approach A

This approach involved using natural dye Madder, its major component Alizarin, Figure 4.5, and sulfonated Alizarin (Alizarin Red S), Figure 4.6, as platform chemicals for the chlorosulfonation reaction. The proposed first reaction step was to react the starting material with
chlorosulfonic acid\textsuperscript{254}. Next was the reaction of the sulfonyl chloride products (2) and/or (3) with vinyl sulfone para ester (VSPE) to generate the sulfonamide dye (4), Figure 4.5\textsuperscript{255}.

Figure 4.5. Reaction approach for the synthesis of the sulfonamide Alizarin analog.

Alizarin Red S (ARS), Figure 4.6 (1), is an anthraquinone derived dye mostly used as a histochemical stain for calcium in biological samples\textsuperscript{256}. The reaction approach, the same shown in Figure 4.5, and the molecular weight of the starting material and products are presented in Figure 4.6.
4.2.2.1. Experimental

4.2.2.1.1. Synthesis parameters

Table 4.4 lists the reactions conditions employed for alizarin 97% (AZN), madder (MDR) and alizarin red S (ARS) as starting materials for the synthesis of the sulfonyl chloride derivative. Further reaction details can be found on the references provided (Table 4.4).
Table 4.4. Experimental conditions for synthesizing the sulfonyl chloride and sulfonamide derivatives of Alizarin.

<table>
<thead>
<tr>
<th>Starting Compound</th>
<th>Procedure</th>
<th>Samples obtained</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.5 g) MDR</td>
<td>(45 mL) dichloromethane (DCM) + (1.5 mL) chlorosulfonic acid (ClSO$_3$H) + room temperature (RT) for 24 h.</td>
<td>B30</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.5 g) MDR</td>
<td>(50 mL) DCM + (5 mL) ClSO$_3$H + heated at 100°C for 18 h.</td>
<td>B31</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.5 g) MDR</td>
<td>(50 mL) DCM + (1.5 mL) ClSO$_3$H + heated at 60°C for 20 h.</td>
<td>B31a</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.2 g) MDR</td>
<td>(25 mL) ClSO$_3$H + stirred at (RT) for 21 h.</td>
<td>B32</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.3 g) MDR</td>
<td>(30 mL) ClSO$_3$H + heated at 50°C under reflux for 48 h.</td>
<td>B33</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.3 g) MDR</td>
<td>(1 g) p-toluene sulfonyl chloride + (3 mL) pyridine + heated at 100°C, under reflux, for 20 h.</td>
<td>B38</td>
<td>Joshi at el. (1951)$^{257}$</td>
</tr>
<tr>
<td>(0.3 g) AZN</td>
<td>(20 mL) ClSO$_3$H + heated at 50°C under reflux for 72 h.</td>
<td>B40</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.5 g) ARS</td>
<td>(20 mL) ClSO$_3$H + heated at 60°C under reflux for 21 h.</td>
<td>B45</td>
<td>Bassin et al. (1991)$^{254}$</td>
</tr>
<tr>
<td>(0.5 g) ARS</td>
<td>(30 mL) ClSO$_3$H + heated at 100°C for 4 h + stirred at room temperature for 24 h.</td>
<td>B47</td>
<td>Bassin et al. (1991) and Chen et al. (2013)$^{254,258}$</td>
</tr>
<tr>
<td>(0.5 g) ARS</td>
<td>(10 mL) DMF+ (20 mL) SO$_2$Cl+ (5 mL) DCM + heated at 70°C for 48 h.</td>
<td>B49</td>
<td>Balaconis et al. (2011)$^{259}$</td>
</tr>
<tr>
<td>(0.5 g) ARS</td>
<td>(25 mL) ClSO$_3$H + heated at 100°C for 20 h.</td>
<td>B50/ B53</td>
<td>Chen et al. (2013)$^{258}$</td>
</tr>
<tr>
<td>(0.5 g) ARS</td>
<td>(20 mL) DMF+ (25 mL) SO$_2$Cl+ (20 mL) DCM + heated at 70°C for 48 h.</td>
<td>B52</td>
<td>Balaconis et al. (2011)$^{259}$</td>
</tr>
<tr>
<td>(2 g) ARS</td>
<td>(100 mL) ClSO$_3$H + heated at 100°C for 20 h.</td>
<td>B58</td>
<td>Chen et al. (2013)$^{258}$</td>
</tr>
<tr>
<td>(0.5 g) B53</td>
<td>Purified + (50 mL) isopropanol + (1.7 g) VSPE + heated at 70°C for 19 h.</td>
<td>B60</td>
<td>-</td>
</tr>
<tr>
<td>(0.1 g) B58</td>
<td>Purified + (12 mL) tetrahydrofuran (THF) + (0.2 g) VSPE + heated at 60°C for 21 h.</td>
<td>B62</td>
<td>-</td>
</tr>
<tr>
<td>(0.1 g) B58</td>
<td>Purified + (12 mL) pyridine + (0.2 g) VSPE + heated at 100°C for 21 h.</td>
<td>B63</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.4 continued

