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Characterization of the textile anthraquinone dye Reactive Blue 4

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Abstract

Analysis and characterization of the textile dye C.I. Reactive Blue 4 (RB4), a commercially important anthraquinone dye, as well as its hydrolysis products, were conducted by spectrophotometry, high performance liquid chromatography (HPLC), as well as advanced mass spectrometric techniques. Although spectrophotometric analysis can be used for the routine quantification of RB4 in aqueous solutions, HPLC analysis is necessary for the quantification of the unhydrolyzed, monohydroxy- and dihydroxy-RB4. The effect of both salt and base at concentrations typically used in reactive dyeing on the dye spectra was also investigated. Although moderate dye aggregation, leading to absorbance depression, was observed at relatively high dye and salt concentrations (1000 mg/l and 100 g/l, respectively), spectrophotometric and HPLC analysis was possible as long as the effect of salt and base was taken into account in the development of standard calibrations. Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) analyses led to the separation and identification of the parent RB4 molecule. LC/ESI-MS analysis of RB4 solutions led to the conclusive identification of the two RB4 hydrolysis products, as well as one impurity. Values of physico-chemical properties of RB4 were estimated using two software packages. The estimated RB4 LC50 value for acute toxicity to fish (1500 mg/l) agreed well with the acute toxicity $EC50_{15\ min}$ value measured by the Microtox® procedure (1108 mg/l). Although the levels of RB4 in the environment are expected in the orders of magnitude lower than these LC50 and EC50 values, the effect of long-term, low-level dye exposure needs to be evaluated.

Keywords: HPLC; LC/ESI-MS; MALDI; Reactive anthraquinone dyes; Spectrophotometry; Textiles; Toxicity

1. Introduction

Reactive dyes are extensively used in the textile industry not only to color mainly cotton, but also wool and polyamide fibers because of their wide variety of color shades, high wet fastness profiles, ease of application, and brilliant colors [1]. A steady increase in reactive dye usage has been observed as a result of the increased cotton use worldwide. Reactive dyeing is typically performed at 30–85 °C and reactive dyebaths contain 25–100 g/l salt (NaCl or Na_2SO_4), and 2–50 g/l Na_2CO_3 (or a combination of Na_2CO_3 and NaOH) [1]. Under typical reactive dyeing conditions, up to 50% of the initial dye remains in the spent dyebath in its hydrolyzed form, which has no affinity for the fabric and results in colored effluent [1,2]. In addition to the presence of dye, high salt concentrations, as well as high pH values (11–13) further complicate the management of spent reactive dyebaths.

Reactive dyes are highly water soluble, are non-degradable under the typical aerobic conditions found in

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conventional, biological treatment systems, and adsorb very poorly to biological solids, resulting in residual color in discharged effluents [3–6]. Reactive dyes released into the environment are a source of public concern because they: (a) cause water bodies to become colored, absorbing and reflecting sunlight, which in turn interferes with the aquatic ecosystem; and (b) may cause chronic and acute toxicity [7,8]. Toxicity data for a wide range of textile dyes have recently been reported [9]. As state and federal environmental regulations became more stringent (requiring lower effluent color limits), reduction or elimination of discharge of colored wastewater is mandated [9–11].

Although azo dyes represent about 60% of all reactive dyes used by the textile industry, other classes of reactive dyes, namely anthraquinone and phthalocyanine dyes, are extensively used either as primary or secondary dyes in commercial trichromatic dyeing formulations [1,12]. However, in sharp contrast to the considerable research that has been conducted on the biotransformation and decolorization of azo dyes, limited information exists on the reductive transformation of both anthraquinone and phthalocyanine reactive dyes. In particular, detailed information is lacking with regard to the behavior of aqueous reactive anthraquinone dyes, as well as the application of advanced analytical/instrumental techniques for the identification and quantification of the parent compounds as well as the dye transformation products.

Our long-term goal is the development of decolorization, closed-loop, in-plant systems to economically renovate and reuse spent reactive dyebaths for minimization of fresh water consumption and reduction of wastewater volume and pollutant concentrations in textile plant discharges. The present report is focused on the analysis and characterization of C.I. Reactive Blue 4 (RB4), a commercially important anthraquinone dye, as well as its hydrolysis products. Analysis and characterization of dye products resulting from the reductive decolorization of this dye will be reported in a subsequent communication.

2. Materials and methods

2.1. Dye

Commercial RB4 (Procion Blue MX-R; Color Index 61205; CAS no. 13324-20-4) was obtained from DyStar LP, Charlotte NC, USA and used without any further purification. The molecular structure of RB4 is shown in Fig. 1. The color of anthraquinone dyes is partially associated with the anthraquinone nucleus and is modified by the type, number, and position of the substituents. Unsubstituted anthraquinone has a pale yellow color and a weak band at ca. 405 nm which is due

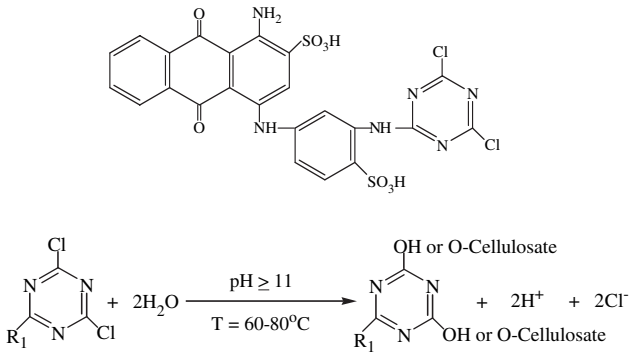

Fig. 1. Chemical structure of unreacted RB4 and hydrolysis of its dichlorotriazinyl reactive group.

to an $n \rightarrow \pi^*$ transition. Electron-withdrawing substituents have little influence on the anthraquinone spectrum. In contrast, electron-donating substituents (e.g., OH^-, $-NH_2$), especially in the α-positions, have bathochromic effects due to a charge transfer band involving the electron lone pair of hydroxyl or amino groups [2]. RB4 has two amino groups (electron-donating groups) in the 1, 4-α-positions, and a sulfonate group in the 2-position (Fig. 1).

