Environmental Pollution 287 (2021) 117299

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Characterizing azobenzene disperse dyes in commercial mixtures and children's polyester clothing☆

Kirsten E. Overdahl [a], David Gooden [b], Benjamin Bobay [b], Gordon J. Getzinger [c], Heather M. Stapleton [a], P. Lee Ferguson [a,c,*]

[a] Nicholas School of the Environment, Box 90328, Duke University, Durham, NC, 27708, United States
[b] Department of Chemistry, Box 90354, Duke University, Durham, NC, 27708, United States
[c] Department of Civil and Environmental Engineering, Box 90287, 121 Hudson Hall, Duke University, Durham, NC, 27708, United States

ARTICLE INFO

Keywords:
Azobenzene disperse dyes
Non-targeted analysis
Human exposure

ABSTRACT

Azobenzene disperse dyes are the fastest-growing class of dyestuffs, yet little is known about dye occurrences, sources, and transformations; azo dyes are also underrepresented in chemical standard catalogs, molecular databases, and mass spectral libraries. Many azo dyes are known to have sensitization, mutagenic, and carcinogenic properties. To fill these knowledge gaps, azo dyes were purified from dyestuffs by Soxhlet extraction and flash chromatography and characterized using ultra-high-performance liquid chromatography (UHPLC) coupled to a high resolution Orbitrap Fusion Lumos mass spectrometer operated in positive electrospray ionization mode, as well as by ^{1}H and ^{13}C NMR. Data were analyzed to identify likely chemical formulas and structures using a weight-of-evidence approach with multiple open-source, *in silico* computational mass spectrometry tools. Nineteen total azobenzene dyes were detected in dyestuffs via a non-targeted analysis approach; the azobenzene dyes Disperse Blue 79:1, Disperse Blue 183:1, Disperse Orange 44, Disperse Orange 73, Disperse Red 50, Disperse Red 73, and Disperse Red 354 were purified from raw dyestuffs. Samples of children's polyester clothing were then analyzed likewise. In clothing, 21 azobenzene disperse dyes were detected, 12 of which were confirmed and quantified via reference standards. Individual dyes in apparel were quantified at concentrations up to 9230 μg dye/g shirt, with geometric means ranging 7.91–300 μg dye/g shirt. Total dye load in apparel was quantified at up to 11,430 μg dye/g shirt. This research supported the development of reference standards and library mass spectra for azobenzene disperse dyes previously absent from standard and spectral libraries. By analyzing the scope and quantities of azo dyes in children's polyester apparel, this study will facilitate a more robust understanding of sources of these potentially allergenic and mutagenic compounds.

1. Introduction

Disperse dyes are a class of substituted anthroquinone- or azobenzene-based dyes used to color synthetic fabrics such as polyester, nylon, and acrylic (Benkhaya et al., 2020a; Waring, 1984). The majority of these dyes (60%) are azobenzene disperse dyes (hereafter referred to as "azo dyes"), high production-volume commodity chemicals that account for roughly 70% of the 9.9 million tons of industrial dye colorants used annually (Dawson, 1991; Drumond Chequer et al., 2011). Azo dyes are used to create the entire range of color shades, and are favored by industries because they are cheap and easy to apply (Malinauskiene et al., 2013). While azo dyes can vary widely in their chemical structures and molecular weights, each azo dye is based on a core substructure of para-aminoazobenzene [Figure SI-1]. Functionalization of the p-aminoazobenzene core with substituents including halogens, (often chlorine or bromine) imparts electronic and, hence, optical properties to the molecules, making them attractive as dyestuffs (Chen et al., 2018; El-Ghamaz et al., 2017; Porobić et al., 2020). These functional groups may also influence the reactivity and potential toxicity of azo dyes. As their name implies, disperse dyes do not chemically bond to synthetic fibers, but rather impart color via non-covalent absorption into polymeric fibers. These dyes must therefore be applied in conjunction with a dispersing or fasting agent (Benkhaya et al., 2020b).

Azo dyes currently represent the most abundant, and the fastest-

☆ This paper has been recommended for acceptance by Charles Wong.

* Corresponding author. Duke University, 121 Hudson Hall, Box 90287, Durham, NC, 27708, United States.
E-mail address: lee.ferguson@duke.edu (P.L. Ferguson).

https://doi.org/10.1016/j.envpol.2021.117299
Received 5 February 2021; Received in revised form 21 April 2021; Accepted 30 April 2021
Available online 15 May 2021

growing, class of dyestuffs (Dawson, 1991). Despite their high and growing use in consumer products, little is known about dye occurrence, transformation, and fate in environmental compartments such as the indoor environment (House of Parliament, 2013; Young, 2016). This lack of knowledge is problematic, as potential for human exposure is high and disperse dyes have been shown to act as electrophilic mutagens in laboratory experiments focused on their occurrence in textile effluents (Balakrishnan et al., 2016; De Aragão Umbuzeiro et al., 2005; Ohe et al., 2004). Disperse dyes are also implicated as contact allergens (Brookstein, 2009; Chung, 2016; Heratizadeh et al., 2017; Seidenari et al., 1990; Susan and Elizabeth, 1988). In fact, p-aminoazobenzene is among the most well characterized mutagens and skin sensitizers (Brüschweiler and Merlot, 2017; Carneiro et al., 2010; Chequer et al., 2011, 2015; Drumond Chequer et al., 2011; Rawat et al., 2018; Rocha et al., 2017; Vacchi et al., 2016, 2017). Because disperse dyes do not bind chemically to fibers, these small lipophilic molecules can potentially migrate onto the skin of a person wearing the garment, especially if textile fastness is poor. Dyes may also be removed from fibers by rubbing or leaching into water (Malinauskiene et al., 2013). Exposure to disperse dyes may occur from skin contact with dyed garments, or subsequently, through inhalation or ingestion, particularly in occupational settings.

Several challenges complicate comprehensive assessment of disperse dye occurrence and exposure in environmental and biological samples. Specifically, disperse dyes are underrepresented in chemical standard catalogs, molecular databases, and mass spectral libraries. Only eight disperse azobenzene dyes are maintained in-stock by distributors of scientific chemicals, although custom synthesis or preparation is available in some cases for selected azobenzene disperse dyes from specialty chemical supply vendors. A search of the term “Disperse” in the open-source database PubChem yields 223 compounds; of these, 133 compounds are annotated as azo dyes. Yet, a 2016 query of the Chemical Abstract Services (CAS) database revealed that the total number of single-component, commercially-available molecules having a core p-aminoazobenzene structure was 4980. While not all azobenzene molecules are utilized as disperse dyes, the breadth of azobenzene disperse dyes indicates that there are almost certainly more azobenzene disperse dyes than are tabulated by common names in PubChem. Lack of available reference standards and analytical spectral libraries present significant barriers to analysis of azobenzene dye presences, sources, and human exposures in the environment. Therefore, there is a need to generate purified reference standards and spectral libraries from raw dyestuffs to support increased monitoring of these compounds in environmental and human-exposure relevant media, and to examine potential exposure sources of azobenzene disperse dyes.

This study[1] aims to address these knowledge gaps via a detailed mass spectrometric analysis of disperse dyes in select dyestuffs and exposure-relevant consumer products. We obtained thirteen raw commercial dyestuff powders from a local textile dye distributor in North Carolina and, from these raw dyestuffs, purified individual azobenzene disperse dyes via flash chromatography. Individual azobenzene dye structures were elucidated via high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy. We then obtained and analyzed children’s polyester athletic apparel to quantify occurrences and concentrations of azobenzene disperse dyes in these materials. Finally, we built an in-house database of known p-aminoazobenzene based compounds and employed this database in suspect screening for comprehensive annotation of disperse azobenzene dyes present in dyestuffs and in children’s athletic apparel. To our knowledge, this study is the most complete examination of azobenzene disperse dyes in commercial products and human-relevant exposure media to date.

2. Materials and methods

2.1. Sample preparation: dyestuffs and analytical standards

Thirteen commercial dyestuff powders were obtained from a textile dyestuff distribution facility in North Carolina in 2018 [Table SI-1]. The dye supplier was unable to provide any technical or chemical information that could guide our preparative separations. For each dyestuff powder, 2 g of powder were weighed and transferred to a cellulose Soxhlet extraction thimble [Cytiva Whatman, 33 × 94mm]. Dyestuff powders were extracted over 12 h via Soxhlet extraction in 300 mL of dichloromethane (DCM), to a final volume of 100 mL. Extracts for each dyestuff powder were examined via thin layer chromatography (TLC) under ultraviolet light to determine the number of individual dye compounds present in each powder [Figure SI-2]. TLC was then used to determine the optimal gradient required to separate individual dyes within each powder extract in subsequent preparative chromatography processes using hexane and ethyl acetate.

Each dichloromethane extract was transferred to a 500 mL round-bottom flask together with 2 g of silica gel [RediSep Rf Gold, 30 μm, 60 Å]. Each silica gel-dye mixture was rotary-evaporated, using a double cold trap with dry ice, under high vacuum with no heat to homogenize the mixture and load the dye extracts onto silica. Silica gel thus loaded was packed into a chromatography cartridge [RediSep Teledyne, 5 g] for flash chromatography separation. Mixtures were then separated using a CombiFlash Rf + chromatography system [Teledyne ISCO]. A full chromatography protocol can be found in Appendix A. Following chromatographic separation, UV traces and visual observations of elution color were used to track fraction collection and compound elution. In total, approximately 75 eluent fractions were collected for each dye mixture. Eluents for each dye mixture were examined via TLC under UV light to identify fractions containing individual dye compounds or mixtures of dyes. Purified fractions were dried, reconstituted, and diluted serially into 75:25 acetonitrile:water (LC-MS grade) to reach 500 ng/mL for HRMS analysis. The preparative chromatographic separations did not have sufficient resolution to enable an assessment of chemical structure through examination of observed elution times.