| (0.1 g) B58 | Purified+ (12 mL) DMF + (0.2 g) VSPE + heated at 100°C for 21 h. | B63a | - |
| (0.5 g) ARS | (1.2 mL) H₂O₂ (34-37%) + (0.3 mL) SO₂Cl in (11 mL) acetonitrile (ACN)+ stirred at RT for 25 h + heated at 60°C for 24 h. | B65 | Bahrami et al. (2009)²⁶⁰ |
| (0.5 g) ARS | (4 mL) ACN + (4 mL) sulfolane+ (1.2 mL) dimethylacetamide (DMA) + (1.2 mL) POCl₃+ heated at 60°C for 1 h. | B67 | Sokolowska-Gadja et al. (1990)²⁶¹ |
| (0.5 g) VSPE | (10 mL) acetone + (15 mL) pyridine + (0.34g) K₂CO₃ + purified B58 in (30 mL) acetone + heated at 40°C for 3 h. | B74 | Sokolowska-Gadja et al. (1990)²⁶¹ |
| (0.5 g) ARS | (10 mL) ACN + (10 mL) sulfolane + (5 mL) DMA + (5 mL) POCl₃+ heated at 60°C for 2 h. | B77a | Sokolowska-Gadja et al. (1990)²⁶¹ |
| (0.5 g) ARS | (10 mL) sulfolane + (5 mL) DMA + (10 mL) POCl₃+ heated at 60°C for 5 h. | B80a | Sokolowska-Gadja et al. (1990)²⁶¹ |
| (0.08 g) B80a | (25 mL) acetone + (0.1 g) VSPE in (10 mL) pyridine + (0.07 g) K₂CO₃ + heated at 40°C for 20 h.+ heated at 60°C for 20 h. | B81 | Sokolowska-Gadja et al. (1990)²⁶¹ |
| (0.07 g) B80a | (16 mL) ACN + (0.06 g) VSPE in (10 mL) pyridine +stirred at RT for 48 h. | B82 | Bahrami et al. (2009)²⁶⁰ |
| (0.05 g) B80a | (0.06 g) VSPE + (16 mL) water + Na₂CO₃ 1 mol/L until pH 8 + stirred at RT for 2 h. | B82a | Deng et al. (2006)²⁶² |

AZN: alizarin; ARS: alizarin red S; MDR: madder
4.2.2.1.2. Analytical procedures

Progress of all reactions was followed by TLC, using a silica gel plate. For the analysis of reaction products having madder as the starting material, eluent system was chloroform/methanol/water (65:30:5). For products obtained from alizarin, the eluent was butanol/acetic acid/water (6:1:2).

Selected samples were also analyzed by HPLC-DAD and mass spectrometry. HPLC-DAD separation was performed using an Atlantis C18, 5µm (150 x 4.6 mm) column, flow rate was 1 mL/min and the volume of sample injected was 10 µL. Solvents 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile (ACN) were used in a gradient elution: 0-8 min: 15-60% ACN, 8-17 min: 60% ACN and 17-18 min: 15% ACN.

Mass spectrometry analysis was performed by the Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer using Electrospray Ionization (ESI) in positive and negative mode. Some samples were directly injected and other were injected after LC separation. Instrument parameters were: gas temperature 350°C, drying gas 10 L/min, nebulizer 30 psi, fragmentor voltage 175 V, capillary voltage 3500V.

4.2.2.2. Results and discussion

For samples B30 to B38, TLC did not show the presence of new bands, observed under the naked eye, suggesting the reactions were not generating new compounds under tested conditions. Chromatograms of B30, B31a and B32, Figure B2, did not show the presence of new peaks in comparison with the starting material madder.
Product mixture containing B33 was filtered and the solid and filtrate obtained were analyzed by HPLC-DAD, Figure B3. The solid portion was solubilized in dichloromethane and methanol but new compounds were not detected in the extracts.