For the type of reactive dye investigated in this study, the reaction between the reactive group (i.e., dichlorotriazinyl) and the fiber involves a nucleophilic addition/elimination substitution mechanism [13]. In the reactive dyeing of cotton (cellulose), the pH value and hence the equilibrium concentration of cellulosate ions [$CellO^-$], are increased in order to result in the formation of a covalent bond between the dye and the fiber. However, due to the competition between the $CellO^-$ and OH^- ions present in the dyebath at elevated pH values, a portion of the dye reacts with OH^- ions instead of the $CellO^-$ ions on the fiber (Fig. 1). It should be emphasized that complete RB4 dye hydrolysis (i.e., substitution of both Cl^- atoms in the dichlorotriazinyl reactive group) may not always be the case, thus leading to partial dye hydrolysis. Preparation of reacted (i.e., hydrolyzed) dye was based on the simulation of typical textile dyebath conditions as follows. Aliquots of 5.0 g dye were dissolved in 50 mM NaOH solution (resulting pH 11.6 $\pm$ 0.1), heated to and kept at 85 °C for 1 h and then diluted to 1 l after cooling while adjusting the pH to 7.0 with 1 N HCl.

2.2. Physico-chemical property estimation

Two software packages were used to estimate several physico-chemical properties of RB4: (a) Estimations Programs Interface (EPI) Suite™, Version 3.11; and (b) SPARC On-Line. EPI Suite™ is an interface program developed by the Syracuse Research Corporation for the U.S. Environmental Protection Agency [14]. The

program uses structural information to estimate several physico-chemical properties such as: melting and boiling points, vapor pressure, octanol–water partition coefficient (K_{ow}), organic carbon partition coefficient (K_{oc}), water solubility, Henry's law constant, biodegradability, bioconcentration factor, hydrolysis constants and environmental distribution. SPARC On-Line is an interface program developed by the University of Georgia, Athens, GA, USA and the U.S. Environmental Protection Agency [15–18]. The program estimates pK_a, hydrolysis, reduction potential, heat of formation, kinetics, and several other physico-chemical properties using structural information. As SPARC On-Line requires the melting point value as input, RB4 property estimation was conducted using the estimated EPI Suite™ melting point of 349.84 °C.

2.3. Analytical methods

2.3.1. Spectrophotometry

All spectrophotometric analyses were carried out using a UV/visible HP model 8453 spectrophotometer equipped with a deuterium and a tungsten lamp, a 1-cm path length, and a diode array detector (Hewlett Packard, Palo Alto, CA, USA). Samples analyzed with the spectrophotometer were first centrifuged for 5 min at 14,000 rpm in 2 ml polypropylene microcentrifuge tubes and, if necessary, the supernatants were diluted with deionized water or a saline solution, depending on the sample matrix. Absorbance scans were carried out at a wavelength range of 200–800 nm and the dye concentration was quantified based on sample absorbance at the maximum dye absorbance wavelength (598 nm) and previously prepared calibration curves.

2.3.2. High performance liquid chromatography (HPLC)

A Hewlett Packard Series 1100 HPLC unit equipped with a diode array detector and a Spherisorb ODS-2 column (25 cm × 4.6 mm I.D., 5 μm; Waters, Milford, MA, USA) was used. The column compartment temperature was set at 40 °C and the injection loop volume was 25.0 μl. A reverse-phased, ion-pairing HPLC method was used. The ion-pairing agent was 1 mM tributyl amine (TBA) with 1 mM acetic acid dissolved in two eluents with different methanol concentrations. Eluent A was 80:20% (v/v) water: methanol and eluent B was 5:95% water:methanol. The eluent flow rate was 0.5 ml/min. The methanol concentration started at 25% and was ramped to 95% over 30 min and held constant for 6 min. In the next 15 min the methanol concentration was ramped down to 25%, resulting in a total sample run time of 45 min. Wavelengths commonly monitored included 230, 250, 280, 485, and 598 nm. Samples used for HPLC analyses were prepared by centrifugation as described above and the resulting sample supernatants were further syringe filtered through 0.22 μm nylon filters (Micron Separations Inc., Westborough, MA, USA) into a 1.8 ml HPLC vials and sealed immediately.

2.3.3. Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS)

A Hewlett Packard 1100 Series HPLC unit equipped with a Luna C-18 column (50 × 2 mm I.D., 3 μm; Phenomenex, Torrance, CA, USA) was used. The column was maintained at room temperature. The injection volume was 10 μl and the eluent flow rate was 0.10 ml/min. The same eluents and ramping times used with the above-described HPLC method were used in this analysis. A Quattro-LC-quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) in negative ion mode with a mass range up to m/z 4000 equipped with an orthogonal Z-spray-electrospray interface was used for LC/ESI-MS detection. The capillary and cone voltages were 2.50 kV and 25 V, respectively. The source block and desolvation (drying) temperatures were 100 and 150 °C, respectively. Nitrogen at a head pressure of 100 psig was used as the desolvation gas at a flow rate of 433 l/h and as a nebulizing gas at a flow rate of 81 l/h. The MassLynx 3.4 application software (Micromass Ltd., Manchester, UK) was used for instrument control and data processing. Samples used for LC/ESI-MS analyses were prepared by centrifugation and filtration as described above. Samples were split, one analyzed by HPLC and the other by LC/ESI-MS in order to compare the results.

2.3.4. Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)

MALDI analyses were performed using a Micromass Time-of-Flight mass spectrometer (TOFSpec) 2E instrument (Manchester, UK) combining MALDI in a linear mode. The matrix – α-cyano-4-hydroxycinnamic acid (CHCA) – was freshly prepared and dissolved at a concentration of 10 mg/ml in 50:50 water:methanol, containing 0.2% formic acid. The undissolved matrix was allowed to settle and only the supernatant was used in sample preparation. Then, 1 μl of a 10 mg/ml dye solution in water was mixed with 1 μl of the matrix mixture, and 1 μl of this preparation was placed onto a MALDI sample plate. Mass analysis was carried out in a negative linear mode using a 337 nm nitrogen laser (Laser Scientific Inc., Mountain View, CA, USA). The acceleration voltage was set to 20 kV and 10 laser shots per single spectrum were collected and averaged. A total of 40 mass spectra were acquired and summed to give the final spectrum. The instrument resolution was approximately 1000 (full-width half-maximum, FWHM). All MALDI spectra were externally calibrated by using adrenocorticotropic hormone (ACTH) fragment 18–39 and the dimer of CHCA. The

MassLynx 3.4 application software was also used for instrument control and data processing. All chemicals were of analytical reagent quality and were obtained from Sigma–Aldrich Chemical (Milwaukee, WI, USA) and solvents were of HPLC grade.

