„BABEŞ-BOLYAI” UNIVERSITY CLUJ-NAPOCA

Faculty of Environmental Sciences and Engineering

CONTRIBUTIONS REGARDING THE INFLUENCE OF TEXTILE DYES ON SOME ENVIRONMENTAL FACTORS

PhD Thesis Abstract

Scientific Advisor:
Prof. Dr. Dumitru RISTOIU

PhD Student:
Florina-Maria ONIŢĂ (COPACIU)

CLUJ-NAPOCA

2012

CONTENT

KEY WORDS:

Textile dyes
Azo dyes
Anthraquinone dyes
Solid Phase Extraction
High Performance Thin Layer Chromatography
Liquid Chromatography ElectroSpray Ionization tandem Mass Spectrometry
Abiotic stress
Photosynthetic parameters
Assimilating pigments
Volatile Organic Compounds
Triticum aestivum L.

INTRODUCTION

Textile industry is one of the most common and important sectors in the world being the biggest consumer of water. The residue accumulation in water in amounts exceeding the natural power of transformation and integration of the environmental factors conducts to the appearance of some imbalances of natural life, leading to the disappearance of plant and animal species, ultimately jeopardizing our own life.

The main objective of this PhD thesis consists in the development of some modern and performant methods for the identification and the quantification of six textile dyes, two anthraquinone dyes (Optilan Blue and Lanasyn Blue) and four azo dyes (Lanasyn Red, Nylosan Red, Nylosan Dark Brown and Lanasyn Dark Brown), and their use for the monitoring of these dyes from wastewater samples, thus contributing to the assessment of their impact on the environment quality and also to the study of their eco-toxicological effects on plants.

The aim of the researches undertaken in this thesis is focused on issues concerning:

- The determination of physicochemical parameters of water quality from the different wastewater samples generated by textile processing industry.
- The development of some isolation methods of the target textile dyes from wastewater samples using solid phase extraction (SPE) technique.
- The development of a high performance thin layer chromatography (HPTLC) method for the identification and quantification of the target textile dyes in order to monitor these dyes of the effluent wastewater samples collected from a textile factory which are discharged into the sewerage network.
- The development of a high performance liquid chromatography method coupled with mass spectrometer detector (LC-ESI/MS-MS) for the identification and quantification of the target textile dyes for their monitoring in the influent and effluent wastewater samples collected from a wastewater treatment plant where the waters from the sewerage networks potentially contain textile dyes.
- The study of the ecotoxicological effects of the target textile dyes on the different performance regarding the plant metabolism in order to identify and to fast assess the toxicity of dyes on wheat plants (*Triticum aestivum* L.). The effects of textile dyes were evaluated on foliage photosynthesis, and the secondary metabolites (photosynthetic pigments, emissions of lipoxygenase pathway products, and emissions of monoterpenes) in wheat.

The first part of the thesis contains a bibliographic study regarding the textile dyes, the analysis techniques used for their isolation, identification and determination from wastewater samples and a few ecotoxicological aspects of the textile dyes.

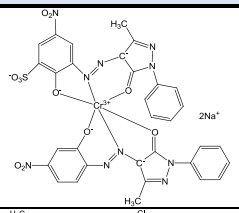
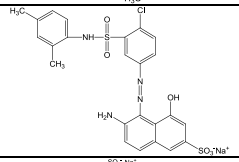
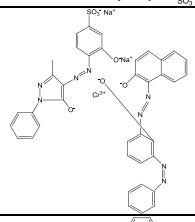
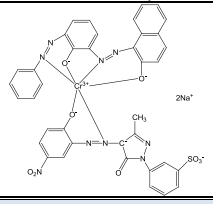
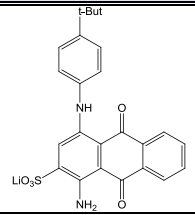
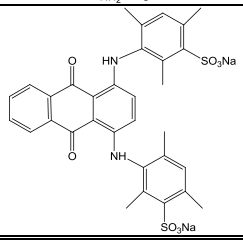
In the second part of the thesis are presented the original contributions referring to the UV-Vis investigations of textile dyes, the determination of physicochemical parameters regarding the monitored textile effluents, the SPE isolation of textile dyes from the wastewater samples, the identification and quantification of dyes by HPTLC and LC-ESI/MS-MS from different wastewater samples. Ecotoxicological studies regarding the action of textile dyes on wheat (*Tritcum aestivum* L.) have been achieved. Also, investigations on the influences of dyes on the photosynthetic parameters and the secondary metabolites mentioned above were done.

4. Presentation and physico-chemical characterization of studied textile dyes

The azo and anthraquinone dyes are the most commonly used dyes in the textile industry due to their properties like adhesion, long lasting and resistance to sunshine and chemical processes *(Lee and Pavlostathis, 2004; Epolito et al., 2005)*.

In Table 4.1 are presented the two classes of the studied textile dyes. That were purchased from Clariant Produkte (Switzerland) AG.

Tabel 4.1. Data of the studied textile dyes.

Trade name/ CAS number/ Abbreviation	**Chemical formula/ Molecular weight (g mol^{-1})**	**Chemical structure**
Azo dyes		
Lanasyn Red M-GA CAS 70209-87-9 **LR**	$C_{32}H_{21}CrN_{10}O_{11}S \cdot 2Na$ 851.61	
Nylosan Red N-2RLB CAS 71873-39-7 **NR**	$C_{24}H_{21}ClN_4O_6S_2 \cdot Na$ 584.02	
Nylosan Dark Brown S-MBL CAS 52587-68-5 **NDB**	$C_{38}H_{25}CrN_8O_7S \cdot 2Na$ 835.69	
Lanasyn Dark Brown M-GLN CAS 70236-62-3 **LDB**	$C_{38}H_{24}CrN_9O_9S \cdot 2Na$ 880.69	
Anthraquinone dyes		
Optilan Blue MF-GL CAS 125328-86-1 **OB**	$C_{24}H_{21}N_2O_5SLi$ 456.44	
Lanasyn Blue F-L 150 CAS 4474-24-2 **LB**	$C_{32}H_{28}N_2O_8S_2 \cdot 2Na$ 678.68	

4.1. UV-Vis study of the textile dyes

Preliminary, UV-Vis measurements were performed in order to establish the maximum of absorption for the HPTLC quantitative analyses of textile dyes. Generally, in the visible range of 400-900 nm, the dyes highlighted. To find the range of the optimum wavelength, UV-Vis spectra of dyes prepared in methanolic solutions were recorded.

Table 4.4. Positions of the maximum absorption in visible and the absorbance of studied textile dyes at the indicated concentration.

Textile dyes	Maximum absorbtion (nm)	Absorbance	Concentration (mg mL^{-1})
Lanasyn Red	502	1.061	0.08
Nylosan Red	511	1.030	0.06
Nylosan Dark Brown	475	1.016	0.08
	567	0.691	
	612	0.575	
Lanasyn Dark Brown	470	1.282	0.06
	615	0.557	
Optilan Blue	588	0.942	0.019
	627	1.040	
Lanasyn Blue	581	0.944	0.048
	626	1.059	

From the UV-Vis spectra of textile dyes, it can be observed that the maxima of absorption were in the range of 470-627 nm.

6. Analysis of the textile dyes by liquid chromatography

Textile industry is one of the most common and essential industrial sectors in the world. The wastewater provided from the textile industry has been characterised with its complexity, composed of dyestuff, surface-active, materials and additives, which are related to colour and effluent toxicity problem *(Eremektar et al., 2007)*.

Many analytical techniques have been developed for the determination of the synthetic dyes from the wastewater samples such as: spectrophotometry *(Şahin et al., 2007)*, thin-layer chromatography *(Umbuzeiro et al., 2005)* and high performance liquid chromatography *(Prevot et al., 2008; Rafaëlly et al., 2008)*.

After critical analysis of the literature data, these dyes have been not studied by these techniques so far.

6.1. Analysis of some dyes in textile effluents by high performance thin layer chromatography technique (HPTLC)

6.1.1. Experimental part. Monitoring of textile dyes by HPTLC

Chemicals and reagents

All textile dyes were purchased from Clariant Produkte (Switzerland) AG [15] and were used for the preparation of the standard solutions. Methanol (≥99.9, HPLC) purchased from Merck (Germany) was used for sample preparation. For the conditioning of SPE cartridges and the elution of dyes, ethanol and 25% ammonia solution purchased from Chimopar (Romania) were used. For the mobile phase, n-butanol and ethyl acetate were purchased from Merck (Germany). Water was purified using a Milli-Q Ultrapure system (Millipore, USA). For SPE studies, four types of cartridges of 500 mg/6 mL (WAX/NH_2, SAX, C18-U and C18-E) purchased from Phenomenex (USA) were tested.

Preparation of stock and working standard solutions

For the quantitative analysis, stock solution (0.2 mg mL^{-1}) of studied dyes was prepared by dissolving 1 mg of each dye in methanol in a volumetric flask of 5 mL and then stored under refrigeration at 4°C in the dark. The working standard solutions in concentration of 20, 30, 40, 50 and 60 μg mL^{-1} respectively were prepared by diluting the stock solution with methanol. These standard solutions were used for the obtaining of the calibration curves.

Sample collection

Samples of wastewater from a textile factory from Romania were collected from effluent directly in polyethylene bottles previously washed three times with ultra-pure water and dried. Then the samples were stored at 4ºC in the refrigerator and analysed within one-week from sampling. The effluent wastewater was monitored for a period of six months.

SPE method

For the SPE extraction of the two target compounds, Nylosan Red N-2RBL and Optilan Blue MF-GL, a device model Supelco purchased from Sigma Aldrich was used and four types of Strata cartridges (C18-U, C18-E, WAX/NH_2 and SAX) were tested *(Copaciu et al., 2012a)*.

The SPE sample processing involved four steps *(Poole et al., 2000; Yoshioka and Ichihashi, 2008)*:

1. **Conditioning** of sorbent to remove the impurities, to activate the sorbent bed and to improve the analyte retention by solvating the functional groups with a proper solvent (5 mL of Milli-Q water followed by 20 mL of 1% acetic acid).
2. **Equilibration** of sorbent with 20 mL of Milli-Q water necessary to prepare the sorbent for the optimized interaction with the analyte.
3. **Loading** sample and retention of textile dyes by the sorbent at a flow rate of 2 mL min^{-1}.
4. **Desorption** of textile dyes from the sorbent with 6 mL of 1% ammonia:ethanol 1:1, (*v/v*) mixture.

The obtained extracts containing the textile dyes were dried by means of a rotary evaporator at 40°C and then solubilised in 2 mL mobile phase.

HPTLC method

The chromatography was performed on HPTLC plates precoated with silica gel Alugram RP-18W/UV254 (20 × 20 cm, 0.15 mm layer, Macherey-Nagel). All standard solutions and wastewater samples (1 μL per band) were applied to the plates as 1 mm bands by means of a Desaga AS 30. The spotted plate was developed by ascending technique on a 5.5 cm distance, in a Desaga twin-trough glass chamber saturated for 30 min. with the n-butanol–ethyl acetate–5% ammonium hydroxide 4:4:1 (*v/v*) mobile phase at 23°C room temperature. After elution the plate was dried at room temperature under a hood. The developed plate was scanned in absorbance mode at 550 nm using a Desaga CD-60 densitometer *(Copaciu et al., 2012a)*.