2.2. Sample preparation: children’s apparel

Fourteen items of children’s apparel were purchased from local department stores in North Carolina, including eleven athletic shirts: red, blue, and black shirts from three different major brand names (Brands A, B, and C), an orange shirt from one major brand name (Brand B), and a black shirt from an off-brand company (Brand E). Apparel examples also included one sleep shirt and pair of pajama pants (Brand D), and one pair of sleep shorts (Brand F) [Table SI-2]. A white shirt was also purchased for use in a matrix-spike recovery experiment. All apparel examples were 100% polyester. For each apparel sample, a 2.5 cm^2 fabric square was weighed and then extracted over 12 h via Soxhlet extraction; a laboratory blank was also prepared for analysis. A full extraction protocol for all shirts (including matrix-spike recovery experiment) can be found in Appendix B.

2.3. Structure elucidation of disperse dyes

All purified dyes (1 mL, in muffled amber glass LC-MS vials) were analyzed by UHPLC [ThermoDionex Ultimate 3000] coupled to high-field orbital trapping mass spectrometry [ThermoFisher Orbitrap Fusion Lumos]. Samples were separated on a reversed phase column

[1] Abbreviations: Dichloromethane (DCM); Disperse Orange A (DOA); Disperse Orange 25 (DO25); Disperse Orange 37 (DO37); Disperse Orange 44 (DO44); Disperse Orange 61 (DO61); Disperse Orange 73 (DO73); Disperse Red 354 (DR354); Disperse Red 50 (DR50); Disperse Red 73 (DR73); Disperse Blue 373 (DB373); Disperse Blue 79:1 (DB79:1); Disperse Blue 183:1 (DB183:1); Disperse Violet 33 (DV33); Disperse Violet 93 (DV93); Disperse Violet 93:Cl (DV93:Cl); high-resolution mass spectrometry (HRMS); nuclear magnetic resonance spectroscopy (NMR); thin-layer chromatography (TLC).

[Thermo Hypersil Gold column, 1.9 μm particle size, 2.1 × 100 mm] over a 25 min water:acetonitrile (LC-MS grade, 0.1% formic acid) gradient. The Orbitrap was operated in ESI(+) mode at 240,000 mass resolution with internal mass calibration to achieve < 2 part per million (ppm) mass accuracy across the mass range of interest. Data-dependent MS/MS acquisition was performed in the Orbitrap via higher energy collisional dissociation (HCD). Ions for MS/MS analysis (approximately 15–20 precursors per second) were dynamically chosen on a per-scan basis. These MS/MS data provided fragment ion data to aid in structure elucidation.

As a first step to assist in annotating structures of purified azobenzene disperse dyes, we built a custom database of all commercially-available p-aminoazobenzene containing compounds available in the CAS chemical structure repository as of 2016. Specifically, three structure lists were created from a p-aminoazobenzene substructure query in SciFinder Scholar (American Chemical Society): a list with all known non-halogenated azobenzenes (n = 4665), a list with chlorinated azobenzenes (n = 220), and a list with brominated azobenzenes (n = 95). For full databases including IUPAC name, formula, monoisotopic mass, CAS number, InChIKey14, and SMILES, please see Appendices D, E, and F. The combined list was first used to build a non-redundant inclusion list of molecular formulas to prioritize MS/MS data acquisition in the mass spectrometer.

Following data acquisition, MS/MS data was then matched against the compiled azobenzene structure database using Compound Discoverer software [ThermoFisher Scientific, version 3.2] to postulate structural candidates for putative dyes in each purified dye extract. Plausibility of structure candidates was then assessed via *in silico* MS/MS tools and rules-based fragmentation interpreters. Experimental isotope fits and MS2 spectra were compared against predicted isotope fits and MS2 spectra for each compound, generated using EnviPat Web 2.4 (Loos et al., 2015). Within Compound Discoverer, a FiSH score was calculated for each predicted compound to evaluate the fit of the predicted structure to the spectral data. FiSH scoring utilizes the rules-based *in silico* MS/MS tool MassFrontier [HighChem, Bratislava, Slovakia] and the expected precursor ion list to predict compound fragment structures for each precursor ion and attempts to match these fragment structures to the centroids in the experimental fragmentation scores of the given precursor ion. The fit score is calculated as the number of fragment structures matched to centroids over the total number of possible fragment structures.

Additional structural characterization of dye molecules in purified dyestuff fractions was performed by Nuclear Magnetic Resonance Spectroscopy (NMR) using either a Bruker 16.4 T spectrometer with a BBO room temperature probe or a Varian 18.8 T spectrometer with a cryogenic probe at room temperature, 25C. The full NMR protocol can be found in Appendix C.

2.4. Suspect screening of dyestuffs and apparel

All dyestuff and apparel samples were analyzed by UHPLC-HRMS over a 43 min chromatographic method designed for optimal compound separation under identical conditions as stated above (Section 2.3). For analyses of clothing extracts, compound masses from the azobenzene database described above were utilized as the precursor ion selection list; precursor ion selection was not employed for analyses of dyestuffs. LC-MS/MS data from Orbitrap analysis of dyestuffs and shirt extracts were applied to commercially-available processing software [ThermoFisher Scientific Compound Discoverer 3.2 (beta)] to aid in identifying azobenzene compounds based on HRMS/MS data. Molecular features with distinct exact mass, unique retention time, and MS/MS data were identified in Compound Discoverer after removal of isotope peaks, blank contaminants, and noise artifacts from the data; rigorous data curation and cleaning was performed using component detection features within Compound Discoverer as well as data processing scripts incorporated into a custom-written Compound Discoverer "node". Specifically, features with exact mass matches to our azobenzene databases were consolidated via dereplication algorithms including RAMClustR to harmonize outputs from the mass list match results (Broeckling et al., 2014). A 5-to-1 sample-to-blank ratio was employed in Compound Discoverer to mark molecular features as background contaminants. Custom-written software (in R) was then used to remove potentially artifactual in-source fragment ions from the detected compound list by examining MS/MS spectra derived from each precursor ion and assessing presence of any observed MS/MS fragment ions in the full-scan MS data at similar retention times. Redundant adduct ions were also collapsed into a single consolidated compound feature in cases where multiple adduct ions were observed for a single compound and adduct relationships were clear from exact m/z differences.

Consolidated features were then processed using custom-written software scripts designed to assign formulas and structures using a weight-of-evidence based analysis approach leveraging multiple open-source, *in silico* computational mass spectrometry tools (Getzinger and Ferguson, 2020). Tentative formula assignments from Compound Discoverer were first validated using Sirius, an open-source tool that utilizes MS/MS spectra to compute fragmentation trees for each possible molecular formula and score these fragmentation trees according to fit to determine the formula that best explains isotope and MS/MS data (Böcker and Dührkop, 2016; Dührkop et al., 2019). Assigned molecular formulas were then passed to four separate *in silico* MS/MS tools (MetFrag, MAGMa, CFM-ID, and CSI:FingerID) to yield possible structural annotations for each molecular feature. MetFrag (Ruttkies et al., 2016; Wolf et al., 2010) and MAGMa (Ridder et al., 2012) accomplish *in silico* fragmentation via exhaustive bond cleavage and generate all possible molecular fragments for a candidate molecule; MAGMa also matches fragments to experimental spectral trees for increased annotation confidence. CFM-ID (Allen et al., 2014) utilizes a machine learning model, trained using mass spectra from curated libraries, to predict mass spectra for predicted structural candidates. CSI:FingerID (Dührkop et al., 2015) is also trained on reference MS/MS spectra, and utilizes the fragment trees generated from Sirius in combination with calculated molecular fingerprints to propose plausible structures. MetFrag and MAGMa are advantageous in that they are not limited by a training set in their generation of molecular fragments; however, because they are not trained on empirical mass spectral data, molecular fragments generated via these tools may not accurately reflect fragmentation processes that actually occur within a mass spectrometer. CFM-ID and CSI:FingerID are machine-learning tools with models based upon empirical spectra and thus perform well for compounds within the applicability domain of the training sets; however, their applicability domains are limited by the training sets on which they are built, which may be problematic when assessing compounds such as azo dyes for which little to no extant mass spectral data exists in open spectral libraries. A combined approach was thus utilized to leverage the strengths and weaknesses of each *in silico* MS/MS tool and to generate multiple lines of evidence for each chemical identification.

For each candidate structure, a score of fit to the fragmentation spectra was determined using each *in silico* MS/MS tool; scores of candidate structures were then ranked by percentile. For each assigned molecular formula, the scores for all structure candidates from each of the four *in silico* MS/MS tools were compared according to percentile rank. The molecular structure with the highest composite percentile rank score was selected as the tentative structure identification for the molecular formula.

2.5. Targeted data analyses of dyestuffs and shirt extracts for azobenzene disperse dyes

Following suspect screening analysis, we utilized our purified reference standards (n = 7), purchased analytical standards of Disperse Orange 25 and Disperse Orange 37 [Sigma; 95% purity], and analytical standards of Disperse Orange 61, Disperse Violet 93, and Disperse Blue

373 provided via a generous gift from Prof. Gisela Umbuzeiro, School of Technology, UNICAMP, Limeira, Brazil to quantify individual azobenzene disperse dyes in dyestuffs and in clothing extracts (total azobenzene disperse dye standards = 12). An eight-point calibration curve was created, ranging from 0 ng/mL to 500 ng/mL, in 75:25 acetonitrile: water; para red d-4 [Toronto Research Chemicals] was used as the internal standard in each sample (50 ng/mL). Results were analyzed using TraceFinder software [ThermoFisher, version 5.0].