2.3.5. Dye toxicity

The RB4 acute toxicity was quantified by the bacterial luminescence-based Microtox® procedure. An Azur Environmental M500 Analyzer with Microtox® Omni software (Strategic Diagnostics Inc., Newark, DE, USA) was used to measure the acute toxicity (15 min incubation time) of an RB4 sample with two controls and eight serial dilutions. The EC50 value was calculated using bacterial luminescence of *Vibrio fischeri* (lot # 3B2159) at 490 nm. Color correction was conducted by subtracting the absorbance of each diluted sample at 490 nm and then using the software built-in color correction function. A zinc sulfate ($ZnSO_4{\cdot}7H_2O$) standard protocol was conducted before using a new bottle of bacterial reagent to ensure its viability. Reacted RB4 dye at 2500 mg/l was filtered through 0.22 μm nylon syringe filters (Micron Separations Inc.) and the pH was adjusted to between 6.9 and 7.1.

3. Results and discussion

3.1. Dye properties

In spite of the fact that RB4 is widely used in the textile industry, little information has been published relative to its physico-chemical properties. Table 1 shows experimentally measured and estimated properties of RB4. Due to the serious lack of experimental values for most of the RB4 properties as seen in Table 1, no conclusions can be drawn as to the accuracy of these estimates. However, these estimates do provide an insight into the overall environmental fate of RB4. Based on these estimates, under normal environmental conditions, RB4 is expected to partition strongly to the aqueous phase due to its high water solubility, low K_{ow} and very low vapor pressure. Based on the estimated pK_a values (Table 1), three predominant, deprotonated, unhydrolyzed RB4 species exist between the pH values of 4 and 14 (Fig. 2). These data show that there is a change in speciation in the range of normal pH values found in the environment, and therefore, pH effects must be considered in any process related to the fate and transformation of RB4.

Table 1
Experimental and estimated property values of unhydrolyzed RB4

Parameter	Experimental value[d]	Estimated value[f]
Molecular weight (g/mol)[a]		637.4
λ_{max} (nm)	598	
pH (units)[b]	6.6 ± 0.1	
COD (mg/l)[c]	390 ± 2	
Carbon content (% dry weight)	29.6 ± 0.2[e]	43.3[a]
Melting point (°C)		349.84[g]
Boiling point (°C)		939.92[g]; 872.88[h]
Vapor pressure (atm)		2.38E-32[g]; 1.42E-33[h]
Water solubility (mol/l)	1.57E-1[i]	5.27E-2[g]; 3.77E-9[g]; 2.64E-8[h]
Henry's law constant (unitless)		1.66E-31[g]; 2.58E-25[g]; 2.19E-27[h]
log K_{ow}		1.72[g]; 4.20[h]
pK_a		0.80, 1.44, 7.83, 12.05[h]

[a] Average mass based on the molecular formula $C_{23}H_{14}O_8N_6S_2Cl_2$.
[b] 50 mg/l unreacted dye solution; Ref. [19].
[c] Chemical oxygen demand value of a 500 mg/l unreacted dye solution; Ref. [19].
[d] Mean ± standard deviation ($n = 3$).
[e] Measured experimentally as DOC of a 100 mg/l unreacted dye solution; Ref. [19].
[f] Values at 25 °C.
[g] Values estimated by EPI Suite™ using two separate methods.
[h] Values estimated by SPARC On-Line.
[i] Ref. [20].

3.2. Assessment of dye environmental fate and impact

With up to 800 mg/l of dye in wastewater dyebath [21], large wastewater flow rates, and ineffective removal by conventional wastewater treatment plants [3–6,22], large quantities of reactive dyes are being released into the environment. However, there are few reports of dyes being detected in the environment [23–26], partly because of difficulties in measuring the low dye concentrations expected in the environment [27]. Reactive dyes should mainly partition to the aqueous phase due to their high water solubility, but can also partition to sediments [25]. When the quantities of dyes being released from textile plants, the ineffective removal by conventional wastewater treatment plants and the general lack of dye detection in the environment are taken into account, there must be some degradation (physico-chemical or biological) taking place in the environment beyond dilution [28]. The degree of bioelimination of reactive dyes is considered low [29]. Very slow environmental degradation of RB4 was also predicted by the EPI Suite™ (LEV3 EPI™), with the following estimated half-lives: air, 0.053 d; water, 150 d; soil, 150 d; and sediment, 600 d. The health risk for many reactive dyes is assumed to be low due to the low dye concentrations found in the environment. The major environmental impact of these dyes is aesthetics.

Relative to the dye toxicity, a rodent must be administered 100 mg into the eye over a 24 h period for a moderate reaction and the oral LD50 for rodents was reported as 8980 mg dye/kg for RB4 [30]. The estimated LC50 for fish over 14 d was estimated as

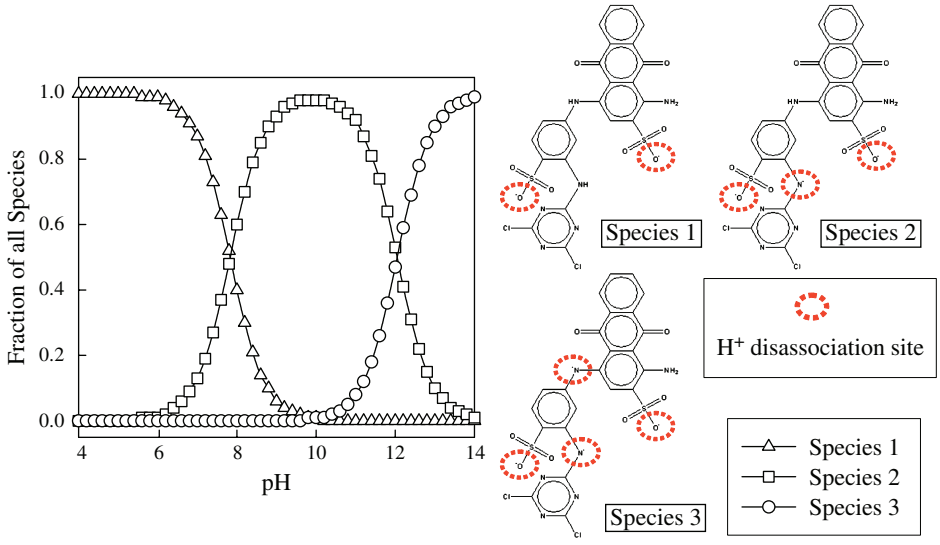

Fig. 2. Predicted unhydrolyzed RB4 speciation between pH 4 and 14 (calculated by SPARC On-Line).