Validation of the SPE-HPTLC procedure

Validation of the SPE-HPTLC procedure was realised for the determination of the studied textile dyes from the wastewater samples collected from the effluent of a Romanian textile factory. In order to validate this procedure, the following parameters were analysed for each dye: selectivity *(Gumustas and Ozkan, 2011)*, linearity *(Mohammad and Zawilla, 2009)*, limits of detection and quantification *(Nayak et al., 2009)*, accuracy *(Nayak et al. 2009)* and precision *(Mehta and Morge, 2008; Gumustas and Ozkan, 2011)*.

6.1.2. Results and discussion on the determination of textile dyes by HPTLC

SPE results

The efficiency of the tested sorbents was estimated by comparing the recovery levels of the eluted dyes on each sorbent. Each extraction was repeated four times and RSD (%) was calculated. The recovery levels are given in Table 6.2.

Table 6.2. Recoveries of studied textile dyes on the four tested SPE cartridges.

Cartridges	Mean recovery[a] ± RSD[b] (%)				
	LB	LDB	LR	NDB	NR
Strata NH_2	108.3 ± 5.0	108.7 ± 3.6	84.8 ± 3.8	109.2 ± 3.2	108.2 ± 2.6
Strata SAX	nd[c]	74.2 ± 2.3	28.5 ± 2.4	61.3 ± 11.8	59.4 ± 5.7
Strata C18-U	nd	22.7 ± 7.5	nd	nd	43.8 ± 0.73
Strata C18-E	nd	20.5 ± 8.0	nd	nd	50.0 ± 3.3

[a]Average of recovery of four extractions; [b]Relative standard deviation; [c]Not detected.

As it can be seen in Table 6.2, the tested Strata NH_2 SPE cartridges assure a good recovery (100%) for all textile dyes, excepting Lanasyn Red with a recovery of 84.8%. For the Strata SAX, the recovery of LDB, NDB and NR dyes was over 59%, excepting LR with a 28.5% value and the antraquinone dye LB not detected because it was stronger retained on cartridge. On Strata C18-U and Strata C18-E cartridges the recoveries were obtained at low values for LDB and NR dyes and the LB, LR and NDB dyes were not detected.

The best results obtained on Strata NH_2 are due to the retention mechanism of polar analytes by either hydrogen bonding or week anion exchange. This sorbent is suitable for extraction of strong and/or weak anions from aqueous samples. The Strata SAX which is a strong anion exchange resin gives lower recovery levels for textile azo dyes due to the strong interactions between the textile dyes and the sorbent.

The antraquinone dye is stronger retained on SAX sorbent and it could not be eluted with the used solvent.

On Strata C18-E has place a non-polar retention mechanism with the surface hydrophobic end-capping for additional hydrophobic retention, minimizing selectivity or eliminating polar silanol interactions. On Strata C18-U has place a primarily non-polar retention mechanism combined with the secondary polar silanol interactions, therefore increased extraction efficiency and enhanced cleanup of hydrophobic compounds that contain hydroxyl or amine moieties take place *(www.phenomenex.com)*. On Strata C18-E and C18-U cartridges, the LB, LR and NDB dyes are not retained due to their polarity. As a result of the SPE study, the Strata NH_2 cartridge was selected for the further determinations of studied textile dyes from wastewater *(Copaciu et al., 2012a).*

Validation results

The validation study demonstrated that the performed SPE-HPTLC procedure for the determination of the five textile dyes from the effluent wastewater samples has a good linearity with a correlation coefficient (r) over 0.99 in the whole concentration range of 20-60 ng per band for each dye; the detection limits between 0.97-6.04 ng per band and the quantification limit between 3.24-20.15 ng per band; the RSD values for the repeatability of sample application are ranged between 0.63-3.5%, and for the repeatability of measurement of peak area are ranged between 0.70-2.6%. The recovery results of all studied textile dye in the spiked wastewater show values over 100% that demonstrate good accuracy *(Copaciu et al., 2012a).*

Analysis of studied dyes from wastewater samples

Determination of the dyes in these wastewater samples is important for the monitoring of the level of water pollution caused by textile industry. So, the described SPE-HPTLC procedure has been applied to five wastewater samples collected monthly from the effluent of a textile factory. The HPTLC chromatogram of wastewater samples is illustrated in Figure 6.8, positions 11-15.

According to the results obtained during the monitored period, the studied textile dyes are not all used in the same time, this fact depending on the factory activity. During the monitored period it was intensively used Nylosan Red and occasionally Lanasyn Blue and Lanasyn Red. The concentrations of the found textile dyes in effluent wastewater are given in Table 6.8.

In Figure 6.8 it is depicted the HPTLC chromatoplate illustrating as follows from left to right: the five individual standard dyes (positions 1-5), the five standard dyes in mixture (positions 6 and 16), the recovery on the four types of tested cartridges (positions 7-10) and five wastewater samples (positions 11-15). The developed plate was scanned in absorbance mode at 550 nm using a Desaga CD-60 densitometer. The overlapped densitograms of a standard dye mixture (position 16) and of a wastewater sample (position 15) are shown in Figure 6.8.

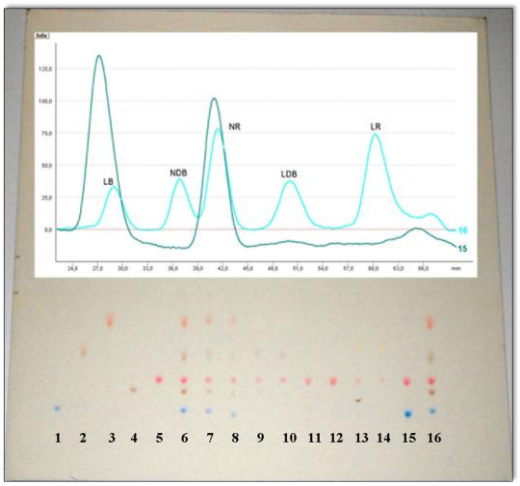


Figure 6.8. The HPTLC chromatoplate and the overlapped densitograms (550 nm) of studied textile dyes. Positions 1-5 of individual standards (60 ng per band): 1-LB, 2-LDB, 3-LR, 4-NDB, 5-NR; Positions 6 and 16: standard mixture (60 ng each dye per band); Positions 7-10: SPE recovery of dyes on Strata cartridges: NH_2 (7); SAX (8); C18-U (9); C18-E (10); and Positions 11-15: wastewater samples from textile effluent.

Table 6.8. Found amount of studied textile dyes in the effluent samples from a textile factory.

Plate band No.	Date of sampling	Concentration (mg L^{-1})				
		Lanasyn Blue	Lanasyn Dark Brown	Lanasyn Red	Nylosan Dark Brown	Nylosan Red
11	04.02.2011	nd	nd	nd	nd	3.7
12	18.02.2011	nd	nd	nd	nd	5.7
13	10.03.2011	nd	nd	nd	nd	1.2
14	15.04.2011	nd	nd	nd	nd	1.0
15	13.05.2011	20.4	nd	nd	nd	7.4
–	17.06.2011	nd	nd	nd	nd	12.0
–	11.07.2011	nd	nd	0.7	nd	7.6
–	25.07.2011	nd	nd	8.1	nd	1.3

The founded concentrations during this monitoring has values between 0.7-20.4 mg L^{-1} and shows that textile effluents resulting from textile processes are not adequately treated before discharge into the sewage system, or environment. Of the two classes of textile dyes investigated, azo dyes are extensively used in textile processing *(Copaciu et al., 2012).*

6.2. Analysis of textile dyes Optilan Blue and Nylosan Red from wastewater by liquid chromatography technique coupled with mass spectrometry (LC-MS/MS)

6.2.1. Experimental part. Determination of textile dyes by LC-MS/MS

Chemicals and materials

The azo dye, Nylosan Red, and the anthraquinone dye, Optilan Blue, used for the preparation of the standard solutions, were purchased from Clariant Produkte AG. Methanol LC-MS Optigrade (≥99.8%) acquired from LGC Standards was used for the sample preparation and the chromatographic elution.

Sample collection

Samples of wastewater from a treatment plant from Romania with a textile factory in its area were collected both in influent and effluent directly in polyethylene bottles, and was monitored for a period of six months. The samples were stored at 4°C away from sunlight and analysed within one-week from sampling.

LC/ESI(-)-MS/MS method

The determination of textile dyes was carried out using a HPLC Agilent 1200 Series coupled with an API 3200 QTRAP mass spectrometer (Applied Biosystems), using a TurboV ionization source in an ESI negative ion mode (LC/ESI(-)-MS/MS). Multiple reaction monitoring (MRM) transitions were followed for quantifying the precursor molecular ions and the related product ions. The system was controlled using the Analyst 5.1 software. A Nucleosil 100 C18 column (25 cm × 0.46 cm, particle size 10 µm) acquired from Teknokroma was used for the HPLC separation of textile dyes. A good separation of dyes was obtained by isocratic elution with a mixture of methanol:water 70:30, (*v/v*) at a flow rate of 0.65 mL min^{-1}. The column oven was set at 25°C.

SPE method

For the SPE extraction of the two target compounds, Nylosan Red and Optilan Blue, a device model Supelco purchased from Sigma Aldrich was used and four types of Strata cartridges (C18-U, C18-E, WAX/NH_2 and SAX) were tested. To quantify the selectivity of the four tested SPE cartridges, 500 mL deionized water sample spiked with 160 ng of each dye were passed through each SPE cartridge.

Validation of SPE-LC/ESI(-)-MS/MS procedure

In order to validate the SPE/LC-ESI(-)-MS/MS procedure for the two target textile dyes the following parameters were studied: linearity, limits of detection and quantification for each dye, accuracy, precision and matrix effect.

6.2.2. Results and discussion regarding the determination of textile dyes by LC-MS/MS

LC/ESI(-)-MS/MS analysis

In order to obtain quantitative data regarding the presence of the investigated textile dyes in wastewater samples a LC/ESI(-)-MS/MS method was developed. Both negative and positive ion modes were tested for the studied dyes using ESI mode. Negative ion mode was finally chosen due to the higher

sensitivity over the investigated dyes. For the establishment of the MRM conditions, the standard solutions for each dye were individually prepared (100 ng mL^{-1} in methanol). The quadrupole 1 scanning was performed for each dye to obtain the precursor ions followed by quadrupole 3 scanning to obtain the product ions.

To obtain the optimal parameters dependent on the studied textile dyes the infusion method was used. Standard solution of each dye was injected directly in MS spectrometer by continuous infusion with a flow rate of 5 µL min^{-1}. The separation of Nylosan Red N-2RBL and Optilan Blue MF-GL with a resolution of 1.83 was obtained by isocratic elution with methanol:water 70:30, (*v/v*) at a 0.65 mL min^{-1} flow rate and 25°C temperature of the column oven. The source dependent parameters were obtained by Flow Injection Analysis (FIA) using a restrictive capillary and a flow rate of 200 µL min^{-1}. A standard solution containing 100 ng mL^{-1} from each dye prepared in mobile phase was injected in LC/ESI(-)-MS/MS system.

The chromatogram corresponding to LC/ESI(-)-MS/MS of the standard mixture of textile dyes using the presented MRM method is shown in Figure 6.12.

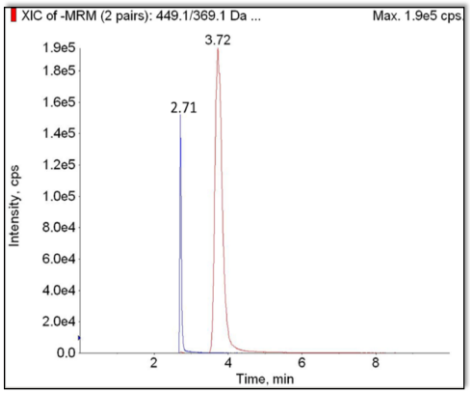


Figure 6.12. LC/ESI(-)-MS/MS chromatogram of standard mixture of textile dyes (100 ng dye mL^{-1}): Nylosan Red, t_R 2.71 min; Optilan Blue, t_R 3.72 min.