3. Results

3.1. Purification of azobenzene disperse dye reference standards

Ten azobenzene disperse dyes were isolated from raw dyestuffs [Table 1]. We used a two prong approach that included HRMS and NMR analyses to confirm chemical structures of isolated compounds. We assessed mass list matches between HRMS predicted molecular weights and molecular formulas and our in-house azo dye databases to determine predicted chemical formulas and chemical structures of each isolated dye identity. For each of the ten isolated compounds, candidate structures were evaluated for fit to the tandem mass spectral data using MassFrontier (Wolf et al., 2010) and FiSH scoring within Compound Discoverer. An example of a fully annotated dyestuff base peak ion chromatogram, azobenzene disperse dye extracted ion chromatogram, MS1 spectrum (experimental and predicted), and FiSH score annotated MS2 spectrum for the dyestuff Permasil Red S2GFL and corresponding extracted dye Disperse Red 354 is shown in Fig. 1. All annotated chromatograms and MS/MS spectral data are shown in Figure SI-4. The octanol water partition coefficient (Log K_{ow}) values for each compound, estimated using JChem [ChemAxon, Boston, MA], were relatively high (range 3.51–6.01), and confirm that these dyes are hydrophobic as expected, given that disperse dyes impart their color through physical adsorption to polymeric fibers (Chakraborty, 2010).

Compound purity was assessed by collecting 1H NMR and 13C NMR spectra [see Appendix C]. We were able to obtain high-quality NMR spectra demonstrating the purity of seven out of ten of our extracts from dyestuffs. Due to a lack of concentration or purity issues, the isolated dyes Disperse Violet 93 Cl, Disperse Violet 33, and Disperse Orange A were not able to be analyzed through NMR and were therefore not used for quantitative analysis. All annotated NMR spectra can be found in Figure SI-3. We further evaluated compound identity by measuring the molar absorptivity of each extract, using a standard cuvette reader [SpectraMax 5.0], to confirm that the absorption spectrum for each dye matched the predicted color of each structure's common dye identity. Molar absorptivities are listed in Table 1; full molar absorptivity plots for each isolated azobenzene disperse dye can be found in Figure SI-5.

3.2. Identification of additional azobenzene disperse dyes in dyestuff

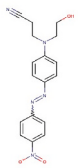
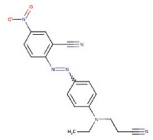
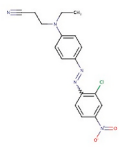
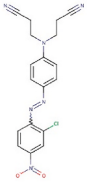
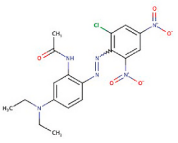
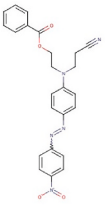
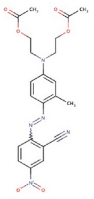
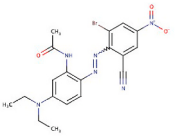
In addition to the ten compounds isolated from dyestuffs, we identified nine azobenzene compounds that we had not been able to purify from the dyestuffs due to low concentrations or because compounds co-eluted too closely to be separated after multiple attempts (but had been detected in TLC analysis). Fig. 2a summarizes results of our HRMS/MS-based suspect screening analysis and shows tentatively identified structural candidates of azobenzene compounds in dyestuffs using our weight-of-evidence approach (Section 2.4), including the percentile rank score generated for each structural candidate from each *in silico* MS/MS tool, as well as metadata including the number of PubChem sources, patents, and references for the structural candidate. In addition to the ten compounds that were isolated (Section 3.1), dyes detected included: Disperse Violet 93, Disperse Blue 373, Disperse Orange 25, and Disperse Orange 61, for which we had previously-obtained standards; Disperse Yellow 163 and Disperse Blue 165, for which we did not have standards but which had common names; and three additional azobenzene compounds without common dye names (CAS #s 96662-24-7, 3025-42-1, and 24112-48-9). It is known that, because chromophore activity is determined by the electronic properties of a compound and its functional groups, azo compounds with similar structures will exhibit similar color behavior (Aljamali, 2015; Choi et al., 2000; Wojciechowski, 1997). To examine the structural relationships of these unnamed compounds to other dyes, compounds (rows) were clustered according to structural similarity via MACCS fingerprints (Anderson, 1984); these relationships are denoted by the dendrogram to the left of the compound names. Structural similarity clustering indicates that CAS # 96662-24-7 is structurally similar to Disperse Red 73, as well as Disperse Orange 25. While chemical fingerprints indicate that 3025-42-1 does not have an immediate structural neighbor, this compound clusters together with orange and red compounds. CAS #s 96662-24-7 and 3025-42-1 are therefore likely to each be orange or red dyes, in concordance with TLC observations of orange-colored spots for these compounds. CAS # 24112-48-9 is shown to structurally similar to Disperse Blue 165, and clusters together with other blue dyes and with violet dyes; CAS # 24112-48-9 is therefore likely a blue dye, in concordance with TLC observations of a blue-colored spot for this compound.

Our results confirm the identities of the primary dye components of black dyestuff (Permasil Black EXNSF 300%.) Given that our initial TLC plates [Figure SI-2] show that orange, violet, and blue components are present in black dyestuff, we expected to see orange and blue dyes as well as Disperse Violet 93 Cl; however, during the structure identification process (Section 2.4), we were able to identify only the chlorine analog of Disperse Violet 93 in black dyestuff. To verify initial TLC results, we analyzed the black dyestuff extract via HPLC [Agilent 1100] with variable wavelength detection at each of the three wavelengths corresponding to orange, violet, and blue dyes (420 nm, 560 nm, and 595 nm respectively); we also analyzed pure samples (1 μg/mL) of Disperse Orange 61, Disperse Violet 93, and Disperse Blue 373 to assess whether each of these colors was present in the dyestuff. Results showed a clear absorbance peak at each of the three wavelengths with corresponding retention times to pure samples, indicating that each of the three colors was present in the black dyestuff [Figure SI-6]. Although the suspect screening yielded no common dye name matches for orange and blue components in the dyestuff, the identifications of CAS #s 96662-24-7 and 24112-48-9 have two of the three high relative abundances in the black dyestuff (area count $\geq 10^8$) and have close structural similarities to Disperse Orange 61 and Disperse Blue 373, respectively. Examination of EICs for CAS #s 96662-24-7 and 24112-48-9 confirmed their presences in black dyestuff. Patent literature identified by PubChem for CAS #s 96662-24-7 and 24112-48-9 indicate that they are actively used as orange and blue ingredients, respectively, in black dyestuff (Hoppe et al., 2016; Price and Hall, 1980).

Fig. 2 provides strong justification for utilizing multiple *in silico* MS/MS tools in a weight-of-evidence approach to annotate chemical structures, rather than relying on one tool only. Given that CSI:FingerID and CFM-ID are among the most recent and rigorous *in silico* tools available for use in annotating structures from MS/MS data, it would have been rational to justify the use of only one or both of these tools. However, as shown in Fig. 2, if we had used only CSI:FingerID or CFM-ID, compounds such as Disperse Orange 44 and Disperse Red 354 would have been mis-annotated without access to the purified chemical standards to verify compound identity. Because few purified reference standards exist for azo dyes, this mis-annotation from CSI:FingerID or CFM-ID would in other cases remain uncorrected without additional lines of evidence, as shown in annotations such as CAS # 96662-24-7. Results shown in Fig. 2 thus support the use of multiple lines of evidence to annotate unknown molecular features from mass spectrometry data, and suggest that relying only on *in silico* MS/MS tools trained on empirical spectra can lead to incorrect structural annotations for compound classes poorly represented in the training sets for those tools.

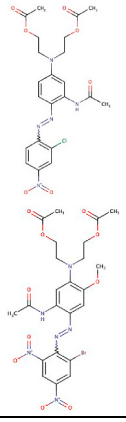
Fig. 3a provides an overview matrix of the occurrence and relative intensity of the tentatively identified azobenzene compounds in dyestuffs colored in a white-to-red gradient according to relative abundance

Table 1
Azobenzene disperse dyes extracted and characterized from raw dyestuffs. Dye names with an asterisk (*) indicate that the compound extract was determined to be pure via NMR analysis. NMR spectra are located in Supplementary Information (Figure SI—4). Log K_{ow} were calculated using the JChem suite (ChemAxon, Boston, MA).

IUPAC Name	Common Name	Dyestuff of Origin	Chemical Structure	Molecular Formula	Monoisotopic Mass (g/mol)	InChIKey-14	Log K_{ow}	λ_{max}
3-[N-(2-hydroxyethyl)-4-[(4-nitrophenyl)diazenyl]anilino] propanenitrile	Disperse Orange A	Permasil Orange RSE		$C_{17}H_{17}N_5O_3$	339.1331	OGHAROMWUKGSDP	3.51	455
2-[[4-[2-cyanoethyl (ethyl)amino] phenyl]diazenyl]-5-nitrobenzonitrile	Disperse Red 73*	Permasil Rubine CKGFL 100%		$C_{18}H_{16}N_6O_2$	348.1335	QEORVDCGZONWCJ	4.41	510
3-[4-[(2-chloro-4-nitrophenyl) diazenyl]-N-ethylanilino] propanenitrile	Disperse Red 50*	Disperse Red 5G		$C_{17}H_{16}ClN_5O_2$	357.0993	NPBDWXMKLFBNIW	5.16	475
3-[4-[(2-chloro-4-nitrophenyl) diazenyl]-N-(2-cyanoethyl) anilino] propanenitrile	Disperse Orange 44*	Permasil Orange H2GFS		$C_{18}H_{15}ClN_6O_2$	382.0945	ZXXVVTBKBDDTSE	4.58	450
IUPAC Name	**Common Name**	**Dyestuff of Origin**	**Structure**	**Molecular Formula**	**Monoisotopic Mass (g/mol)**	**InChIKey-14**	**Log K_{ow}**	**λ_{max}**
N-[2-[(2-chloro-4,6-dinitrophenyl)diazenyl]-5-(diethylamino)phenyl] acetamide	Disperse Violet 93, Cl	Permasil Black EXNSF 300%		$C_{18}H_{19}ClN_6O_5$	434.1105	YREBUQFUGONNSP	4.92	560
4-Nitro-4'-((2-cyanoethyl) (2-benzoyloxyethyl)amino) azobenzene	Disperse Orange 73*	Permasil Orange RSE		$C_{24}H_{21}N_5O_4$	443.1593	SCLYXZFZPIKFAH	6.01	445
2-[N-(2-acetyloxyethyl)-4-[(2-cyano-4-nitrophenyl) diazenyl]-3-methylanilino]ethyl acetate	Disperse Violet 33	Permasil Rubine S2GFL		$C_{22}H_{23}N_5O_6$	453.1648	YAOMDDRLRVOOIC	4.30	525
N-[2-[(2-bromo-6-cyano-4-nitrophenyl)diazenyl]-5-(diethylamino)phenyl] acetamide	Disperse Blue 183:1*	Permasil Blue P2BR		$C_{19}H_{19}BrN_6O_3$	458.0702	KTXMBTRTVMMWQS	5.00	600
IUPAC Name	**Common Name**	**Dyestuff of Origin**	**Structure**	**Molecular Formula**	**Monoisotopic Mass (g/mol)**	**InChIKey-14**	**Log K_{ow}**	**λ_{max}**
2-[3-acetamido-N-(2-acetyloxyethyl)-4-[(2-chloro-4-nitrophenyl)diazenyl] anilino] ethyl acetate	Disperse Red 354*	Permasil Rubine S2GFL		$C_{22}H_{24}ClN_5O_7$	505.1364	ORDDDNUMJXPGOC	3.77	505