1500 mg/l (2.35E-3 mol/l) for RB4 (EPI Suite™). The acute toxicity, $EC50_{15\ min}$ value of reacted RB4 determined by Microtox® procedure was equal to 1108 mg/l (95% confidence range 963–1274 mg/l; $r^2 = 0.977$). This value is similar to the above-reported LC50 value for fish obtained from the EPI Suite™. Both the values are well above the expected dye concentrations in the environment (low mg/l), but are within the same order of magnitude of the typical dye concentration in spent reactive dyebaths. Therefore, potential detrimental environmental effects of RB4 in locations where concentrated dyebaths may be disposed off must be considered. In addition, the effect of long-term, low-level dye exposure is presently unknown and should be determined [29].

3.3. UV/visible spectrophotometric analysis

Fig. 3 shows that although UV/visible spectrophotometric analysis can be used to monitor RB4, it cannot distinguish between the unhydrolyzed and hydrolyzed form of RB4. However, it is important to determine which dye species are present in the solution because different species can have different reactivity, thus affecting the effectiveness of various decolorization processes and their optimal conditions. Therefore, it is necessary to use advanced analytical techniques (e.g., HPLC) in order to distinguish between hydrolyzed and unhydrolyzed reactive dyes. Beydilli [31] and Lee [32] have shown that different bases (NaOH and Na_2CO_3) result in a different extent of dye hydrolysis for reactive dyes. NaOH was used in this study based on Lee's research [32], which showed that NaOH resulted in a higher extent of RB4 dye hydrolysis. The pH is also an important factor in dye quantification, because the typical pH in reactive dyebaths is higher than 10, but different decolorization processes may have different optimal pH values. Therefore, the pH effect on 100 mg/l of unreacted RB4 was analyzed for pH values from 3.5 to 12.3. Fig. 4A shows that there was no spectral shift, but the absorbance in the visible region at the maximum wavelength generally increased as the pH increased. The change in absorbance for 100 mg/l of unreacted RB4 at the maximum wavelength varied from −5.5% to +11.8% when compared to the unadjusted pH of 6.4 as seen in Fig. 4B. There was statistically no difference in the absorbance from pH 6 to 10.5. Similar results were obtained with NaOH-reacted RB4 [32].

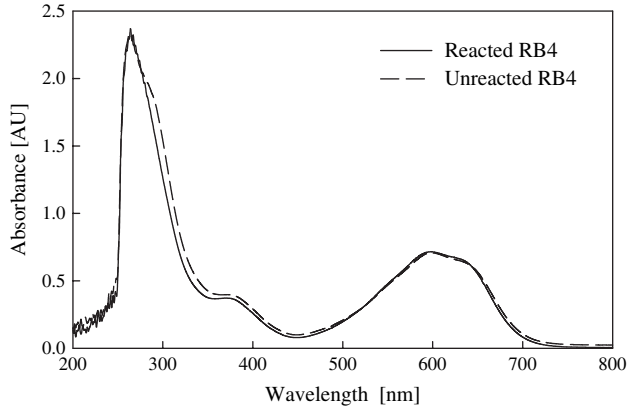

Fig. 3. UV/visible spectra of reacted and unreacted RB4 (100 mg/l).

3.4. HPLC analysis

HPLC was used to separate and identify dye components, specifically the unhydrolyzed and hydrolyzed RB4 species. Fig. 5A–C shows the chromatogram at 598 nm for 300 mg/l of reacted and unreacted RB4,

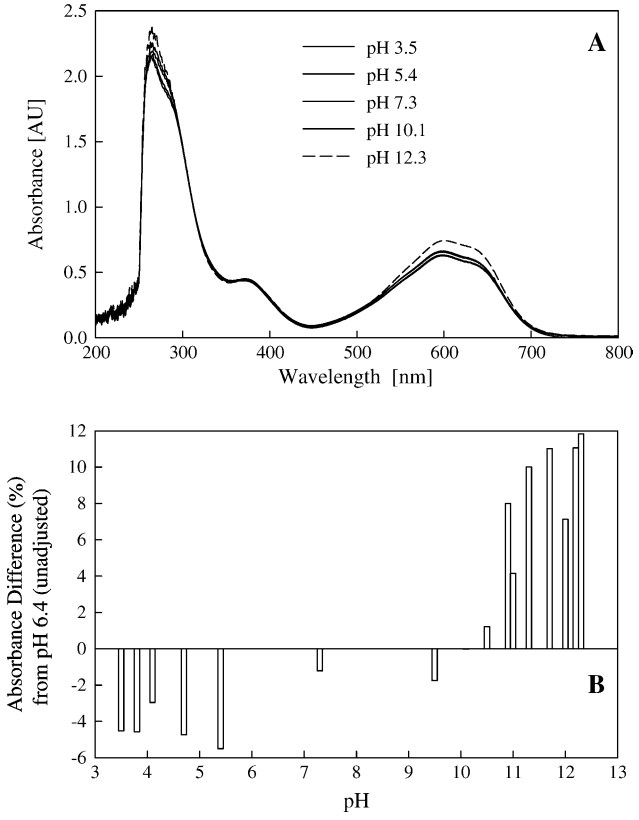

Fig. 4. pH effect on UV/visible spectra (A) and 598 nm absorbance (B) for 100 mg/l of unreacted RB4.

whereas Fig. 5D and E shows the spectra for several peaks identified on these chromatograms. Dye hydrolysis conditions converted all of the unreacted RB4 dye to reacted dye resulting in 30% monohydrolyzed and 52% dihydrolyzed, whereas major impurities accounted for 18% in this particular reacted RB4 stock solution. Impurities in this context are defined as dye components that do not have the complete characteristic spectra of unhydrolyzed, monohydrolyzed or dihydrolyzed RB4 dye, but do have absorbance at 598 nm. HPLC analysis of another reacted RB4 stock solution resulted in 17% monohydrolyzed, 65% dihydrolyzed, and 18% major impurities, which demonstrates that the extent of dye hydrolysis is variable. HPLC analysis also determined that the unreacted RB4 was 65% unhydrolyzed, 14% monohydrolyzed and 15% dihydrolyzed with 6% major impurities with absorbance at 598 nm. It should be noted that these dye hydrolysis product fractions are estimates based on the assumption that the molar absorption coefficient is similar for dihydrolyzed, monohydrolyzed and unhydrolyzed dye and impurities, which cannot be verified due to the unavailability of such dye compounds. The hydrolyzed and unhydrolyzed RB4 were identified based on their characteristic spectra and HPLC retention time. The shorter the retention time, the more hydrophilic the compound should be; therefore, the dihydrolyzed dye would have the shortest retention time followed by the monohydrolyzed and