SPE method

The SPE study on Strata reversed phase (Strata C18-E and Strata C18-U) and on Strata ion exchange (Strata WAX/NH_2 and Strata SAX) showed that polar compounds can be retained by different mechanisms (reversed phase and anion exchange respectively). The efficiency of the tested cartridges was estimated by comparing the recovery levels of the eluted dyes on each type of cartridge. Each extraction was repeated four times and RSD was calculated for each cartridge. The recovery levels are given in Table 6.10 *(Copaciu et al., 2012b)*.

Table 6.10. Recoveries of studied textile dyes on the four tested SPE cartridges.

Cartridge	Added amount of each dye (ng)	Nylosan Red			Optilan Blue		
		Mean[a] found amount (ng)	Recovery Mean[b] ± SD[c] (%)	RSD[d] (%)	Mean found amount (ng)	Recovery Mean ± SD (%)	RSD (%)
Strata NH_2	160	166.9	104.3 ± 6.0	5.7	175.2	109.5 ± 3.3	3.0
Strata SAX	160	92.8	58.0 ± 6.9	11.8	128.8	80.5 ± 5.4	6.7
Strata C18-U	160	152.7	95.4 ± 9.4	9.9	139.7	87.3 ± 9.8	11.2
Strata C18-E	160	135.6	84.7 ± 6.0	7.0	162.6	101.6 ± 8.9	8.7

[a]Average of found amount for four extractions; [b]Average of recovery of four extractions; [c]Standard deviation; [d]Realtive standard deviation.

As it can be seen from Table 6.10 the tested SPE cartridges assure a good recovery (over 84%) for the both textile dyes, excepting the Strata SAX cartridge. The best results obtained on Strata NH_2 are due to the retention mechanism of polar analytes by either hydrogen bonding or week anion exchange. It is suitable for extraction of strong and/or weak anions from aqueous samples. The Strata SAX gives lower recovery levels for both dyes (58.0% for Nylosan Red N-2RBL and 80.5% for Optilan Blue MF-GL) that is a consequence of the strong interactions between the two textile dyes and the Strata SAX sorbent which is a strong anion exchange resin. Regarding the Strata C18 tested sorbents, the dye recoveries have good values due to the non-polar interactions between the functional groups of dyes and those of sorbents. On Strata C18-E has place a non-polar retention mechanism with the surface hydrophobic end-capping for additional hydrophobic retention and selectivity minimizing or eliminating polar silanol interactions. Desalting a matrix, as ions and polar molecules are not retained and washed off of the sorbent. On Strata C18-U has place a primarily non-polar retention mechanism combined with secondary polar silanol interactions, therefore increased extraction efficiency and enhanced cleanup of hydrophobic compounds that contain hydroxyl or amine moieties take place *(www.phenomenex.com)*.

Validation SPE-LC/ESI(-)-MS/MS procedure

The validation study demonstrated that the performed SPE-LC/ESI(-)-MS/MS procedure for the determination of the two textile dyes from the wastewater samples has a good linearity with a correlation coefficient over 0.99 on the whole range of 1-100 ng mL^{-1} concentration and the limit of detection to 0.28 ng mL^{-1}. To show the precision of the procedure, the RSD values for the intra-day and inter-day were calculated. RSD values for the intra-day precision are ranged between 0.8-2.6% for Nylosan Red N-2RBL and 0.7-2.4% for Optilan Blue MF-GL, and for the inter-day precision the results are ranged between 1.0-1.4% for Nylosan Red and 1.0-2.1% for Optilan Blue. The recovery of the two textile dyes in the spiked cases is good, obtaining values ranged between 92.5-97.7%.

The matrix effect was evaluated for each textile dye in wastewater sample. For the analysed wastewater sample, the ratio between the slope of the calibration curve in solvent and in matrix for each studied textile dye was between -20% and +20% (Nylosan Red N-2RBL -8.6% and Optilan Blue MF-GL -13.8%) *(Sur and Dunemann, 2004; Kmellár et al., 2008)* that indicates no matrix effect *(Copaciu et al., 2012b)*.

Analysis of studied dyes from wastewater samples

The determination of textile dyes in the wastewater has great importance for the monitoring of the water pollution caused by the textile industry. The described SPE-LC/ESI(-)-MS/MS procedure has been applied for the determination from wastewater of Nylosan Red and Optilan Blue, two textile dyes very often used in the last time for dyeing in the textile industry. Eight wastewater samples collected from a treatment plant with a textile factory in its area have been analysed. The results are given in Table 6.15.

The chromatograms corresponding to LC/ESI(-)-MS/MS analysis of the wastewater samples collected in 05.02.2011 from influent and effluent of the monitored treatment plant are presented in Figure 6.14. In these samples we found Nylosan Red, in influent 81.2 ng L^{-1} and in effluent 1.8 ng L^{-1}.The results of this study show the presence of the two dyes in the wastewater samples according to their occasional use in the textile industry. Also the results show a good purge of the wastewater by the treatment plant, the red textile dye being found in traces in effluent for a single wastewater sample *(Copaciu et al., 2012b)*.

Table 6.15. Amount of studied textile dyes in the influent and effluent samples collected from a wastewater treatment plant having a textile factory in its area.

Date of sampling	Wastewater sample	Amount (ng L^{-1})	
		Nylosan Red	Optilan Blue
05.02.2011	Influent	81.2	nd
	Effluent	1.8	nd
11.03.2011	Influent	264.8	nd
	Effluent	nd[a]	nd
16.04.2011	Influent	nd	nd
	Effluent	nd	nd
14.05.2011	Influent	24.3	84.0
	Effluent	nd	nd
18.06.2011	Influent	36.8	116.0
	Effluent	nd	2.7
12.07.2011	Influent	nd	nd
	Effluent	nd	nd
26.07.2011	Influent	nd	nd
	Effluent	nd	nd

[a]Not detected

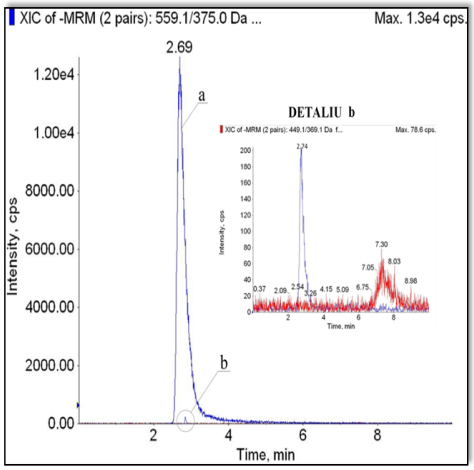


Figure 6.14. LC/ESI(-)-MS/MS chromatograms of influent (a) and effluent (b) wastewater samples from a treatment plant. Nylosan Red found dye: (a) 81.2 ng L^{-1} and (b) 1.8 ng L^{-1}.

6.3. Analysis of textile dyes chromium complexes Nylosan Dark Brown, Lanasyn Dark Brown and Lanasyn Lanasyn Red from wastewater by LC-MS/MS

6.3.1. Experimental part. Determination of textile dyes by LC-MS/MS

Chemicals and materials

The textile dyes chromium complexes Nylosan Dark Brown, Lanasyn Dark Brown and Lanasyn Red used for the preparation of the standard solutions, were purchased from Clariant Produkte AG. For the sample preparation and the chromatographic elution, methanol LC-MS Optigrade (≥99.8%) from LGC Standards, acetonitrile LC-MS from Merck, ammonium formate (97%) and formic acid (≥95%) from Sigma-Aldrich were used.

LC/ESI(-)-MS/MS method

The determination of textile dyes was carried out using a HPLC Agilent 1200 Series coupled with an API 3200 QTRAP mass spectrometer (Applied Biosystems) using a TurboV ionization source in an ESI negative ion mode (LC/ESI(-)-MS/MS). A Phenomenex Luna column 3u C18 (2) 100A (50 × 2 mm) setted at 40°C was used for the HPLC separation of textile dyes. A good separation of dyes was obtained by isocratic elution with a mixture of methanol:acetonitrile:water (ammonium formate 2 mM and formic acid 0.2%) 47:23:30 (*v/v*) at a flow rate of 0.15 mL min^{-1}.

SPE method

For the SPE extraction of the Nylosan Dark Brown, Lanasyn Dark Brown and Lanasyn Red, a device model Supelco purchased from Sigma Aldrich was used and four types of Strata cartridges (C18-U, C18-E, WAX/NH_2 and SAX) were tested. To quantify the selectivity of the tested SPE cartridges, 500 mL deionized water sample spiked with 120 ng LR, 200 ng NDB and 200 ng LDB were passed through each SPE cartridge.

Validation of SPE-LC/ESI(-)-MS/MS procedure

In order to validate the SPE/LC-ESI(-)-MS/MS procedure for the three mentioned textile dyes, the following parameters were studied: linearity, limits of detection and quantification for each dye, accuracy, precision and matrix effect.

6.3.2. Results and discussions regarding the determination of textile dyes by LC-MS/MS

LC/ESI(-)-MS/MS analysis

In order to obtain quantitative data regarding the presence of the investigated textile dyes in wastewater samples a LC/ESI(-)-MS/MS method was developed. Both negative and positive ion modes were tested for the studied dyes using ESI mode. Finally, due to the higher sensitivity over the investigated dyes negative ion mode was chosen. For the establishment of the MRM conditions, the standard solutions for each dye were individually prepared (400 ng mL^{-1} in acetonitrile). To obtain the precursor ions, the quadrupole-1 scanning was performed for each dye followed by the quadrupole-3 scanning for the product ions. The optimal parameters dependent on the studied textile dyes were obtained by the infusion method. The standard solution of each dye was injected directly in MS spectrometer by continuous infusion at a flow rate of 5 μL min^{-1}. The source dependent parameters were obtained by Flow Injection Analysis (FIA) using a restrictive capillary and a flow rate of 200 μL min^{-1}. A standard solution containing 400 ng mL^{-1} from each dye prepared in mobile phase was injected in LC/ESI(-)-MS/MS system.

The chromatogram corresponding to LC/ESI(-)-MS/MS of the standard mixture of textile dyes is shown in Figure 6.18.

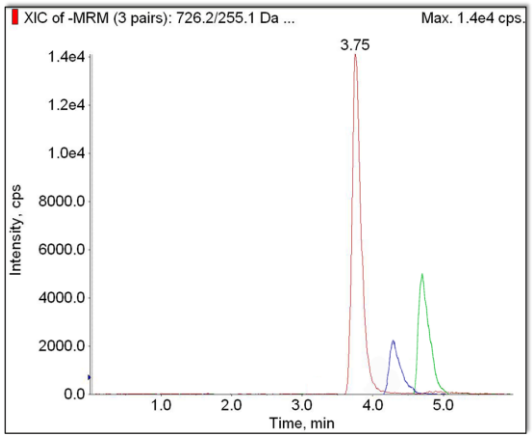


Figure 6.18. LC/ESI(-)-MS/MS chromatogram of standard mixture of textile dyes:
Lanasyn Red, t_R = 3.75 min (100 ng mL^{-1});
Lanasyn Dark Brown, t_R = 4.34 min (300 ng mL^{-1});
Nylosan Dark Brown, t_R = 4.79 min (50 ng mL^{-1}).