(*continued on next page*)

Table 1 (*continued*)

IUPAC Name	Common Name	Dyestuff of Origin	Chemical Structure	Molecular Formula	Monoisotopic Mass (g/mol)	InChIKey-14	Log K_{ow}	λ_{max}
2-[5-acetamido-N-(2-acetyloxyethyl)-4-[(2-bromo-4,6-dinitrophenyl)diazenyl]-2-methoxyanilino]ethyl acetate	Disperse Blue 79:1*	Permasil Navy GRL 200%		$C_{23}H_{25}BrN_6O_{10}$	624.0816	JSRUDOBCTLPTFO	3.72	580

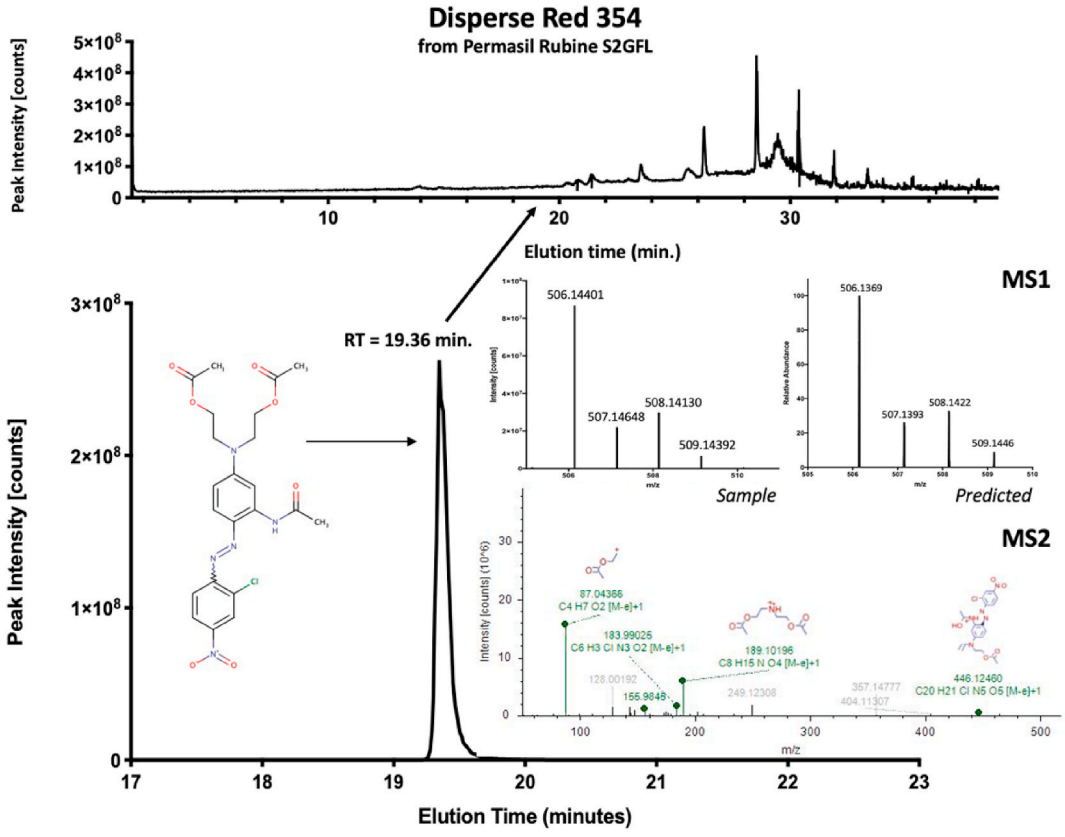


Fig. 1. Base peak ion chromatogram for Permasil Rubine S2GFL (top) and extracted ion chromatogram (EIC) for Disperse Red 354 (bottom, m/z 506.1401). The EIC for DR354 is shown with the experimental MS1 spectrum, predicted MS1 spectrum (generating using EnviPat), and FiSH-scored (MassFrontier, Compound Discoverer 3.2) MS2 spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by peak area. The most commonly detected azo dye was Disperse Violet 93 Cl, which was detected in five dyestuffs; Disperse Blue 373 and Disperse Red 50 were each detected in three dyestuffs. All other azobenzene compounds were detected in only one or two dyestuffs. Specific quantities of individual azo dyes in dyestuffs can be found in the Supplementary Information [Table SI-3]. As might be expected, common-named dyes were largely detected in dyestuffs of corresponding colors; primary color dyes (red, yellow, blue) were also detected in dyestuffs of corresponding secondary colors such as orange and violet. Relative abundance results indicate a high variance of percent composition within dyestuffs: some dyestuffs contained far less dye compound(s) on a per-mass basis than other dyestuffs. Further analysis of dyestuff percent composition can be found in Figure SI-7.

3.3. Identification and quantitation of azobenzene disperse dyes in children's athletic apparel

We extracted 14 children's athletic apparel samples [Table SI-2] and assessed occurrence of azobenzene disperse dyes via our weight-of-evidence suspect screening approach (Section 2.4). Our suspect

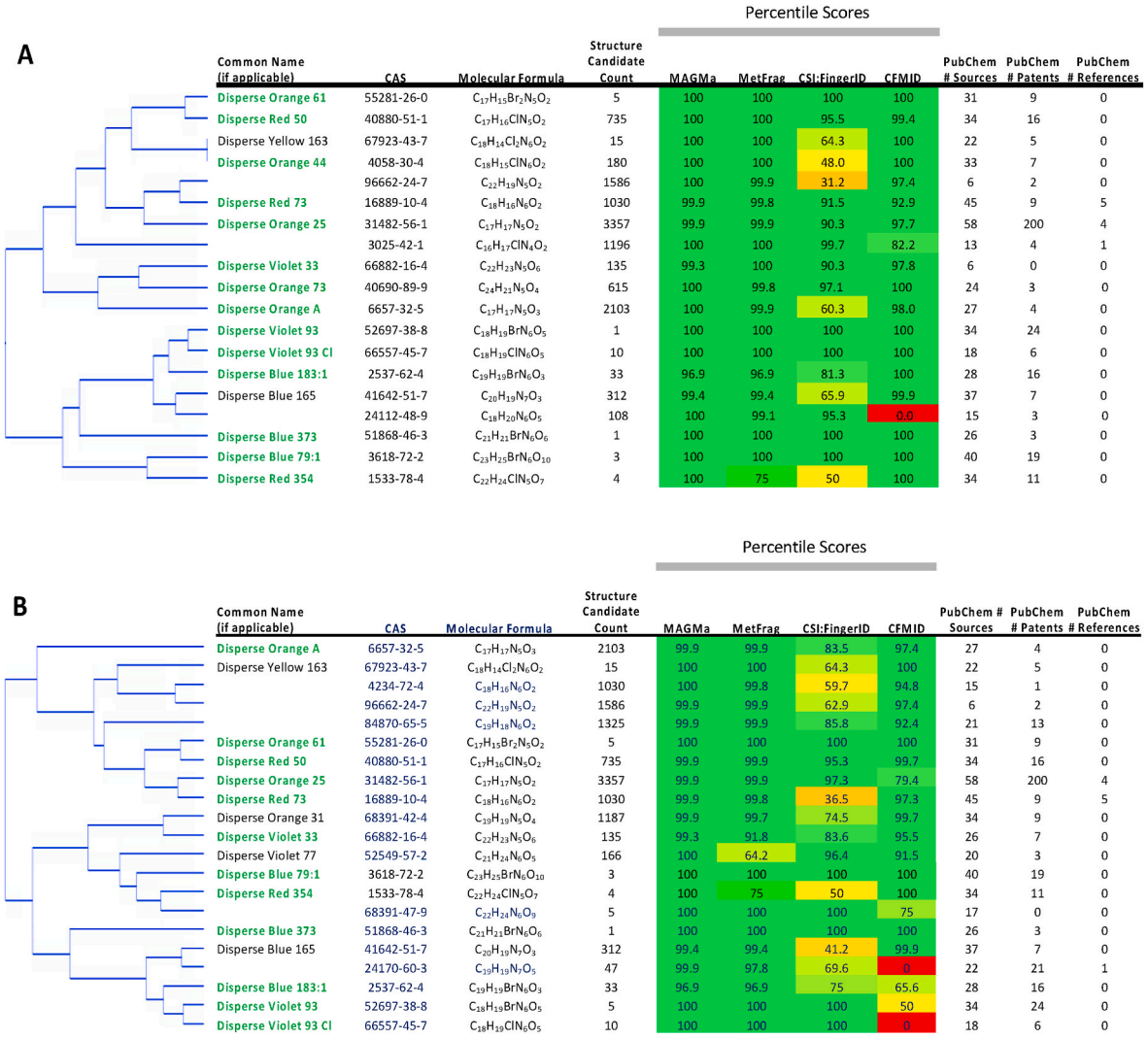
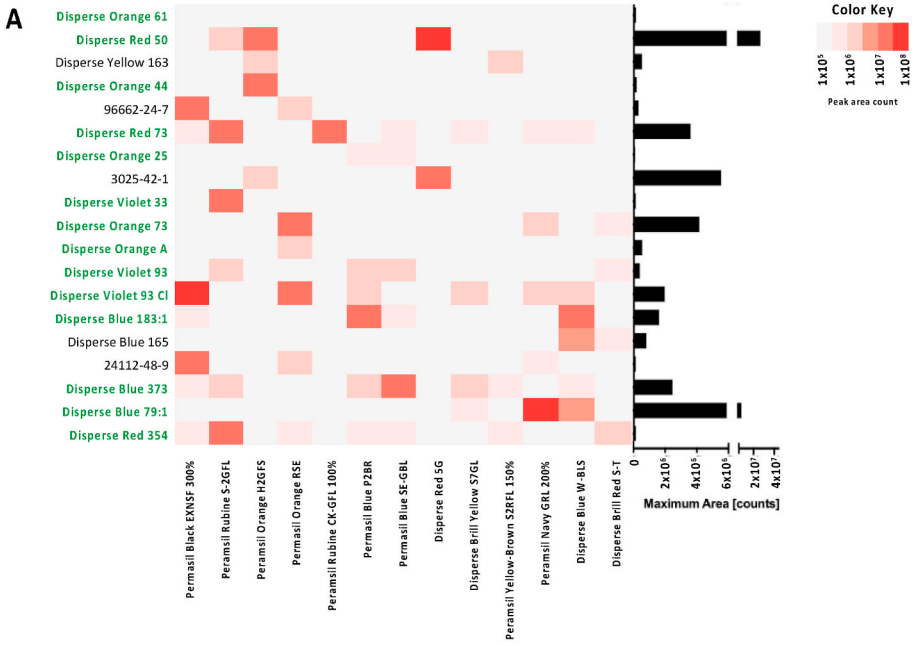