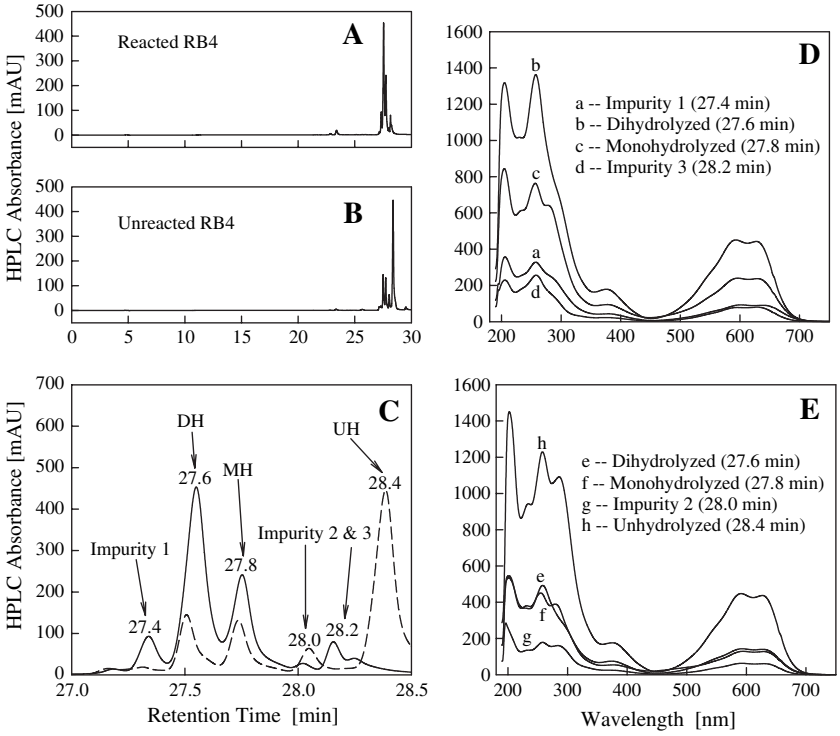

Fig. 5. HPLC analysis of reacted and unreacted RB4 (300 mg/l): total chromatograms at 598 nm (A and B), enlarged chromatograms from 27.0 to 28.5 min retention time (C), spectra of reacted RB4 components (D), and spectra of unreacted RB4 components (E) (DH, dihydrolyzed; MH, monohydrolyzed; UH, unhydrolyzed).

unhydrolyzed dye. The more hydrolyzed the dye is, the more hydrophilic it becomes because, as the dye hydrolyzes, it looses a chloride and gains a hydroxyl group, making it more hydrophilic. The hydrophobicity of RB4 species based on estimated K_{ow} values (EPI Suite™) followed a descending series (log K_{ow} values in parenthesis): unhydrolyzed RB4 (1.72) > monohydroxy-RB4 (0.99) > dihydroxy-RB4 (0.62), which is consistent with the reverse order of HPLC elution of these dye species. Fig. 5C shows that there are two impurities with absorbance at 598 nm for reacted RB4 dye, and one impurity for unreacted RB4 dye. These peaks are most likely due to the loss or gain of a functional group that affects the hydrophilicity of the dye, while not affecting the dye's chromophore. Monohydrolyzed and dihydrolyzed dye peaks were confirmed with mass spectrometric analysis, but only one of the impurities could be identified (see Section 3.6.1).

3.5. Effects of salt and base on dye aggregation and analysis

Salt and base are used in the reactive dyeing process to increase the ionic strength, decrease the electronic double layer thickness and favor the transfer of dye aggregates from the aqueous solution to the fiber [33]. Reactive dyebaths used in our studies contain 100 g/l NaCl, 3 g/l Na_2CO_3 and 1 g/l NaOH (calculated ionic strength of 1.82 M). In order to quantify the effect of salt and base on the quantitative analysis of RB4, a 100 mg/l RB4 solution was amended with either salt (100 g/l NaCl), base (3 g/l Na_2CO_3 and 1 g/l NaOH), or both. The UV/visible absorbance of the RB4 solution at 598 nm decreased by 19.7, 11.4, and 20.1% after the addition of salt, base, or both salt and base, respectively, and centrifugation. These results are consistent with a similar report by Hamlin et al. [33] who observed the same effect with two azo dyes, Reactive Red 180 and Reactive Red 2, as well as with the results of Lee [32], who observed the dye aggregation for RB4 solutions and even a much higher degree of aggregation for Reactive Blue 19, another anthraquinone dye with a vinyl sulfone reactive group. Dye aggregation was more noticeable at higher dye concentrations (>1000 mg/l) in the presence of salt and salt–base. Bredereck and Schumacher [34] reported that for 12 reactive azo dyes, dye aggregation was enhanced not only by the addition of salt, but also by increasing the dye concentration. HPLC analysis of RB4 solutions showed a similar effect of salt and base addition on the dye concentration after centrifugation and filtration. The monohydrolyzed dye was affected more than the dihydrolyzed dye. It is important to note that despite these effects, RB4 dye solutions containing salt and base can still be quantified with spectrophotometry and HPLC if separate calibration curves are developed taking into account the effect of salt and base.

3.6. Mass spectrometric analysis

3.6.1. Liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS)

Similar to other previous works [35,36], preliminary experiments with the electrospray ionization (ESI) technique for the analysis of RB4 solutions demonstrated that the best sensitivity and signal stability was achieved using the negative-ion ESI (data not shown). Holčapek et al. [36] reported that the signal using positive-ion ESI was obtained only for certain compounds with proton acceptor groups such as hydroxyl, amino or carbonyl. Ràfols and Barceló [35] did not obtain any peaks for mono- and disulfonated azo dyes in the positive-ion ESI. Therefore, the negative-ion ESI mode was selected as the standard ionization technique for the determination of RB4. Mass spectra of disulfonated dyes generally showed decationized molecular ions with different charges, i.e. $[M-H]^-$ and $[M-2H]^{2-}$ [32]. Also, Lee [32] reported that adducts with sodium cations were also observed, including monocharged ions, $[M-H]^-$, $[M-2H+Na]^-$, and dicharged ions, $[M-2H]^{2-}$, $[M-3H+Na]^{2-}$, $[M-4H+2Na]^{2-}$. In addition to the previously reported ESI-MS results, an isotope model included in the LC/ESI-MS MassLynx 3.4 application software was used to predict the fragment ions as well as the isotope abundance patterns. Predicted ESI-MS fragment ions for RB4 dye are listed in Table 2. Only the major m/z peak value for each compound based on the predicted isotope modeling is shown.