SPE method

The efficiency of the tested cartridges was estimated by comparing the recovery levels of the eluted dyes on each type of cartridge. Each extraction was repeated four times and *RSD* (%) was calculated for each tested cartridge. The recovery levels are given in Table 6.17. The Strata NH_2 and SAX cartridges don't have provided a good recovery for NDB and LDB textile dyes in comparison with the Strata C18 ones (the recovery around 100%) excepting Lanasyn Red (≈70%) where the results have been better. The best results obtained on Strata C18-E are due to the retention mechanism of polar analytes by either hydrogen bonding or week anion exchange. On Strata C18-U cartridge has been obtained for NDB and LDB dyes recovery degrees over 80%, having place a primarily non-polar retention mechanism combined with secondary polar silanol interactions that increased the extraction efficiency. Due to the retention mechanism of polar analytes by either hydrogen bonding or week anion exchange, on Strata NH2 cartridge the textile dyes were partially retained (45.5% NDB and 58.8% LDB). On Strata SAX cartridges, the NDB and LDB dyes could not be eluted being retained on the adsorbent due to the strong interactions between the two textile dyes and the adsorbent that is a strong anion exchange resin *(www.phenomenex.com).*

Tabel 6.17. Recoveries of studied textile dyes on the tested SPE cartridges.

Cartridge	Nylosan Dark Brown				Lanasyn Dark Brown				Lanasyn Red			
	Added amount (ng)	Mean[a] found amount (ng)	Recovery Mean[b] ± SD[c] (%)	RSD[d] (%)	Added amount (ng)	Mean found amount (ng)	Recovery Mean[b] ± SD (%)	RSD (%)	Added amount (ng)	Mean found amount (ng)	Recovery Mean[b] ± SD (%)	RSD (%)
Strata NH_2	200	91.1	45.5 ± 1.3	2.8	200	117.7	58.8 ± 3.3	5.6	120	89.2	74.3 ± 1.0	1.4
Strata SAX	200	nd[e]	nd	nd	200	nd	nd	nd	120	83.8	69.8 ± 1.5	2.1
Strata C18-U	200	166.8	83.4 ± 3.1	3.8	200	170.5	85.2 ± 2.0	2.4	120	80.6	67.1 ± 1.0	1.4
Strata C18-E	200	187.2	93.6 ± 4.3	4.6	200	204.8	102.2 ± 3.4	3.3	120	85.2	71.0 ± 1.2	1.6

[a]Average of found amount for four extractions; [b]Average of recovery of four extractions; [c]Standard deviation; [d] Realtive standard deviation; [e]Not detected.

Validation of the SPE-LC/ESI(-)-MS/MS procedure

The validation study demonstrated that the performed SPE-LC/ESI(-)-MS/MS procedure for the determination of the two textile dyes from the wastewater samples has a good linearity with a correlation coefficient over 0.99 on the whole concentration range of 1-100 ng mL^{-1} for the Lanasyn Red and of 50-300 ng mL^{-1} for the Nylosan Dark Brown and Lanasyn Dark Brown. To show the precision of the procedure, the RSD values for the intra-day and inter-day were calculated. RSD values for the intra-day / inter-day precision: 0.66-1.4% / 0.84-1.5% for Nylosan Dark Brown, 0.63-1.6% / 0.82-1.2% for Lanasyn Dark Brown and 1.0-2.3% / 1.1-1.2% pentru Lanasyn Red. The recovery of the two textile dyes (NDB and LR) in the spiked cases is good, obtaining values ranged between 93.1-99.7%.

The matrix effect was evaluated for each textile dye from wastewater sample through the ratio between the slope of the calibration curve in solvent and in matrix for each studied textile dye. This ratio was between -20% and +20% (Nylosan Dark Brown 8.9%, Lanasyn Dark Brown -7.3% and Lanasyn red -5.3%) that indicates no matrix effect according to *Sur and Dunemann, 2004; Kmellár et al., 2008*.

Analysis of studied dyes from wastewater samples

The described SPE-LC/ESI(-)-MS/MS procedure has been applied for the determination from wastewater of the Nylosan Dark Brown, Lanasyn Dark Brown and Lanasyn Red, three textile dyes very often used in the last time for dyeing in the textile industry. Twelve wastewater samples collected from a treatment plant with a textile factory in its area have been analysed. The results are given in Table 6.22.

Table 6.22. Amount of the studied textile dyes in influent and effluent samples collected from a wastewater treatment plant having a textile factory in its area.

Date of sampling	Wastewater sample	Amount (ng L^{-1})		
		Nylosan Dark Brown	Lanasyn Dark Brown	Lanasyn Red
19.02.2011	Influent	150.1	nd[a]	156.0
	Effluent	22.8	nd	74.0
26.03.2011	Influent	nd	nd	89.0
	Effluent	nd	nd	nd
30.04.2011	Influent	nd	200.6	nd
	Effluent	nd	nd	nd
27.05.2011	Influent	24.3	nd	183.0
	Effluent	nd	nd	89.6
04.06.2011	Influent	nd	nd	244.0
	Effluent	nd	nd	nd
26.07.2011	Influent	nd	nd	nd
	Effluent	nd	nd	nd

[a]Not detected

Using the presented procedure, in the influents of wastewater treatment plant were found concentrations between 24.3-244.0 ng L^{-1} of the three textile dyes of chromium complexes and in the corresponding effluents concentrations between 22.8-89.6 ng L^{-1}. The LC/ESI(-)-MS/MS chromatograms of the wastewater samples collected in 04.06.2011 from influent of the monitored treatment plant are presented in Figure 6.22.

The results of this study indicate the presence of the three dyes in the wastewater samples according to their occasional use in the textile industry. Also the results show a good purge of the wastewater treatment plant, the textile dyes being found in effluents in traces.

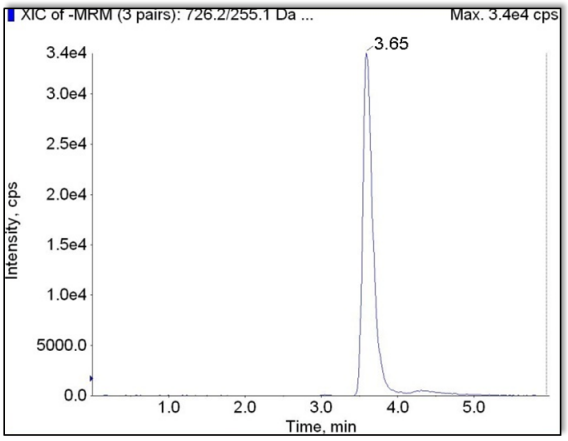


Figura 6.22. LC/ESI(-)-MS/MS chromatogram of influent wastewater sample from a treatment plant. Lanasyn Red found dye: 244.0 ng L^{-1}.

7. Ecotoxicological studies regarding the action of textile dyes action on *Triticum aestivum* L. plants

In the current study, we investigated the effects of two anthraquinone dyes (Optilan Blue (OB) and Lanasyn Blue (LB)) and four azo dyes (Lanasyn Red (LR), Nylosan Red (NR), Nylosan Dark Brown (NDB) and Lanasyn Dark Brown (LDB)) at two different concentrations (0.5 mg L^{-1} and 1.5 mg L^{-1}) on wheat (*Triticum aestivum* L.). The applied concentrations correspond to moderately high concentrations found in the environment. We hypothesized that textile dyes reduce plant photosynthesis and secondary metabolites contents in dose-dependent manner and lipoxygenase pathway volatiles products and monoterpenes constitute a sensitive indicator of the toxicity of these abiotic stressors.

7.1. *Triticum aestivum* L. and the stress application

Wheat (*Triticum aestivum* L.) seeds (cv. Lovrin, source: Fundulea, Romania) were used for the experiment and sown in plastic pots filled with commercial garden soil including slow release NPK fertilizer with microelements (Biolan, Finland). The sowing depth was 1 cm. The plants were grown in a growth chamber (floor area 0.29 m^2, height 0.42 m, Percival, IA) under a light intensity of 1000 µmol m^{-2} s^{-1} provided for a 12 h light period and day/night temperatures of 25°C/18°C. All textile dyes (two anthroquinone dyes and four azo dyes) were purchased from Clariant Produkte.

The experiment was started when the second emerged leaf had reached at least 50% emerges (Zadoks growth stage of 1.2 *(Zadoks et al., 1974)*), 14 days after sowing the seeds. The plants were periodically watered with aqueous solutions of OB, LB, LR, NR, NDB and LDB at concentrations of either 0.5 mg L^{-1} or 1.5 mg L^{-1}, while the control treatment received distilled water. The measurements were conducted after 7 days since the start of the treatment when the plants have been watered daily with a total of 0.5 L of given dye solution, and at 14 days since the start of the treatment when they were watered with 1 L of given dye solution.

7.2. Analysis of photosynthetic parameters of wheat plants treated with textile dyes

7.2.1. Photosynthesis measurements

The monitoring of plants photosynthetic parameters were performed using the GFS 3000 Portable Gas Exchange System (Walz, Effeltrich, Germany). The system has an environmental-controlled cuvette with a 8 cm^2 window area and is equipped with full window leaf chamber fluorimeter for sample illumination and chlorophyll fluorescence measurements. The measurements were performed at a chamber CO_2 concentration of 385 μmol mol^{-1}, photosynthetic quantum flux density was kept at 1000 μmol m^{-2} s^{-1}, leaf temperature at 25ºC and chamber relative humidity at 70%. The air flow rate was 750 μmol s^{-1}.

The leaf were enclose in the cuvette and it was left to stabilize until steady-state values of net assimilation rate (*A*) and stomatal conductance to water vapor (g_s) (stomata opened) were obtained. The rates of net assimilation (*A*) and stomatal conductance to water vapor (g_s) were calculated from these measurements according to von Caemmerer and Farquhar (1981).

7.2.2. Results and discussion regarding the effects of textile dyes on photosynthetic parameters of wheat plants

Results

For the treatments with 0.5 L solutions of given textile dye, no significant differences in net assimilation rate (*A*) were observed between the two applied concentrations (0.5 mg L^{-1} and 1.5 mg L^{-1}) and control (Figure 7.3a). In contrast, under severe stress, (1 L of dye solution at 1.5 mg L^{-1} concentration), the net assimilation rate significantly decreased from 19.6 ± 0.3 μmol m^{-2} s^{-1} in control plants to 17.1 ± 0.8 μmol m^{-2} s^{-1} for OB , 16.6 ± 0.2 μmol m^{-2} s^{-1} for LR and 15.8 ± 0.3 μmol m^{-2} s^{-1} for NR (Figure 7.3b). Net assimilation rate also decreased with increasing the volume of NR solutions in concentration of 0.5 mg L^{-1}.

Stomatal conductance to water vapor decreased only in the case of the NDB treatment, after the plants were watered with a volume of 0.5 L NDB solution, in concentration of 1.5 mg L^{-1} (Figure 7.3c). After watering the plants with 1 L of all investigated azo dye solutions, in concentration of 1.5 mg L^{-1}, the stomatal conductance to water vapor decreased on average (± SE) to a level of 68 ± 5 % (LR), 65.3 ± 2.2 % (NR), 65.7 ± 3.9 % (NDB) and 77.3 ± 2.5 % (LDB) compared to the control (Figure 7.3d).