Fig. 2. Azobenzene disperse dyes tentatively identified in dyestuffs (**2A**) and in children's polyester apparel (**2B**) through our weight-of-evidence approach using the computational *in silico* MS/MS tools MAGMa, MetFrag, CSI:FingerID, and CFMID; percentile scores of fit to the compound fragmentation spectra (derived as described in Section 2.4) are shown, generated by each of the four *in silico* MS/MS tools, and are color coded from red (0^{th} percentile score of fit predicted by the *in silico* MS/MS tool) to green (100th percentile score of fit predicted by the *in silico* MS/MS tool). Numbers of sources, patents, and references in PubChem are also included as additional metadata for substantiation of compound annotation. Common names highlighted in green are those verified by matching with reference spectra from purified dyes in Table 1; compounds with no common name listed are not known by any azo dye name in open-source databases. Compounds (rows) are clustered according to structural similarity via MACCS fingerprints (Anderson, 1984) to examine structural relationships between azo dyes, as denoted by the dendrogram (in blue) to the left of the compound names. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

screening analysis of azobenzene compounds in children's athletic apparel yielded 21 detected azobenzene compounds, 12 of which we were able to confirm via spectral matching with our standards (purified, purchased, and gifted) [Fig. 2b]. As was shown in the dyestuff data [Fig. 2a], annotated structures presented in Fig. 2b justify the use of multiple lines of evidence rather than relying on a single *in silico* MS/MS annotation tool such as CSI:FingerID: compounds in apparel such as Disperse Red 73 and Disperse Violet 93 Cl would have likely been mis-annotated without purified standards available to verify compound identity. Commonly detected dyes with high relative abundances included Disperse Blue 373, Disperse Red 354, Disperse Violet 93, and Disperse Violet 93 Cl [Fig. 3b]. Other compounds, such as Disperse Blue 79:1, Disperse Blue 183:1, Disperse Orange 25, Disperse Orange 61, and Disperse Red 50 were frequently detected in apparel but showed high relative abundance in only one or two apparel samples. We then used our purified standards to quantify azo dyes in apparel where possible. Of our 14 apparel samples, seven samples contained dyes for which we had standards. Fig. 4 depicts the relative quantities of each dye within the total amount of quantifiable disperse dye, listed to the right of each bar. For specific measurements in apparel, refer to Table SI-4. We quantified total levels of dyes in apparel samples up to 11,430 μg dye/g shirt (Red Shirt A), and individual levels of dyes in apparel at up to 9230 μg dye/g shirt (DR354 in Red Shirt A). Geometric means of dyes in apparel ranged from 7.91 μg dye/g shirt (DO44) to 300 μg dye/g shirt (DR354) [Table SI-4]. Due to limits of detection, only the most abundant dyes in apparel from Fig. 3b were quantified. Instrument limits of detection (LOD), reported in Table SI-4, were calculated as the blank signal plus three standard deviations of the blank; where the calculated LOD was lower than the lowest calibration sample 1 ng/mL, 1 ng/mL was instead used as the LOD. Recoveries for each compound were measured after matrix-spike experiments with white apparel swatches and ranged from 68.9% ± 1.9% (Disperse Orange 73) to 101% ± 6.9% (Disperse Red 50); percent recoveries for each dye are shown in Table SI-5.

Apparel samples (columns) in Fig. 3b are clustered by similarity. All black shirts cluster together: each black shirt contains a blue, a violet, and an orange dye, in accordance with our results for black dyestuff in



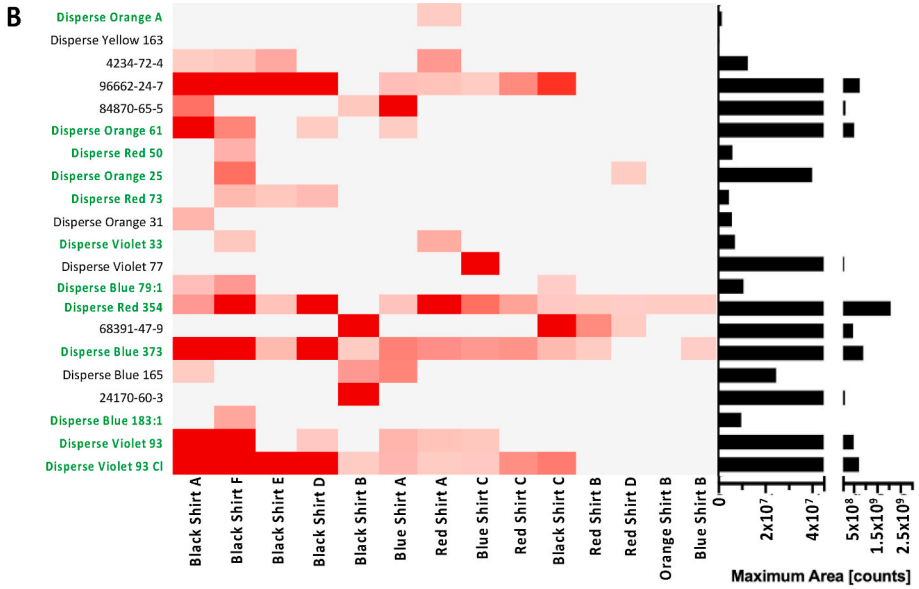


Fig. 3. Relative abundances, measured via peak area, of azobenzene compounds tentatively identified in dyestuffs (**3A**) and of tentatively identified azobenzene compounds in children's polyester apparel (**3B**). Peak areas ranging from 10^5 to 10^8 are colored in a gradient from white to deep red according to peak intensity, as shown in the legend. Columns (dyestuffs in 3 A, apparel samples in 3 B) are clustered according to abundance similarity; rows (tentatively confirmed azobenzenes) are clustered according to molecular fingerprint similarity. Azobenzene compounds were identified using our weight-of-evidence approach (Fig. 2) and are clustered according to structural similarity via MACCS fingerprints (Anderson, 1984) identically as shown in Fig. 2. The black bar charts on the right display the maximum detected peak area per compound across all dyestuff samples (4 A) or apparel samples (4 B). As in Fig. 2, compounds listed in green are those for which we were able to definitively confirm and identity with an authentic reference compound. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Section 3.1. Dyes appear to be in common among red and blue apparels as well: Disperse Violet 93 (Br and Cl analogs), Disperse Blue 373, and Disperse Red 354 are each detected in Blue Shirt A, Blue Shirt C, Red Shirt A, and Red Shirt C with high relative abundances. Notably, similarity clustering is also observed for shirts within a given brand. All apparel from Brand A, for example, contain DB183:1, DB373, DO25, DO61, DR354, DR50, DV93, and DV93 Cl. The three apparel samples from Brand C cluster entirely together, displaying common dyes and common abundances of these dyes with the exception of one or two dyes per apparel sample. This suggests that Brand C uses a common dyestuff "recipe" to color their apparel, changing abundances of key dye colors as necessary. Brand B showed minimal detections of azobenzene disperse dyes, suggesting that Brand B may use other materials such as anthraquinone disperse dyes to color their polyester apparel.