RB4 dye solutions at or less than 300 mg/l did not have reproducible retention time or m/z peaks of the dye components above the baseline intensity of the eluent. Therefore, a stock dye solution of 5000 mg/l was used for LC/ESI-MS analysis. Fig. 6 shows the total LC/ESI-MS chromatogram for reacted RB4 at 5000 mg/l. Four major peaks were identified (retention time 26.9, 27.8,

Table 2
Predicted ESI-MS fragment ions for unhydrolyzed and hydrolyzed RB4 dye components based on an isotope model (MassLynx 3.4 application software)[a]

Compound	Fragment ion	Mass/charge (m/z)
Unhydrolyzed RB4	$[[M-2H]^{2-}$	317
($M = 636$[b])	$[[M-SO_3]-H]^-$	555
Monohydrolyzed RB4	$[[M-Cl+OH]-2H]^{2-}$	308
($M = 618$[b])	$[[M-Cl+OH-SO_3]-H]^-$	537
Dihydrolyzed RB4	$[[M-2Cl+2OH]-2H]^{2-}$	299
($M = 600$[b])	$[[M-2Cl+2OH-SO_3]-H]^-$	519.1

[a] Only expected major intensity peaks based on the isotope model are shown.
[b] Monoisotopic mass.

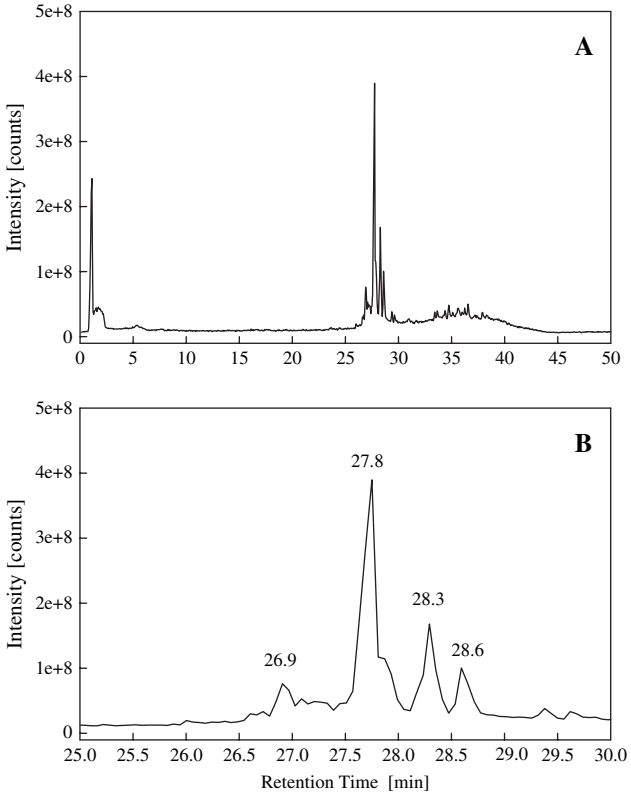

Fig. 6. LC/ESI-MS analysis of reacted RB4 (5000 mg/l) in negative ion mode of operation: total LC chromatogram (A) and enlarged chromatogram from 25 to 30 min retention time (B).

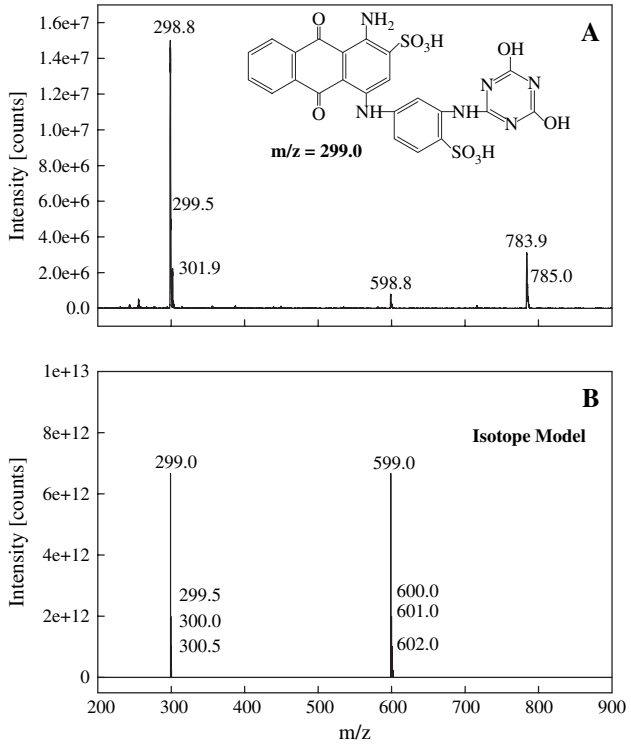

Fig. 7. LC/ESI-MS analysis of reacted RB4 (5000 mg/l) in negative ion mode of operation: ESI-MS spectra of 27.8 min retention time peak (A) and isotope model predicted MS spectra for dihydrolyzed RB4 (B).

28.3 and 28.6 min), which correspond to four previously observed HPLC peaks (retention time 27.4, 27.6, 27.8 and 28.2 min). While the retention times were similar, there was no constant correlation factor between the two sets of retention times because of the different columns and flow rates used for the HPLC and LC/ESI-MS analysis. Peaks at retention times below 2 min and above 32 min are most likely eluent peaks, not dye components because they were present in D.I. water blank control runs.

Fig. 7A shows the ESI-MS m/z spectra for dihydrolyzed dye (retention time 27.8 min). The major m/z peaks of 298.8 and 598.8 match that of the isotope model prediction of 299.0 (double charged) and 599.0 (single charged) shown in Fig. 7B. Each peak also matched the predicted isotope abundance pattern. For example, the 299.0 peak has isotope abundance peaks every 0.5 m/z, which matched the pattern for the predicted spectra. While not every isotope pattern peak (i.e., 300.0 and 300.5) was observed, the pattern obtained was consistent with the predicted one. The isotope abundance pattern of small peaks (i.e., 598.8 m/z) is difficult to distinguish. The other major peak of m/z 783.9 is most likely a dimer of dihydrolyzed dye, but could also be an impurity. Fig. 8A shows the m/z spectra for monohydrolyzed dye (retention time 28.3 min). The major m/z peaks of 308.0 and 616.7 match that of the isotope model prediction of 308.0 (double charged) and 617.0 (single charged), as well as the predicted isotope abundance pattern shown in Fig. 8B. The other major peaks at m/z values of 268.5, 329.4, 494.6, 535.1 and 802.0 are most likely dimers or impurities.