Because the synthesis starting from madder was not successful, this work moved to the use of synthetic alizarin as the starting compound, hypothesizing its higher purity would aid the reaction. However, no new products were observed in sample B40, Figure B4.

Next step was trying to facilitate the synthesis by starting with a sulfonated compound (ARS) to be chlorinated instead of adding the chlorosulfonyl group to the aromatic ring. Chromatograms generated at alizarin red S maximum absorption wavelength (420 nm), Figure B5, suggested reaction was unsuccessful in producing samples B45 and B47.

Samples B62, B63, B63a, B68 and B74 were expected to be the sulfonamide derivative of ARS, Figure 4.6 (3), with [M-H]=581.9835, which was not identified in the spectra in Figures A2-A6. The absence of this expected peak was also true for sample B82, Figure A9.

ESI/MS spectra for B77a and B80a, Figures A7 and A8, respectively, did not indicate the presence of the expected sulfonyl chloride derivative of ARS, Figure 4.6 (2), with [M-H]=336.9574.

Samples B50, B53 and B58 will be discussed in further details in the next section. TLC for the remaining products, listed on Table 4.4 and not discussed in this section, did not show the presence of new bands and those compounds were not further analyzed.

The method described by Balconis et al (2011)259 was not specific when describing the molar ratio of the reactants used, so it was difficult to try to replicate the experiment, and the conditions tested in this work were not successful in synthesizing the sulfonyl chloride derivative. Moreover, a mixture of DMF and thionyl chloride is used and these compounds react with each
other to form N,N-dimethylchlororosulfitemethaniminium chloride, which can be interfering with the target reaction\textsuperscript{263}.

4.2.2.2.1. **Synthesis of a sulfonyl chloride derivative of Alizarin Red S**

Samples B50, B53 and B58 were prepared using the same reaction conditions. B58 is a “scale-up” version of B50/B53. The method used, reported by Chen et al. (2013)\textsuperscript{258}, generated a product with MS spectrum, **Figure 4.7**, containing ions \( m/z \) 336.9544 and \( m/z \) 338.9532 in a 3:1 ratio, indicating the presence of one chlorine in the molecule, and exact mass matching that expected for the desired sulfonyl chloride compound (\( m/z \) 336.9574). B58 was purified by column chromatography, **Figure 4.8 A**. A silica gel nylon column with mobile phase butanol/ acetic acid/ water (6:1:2) were the purification conditions. Fraction 3 corresponds to the unreacted dye ARS and fractions 2 and 1 contained B58.

To further characterize the sample, the purified compound was analyzed by NMR. Pulsed field NMR experiments were performed on a Bruker AVANCE 500 MHz Spectrometer (1996) equipped with an Oxford Narrow Bore Magnet (1989), HP XW 4200 Host Workstation, and Topspin 1.3 Software. Homonuclear (1H-1H) 2D COSY, TOCSY and heteronuclear (1H-13C) 2D HSQC experiments were recorded on a 500MHz Bruker Advance system operating at 500.18 MHz for \(^1\text{H}\) nuclei and 125.5 MHz for \(^{13}\text{C}\) nuclei equipped with room temperature inverse detection probe. Solvent was DMSO-d6. Even though the sample had been previously purified, the spectrum was not clear, making it difficult to confirm dye structure.
Figure 4.7.  (-) ESI/MS spectra for B58.

Figure 4.8.  Silica gel column with mobile phase butanol/ acetic acid/ water (6:1:2) used to purified B58 and (B) the three major fractions obtained.
The procedure to obtain B58 involved reacting ARS with chlorosulfonic acid at 100°C, then cooling and pouring the solution on ice. This is a very exothermic work up step. Furthermore, the goal was to apply the reaction method optimized using the synthetic alizarin to the synthesis of new dyes starting from a natural dye. Using ARS as starting component would add another step to the procedure as the component present in Madder is Alizarin. Considering these factors and the inability to get a clear NMR spectrum to further aid in the structure elucidation, another synthesis approach, described below, was evaluated.

4.2.2. Synthesis of a disperse-reactive alizarin analog for synthetic and natural fibers

The new strategy was to synthetize a dichlorotriazine derivative of alizarin and convert this product to a vinylsulfone dye. Therefore, the final product was expected to be a hetero functional bireactive dye, Figure 4.10.