Of the apparel samples in which azo dyes were quantified, DR354 was quantified in every sample except one (Black Shirt E), whose total amount of quantifiable disperse dye is instead entirely comprised of Disperse Red 73. Given that TLC [Figure SI-2] and HPLC [Figure SI-6] analyses of black and most blue dyestuffs did not show a red dye, we did not expect to see red dyes present in blue or black shirts. Yet, DR354 makes up roughly 40% of the total 241 μg dye/g shirt that we quantified in Blue Shirt C. Where black apparel is concerned, DR354 makes up roughly 85% of the 1103 μg dye/g shirt quantified in Black Shirt F, and makes up more than 95% of the 1304 μg dye/g shirt quantified in Black Shirt D. Fig. 2b shows that when detected, azo dyes are clustered according to MACCS fingerprint similarity: DR354 does not cluster near the other red compounds, and instead clusters with blue and violet compounds such as Disperse Blue 79:1 and Disperse Violet 77. Experimentally determined maximum absorbance wavelengths [Table 1] also reveal that DR354's λ_{max} is 505 and DR73's λ_{max} is 510, which are closer to DV33's 525 λ_{max} than to the λ_{max} of any other purified red dyes. Thus, it may be that DR354 and DR73 function in certain black dyestuff mixtures as an ingredient in the "violet component" of the dyestuff.

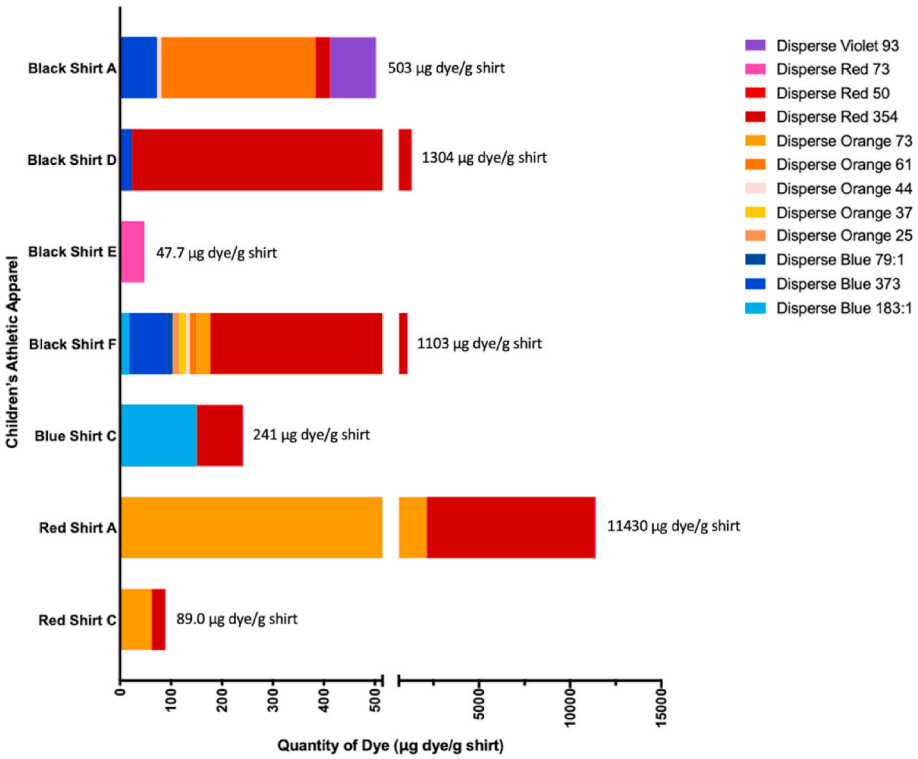


Fig. 4. Quantitative measurements of purified reference dyes in each shirt extract. All values are reported in µg dye/g shirt.

4. Discussion

4.1. Clothing is a source of human exposure to chemicals

This study sought to address the knowledge gaps in sources, quantities, and possible exposure paths of azobenzene disperse dyes in dyestuffs and apparel. A growing body of evidence identifies clothing as an important source and mediator of human exposure to chemicals and particles, including chemical byproducts of clothing manufacture and chemicals that adhere to clothing during use and care (Heratizadeh et al., 2017; Iadaresta et al., 2018; Licina et al., 2019; Nicolai et al., 2020; Rovira and Domingo, 2019). For example, it is known that clothing can act as a sorbent of semivolatile organic compounds (SVOCs) such as phthalates, brominated flame retardants, and organophosphate esters, and that these chemicals can then be released during laundering (Rovira and Domingo, 2019). Quinoline, an aromatic compound involved in the manufacture of dyes and a possible human carcinogen, was found by Luongo et al. (2014) to be present in almost all polyester garment samples analyzed, at concentrations up to 1.9 mg (Luongo et al., 2014). Despite the widespread realization that clothing, particularly synthetic clothing, is an important source of human exposure to chemicals, to our knowledge few studies have evaluated the presences of azobenzene disperse dyes in cloth and apparel.

4.2. Clothing may be a significant source of human exposure to azo dyes

Recently, Dhungana et al. (2019) examined the presence and relative abundances of brominated azobenzene dyes in a pooled cloth sample and confirmed detection of Disperse Orange 61 and Disperse Blue 373. In addition, they tentatively identified chemical formulas that may correspond to brominated azo dyes, although no standards or spectra were available at the time with which to verify these results (Dhungana et al., 2019). We compared this list of tentative identifications (chemical formula and m/z for the M + H ion) in the pooled cloth to our own list of purified compounds and found five matches to our list of purified azobenzene dyes. In addition to Disperse Orange 61 ($C_{17}H_{15}Br_2N_5O_2$, m/z 478.9598) and Disperse Blue 373 ($C_{21}H_{21}BrN_6O_6$, m/z 532.0721) which Dhungana et al. had already identified with reference standards, we matched Disperse Violet 93 ($C_{18}H_{19}BrN_6O_5$, m/z 478.0611) and Disperse Blue 79:1 ($C_{23}H_{25}BrN_6O_{10}$, m/z 624.0817). All of these compounds were identified in our children's apparel extracts and the relative magnitudes of these detections parallel our results. Additionally, the formula $C_{17}H_{16}BrN_5O_2$, with m/z 401.0491 reported in Dhungana et al. matches the predicted brominated analog of Disperse Red 50 ($C_{17}H_{16}ClN_5O_2$), providing another example of compounds with halogenated analogs (our example in this paper being the brominated and chlorinated analogs of Disperse Violet 93). These examples indicate that as screening for azobenzene disperse dyes continues, it will be critical to consider halogenated analogs of each possible azo dye in order to ensure that compounds do not go undetected.

To date, azo dyes have been identified and assessed in environmental and commercial materials by focusing on the aromatic amine breakdown products of suspected parent azo dyes: while azo dyes are established in the literature as skin sensitizers, many aromatic amines released by azo dyes are suspected mutagens and carcinogens (Korinth et al., 2007; Leucea, 2010; Platzek et al., 1999; Rovira and Domingo, 2019; Sun et al., 2017; T, 2013). Brüschweiler and Merlot (2017) identified 40 non-regulated aromatic amines in textiles after performing reductive cleavage on fabrics (Brüschweiler and Merlot, 2017; Rovira and Domingo, 2019); using a similar experiment, Crettaz et al. (2020) quantified non-regulated aromatic amines in 48% of 150 samples (Crettaz et al., 2020). Brüschweiler et al. (2014) identified 15 non-regulated aromatic amine cleavage products from azo dyes with toxicity data suggesting carcinogenic or genotoxic activity; included in this list is 4-nitroaniline (Brüschweiler et al., 2014). While the present study assessed azo dyes rather than aromatic amine cleavage products, it is important to consider the possibility of aromatic amine release from the azo dyes assessed here. 22 aromatic amines are suspected or known carcinogens, and are banned in the European Union under REACH (Stingley et al., 2010); included in this list is p-aminoazobenzene, the core structure of each azo dye examined in this study [Figure SI-1]. 426 different azo dyes are capable of producing one or more of these 22

banned aromatic amines, and despite REACH legislation, these compounds are present in commerce at toxicologically relevant concentrations (Brüschweiler et al., 2014); these concentrations in clothes are known to correlate to human exposure levels of concern (Zeilmaker et al., 1999). Many other aromatic amines remain unregulated under REACH: an additional 470 azo dyes can be cleaved into 397 non-regulated aromatic amines (Brüschweiler et al., 2014; Brüschweiler and Merlot, 2017). We predicted reductive cleavage products for each azo dye included in this study [see Table SI-5] and determined that of the azo dyes examined in this study, three dyes (DOA, DO73, and DO25) could potentially be reductively cleaved to produce 4-nitroaniline. An additional nine dyes (DR50, DO44, DV93, DV93 Cl, DR354, DB79:1, DB373, DO37, and DO61) could potentially be cleaved to produce halogenated analogs of 4-nitroaniline such as 2,6-dichloro-4-nitroaniline and 2,6-dibromo-4-nitroaniline. These results demonstrate that azo dyes with potential to release potentially toxic amines are present in United States commerce and in children's clothing, and suggest that traditional screening efforts must look beyond known and commonly-monitored aromatic amines. Future research should prioritize identification of the azo dyes from which these amines can be released in order to understand the scope of human exposure and potential risks from use of this class of compounds.

While azo dyes are not applied exclusively to clothing, previous work indicates that clothing is by far the most relevant source of human exposure to azo dyes. Out of the pooled cloth, TV casing, and carpet samples that Dhungana et al. tested, prominent peak areas for brominated azo dyes were detected only in the pooled cloth sample (Dhungana et al., 2019); Zeilmaker et al. (2000) determined that where dermal exposure is concerned, other household textiles such as towels do not make contact with human skin for long enough to lead to toxicologically significant exposure to azo dyes and their derivatives (Zeilmaker, M.J., Van Kranen, H.J., Van Veen, M.P., Janus, 2000). Our present study demonstrates that the range of total azo dye concentrations in children's clothing (47.7 μg/g – 11,430 μg/g) can account for up to 1.1% of the total clothing mass [Figure SI-8]; work by Crettaz et al. (2020), however, suggests that this percentage is toxicologically relevant. Crettaz et al. detected p-phenylenediamine, an aromatic amine released from numerous azo dyes, at a range of toxicologically relevant concentrations between 67 μg/g and 953 μg/g in nine polyester samples, and calculated that these concentrations correspond to only 0.2–2.7% of the theoretical mean dye load on garments (Crettaz et al., 2020). The predominant route of azo dye uptake into the body is considered to be dermal (Kaefferlein et al., 2009); dermal exposure can also result in azo dyes being reductively cleaved by skin bacteria to yield aromatic amines (Korinth et al., 2007; Platzek et al., 1999; Sun et al., 2017). In the context of this collective work by others, our results demonstrate that clothing may indeed be a significant source of human exposure to azo dyes, which raises concerns about the potential for allergic reactions and may account for why some individuals react to clothing if not washed before wearing (Licina et al., 2019; Su and Horton, 1998).