Fig. 8C shows the m/z spectra for impurity 1 (retention time 26.9 min). The major peaks at m/z values of 393.1 and 590.1 do not match the predicted RB4 dye masses. Because this impurity has absorbance at 598 nm, it is either an undisclosed dye compound added to the dye formulation, or a result of incomplete conversion of reactants during the production of RB4. Although the 393.1 m/z peak is similar to that expected for Acid Blue 25 (m/z 393.1 as calculated by the isotope model), the possibility that the impurity may be related to Acid Blue 25 was discounted because the isotope abundance pattern and HPLC retention time did not match that of Acid Blue 25. Fig. 9A shows the m/z spectra for impurity 3 (retention time 28.6 min). The major peak at m/z 317.9 appears to match one of the predicted isotope peaks of unhydrolyzed dye, 317.0 (double charged) in Fig. 9B. However, firstly, the impurity does not contain the single charged species with a predicted peak at m/z 635.0 as was the case for both di- and monohydrolyzed dye. Secondly, the measured m/z value of 317.9, was slightly higher than the predicted m/z value of 317.0 (double charged). This is significant because the m/z values for the peaks of

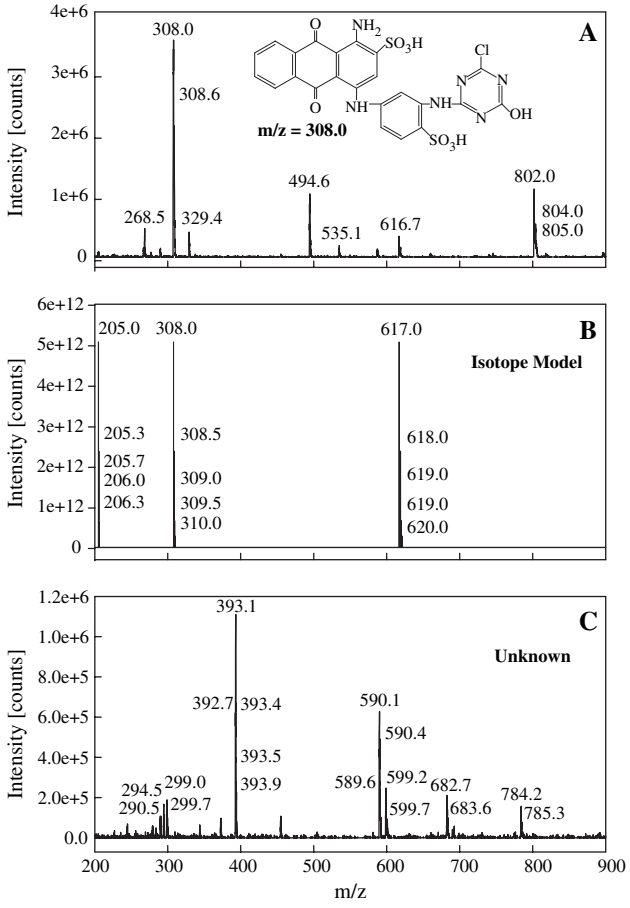

Fig. 8. LC/ESI-MS analysis of reacted RB4 (5000 mg/l) in negative ion mode of operation: ESI-MS spectra of 28.3 min retention time peak (A), isotope model predicted MS spectra for monohydrolyzed RB4 (B), and MS spectra of 26.9 min retention time peak (C).

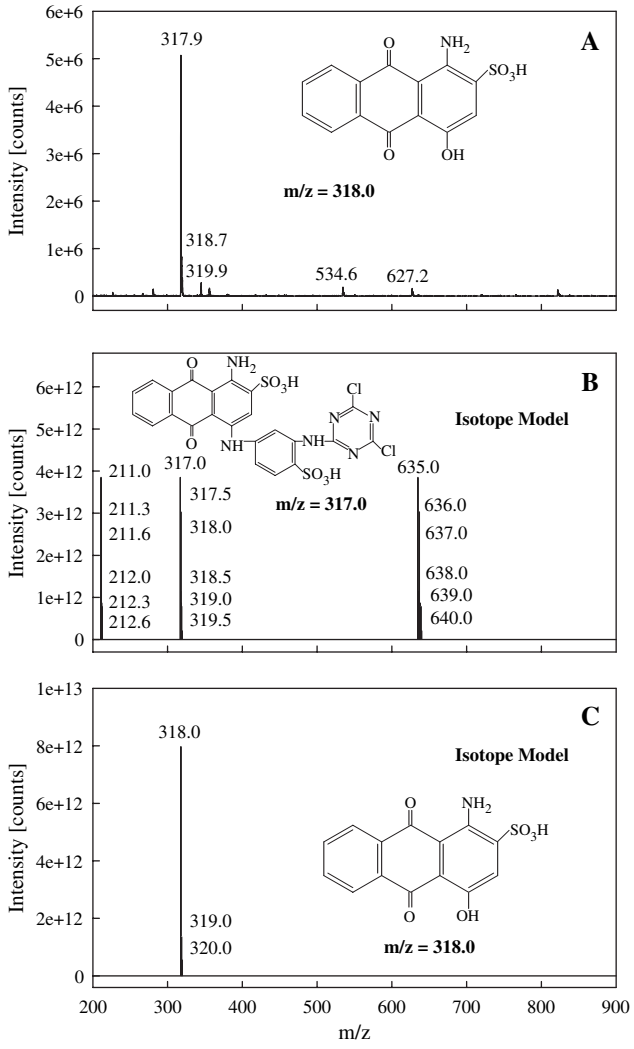

Fig. 9. LC/ESI-MS analysis of reacted RB4 (5000 mg/l) in negative ion mode of operation: ESI-MS spectra of 28.6 min retention time peak (A), isotope model predicted MS spectra for unhydrolyzed RB4 (B), and isotope model predicted MS spectra for 1-amino-4-hydroxy-anthraquinone-2-sulfonic acid (C).

di- and monohydrolyzed dye were equal to or slightly less than the predicted isotope m/z values. Thirdly, this impurity has a different HPLC retention time (28.2 min) than the unhydrolyzed dye (28.4 min) and unhydrolyzed dye was not detected after dye hydrolysis. Therefore, based on these observations it is concluded that this impurity is not an unhydrolyzed dye, but must be a similar impurity as impurity 1 because it also has absorbance at 598 nm. A search of similar structures was conducted and it was discovered that the spectra in Fig. 9A matched that of 1-amino-4-hydroxyanthraquinone-2-sulfonic acid, a sulfonated dye intermediate [36] as shown in Fig. 9C. Based on this peak m/z value and isotope abundance pattern match, and the 598 nm absorbance of the impurity, it was concluded that this impurity is 1-amino-4-hydroxyanthraquinone-2-sulfonic acid.