The vinyl sulfone para ester compound is highly soluble in water, and poorly soluble in acetone, acetonitrile, tetrahydrofuran, dimethylacetamide and other solvents tested for the reaction. Therefore, we modified the compound following the reaction shown on Figure 4.9.

![Figure 4.9. Scheme for the chemical modification of VSPE.](image)

A

B

\[
\begin{align*}
\text{SO}_2\text{CH}_2\text{CH}_2\text{OSO}_3\text{H} & \quad \text{MW= 281.30 g/mol} \\
\text{SO}_2\text{CH}=\text{CH}_2 & \quad \text{MW= 183.23 g/mol}
\end{align*}
\]
4.2.2.1. Experimental section

4.2.2.1.1. Materials

All the chemical used for synthesis and dyeing were obtained from Sigma Aldrich, Fisher Scientific and VWR International. Alizarin, supplied as 97%, vinyl sulfone para ester, and cyanuric chloride, was used without further purification. Polyester (knit Dacron 56), bleached knit cotton, silk, wool, woven nylon and the multifiber strip were kindly provided by Ms Judy Elson in the College of Textiles at North Carolina State University. Natural dye Madder (CI Natural Red 8), and the dispersing agents Irgasol FL (lignin sulfonate), Dohnen dispersant DA-GD, Sera Gal N-LP (nonionic leveling agent) and Novadye NT9, were kindly supplied by Mr Jeffrey Krauss in the pilot plant at the College of Textiles, North Carolina State University.
4.2.2.1.2. Synthesis

Synthesis of intermediate compound, **Figure 4.10 (B118c)**, involved the solubilization of 4.2x10⁻³ mol alizarin in 80 mL of THF, followed by the addition of 4.2x10⁻³ mol K₂CO₃. A cyanuric chloride solution, 4.2x10⁻³ mol in 10 mL THF was added dropwise, over 10 minutes, to the alizarin solution, at 0°C. Reaction was followed by TLC, using silica gel and the eluent system toluene/ethyl acetate (2:1). After 5 hours, the product was purified by column chromatography using the same eluent.

Due to the high reactivity of the sulfonyl chloride intermediate, B118c, with hydroxylated compounds and solvents, the second step of the reaction had to be run in dry ethyl acetate. Reactant on Figure 4.10 (7) was obtained by adding a 4% NaOH solution to and aqueous vinylsulfone para ester (VSPE) solution, until pH 10.5, and extracting the resulting compound with ethyl acetate. This procedure was necessary due to the solubility of VSPE.

For the synthesis of B120, 2.5x10⁻⁴ mol of the purified product B118c was mixed with 20 mL of ethyl acetate and added to a solution of 2.5x10⁻⁴ mol of reactant on **Figure 4.10 (7)** in 10 mL of ethyl acetate. Solution was heated at 40°C and stirred for 72 h.

4.2.2.1.3. Characterization of synthesis products

HPLC-DAD separation was performed using a XBridge Waters C18 3.5µm (150 x 4.6 mm) column, flow rate was 0.8 mL/min and the volume of sample injected was 10 µL. Solvents 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile (ACN) were used in a gradient elution: 0-6 min: 60% ACN, 6-8 min: 60-90% ACN and 16-17 min: 90-60% ACN.

Mass spectrometry and NMR analysis were performed using the parameters described on sections 4.2.2.1.2. and 4.2.2.2.1., respectively.
4.2.2.1.4. Preparation of dye suspensions

The needed amount of synthesized dye (B120) for a 1% (owf) shade was weighed and added to 50 mL of water. The required amount for a 1% (owf) proportion of dispersing agent was added and the dispersion was sonicated for 10 min and stirred for 30 min. For the study of the effect of dye % shade on color strength, dispersions were prepared with 1% (owf) Irgasol FL dispersing agent and the amount of dye required to reach the desired shade. Alizarin and madder dispersions were prepared in similar way for comparison. The effect of the 4 types of dispersing agents, Irgasol FL (I), Novadye NT (N), SeraGal N-LP (S) and Dohmen DA-GD (D), on the color strength was evaluated.

For dyeing cotton dye dispersions were prepared in similar way, but with the addition of the amount of sodium carbonate (Na$_2$CO$_3$) needed for a 2% (owf) concentration.