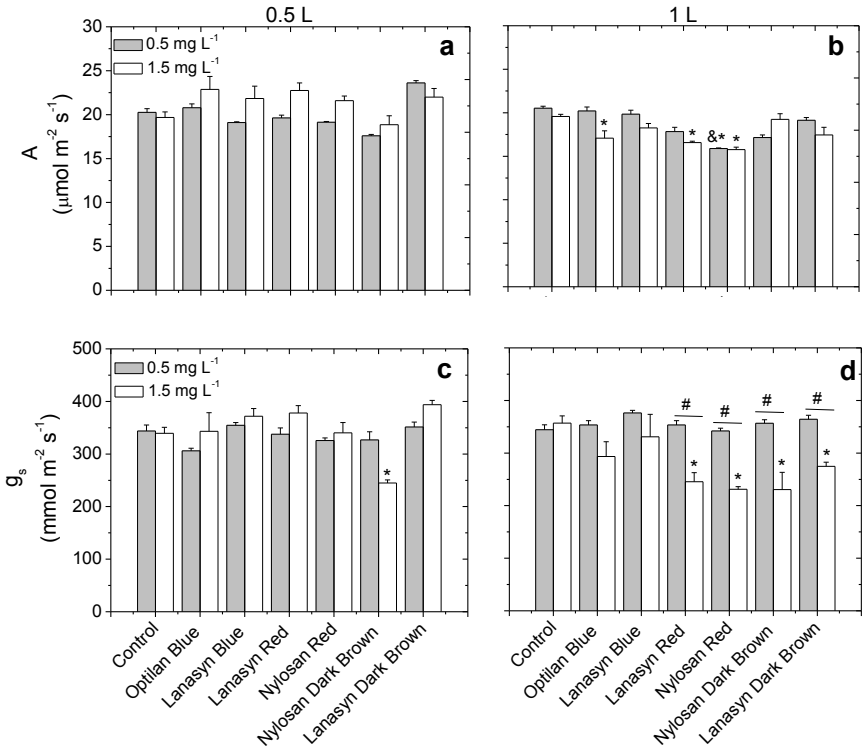


Figure 7.3. Changes in net assimilation rate (*A*) (a, b) and stomatal conductance to water vapor (g_s) per unit projected leaf area (c, d), in *Triticum aestivum* L. cv. "Lovrin" plants treated with six textile dye solutions (Table 1 for information of the dyes used) in concentrations of either 0.5 mg L^{-1} or 1.5 mg L^{-1}. The measurements were performed after the plants were watered with a total volume of textile dyes solutions of either 0.5 L (a and c) or 1 L (b and d). Each value is the mean (+ SE) of three independent replicate experiments with a different plant. The symbols above the columns represent: * = statistically significant difference ($P < 0.05$) between the given textile dye treatment and control; & = significant difference between the volume of textile dye solution used for plant treatment (0.5 L vs. 1 L) at the same concentration (either 0.5 mg L^{-1} or 1.5 mg L^{-1}), and # = significant difference between the two concentrations applied (0.5 mg L^{-1} vs. 1.5 mg L^{-1}) at the same volume of textile dyes solution used for plants watering (either 0.5 L or 1 L).

Discussions

Photosynthesis, the most fundamental biological process, is affected by a variety of biotic and abiotic stresses (*(Mittler, 2006)* for a review). In our study, we applied the textile dyes at realistic concentrations that can be occasionally observed in the environment. These concentrations resulted in significant effects of some textile dyes on foliage net assimilation rate (Figure 7.3a and 7.3b). In common with the azo dyes (LR, NR and LDB) that reduced photosynthesis is that all of them contain chromium in their structure. Chromium is known to affect plant photosynthesis but the mechanism is not well understood *(Shanker et al., 2005, 2009)*. Chromium can act non-specifically by inducing oxidative stress and thereby impairing photosynthesis *(Dey et al., 2009; Ali et al., 2011)*. A recent study has also hypothesized some interactions between chromium valence and the effect upon photosynthetic machinery *(Lopez-Luna et al., 2009)*. Inhibition of photosynthetic electron transport processes by chromium and a diversion of electrons from the electron-donating side of PS I to Cr(VI) can be responsible for decreased assimilation rates in plants treated with azo dyes which contained chromium *(Diwan et al., 2012)*.

However, it is important to consider that in all of these studies, the concentrations applied were far higher than in our work. For example *(Rodriguez et al., 2012)* demonstrated a decrease of assimilation rates at a value less than 0.4 μmol m^{-2} s^{-1} in *Pisum sativum* exposed to at 2000 mg L^{-1} chromium.

All azo dyes at the higher concentration of 1.5 mg L^{-1} significantly reduced stomatal conductance (Figure 7.3c and 7.3d). This is consistent with previous observations demonstrating reduced transpiration rates and stomatal conductance in leaves of *Brassica oleracea (Chatterjee and Chatterjee, 2000),* and in *Hordeum vulgare (Ali et al., 2011)* in response to treatments with that chromium salts. Such reductions in stomatal conductance by heavy metals have been associated with impaired aquaporin activity *(Eckert et al., 1999; Kholodova et al., 2011)*. In addition, reduction of stomatal conductance is a typical stress response triggered by increased concentrations of stress hormone abscisic acid *(Antoni et al., 2011)*.

7.3. Analysis of some volatile secondary metabolites (LOX and monoterpenes) emitted by wheat plants treated with textile dyes

7.3.1. Sampling and analysis of some LOX and monoterpenes

The sampling of volatile organic compounds (VOC) was realized using the same gas-exchange system as for the gas exchange measurements. VOC sampling was performed via the cuvette outlet with a flow rate of 200 mL min^{-1} for 15 min using a constant flow air sample pump (1003-SKC, SKC Inc., Houston, TX, USA). The sampling was started after steady-state values of gas-exchange characteristics were achieved. The exhaust air was sampled in a multibed stainless steel cartridge (10.5 × 3 cm, Supelco, Bellefonte, PA, USA) filled with Carbopack adsorbents (C 20/40 mesh, C 40/60 mesh, and X 20/40 mesh) optimized for the quantitative analyses of the lipoxygenase pathway products (LOX) and monoterpenes *(see Copolovici et al., 2009; Niinemets et al., 2010; Niinemets et al., 2011)*.

7.3.2. Results and discussion regarding the effects of the textile dyes on the emission of LOX and monoterpenes in wheat plants

Results

To gain further insight into the effects of dyes, emission rates of lipoxygenase pathway volatiles (LOX, also green leaf volatiles) and monoterpenes were analyzed. The LOX products found in leaf emissions were 1-hexanol, (*Z*)-3-hexenol, (*Z*)-2-hexenal, and (*Z*)-3-hexenyl acetate. The monoterpenes detected were α-pinene, β-pinene, camphene, limonene, 3-carene, *p*-cymene, and *β* -phellandrene. As no qualitatively different patterns were observed for single compounds of given compound class, we present the results for the emissions of the sum of all LOX products and for the sum of all monoterpenes.

Both total LOX (Φ_{LOX}) and monoterpene ($\Phi_{Monoterpene}$) emissions were low in control treatments (Figure 7.5).

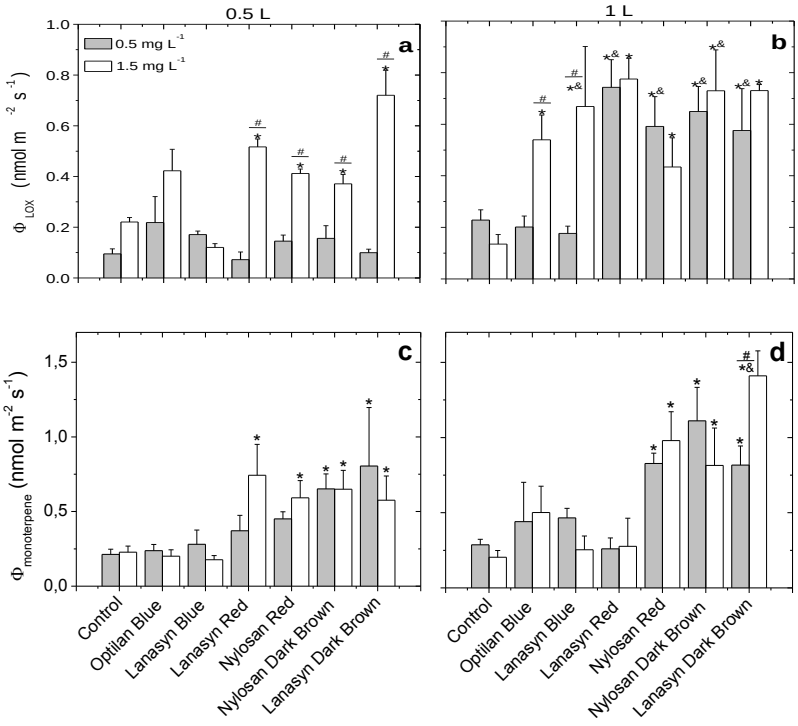


Figure 7.5. Modifications of the emission rates of lipoxygenase pathways products (LOX) (a and b) and monoterpenes (c and d) from *Triticum aestivum* L. leaves in response to treatments with six textile dye solutions. The measurements were performed after the plants had been watered with a total volume of textile dyes solutions of 0.5 L (a and c) and 1 L (b and d) in concentration of 0.5 mg L^{-1} and 1.5 mg L^{-1}. The statistical significance as in Figure 7.3.

After the plants were watered with a volume of 0.5 L of azo textile dye solutions, significantly increased LOX product (sum of all LOX products) emissions (on average by 122%) were observed only for the concentration of pollutants of 1.5 mg L^{-1} (Figure 7.5.a). At the same concentration and treatment, anthraquinone dyes not influence the emission of LOX (P>0.1).

The abiotic stress induced by all the investigated textile dyes (1.5 mg L^{-1}, 1 L solution) resulted in a drastic enhancement of total LOX emissions up to a level of 0.54 ± 0.10 nmol m^{-2} s^{-1} (OB), 0.67 ± 0.23 (LB), 0.77 ± 0.08 (LR), 0.43 ± 0.11 (NR), 0.72 ± 0.16 (NDB) and 0.73 ± 0.03 nmol m^{-2} s^{-1} (LDB). At the lower concentration of 0.5 mg L^{-1}, the treatment with 1 L an enhancement of emissions was only observed for azo textile dyes (P < 0.05). For LB (1.5 mg L^{-1}), LR (0.5 mg L^{-1}), NR (0.5 mg L^{-1}), NDB (0.5 mg L^{-1}, 1.5 mg L^{-1}) and LDB (0.5 mg L^{-1}) treatments with 1 L solution, the total LOX emissions increased significantly relative to the treatments with 0.5 L solution with the same concentration (Figure 7.5.b and a).

In treatments with 0.5 L at the higher concentration of 1.5 mg L^{-1}, total monoterpene emissions increased significantly in all treatments with azo dyes. In the case of the lower concentration of 0.5 mg L^{-1}, total monoterpene emissions increased only in the treatments with NDB and LDB (Figure 7.5c). A larger volume of azo dye solutions used to water the plants (1 L) resulted in increased monoterpene emissions for NR, NDB and LDB treatments, at both concentrations used (0.5 and 1.5 mg L^{-1}). The increase of total monoterpene emissions was more severe for LDB azo dye at 1.5 mg L^{-1} concentration in comparison with the lowest concentration used (0.5 mg L^{-1}) (Figure 7.5d).