4.3. Clothing may be a significant source of household exposure to azo dyes

Literature documenting the "washout effect" of chemical treatments on apparel suggests that children's clothing may also be an important source of azo dye release into the environment. Zheng and Salamova (2020) examined the washout effect for melamine, another class of aromatic amines, and determined that melamine concentrations in clothing decreased by up to 90% when washed in cool and warm water, and decreased by up to 97% when washed with a detergent, after a single washing (Zheng and Salamova, 2020). Similarly, Luongo et al. (2016) investigated the washout effect for benzothiazoles and quinolines and found that benzothiazoles decreased by 50% after ten washes, while quinolines decreased by 20%; 0.5 g and 0.24 g, respectively, were emitted to households during one washing (Luongo et al., 2016). Previous literature has established that disperse dyes can migrate off of fibers onto water or skin by rubbing and by exposure to water, particularly if textile fastness is poor (Malinauskiene et al., 2013). Given that azobenzene disperse dyes can only operate in the dyeing process in the presence of dispersing agents such as detergents, it is plausible that when clothes treated with azobenzene disperse dyes come back into contact with detergents, this migration process off of clothes will be exacerbated, increasing the likelihood of releasing azobenzene disperse dyes into the home environment. Washout effect studies (Luongo et al., 2016; Zheng and Salamova, 2020) suggest that laundering of clothing can be an important source of release of these compounds to households. It is of critical importance to prioritize examination of chemical treatments and dye migration off of clothing in order to better understand the pathways by which these compounds may migrate into the environment (indoor and outdoor) and engage in pathways of human exposure.

5. Conclusion

To our knowledge, this study is the most complete examination of azo dyes in human exposure sources to date. We identified 19 different azobenzene disperse dyes in 13 raw dyestuff powders, demonstrating that commercial dyestuffs are often comprised of multiple azo dyes. We were able to isolate ten of these dyes and seven were sufficiently pure to be used as reference standards. Similarly, we identified 21 different azo dyes in children's apparel samples, 12 of which we were able to confirm and quantify using purified, purchased, or gifted reference standards. Azo dyes were found to be present in apparel at total levels up to 11,430 μg dye/g shirt and at individual levels up to 9230 μg dye/g shirt.

Given our results presented here, the concentrations of azo dyes detected in children's clothing in this study raise questions and concerns. Further research is warranted to understand the health risks that these compounds may pose to children when applied in clothing, particularly where suspected allergic sensitization potential of these compounds is concerned. To mitigate exposure to azo dyes in clothing, it may be best for parents to wash all clothing before children wear apparel.

Credit author statement file

Kirsten E. Overdahl: Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, David Gooden: Investigation, Resources, Benjamin Bobay: Investigation, Resources, Gordon J. Getzinger: Software, Data curation, Heather M. Stapleton: Methodology, Validation, Resources, Writing – review & editing, Supervision, Funding acquisition, P. Lee Ferguson: Methodology, Software, Validation, Resources, Writing – review & editing, Supervision, Funding acquisition

Funding

This work was supported by grants from the National Institute of Environmental Health Sciences (R01 ES016099 and U2C ES030851); and by a generous donation from the Falk family.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank Dr. Gisela de Aragão Umbuzeiro for supplying the analytical standards Disperse Orange 61, Disperse Violet 93, and Disperse Blue 373 utilized in this study (BEX 14540/13–4). The authors also wish to thank Santhosh Kalpathy, PhD, for his assistance in

purifying analytical reference standards from dyestuffs utilized in this study; Taylor Hoxie, for her assistance with Soxhlet extractions of children's apparel; and Nicholas Herkert, PhD, for assistance proof-reading this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117299.

References

Aljamali, N.M., 2015. Review in azo compounds and its biological activity. Biochem. Anal. Biochem. https://doi.org/10.4172/2161-1009.100016.

Allen, F., Greiner, R., Wishart, D., 2014. Competitive fragmentation modeling of ESI-MS/MS spectra for putative metabolite identification. Metabolomics. https://doi.org/10.1007/s11306-014-0676-4.

Anderson, S., 1984. Graphical representation of molecules and substructure-search queries in MACCStm. J. Mol. Graph. 2, 83–90. https://doi.org/10.1016/0263-7855(84)80060-0.

Balakrishnan, V.K., Shirin, S., Aman, A.M., de Solla, S.R., Mathieu-Denoncourt, J., Langlois, V.S., 2016. Genotoxic and carcinogenic products arising from reductive transformations of the azo dye. Disperse Yellow 7. https://doi.org/10.1016/j.chemosphere.2015.11.119. Chemosphere.

Benkhaya, S., M' rabet, S., El Harfi, A., 2020a. A review on classifications, recent synthesis and applications of textile dyes. Inorg. Chem. Commun. https://doi.org/10.1016/j.inoche.2020.107891.

Benkhaya, S., M'rabet, S., El Harfi, A., 2020b. Classifications, properties, recent synthesis and applications of azo dyes. Heliyon. https://doi.org/10.1016/j.heliyon.2020.e03271.

Böcker, S., Dührkop, K., 2016. Fragmentation trees reloaded. J. Cheminf. https://doi.org/10.1186/s13321-016-0116-8.

Broeckling, C.D., Afsar, F.A., Neumann, S., Ben-Hur, A., Prenni, J.E., 2014. RAMClust: a novel feature clustering method enables spectral-matching-based annotation for metabolomics data. Anal. Chem. https://doi.org/10.1021/ac501530d.

Brookstein, D.S., 2009. Factors associated with textile pattern dermatitis caused by contact allergy to dyes, finishes, foams, and preservatives. Dermatol. Clin. https://doi.org/10.1016/j.det.2009.05.001.

Brüschweiler, B.J., Merlot, C., 2017. Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. Regul. Toxicol. Pharmacol. https://doi.org/10.1016/j.yrtph.2017.06.012.

Brüschweiler, B.J., Küng, S., Bürgi, D., Muralt, L., Nyfeler, E., 2014. Identification of non-regulated aromatic amines of toxicological concern which can be cleaved from azo dyes used in clothing textiles. Regul. Toxicol. Pharmacol. https://doi.org/10.1016/j.yrtph.2014.04.011.

Carneiro, P.A., Umbuzeiro, G.A., Oliveira, D.P., Zanoni, M.V.B., 2010. Assessment of water contamination caused by a mutagenic textile effluent/dyehouse effluent bearing disperse dyes. J. Hazard Mater. https://doi.org/10.1016/j.jhazmat.2009.09.106.

Chakraborty, J.N., 2010. Chapter 18: dyeing with disperse dye. Fundamentals and Practices in Colouration of Textiles. https://doi.org/10.1533/9780857092823.192.

Chen, B., Ni, S., Sun, L., Luo, X., Zhang, Q., Song, Y., Zhong, Q., Fang, Y., Huang, C., Chen, S., Wu, W., 2018. Intramolecular charge transfer tuning of azo dyes: spectroscopic characteristic and third-order nonlinear optical properties. Dyes Pigments. https://doi.org/10.1016/j.dyepig.2018.05.063.

Chequer, F.M.D., Lizier, T.M., de Felício, R., Zanoni, M.V.B., Debonsi, H.M., Lopes, N.P., Marcos, R., de Oliveira, D.P., 2011. Analyses of the genotoxic and mutagenic potential of the products formed after the biotransformation of the azo dye Disperse Red 1. Toxicol. Vitro. https://doi.org/10.1016/j.tiv.2011.05.033.

Chequer, F.M.D., Lizier, T.M., de Felício, R., Zanoni, M.V.B., Debonsi, H.M., Lopes, N.P., de Oliveira, D.P., 2015. The azo dye Disperse Red 13 and its oxidation and reduction products showed mutagenic potential. Toxicol. Vitro. https://doi.org/10.1016/j.tiv.2015.08.001.

Choi, J.H., Hong, S.H., Lee, E.J., Towns, A.D., 2000. Structure-wet fastness relationships of some blue disperse dyes for polyester. J. Soc. Dye. Colour. https://doi.org/10.1111/j.1478-4408.2000.tb00046.x.

Chung, K.T., 2016. Azo dyes and human health: a review. J. Environ. Sci. Health Part C Environ. Carcinog. Ecotoxicol. Rev. https://doi.org/10.1080/10590501.2016.1236602.

Crettaz, S., Kämpfer, P., Brüschweiler, B.J., Nussbaumer, S., Deflorin, O., 2020. Survey on hazardous non-regulated aromatic amines as cleavage products of azo dyes found in clothing textiles on the Swiss market. J. fur Verbraucherschutz und Leb. https://doi.org/10.1007/s00003-019-01245-1.

Dawson, J.F., 1991. Azobenzene disperse dyes-past development and future prospects. J. Soc. Dye. Colour. https://doi.org/10.1111/j.1478-4408.1991.tb01285.x.

De Aragão Umbuzeiro, G., Freeman, H.S., Warren, S.H., De Oliveira, D.P., Terao, Y., Watanabe, T., Claxton, L.D., 2005. The contribution of azo dyes to the mutagenic activity of the Cristais River. Chemosphere. https://doi.org/10.1016/j.chemosphere.2004.11.100.