4.2.2.1.5. Dyeing procedure

Each fabric (1 g), nylon, wool, silk, polyester was dyed in individual dyebaths using an Ahiba IR™ Nuance machine. A 50:1 liquor ratio was used, and dispersions were kept at pH 5.5-6.5. The dyebath was heated steadily at 2°C/min until the 130°C temperature was achieved, then held at that temperature for 60 min. After dyeing, the dyebath was cooled at 2°C/min. Reduction clearing was conducted to remove surface dye from the samples, by stirring the fabric, at 90°C, for 10 min, in the scouring bath, which was prepared with 10 g of Na$_2$CO$_3$ and 20 g of NaOH. Samples were rinsed, washed with 5 g of Tergal detergent and thoroughly rinsed with warm water.

For cotton, the pad-dry-cure application method was used. 1 g of bleached cotton was soaked in the dye dispersion for 10 minutes. Fabric went through the padding machine, was dried at 110°C for 5 minutes and cured at 199°C for 2 minutes. Scouring was conducted.
4.2.2.1.6. Color measurement

The spectral reflectance properties of the dyed samples were measured with X-Rite, color i7 spectrophotometer. Each sample was folded twice and 3 different areas of each sample were analyzed to give the average color value. CIELAB values (under illuminant D65). K/S values were calculated. K/S values are obtained from the Kubelka-Munk equation:

$$\frac{K}{S} = \frac{(1 - R)^2}{2R}$$

4.2.2.1.7. Lightfastness testing

The light fastness of polyester dyed samples was tested by exposing the samples to an irradiation of 51310.8 kJ/m², simulating a 26°N latitude, average Summer temperature of 34°C and 79% relative humidity, for 48 hours.

4.2.2.2. Results and discussion

4.2.2.2.1. Dye synthesis

The goal of this study was to chemically modify the Alizarin structure producing a new dye with better affinity for a variety of fibers. Because the synthesized dye has two reactive groups, we anticipated its successful application to cotton.

Purified dichlorinated product, B118c (m/z 385.9752), Figure 4.13 A, was obtained in a 76% yield. Chromatogram, Figure 4.11, shows the appearance of several peaks in comparison with alizarin. Product B120 (m/z 535.0496), Figure 4.13 B, was obtained in 37% yield, after purification. Yield was very modest, but this value is for the purified dye and it is expected that optimization can be performed in order to increase the amount of final product obtained. Although not ideal, low yields have been reported for the chemical modification of Alizarin.
Figure 4.11. Comparison between the chromatograms for (a) Alizarin and (b) B118c, at 430 nm.

Three peaks are present in product B118c chromatogram, Figure 4.11 (b), indicating the presence of hydrolyzed reaction by-products. Figure 4.12.

Figure 4.12. By-products of the reaction for the synthesis of B118c.
Figure 4.13. (A) (-) ESI/MS showing the presence of the dichlorinated product $m/z$ 385.9754 and (B) (+) ESI/MS showing the presence of the desired monochlorinated product $m/z$ 535.0473.
4.2.2.2. Dyeing properties

Commission International de l’Eclairage (CIE) L* a* b* color space was used to measure color differences. L* indicates lightness, a* is the red/green coordinate, and b* is the yellow/blue coordinate. The ΔE* value is the total color difference between the analyzed sample and the standard. The dispersing agent Novadye NT was responsible for the major color difference between PET samples dyed with B120 and with Alizarin (used as the standard for calculations), Figure 4.16 A. Δa* and Δb* are all negative for B120, indicating samples are greener and less yellow. Samples also became lighter (ΔL* positive), and there was not a very significant influence of the dispersing agent used on lightness as ΔL* values did not change much. For comparison, Figure 4.14 shows the PET samples dyed with 1% (owf) B120 and the four dispersing agents tested. The same trend was observed in comparison with madder dyed PET, Figure 4.15. Samples became greener, less yellow and lighter, in comparison with the PET samples dyes with Alizarin, Figure 4.16 B. However, the dispersing agents used did not cause a significant overall color difference between the samples.

Figure 4.14. PET samples dyed using 1% (owf) of B120 and 1% (owf) of dispersing agents (A) Irgasol FL, (B) Sera Gal N-LP, (C) Dohmen DA-GD and (D) Novadye NT (N).
**Figure 4.15.** PET samples dyed using 1% (owf) of Madder and 1% (owf) of dispersing agents (A) Irgasol FL, (B) Novadye NT (N) and (C) Sera Gal N-LP.