Discussions

We examined the induction of emissions of lipoxygenase pathway products (LOX, green leaf volatiles) and monoterpenes in response to the textile dye solutions. LOX volatiles are formed from polyunsaturated fatty acids that are released from plant membranes during stress *(Feussner and Wasternack, 2002)*. LOX emissions are associated with stress signalling in plants, and enhanced LOX emissions have been reported in response to heat and cold stress *(Copolovici et al., 2012)*, drought *(Permyakova et al., 2012)*, ozone *(Beauchamp et al., 2005)*, and high light *(Loreto et al., 2006)* stress as well as after leaf herbivory or mechanical damage *(Holopainen, 2011)*. Our study demonstrates that LOX emissions were also importantly induced by textile dyes (Figure 7.5), indicating that treatment with these dyes constituted a stress for the plants. LOX emissions were already elevated at the lower concentration and volume of solution of textile dyes used in the treatments (0.5 mg L^{-1}, 0.5 L) (Figure 7.5a), and LOX emission rates were increasing with increasing the stress severity (Figure 7.5b).

Leaves of *Triticum aestivum* are not accumulating monoterpenes, and this species is not a constitutive monoterpene emitter under physiological conditions *(Kesselmeier and Staudt, 1999)*. This was confirmed by very low terpenoid emissions, close to the detection limit, in control plants of *T. aestivum* in our study. However, important monoterpene emission can be induced in most species by biotic or abiotic stresses (review in *(Loreto and Schnitzler, 2010; Niinemets, 2010)*. Compared with stress-induced LOX emissions, terpene emissions are usually observed after some delay that is needed for expression of specific terpene synthase genes.

In our study, treatments with azo dyes increased monoterpene emission rates, but there were no statistical differences between the two concentration of pollutant used (Figure 7.5c and 7.5d). This non-dose dependent response might indicate a threshold-type response (presence/non presence of the stress) as may be observed for some compounds under moderate stress treatments *(Niinemets, 2010)*. However, this evidence may also indicate enhanced substrate limitations for monoterpene synthesis under more severe stress. Monoterpenes are formed in plant plastids by 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway *(Lichtenthaler, 1999; Nagegowda, 2010)*. The same pathway is used for carotenoid formation and for synthesis of phytol tail of chlorophyll. Stress-driven induction of monoterpene emission indicates shift of MEP pathway towards monoterpene synthesis, but the stronger reduction of carotenoid contents under more severe stress (Figure 7.8) and constancy of monoterpene emissions might indicate overall greater substrate limitations for isoprenoid synthesis.

7.4. Analysis of some assimilating pigments (chlorophylls and carotenoids) of wheat plants treated with textile dyes

7.4.1. Extraction and analysis of pigments in wheat plants

Pigments extraction

The pigment extraction protocol follows *Niinemets et al. (1998)* with minor modifications. Leaf samples of 3 cm^2 were taken after gas-exchange measurements and immediately frozen in liquid nitrogen. The samples were ground in dim light in liquid nitrogen in the presence of magnesium carbonate (Sigma Aldrich, Steinheim, Germany), extracted on ice with 100% acetone HPLC grade (Sigma Aldrich, Steinheim, Germany), and centrifuged with a Hettich 320 R Universal centrifuge (Hettich GmbH,

Tuttlingen, Germany) at 0°C and 9500 g for 3 min and the supernatant was decanted. The pellet was further extracted with a small amount of acetone until the residue remained colorless, but the re-extraction was repeated at least twice. The extracts were pooled and brought to a final volume of 1 mL acetone and then filtered through a 0.45 µm PTFE membrane filter (VWR International, Radnor, PA, USA).

Pigment analyses by high pressure liquid chromatography (HPLC)

The determination of pigments (carotenoid and chlorophyll pigments) was carried out by an Agilent 1200 Series high pressure liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD) according to Niinemets et al. (1998) with modifications outlined below. A Zorbax Eclipse XDB-C18 reversed-phase column (4.6 mm i.d. × 150 mm column length, 5 µm particle size, Agilent Technologies, Santa Clara, CA, USA) was used and the column was thermostated at 10 ± 0.1°C. The solvents used for the chromatographic elution consisted of ultra-pure water with Hepes (0.1 M, pH = 8) (solvent A) and acetone HPLC grade (solvent B) (Sigma-Aldrich, Steinheim, Germany). The mixture of 25% solvent A and 75% solvent B was run isocratically for the first 7.5 min, followed by a 9.5 min linear gradient to 100% B, which was run isocratically for 3 min. The eluent composition was further changed to 25% A and 75% B by a 2 min linear gradient, and the column was equilibrated for 8 min before the next sample was injected. The pigments were eluted at a flow rate of 1.5 ml min^{-1}.

HPLC detection was performed simultaneously at 430 nm to measure chlorophyll *a*, 450 nm for neoxanthin and violaxanthin, and 455 nm for the rest of the pigments (chlorophyll *b*, β-carotene, zeaxanthin and lutein).

The HPLC was calibrated using purified or commercially available pigment standards. Chlorophyll *a*, chlorophyll *b*, and β-carotene, were obtained from Sigma Aldrich (Steinheim, Germany) and zeaxanthin and lutein from Fluka (Steinheim, Germany).

7.4.2. Results and discussions regarding the effects of the textile dyes on chlorophyll and carotenoid content in wheat plants

Results

Watering the plants with a volume of 0.5 L there are not any statistical differences in total chlorophyll content and in chlorophyll *a*/*b* ratio for both dye concentration (Figure 7.8a and 7.8c). In treatments with 1 L of dye solution, a significant reduction in total chlorophyll content was observed in treatments with 1.5 mg L^{-1} of azo dyes (Figure 7.8b). This treatment resulted in increased chlorophyll *a*/*b* ratio for all dyes (Figure 7.8d).

The carotenoid pigments that were analyzed in these experiments were zeaxanthin, lutein and β-carotene. The treatment with 0.5 L solution of textile dyes at concentration 0.5 mg L^{-1} did not affect carotenoid contents (Figure 7.9.a). However, after application of 1 L solution, this concentration resulted in reduced β -carotene contents only for the azo dyes. Treatments with the 0.5 L dye solutions at the higher concentration of 1.5 mg L^{-1} resulted in reduced lutein and β -carotene contents for all of the dyes. A treatment with 1 L solution at the higher concentration led to decreased contents of all carotenoids. The effect of the decrease of β-carotene was higher for the azo dyes (on average ± SE of 26.7 ± 1.4 mg m^{-2}) than for anthraquinone dyes (in average 34.1 ± 0.6 mg m^{-2}) (Figure 7.9b and 7.9d).

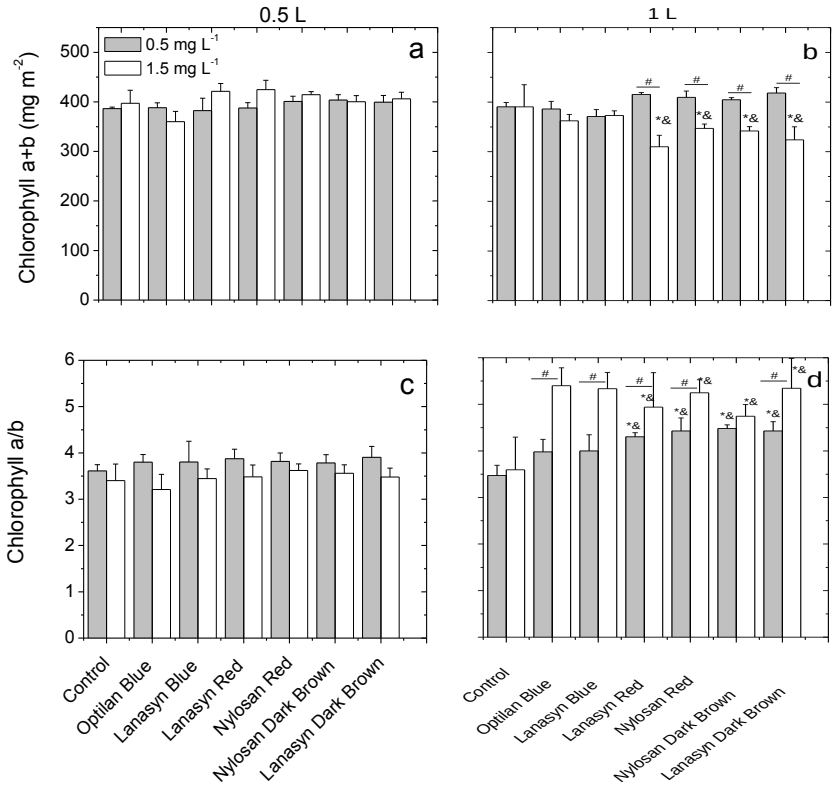


Figure 7.8. Changes in chlorophyll *a* and *b* contents in foliage of *Triticum aestivum* L.due to stress induced by textile dyes. The measurements were carried out after the plants had been watered with a total volume of textile dyes solutions of 0.5 L (a and c) and 1 L (b and d) in concentration of either 0.5 mg L^{-1} or 1.5 mg L^{-1}. Statistical significance among the treatments reported as in Figure7.3.

Discussions

Chlorophylls, the light-collecting photosynthetic pigments, sensitively respond to stress, and chlorophyll content is often measured to assess the cumulative impact of environmental stress *(Młodzińska, 2009)*. Several studies have demonstrated decreases in chlorophyll content in different species under the impact of heavy metals *(Zengin and Munzuroglu, 2005; Vernay et al., 2007; Subrahmanyam, 2008)*, but little is known of the impact of textile dyes on chlorophyll content. To our knowledge, reduction of chlorophyll content in response to textile wastewater has been only reported in *Cicer arietinum (Garg and Kaushik, 2006)*. In our study, chlorophyll content declined progressively with increasing concentrations and volume of the textile dyes studied (Figure 7.8a and 7.8b). The most important reductions were observed in treatments with chromium-containing azo dyes.

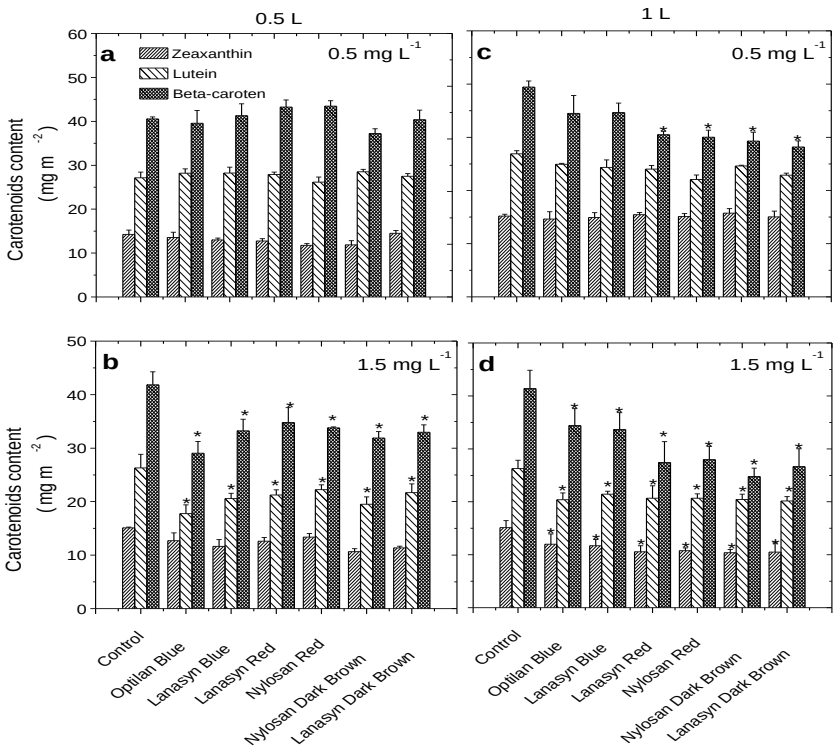


Figure 7.9. Changes in carotenoid contents in Triticum aestivum leaves in response to stress induced by textile dyes. The measurements were performed after the plants had been provided a total volume of textile dyes solutions of 0.5 L (a and b) and 1 L (c and d) in concentration of either 0.5 mg L^{-1} or 1.5 mg L^{-1}. Statistically significant difference shown as in Figure 7.3.