Dhungana, B., Peng, H., Kutarna, S., Umbuzeiro, G., Shrestha, S., Liu, J., Jones, P.D., Subedi, B., Giesy, J.P., Cobb, G.P., 2019. Abundances and concentrations of brominated azo dyes detected in indoor dust. Environ. Pollut. https://doi.org/10.1016/j.envpol.2019.05.153.

Drumond Chequer, F.M., Junqueira, D., de Oliveir, D.P., 2011. Azo dyes and their metabolites: does the discharge of the azo dye into water bodies represent human and ecological risks?. In: Advances in Treating Textile Effluent https://doi.org/10.5772/19872.

Dührkop, K., Shen, H., Meusel, M., Rousu, J., Böcker, S., 2015. Searching molecular structure databases with tandem mass spectra using CSI:FingerID. Proc. Natl. Acad. Sci. U.S.A. https://doi.org/10.1073/pnas.1509788112.

Dührkop, K., Fleischauer, M., Ludwig, M., Aksenov, A.A., Melnik, A.V., Meusel, M., Dorrestein, P.C., Rousu, J., Böcker, S., 2019. Sirius 4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nat. Methods. https://doi.org/10.1038/s41592-019-0344-8.

El-Ghamaz, N.A., El-Bindary, A.A., El-Boz, R.A., 2017. Electrical and optical properties of new azo dyes derived from m-aminophenol. Synth. Met. https://doi.org/10.1016/j.synthmet.2017.02.005.

Getzinger, G.J., Ferguson, P.L., 2020. Illuminating the exposome with high-resolution accurate-mass mass spectrometry and nontargeted analysis. Curr. Opin. Environ. Sci. Heal. https://doi.org/10.1016/j.coesh.2020.05.005.

Heratizadeh, A., Geier, J., Molin, S., Werfel, T., 2017. Contact sensitization in patients with suspected textile allergy. In: Data of the Information Network of Departments of Dermatology (IVDK) 2007–2014. Contact Dermatitis. https://doi.org/10.1111/cod.12760.

Hoppe, M., Himeno, K., Sekioka, R., inventors, 2016. DyStar Colours Distribution GmbH, Assignee.Disperse Azo Dye Mixtures. United States reissued patent US. RE46,018 E.

House of Parliament, 2013. The Environmental, Health and Economic Impacts of Textile Azo Dyes. House of Parliament-Parliament of Science and Technology, pp. 1–5.

Iadaresta, F., Manniello, M.D., Östman, C., Crescenzi, C., Holmbäck, J., Russo, P., 2018. Chemicals from textiles to skin: an in vitro permeation study of benzothiazole. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-018-2448-6.

Kaefferlein, H.U., Slowicki, A., Bruening, T., 2009. Azo dyes - part 2: toxicology and regulatory aspects. Gefahrst. Reinhalt. Luft.

Korinth, G., Schaller, K.H., Drexler, H., 2007. Percutaneous absorption of aromatic amines and the risk assessment resulting from the dermal pathway. Occup. Environ. Med. https://doi.org/10.1136/oem.2006.027755.

Leucea, D., 2010. The effects of azo dyes on human health. Industria Textila.

Licina, D., Morrison, G.C., Bekö, G., Weschler, C.J., Nazaroff, W.W., 2019. Clothing-mediated exposures to chemicals and particles. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.9b00272.

Loos, M., Gerber, C., Corona, F., Hollender, J., Singer, H., 2015. Accelerated isotope fine structure calculation using pruned transition trees. Anal. Chem. https://doi.org/10.1021/acs.analchem.5b00941.

Luongo, G., Thorsén, G., Östman, C., 2014. Quinolines in clothing textiles-A source of human exposure and wastewater pollution? Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-014-7688-9.

Luongo, G., Avagyan, R., Hongyu, R., Östman, C., 2016. The washout effect during laundry on benzothiazole, benzotriazole, quinoline, and their derivatives in clothing textiles. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-015-5405-7.

Malinauskiene, L., Bruze, M., Ryberg, K., Zimerson, E., Isaksson, M., 2013. Contact allergy from disperse dyes in textiles-a review. Contact Dermatitis. https://doi.org/10.1111/cod.12001.

Nicolai, S., Tralau, T., Luch, A., Pirow, R., 2020. A scientific review of colorful textiles. J. fur Verbraucherschutz und Leb. https://doi.org/10.1007/s00003-020-01301-1.

Ohe, T., Watanabe, T., Wakabayashi, K., 2004. Mutagens in surface waters: a review. Mutat. Res. https://doi.org/10.1016/j.mrrev.2004.08.003.

Platzek, T., Lang, C., Grohmann, G., Gi, U.S., Baltes, W., 1999. Formation of a carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. Hum. Exp. Toxicol. https://doi.org/10.1191/096032799678845061.

Porobić, S.J., Božić, B., Dramićanin, M.D., Vitnik, V., Vitnik, Ž., Marinović-Cincović, M., Mijin, D., 2020. Absorption and fluorescence spectral properties of azo dyes based on 3-amido-6-hydroxy-4-methyl-2-pyridone: solvent and substituent effects. Dyes Pigments. https://doi.org/10.1016/j.dyepig.2019.108139.

Price, R., Hall, N., inventors, 1980. Imperial Chemical Industries Limited, assignee. Process for the manufacture of aromatic nitriles. United States patent US 4, 192,800.

Rawat, D., Sharma, R.S., Karmakar, S., Arora, L.S., Mishra, V., 2018. Ecotoxic potential of a presumably non-toxic azo dye. Ecotoxicol. Environ. Saf. https://doi.org/10.1016/j.ecoenv.2017.10.049.

Ridder, L., Van Der Hooft, J.J.J., Verhoeven, S., De Vos, R.C.H., Van Schaik, R., Vervoort, J., 2012. Substructure-based annotation of high-resolution multistage MSn spectral trees. Rapid Commun. Mass Spectrom. https://doi.org/10.1002/rcm.6364.

Rocha, O.P., Cesila, C.A., Christovam, E.M., Barros, S.B. de M., Zanoni, M.V.B., de Oliveira, D.P., 2017. Ecotoxicological risk assessment of the "Acid Black 210" dye. Toxicology. https://doi.org/10.1016/j.tox.2016.04.002.

Rovira, J., Domingo, J.L., 2019. Human health risks due to exposure to inorganic and organic chemicals from textiles: a review. Environ. Res. https://doi.org/10.1016/j.envres.2018.09.027.

Ruttkies, C., Schymanski, E.L., Wolf, S., Hollender, J., Neumann, S., 2016. MetFrag relaunched: incorporating strategies beyond in silico fragmentation. J. Cheminf. https://doi.org/10.1186/s13321-016-0115-9.

Seidenari, S., Manzini, B.M., Danese, P., Motolese, A., 1990. Patch and prick test study of 593 healthy subjects. Contact Dermatitis. https://doi.org/10.1111/j.1600-0536.1990.tb04777.x.

Stingley, R.L., Zou, W., Heinze, T.M., Chen, H., Cerniglia, C.E., 2010. Metabolism of azo dyes by human skin microbiota. J. Med. Microbiol. https://doi.org/10.1099/jmm.0.012617-0.

Su, J.G., Horton, J.J., 1998. Allergic contact dermatitis from azo dyes. Australas. J. Dermatol. https://doi.org/10.1111/j.1440-0960.1998.tb01243.x.

Sun, J., Jin, J., Beger, R.D., Cerniglia, C.E., Chen, H., 2017. Evaluation of metabolism of azo dyes and their effects on Staphylococcus aureus metabolome. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1007/s10295-017-1970-8.

Susan, E.F., Elizabeth, A.D., 1988. Sensitization to dyes in textiles and other consumer products. Cutan. Ocul. Toxicol. https://doi.org/10.3109/15569528809052329.

Vacchi, F.I., Vendemiatti, J.A.S., Brosselin, V., Ferreira da Silva, B., Maria, M.V., DeMeo, M., Bony, S., Devaux, A., Umbuzeiro, G.A., 2016. Combining different assays and chemical analysis to characterize the genotoxicity of waters impacted by textile discharges. Environ. Mol. Mutagen. https://doi.org/10.1002/em.22034.

Vacchi, F.I., Vendemiatti, J.A. de S., da Silva, B.F., Zanoni, M.V.B., Umbuzeiro, G. de A., 2017. Quantifying the contribution of dyes to the mutagenicity of waters under the influence of textile activities. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2017.05.103.

Waring, D.R., 1984. Heterocyclic dyes and pigments. In: Comprehensive Heterocyclic Chemistry. https://doi.org/10.1016/B978-008096519-2.00012-6.

Wojciechowski, K., 1997. Structure - property relationships in azo disperse dyes, derivatives of naphthalimide. Dyes Pigments. https://doi.org/10.1016/S0143-7208(96)00039-3.

Wolf, S., Schmidt, S., Müller-Hannemann, M., Neumann, S., 2010. In silico fragmentation for computer assisted identification of metabolite mass spectra. BMC Bioinf. https://doi.org/10.1186/1471-2105-11-148.

Young, S., 2016. Occurrence and Formation of Emerging Disinfection Byproducts in Beverages and Over-the Counter Medications. The University of Arizona. Retrieved from. https://search.proquest.com/-docview/1870787671?accountid=146813.

Zeilmaker, M.J., Kroese, E.D., van Harpen, P., Bremmer, H.J., van Kranen, H.J., Wouters, M.F.A., Janus, J.A., 1999. Cancer Risk Assessment of Azo Dyes and Aromatic Amines from Garments and Footwear.

Zeilmaker, M.J., Van Kranen, H.J., Van Veen, M.P., Janus, J.A., 2000. Cancer risk assessment of azo dyes and aromatic amines from tattoo bands, folders or paper, bed clothes, watch straps, and ink. Res. Man Environ.

Zheng, G., Salamova, A., 2020. Are melamine and its derivatives the alternatives for per- and polyfluoroalkyl substance (PFAS) fabric treatments in infant clothes? Environ. Sci. Technol. https://doi.org/10.1021/acs.est.0c03035.