**Figure 4.16.** CIELAB values differences for PET sample dyed with the 1% (owf) synthesized dye B120, using Irgasol FL (I), Novadye NT (N), SeraGal N-LP (S) and Dohmen DA-GD (D), 1% (owf), dispersing agents, in comparison with (A) PET dyed with Alizarin; and (B) PET dyed with Madder (MD).
Comparing PET samples dyed with alizarin, B120 and madder, Figure 4.17, showed that dyeing with Alizarin led to a darker shade and dyeing with B120 led to the lighter shade, which agrees the CIELAB values in Figure 4.16 A. Sample B120 became lighter (positive ΔL*). Moreover, the color difference (ΔE*) is larger when comparing the pair PET dyed with Alizarin (standard)/PET dyed with B120, Figure 4.16 A, with the pair PET dyed with Madder (standard)/PET dyed with B120, Figure 4.16 B.

![Figure 4.17](image)

**Figure 4.17.** PET samples dyed using 1% (owf) of Irgasol FL and 1% (owf) (A) Alizarin, (B) B120 and (C) Madder.

When applied to wool, nylon and silk, the colors obtained were significantly different from the color obtained for the PET samples, Figure 4.18. Alizarin applied to PET was the standard for the comparison with nylon, silk and wool dyed with Alizarin. Likewise, PET dyed with B120 was the standard to measure the color change of nylon, silk and wool dyed with B120. All samples became darker, bluer and redder, and larger ΔE* were calculated, agreeing with the big color difference observed, Figure 4.19.
Figure 4.18. 1% Irgasol FL used in the dispersion to create a 1% shade to silk dyed with (A) Alizarin and (B) B120, wool dyed with (C) Alizarin and (D) B120, nylon dyed with (E) Alizarin, (F) B120 and (G) Madder.

Figure 4.19. CIELAB results obtained for the comparison between PET dyed at 1% (owf) shade using 1% of Irgasol FL, and nylon, silk and wool, dyed with Alizarin (AZN), B120 and Madder (MD).
Figure 4.20. K/S values for wool, silk, nylon and PET samples dyes at 1% (owf) shade with Madder, Alizarin and B120, using 1% of Irgasol FL as dispersing agent.

A bathochromic shift in absorption resulted in the color change of the samples, going from yellow to purple (Alizarin), yellow to orange (B120) and yellow to brown (Madder). Alizarin varies in aqueous solubility and color according to the pH conditions with a blue color being observed in strong alkali. Alkali reduces inter-molecular hydrogen bond, decreasing the extent of aggregation, and leads to absorbance at longer wavelengths. This process is due to the ionization of the -OH in position 2 leading to the formation of a more electron-rich conjugated π systems\textsuperscript{205}. However, the present dyeing was not done at an alkaline pH, so the color shift observed is probably due to the nature of interaction with different fiber groups. The brown color obtained for madder shows the influence of the other components present in the natural dye on the dyeing of nylon.

Highest color strength (K/S), Figure 4.20, for B120 was obtained when dye was applied to wool. At neutral pH there is a higher nucleophilicity of the nucleophilic sites in wool. The nonionic vinyl sulfone group in the dye has increased substantively for the amino group in wool\textsuperscript{266},
which is probably leading to higher fixation to the fiber, and therefore, higher K/S values. Color strength for B120 on PET increased with the increase of % shade, **Figure 4.21.**

![Graph showing K/S values for different % shades](image)

**Figure 4.21.** Color strength of B120 applied to PET at 1-4% (owf) shades, using 1% Irgasol FL.

Dyeing applying B120 and Alizarin to cotton using the high temperature dyeing and the pad-dry-cure method, typically employed for the dyeing of cotton, did not result in a dyed fabric. After scouring all the surface dye was removed and the fabric was colorless. Viscose (rayon) is another example of fabric that cannot be dyed by Alizarin or B120, **Figure 4.22.** From the multifiber strip, B120 was also successful in dyeing polypropylene, Dacron 54 and modacrylic.
4.2.2.2.3. Lightfastness evaluation

The results of fastness tests of Alizarin and B120 on polyester are given in Table 4.5. CIELAB values for PET dyed with Alizarin was compared before and after exposure to light, and the same procedure was adopted for B120. The highest color difference ($\Delta E^*$) between the samples was observed for Alizarin, and the lowest value was obtained for B120 1%. Increasing dye shade seems to increase the adverse effect of light on the dye. Although the difference was very modest, the newly synthesized dye showed better light fastness than the starting material Alizarin. Light fastness of the three samples was 5 according to the gray scale standard, and a difference could not be observed.
Table 4.5. Difference between CIELAB values for Alizarin and B120 samples before and after exposure to light.