The direct effect of chromium on chlorophyll metabolism is associated with reduction of metal co-factors in enzymes responsible for chlorophyll synthesis or what followed by the indirect effect due to the depletion of Fe in chlorophylls *(Vernay et al., 2007)*. Heavy metals interfere with the synthesis of amino levulinic acid and protochlorophyllide reductase complex *(Garg and Kaushik, 2006)*. Furthermore, Fe-deficiency results in destruction of the lamellar system of thylakoids and changes in lipid and protein composition of membranes *(Garg and Kaushik, 2006)*. Chlorophyll *a*/*b* ratio significantly increased for all textile dyes (Figure 7.8d) especially in the case of azo dyes. As this ratio characterizes the pigment distribution between the reaction centers and light harvesting complexes, a high ratio of chlorophyll *a*/*b* in plant leaves treated with azo dyes suggest reduction of light-harvesting complexes of PSII relative to reaction centers.

Carotenoids in plants are integral constituents of pigment-binding protein complexes in chloroplasts, but also have important antioxidative functions as scavengers of reactive oxygen species (ROS), including free radicals and peroxy-radicals in plant membranes *(Havaux, 1998; Collins, 2001; Gill and Tuteja, 2010)*. However, under severe stress, carotenoid content becomes reduced due to destruction by ROS *(Munne-Bosch and Alegre, 2000)*. On the other hand, abiotic stress specifically increases zeaxanthin content that is formed from violaxanthin in xanthophyll cycle *(Demmig-Adams and Adams, 2006)*.

In our study, decreases in *β*-carotene and lutein content were observed for plants treated with different textile dyes (Figure 7.9), and a stronger effect was observed for the plants stressed with azo dyes, further suggesting that treatments with azo dyes resulted in a stronger stress. On the other hand, zeaxanthin content decreased only in treatments with the higher concentration of the stressor.

7.5. Analysis of non-volatile secondary metabolites (total flavonoid content) from wheat plants treated with textile dyes

7.5.1. Extraction and analysis of total flavonoid content from wheat plants

Extraction of total flavonoid content from wheat plants

The extraction procedure for the determination of total flavonoid content followed the method of *Soran et al. (2012) and Opriş et al. (2011)*. The plant material was powdered in liquid nitrogen and 5 mL 80:20 ethanol:water mixture (v/v) was added. For maceration, the extracts were kept for 10 min in an oven (Model UNE 200, Memmert) at 35ºC, and then were sonicated with Bandelin Sonorex for 30 min at the same temperature. Each extract was filtered through a 0.45 µm PTFE membrane filter and was brought to a final volume of 5 mL with the same solvent mixture.

Ultraviolet–visible spectrophotometry (UV-Vis) total flavonoids analysis

The determination of total flavonoid content in wheat, treated with the textile dye and at two concentrations was performed using UV-Vis technique. The method used was according to *Romanian Pharmacopoeia (1993)*, and the quantitative determination was expressed as rutin.

The method is based on the reaction of total flavonoids with aluminum chloride (Sigma Aldrich, Germany) resulting a yellow compound. Its maximum of absorbance was identified at 420 nm, being characteristic for flavonoids, expressed as rutin.

7.5.2. Results and discussions regarding the effects of the textile dyes on total flavonoids content in wheat plants

Results

The experimental results showed that treatment of wheat plants with textile dye solutions significantly influence the decrease of total content of flavonoids in these plants (Figure 7.12). For all textile dyes investigated at concentrations of 1.5 mg L^{-1} for both volumes applied (Figure 7.12a and 7.12b), the total content of flavonoids has decreased comparing to control. Regarding the LB, NDB and LDB, even at 0.5 mg L^{-1} concentration, a decrease in the total content of flavonoids was observed. Within these results the toxicity of azo dyes (NDB and LDB) which contain chromium in their structure it is clearly evidenced (Figure 7.12b).

Discussions

In relation of plants with the environment, the secondary metabolites of plants are of a great importance. From this class the flavonoids are phenolic compounds, which are widely present in plants and prokaryotes. The phenolic compounds act as indicators of stress, protect plants against various biotic and abiotic stresses *(Samanta et al., 2011; Rezanejad, 2012, Di Ferdinando et al., 2012).*

The results presented in these studies show a drastic decrease in the total content of flavonoids in wheat plants exposed to treatments with textile dyes in different volumes and concentrations.

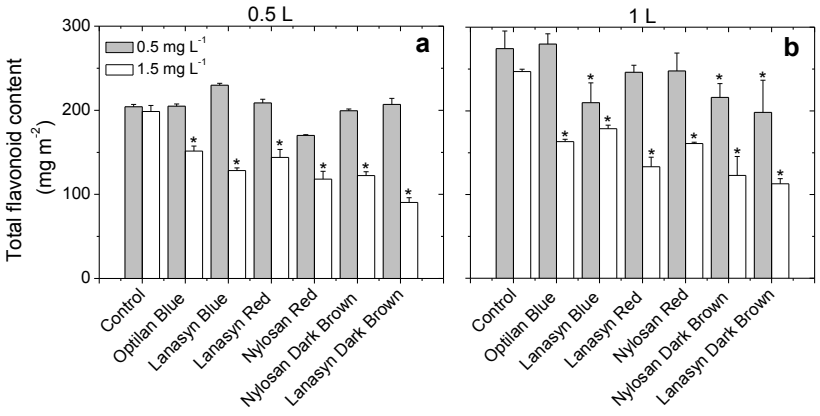


Figure 7.12. Changes in total flavonoid content from *Triticum aestivum* L. plants in response to stress induced by textile dyes. The measurements were performed after the plants were watered with a total volume of textile dyes solutions of 0.5 L (a) and 1 L (b) in concentration of 0.5 mg L^{-1} and 1.5 mg L^{-1}. Statistically significant difference shown as in Figure 7.3.

CONCLUSIONS

The purpose of this thesis was to elaborate some modern and performance procedures for the isolation, identification and quantification of six textile dyes, two anthraquinone (Optilan Blue and Lanasyn Blue) and four azo (Lanasyn Red, Nylosan Red, Nylosan Dark Brown and Lanasyn Dark Brown) and their application for the determination of these dyes in wastewater samples. The results of this research contribute to the evaluation of the impact of these textile dyes over the environmental quality and of the ecotoxicological effects over the wheat plants.

To perform the objectives of the present thesis, the following stages have been browsed:

1. The elaboration of a literature study referring to:
   - The textile dyes and the importance of their monitoring in different environmental factors;
   - The analysis techniques used to study the textile dyes from the wastewater samples;
   - The ecotoxicology of textile dyes.
2. The selection of the six textile dyes frequently used in present in the textile industry.
3. The registering of the UV-Vis spectra of the selected textile dyes in order to establish the wavelength of maximum absorbance used in the HPTLC analyses.
4. The determination of the regulated physicochemical parameters of wastewater from textile industry processing:
   - A seasonal variation for some physicochemical parameters given by the production system or the manufacture of textile fibers was observed.
   - These parameters had increased values during the cold season comparing to the warm season that can be explained through an intense production regime in the cold period and a reduced action of the sun.
   - According to the actual regulation NTPA 002/2005, the maximum limits admitted for temperature, total suspended solids, total phosphorus and detergent content were not overcome, but the maximum limits of pH, biochemical oxygen demand, chemical oxygen demand and ammonium were strongly affected.
   - For the total nitrogen content there are no maximum limits given by the accepted rules for the water discharge in the sewerage networks, but its monitoring is essential because the nitrogen has long-term negative effects.
   - The effluents from the textile industry process evacuated in the sewerage network represent a major water pollutant because they have also contribution to the increase of the oxygen consumption and of the nutrient loading of the water that leads in time at a destabilized aquatic ecosystem.
5. A fast and sensitive SPE-HPTLC procedure was developed and applied to the monitoring of five textile dyes (one anthraquinone dye, Lanasyn Blue, and four azo dyes - Nylosan Dark Brown, Nylosan Red, Lanasyn Dark Brown and Lanasyn Red) from the effluents of textile factories.
   - In order to isolate these textile dyes from wastewater by solid phase extraction, four types of Strata cartridges (WAX/NH_2, SAX, C18-U and C18-E) were tested. The best recovery degrees around 100% were obtained on Strata WAX/NH_2 cartridges excepting LR (84%). All obtained results on this cartridge showed that the elaborated SPE method is efficient and reproducible and permits the extraction of the five textile dyes from the liquid matrix up to mg L^{-1}.

- For the quantitative determination of the studied textile dyes by HPTLC, their densitograms at 550 nm were registered and used at the achievement of calibration curves.
- This procedure was validated with good results on selectivity, linearity, limits of detection, limits of quantification, accuracy and precision.
- These dyes used today in the textile industry were determined at ppm level in the monitored textile effluents by the elaborated SPE-HPTLC procedure that indicates the necessity of their removal from the effluents before the discharge in different emissaries or in the sewerage networks.

6. A SPE-LC/ESI(-)MS/MS procedure was developed for the trace analysis of the anthraquinone dye Optilan Blue and of the azo dye Nylosan Red from different wastewater samples (influent and corresponding effluent).
   - The optimum cartridge used for the isolation of these two textile dyes up to ng L^{-1} from wastewater samples was Strata NH_2, the elaborated SPE method being efficient and reproducible.
   - During the validation of the SPE-LC/ESI (-)-MS/MS procedure good results were obtained for accuracy, precision, linearity and detection limits (0,28 ng mL^{-1}Nylosan Red; 0,43 ng mL^{-1}Optilan Blue).
   - The presence of these dyes at the ng L^{-1} level in the wastewater samples collected from the influent of a wastewater treatment plant indicates the necessity of dye removal from the effluents of the textile factories. The absence of the colorants in the effluents shows the efficiency of the treatment station of wastewaters.
7. A SPE-LC/ESI(-)-MS/MS procedure was developed for the trace determination of three textile dyes chromium complexes (Nylosan Dark Brown, Lanasyn Dark Brown and Lanasyn Red) from different wastewater samples.
   - The optimum cartridge for the extraction of these three textile dyes chromium complexe was Strata C18-E. The elaborated SPE method from wastewater samples is efficient and reproducible.
   - The procedure SPE-LC/ESI (-)-MS/MS was validated with good results on accuracy, precision, linearity and limit of detection (12.0 ng mL^{-1}Nylosan Dark Brown; 15.5 ng mL^{-1}Lanasyn Dark Brown and 0.94 ng mL^{-1}Lanasyn Red).
   - The presence of these dyes at ng L^{-1} level in wastewater samples collected from the influent of a wastewater treatment plant indicates the necessity of a more efficient removal of these dyes from the effluents of textile factories.
8. A study regarding the ecotoxicological effects of the investigated textile dyes over different performances of plants was realized, namely: foliage photosynthesis, assimilation pigments, secondary volatile metabolites (emissions of lipoxigenase pathway products and emissions of monoterpenes) and of some secondary non-volatile metabolites (flavonoids) over the wheat plants (*Triticum aestivum* L.)
   - The ecotoxicological effects of textile dyes over the assimilation rate of CO_2 were about 10-15%, being more pronounced for the dyes containing chromium in their structure.
   - Changes in the stomatal conductance of water vaporization were overall with a possible inhibition of the light enzimatic reactions, especially for the azo dyes.
   - The volatile organic compounds, especially LOX and monoterpenes emitted by the green leafs of wheat plants, were the most sensible at the pollution with textile dyes.