3.6.2. Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)

Based on the LC/ESI-MS results, negative ion mass spectrometry was used. The MALDI-TOF mass spectra of unreacted RB4 are shown in Fig. 10. Similar to the results from ESI-MS analysis, the MALDI-MS analysis for unreacted RB4 showed a major base peak $[M-H]^-$ at m/z 636 and $[[M-2Cl+2OH]-H]^-$ at m/z 600 corresponding to unhydrolyzed RB4 and hydrolyzed RB4, respectively, in good agreement with the calculated monoisotopic molar mass of RB4. These results confirm the presence of hydrolyzed RB4 in the unreacted dye solution consistent with the above-presented results of both HPLC and LC/ESI-MS analyses. In addition, some RB4 hydrolysis during the MALDI analysis is possible. The presence of the ^{37}Cl isotopic variant was shown by the signal at m/z 622, 636, 658, 680, and 1339 demonstrating that Cl was strongly bound in RB4. The major signal at m/z 658 can be explained by the formation of an Na^+ salt of the one-linker arm species at m/z 636 i.e., $[M-2H+Na]^-$. The signals at m/z 622 and 680 were attributed to a Na adduct of 600 and

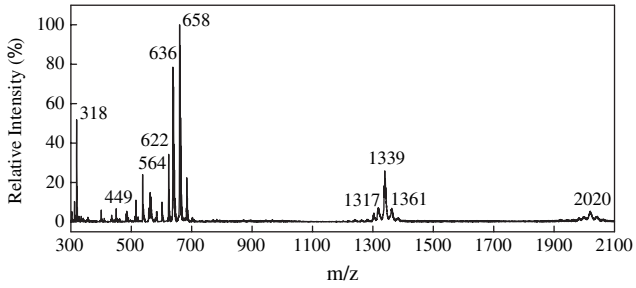

Fig. 10. MALDI-TOF-MS spectra of unreacted RB4 (linear negative ion mode).

658 species, respectively. The calculated masses of minor fragment ions were $[M-2H]^{2-}$ (m/z 318), $[M-H-2Cl-2SO_3H+2Na]^-$ (m/z 449), $[M-H-2Cl]^-$ (m/z 564). Other peaks at m/z values higher than 1000 could be due to dimer species. The signals at m/z values of 1317, 1339, 1361, and 2020 were $[2M-3H+2Na]^-$, $[2M-4H+3Na]^-$, $[2M-5H+4Na]^-$, and $[3M-6H+5Na]^-$, respectively. Beside the identified peaks, a series of unidentified peaks with smaller intensities at m/z 514, 536, 563 were observed and attributed to impurities. It is noteworthy that these m/z values do not match those of dye impurities based on LC/ESI-MS analysis, which may be attributed to the fact that reacted and unreacted RB4 dye solutions were used for the LC and MALDI analyses, respectively. It should be noted that MALDI-MS data are based on average masses, whereas the LC/ESI-MS data are based on monoisotopic masses. In addition, MALDI generally detects only singly charged species, whereas LC/ESI-MS detects multiply charged species. The fragment ions obtained from MALDI-MS analysis of RB4 are shown in Table 3. The negative ion MALDI mass spectrum of RB4 showed a similar profile to that observed in the ESI-MS analysis, except that the relative abundance ratios were different.

Table 3
Fragment ions and relative abundance of unreacted RB4 by MALDI-MS in negative ion mode of operation

Fragment ion	Mass/charge (m/z)[a]	Relative abundance
$[M-H]^-$	636	78
$[M-2H]^{2-}$	318	52
$[M-H-2Cl-2SO_3H+2Na]^-$	449	6
$[M-H-2Cl]^-$	564	15
$[[M-2Cl+2OH]-H]^-$	600	10
$[[M-2Cl+2OH]-2H+Na]^-$	622	34
$[M-2H+Na]^-$	658	100
$[M-3H+2Na]^-$	680	22
$[2M-3H+2Na]^-$	1317	7
$[2M-4H+3Na]^-$	1339	26
$[2M-5H+4Na]^-$	1361	7
$[3M-6H+5Na]^-$	2020	5

[a] Based on average, monoisotopic mass.

The signal could be contributed to the loss or addition of SO_3 from the original signal, therefore the linker arms and sulfonic acid groups are labile in MALDI-MS. Based on these results, MALDI-MS could be employed for the separation and molecular weight determination of individual compounds in RB4 solutions. The use of MALDI-MS for the analysis of dye solutions has been previously reported [37,38]. Sullivan and Gaskell [39] demonstrated that MALDI-MS was a suitable alternative method for the determination of the molecular weight of dyes containing one to six sulfonic acid groups.

4. Conclusions

Estimated values of the physico-chemical properties of the RB4 dye were presented and discussed, but their accuracy was not confirmed due the lack of experimental data. Based on these estimates, the environmental behavior of RB4 was discussed. The measured RB4 EC50 (1108 mg/l) acute toxicity based on the Microtox® procedure agreed well with the LC50 value for acute toxicity to fish (1500 mg/l) estimated by the EPI Suite™. Although the levels of RB4 in the environment are expected in the orders of magnitude lower, the effect of long-term, low-level dye exposure needs to be evaluated.

The maximum absorbance of RB4 in the visible region was measured at 598 nm, and did not shift after dye hydrolysis. Therefore, although spectrophotometry can be used to quantify dye concentrations, it cannot distinguish between the hydrolyzed and unhydrolyzed dye forms. In contrast to the spectrophotometric analysis, HPLC analysis using TBA as the ion-pairing agent resulted in successful separation and quantification of both hydrolyzed and unhydrolyzed RB4, even at high concentrations of salt and base. The effect of salt and base on the analysis of RB4 solutions was further investigated. While high concentrations of salt and base do depress the measured absorbance at the visible wavelength due to dye aggregation, they do not alter the overall spectra. Therefore, even at high concentrations of salt and base, both spectrophotometric and HPLC techniques can be used to quantify RB4 dye solutions.

LC/ESI-MS analysis of RB4 solutions successfully led to the conclusive identification of di- and monohydrolyzed RB4 dye. The presence of two major impurities in reacted RB4 dye solutions was also confirmed. One of these impurities was identified as 1-amino-4-hydroxyanthraquinone-2-sulfonic acid. In addition, MALDI-MS could be employed for the separation and molecular weight determination of individual compounds in RB4 solutions.

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