<table>
<thead>
<tr>
<th>Dye</th>
<th>ΔL*</th>
<th>Δa*</th>
<th>Δb*</th>
<th>ΔE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alizarin</td>
<td>-4.46</td>
<td>0.83</td>
<td>-6.49</td>
<td>7.92</td>
</tr>
<tr>
<td>B120 1%*</td>
<td>0.45</td>
<td>-0.68</td>
<td>1.06</td>
<td>1.34</td>
</tr>
<tr>
<td>B120 3%*</td>
<td>-3.67</td>
<td>-0.3</td>
<td>-3.08</td>
<td>4.80</td>
</tr>
</tbody>
</table>

*Starting dyebath concentrations.

4.2.2.3. Conclusions

The research herein demonstrated the synthesis of a vinyl sulfone reactive-disperse dye (m/z 585.0496) which showed excellent light fastness on PET. The natural anthraquinoid analog was synthesized in a 2-step reaction starting from Alizarin, and successfully applied to nylon, silk, wool and polyester, with the potential of being used for the coloration of polypropylene and modacrylic fabrics as evidenced by the dyed multifiber strip.

Using the dispersing agent Novadye NT led to the highest color difference and a lighter color in comparison with Alizarin, suggesting lower fixation rate. Color strength was found to increase with the % B120 dye shade, as expected. In comparison with the parent compound, B120 had slightly better K/S value when applied to silk, using Irgasol FL as dispersing agent. For B120, the highest color strength value (4.83) was measured for wool, probably due to the formation of an amino bond between the vinyl sulfone group on the dye and the amino group on wool.

Although, synthesis yield was modest (37%), and K/S values were not as good for PET, nylon and wool, in comparison with the parent dye, this novel dye can be used as an extension of natural dyes. It can be applied to several fibers without the use of the metallic mordants typically used for natural dyes and are more likely to produce reproducible shades, which is a big problem
with natural dyes. Interestingly, this dye can also be applied to polypropylene, a quite unexpected result.
CHAPTER 5. Future work

Next steps towards obtaining more information, to complement the information presented in this work would be:

1. Perform LC-DAD-MS analysis of the degradation products obtained from the chlorination of Logwood, Weld, Cochineal and Fustic natural dyes, and compare the degradation pathway of these dyes with that of Madder.

2. Optimize the reaction conditions for the synthesis of the novel B120 dye to improve reaction yield and, NMR spectra quality. Further, extend this work to the sulfatoethylsulfone (SES) form, to achieve dye uptake on cotton.

3. Apply the optimized synthesis method using madder as the starting material and compare the dyeing properties with B120.

4. Perform ecotoxicity and mutagenicity evaluation of B120.

5. Analyze the exhaustion behavior of B120 to investigate its dyeing mechanism on wool, silk and nylon.
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APPENDICES
Figure A1. (A) (-) ESI/MS full scan of P59 and (B) close up of the m/z 456.9118 ion.
Figure A2.  (-) ESI/MS spectrum for B62.

Figure A3.  (-) ESI/MS spectrum for B63.
Figure A4. (-) ESI/MS spectrum for B63A.

Figure A5. (-) ESI/MS spectrum for B68.
Figure A6. (-) ESI/MS spectrum for B74.

Figure A7. (-) ESI/MS spectrum for B77a.
Figure A8.  (-) ESI/MS spectrum for B80a.

Figure A9.  (-) ESI/MS spectrum for B82.
APPENDIX B: HPLC-DAD

Figure B1. Comparison of the elution profiles for Indigo carmine and B20 at 608 nm.

Figure B2. Comparison of the chromatograms for (a) Madder, (b) B30, (c) B31a and (d) B32, at 430 nm.
Figure B3. Comparison of the chromatograms for (a) Madder, (b) B33 solid in methanol, (c) B33 solid in dichloromethane and (d) B33 filtrate, at 430 nm.

Figure B4. Comparison of the chromatograms for (a) Alizarin, (b) B40 in methanol and (c) B40 in dichloromethane, 430 nm.
Figure B5. Comparison of the chromatograms for (a) ARS, (b) B45 and (c) B47, 420 nm, and the DAD absorption spectrum for peak with rt=7.059 min.