- The content of assimilation pigments, chlorophylls and carotenoids, has been decreased dramatically at the treatments with azo dyes, especially in the case of azo dyes chromium complexes.
- The reducing of the carotenoid content, especially zeaxanthin, suggests that the plant was under a chronic stress caused by the treatment with the investigated textile colorants in our case.
- The decreased content of carotenoids can be partially responsible for the net assimilation rate that is registered in our study.
- In the case of total flavonoid content, the obtained results demonstrate that the decrease of this content may be associates with the defensive role of the flavonoids under stress conditions.

SELECTIVE REFERENCES

1. Ali S., Zeng F., Qiu L., Zhang G., **2011**. The effect of chromium and aluminum on growth, root morphology, photosynthetic parameters and transpiration of the two barley cultivars. *Biologia Plantarum* 55, 291-296.
2. Antoni R., Rodriguez L., Gonzalez-Guzman M., Pizzio G.A., Rodriguez P.L., **2011**. News on ABA transport, protein degradation, and ABFs/WRKYs in ABA signalling. *Current Opinion in Plant Biology* 14, 547-553.
3. Beauchamp J., Wisthaler A., Hansel A., Kleist E., Miebach M., Niinemets Ü., Schurr U., Wildt J., **2005**. Ozone induced emissions of biogenic VOC from tobacco: relationships between ozone uptake and emission of LOX products. *Plant, Cell and Environment* 28, 1334-1343.
4. Chatterjee J., Chatterjee C., **2000**. Phytotoxicity of cobalt, chromium and copper in cauliflower. *Environmental Pollution* 109, 69-74.
5. Collins A.R., **2001**. Carotenoids and genomic stability. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 475, 21-28.
6. **Copaciu F.**, Coman V., Vlassa M., Opriş O., **2012a**. Determination of some textile dyes in wastewater by solid phase extraction followed by high performance thin layer chromatography. *Journal of Planar Chromatography-Modern TLC*, accepted No. 1550.
7. **Copaciu F.**, Coman V., Simedru D., Beldean-Galea S., Opriş O., Ristoiu D., **2012b**. Determination of two textile dyes in wastewater by solid phase extraction and liquid chromatography/electrospray ionization tandem mass spectrometry analysis, *Journal of Liquid Chromatography & Related Technologies*, ID: 695312 DOI:10.1080/10826076.2012.695312.
8. Copolovici L., Kännaste A., Niinemets Ü., **2009**. Gas chromatography-mass spectrometry method for determination of monoterpene and sesquiterpene emissions from stressed plants. *Studia Universitatis Babeş-Bolyai Seria Chemia* 54, 329-339.
9. Copolovici L., Kännaste A., Pazouki L., Niinemets Ü., **2012**. Emissions of green leaf volatiles and terpenoids from Solanum lycopersicum are quantitatively related to the severity of cold and heat shock treatments. *Journal of Plant Physiology* 169, 664-672.
10. Demmig-Adams B., Adams W.W.III. **2006**. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation: Tansley review. *The New Phytologist* 172, 11-21.
11. Dey S.K., Jena P.P., Kundu S., **2009**. Antioxidative efficiency of Triticum aestivum L. exposed to chromium stress. *Journal of Environmental Biology* 30, 539-544.
12. Di Ferdinando M., Brunetti C., Fini A., Tattini M., **2012**. Flavonoids as antioxidants in plants under abiotic stresses, in: Ahmad P., Prasad M.N.V. (Eds.), Abiotic Stress Responses in Plants: Metabolism, Productivity and Sustainability. Springer, New York, p. 159–179.
13. Diwan H., Ahmad A., Lqbal M., **2012**. Chromium-induced alterations in photosynthesis and associated attributes in Indian mustard. *Journal of Environmental Biology* 33, 239-244.
14. Eckert M., Biela A., Siefritz F., Kaldenhoff R., **1999**. New aspects of plant aquaporin regulation and specificity. *Journal of Experimental Botany* 50, 1541-1545.
15. Epolito W.J., Lee Y.H., Bottomley L.A., Pavlostathis S.G., **2005**. Characterization of the textile anthraquinone dye Reactive Blue 4. *Dyes and Pigments* 67, 35-46.

16. Eremektar G., Selcuk H., Meric S., **2007**. Investigation of the relation between COD fractions and the toxicity in a textile finishing industry wastewater: Effect of preozonation. *Desalination* 211, 314-320.
17. Feussner I., Wasternack C., **2002**. The lipoxygenase pathway. *Annual Review of Plant Biology* 53, 275-297.
18. Garg V.K., Kaushik P., **2006**. Influence of short-term irrigation of textile mill wastewater on the growth of chickpea cultivars. *Chemistry and Ecology* 22, 193-200.
19. Gill S.S., Tuteja N., **2010**. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48, 909-930.
20. Gumustas M., Ozkan S.A., **2011**. The role of and the place of method validation in drug analysis using electroanalytical techniques. *The Open Analytical Chemistry Journal* 5, 1-21.
21. Havaux M., **1998**. Carotenoids as membrane stabilizers in chloroplasts. *Trends in Plant Science 3*, 147-151.
22. Holopainen J.K., **2004**. Multiple functions of inducible plant volatiles. *Trends in Plant Science* 9, 529-533.
23. Kesselmeier J., Staudt M., **1999**. Biogenic volatile organic compounds (VOC): An overview on emission, physiology and ecology. *Journal of Atmospheric Chemistry* 33, 23-88.
24. Kholodova V., Volkov K., Abdeyeva A., Kuznetsov V., **2011**. Water status in Mesembryanthemum crystallinum under heavy metal stress. *Environmental and Experimental Botany* 71, 382-389.
25. Kmellar B., Fodor P., Pareja L., Ferrer C., Martinez-Uroz M.A., Valverde A., Fernandez-Alba A.R., **2008**. Validation and uncertainty study of a comprehensive list of 160 pesticide residues in multi-class vegetables by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 1215, 37-50.
26. Lee Y.H., Pavlostathis S.G., **2004**. Decolorization and toxicity of reactive anthraquinone textile dyes under methanogenic conditions. *Water Research* 38, 1838–1852.
27. Lichtenthaler H.K., **1999**. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 50, 47-65.
28. Lopez-Luna J., Gonzalez-Chavez M.C., Esparza-Garcia F.J., Rodriguez-Vazquez R., **2009**. Toxicity assessment of soil amended with tannery sludge, trivalent chromium and hexavalent chromium, using wheat, oat and sorghum plants. *Journal of Hazardous Materials* 163, 829-834.
29. Loreto F., Barta C., Brilli F., Nogues I., **2006**. On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. *Plant Cell and Environment* 29, 1820-1828.
30. Loreto F., Schnitzler J.-P., **2010**. Abiotic stresses and induced BVOCs. *Trends in Plant Science* 15, 154-166.
31. Mehta B.H., Morge S.B., **2008**. HPTLC-densitometric analysis of candesartan cilexetil and hydrochlorothiazide. *Journal of Planar Chromatography-Modern TLC* 21, 173-176.
32. Mittler R., 2006. Abiotic stress, the field environment and stress combination. *Trends in Plant Science* 11, 15-19.
33. Młodzińska E., **2009**. Survey of plant pigments: molecular and environmental determinants of plant colors. *Acta Biologica Cracoviensia Series Botanica* 51, 7-16.

34. Mohammad M.A-A., Zawilla N.H., **2009**. Thin layer and column-chromatographic methods for simultaneous analysis of ambroxol hydrocloride and doxycycline hyclate in a binary mixture. *Journal of Planar Chromatography-Modern TLC* 22, 201-206.
35. Munne-Bosch S., Alegre L., **2000**. Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in Rosmarinus officinalis plants. Rosmarinus officinalis plants. *Planta* 210, 925-931.
36. Nagegowda D.A., **2010**. Plant volatile terpenoid metabolism: Biosynthetic genes, transcriptional regulation and subcellular compartmentation. *FEBS Letters* 584, 2965-2973.
37. Nayak P.S., Upadhyaya S.D., Upadhyaya A., **2009**. HPTLC method for analysis of Winhaferin-A in Ashwagandha (Withania somnifera). *Journal of Planar Chromatography-Modern TLC* 22, 197-200.
38. Niinemets Ü., Bilger W., Kull O., Tenhunen J.D., **1998**. Acclimation to high irradiance in temperate deciduous trees in the field: changes in xanthophyll cycle pool size and in photosynthetic capacity along a canopy light gradient. *Plant, Cell and Environment* 21, 1205-1218.
39. Niinemets Ü., **2010**. Mild versus severe stress and BVOCs: thresholds, priming and consequences. *Trends in Plant Science* 15, 145-153.
40. Niinemets Ü., Copolovici L., Hüve K., **2010**. High within-canopy variation in isoprene emission potentials in temperate trees: Implications for predicting canopy-scale isoprene fluxes. *Journal of Geophysical Research* 115, G04029, doi:10.1029/2010JG001436.
41. Niinemets Ü., Kuhn U., Harley P.C., Staudt M., Arneth A., Cescatti A., Ciccioli P., Copolovici L., Geron C., Guenther A.B., Kesselmeier J., Lerdau M.T., Monson R.K., Peñuelas J., 2**011**. Estimations of isoprenoid emission capacity from enclosure studies: measurements, data processing, quality and standardized measurement protocols. *Biogeosciences* 8, 2209-2246.
42. Opriş O., **Copaciu F.**, Coman V., Ristoiu D., **2011**. UV-Vis study regarding the influence of two potential environmental pollutants on the total flavonoid content in *Triticum aestivum* L. and *Secale cereale* L. *Studia Universitatis Babeş-Bolyai Seria Chemia* LVI 4, 17-25.
43. Permyakova M.D., Permyakov A.V., Osipova S.V., Pshenichnikova T.A., **2012**. Lipoxygenase from the leaves of wheat grown under different water supply conditions. *Applied Biochemistry and Microbiology* 48, 77-82.
44. Poole C.F., Gunatilleka A.D., Sethuraman R., **2000**. Contributions of theory to method development in solid-phase extraction. *Journal of Chromatography A* 885, 17-39.
45. Prevot A.B., Fabbri D., Pramauro E., Baiocchi C., Medana C., **2008**. High-performance liquid chromatography coupled to ultraviolet diode array detection and electrospray ionization mass spectrometry for the analysis of intermediates produced in the initial steps of the photocatalytic degradation of sulfonated azo dyes. *Journal of Chromatography A* 1202, 145–154.
46. Rafaëlly L., Héron S., Nowik W., Tchapla A., **2008**. Optimisation of ESI-MS detection for the HPLC of anthraquinone dyes. *Dyes and Pigments* 77, 191-203.
47. Rezanejad F., **2012**. Air pollution effects on flavonoids in pollen grains of some ornamental plants. *Turkish Journal of Botany* 36, 49-54
48. Rodriguez E., Santos C., Azevedo R., Moutinho-Pereira J., Correia C., Dias M.C., **2012**. Chromium (VI) induces toxicity at different photosynthetic levels in pea. *Plant Physiology and Biochemistry* 53, 94-100.

49. Romanian Pharmacopoeia. **1993**. Medical Publishing, X-th Edition, Bucharest.
50. Samanta A., Das G., Das S.K., **2011**. Roles of flavonoids in plants. *International Journal of Pharmaceutical Sciences and Technology* 6, 12-35.
51. Shanker A.K., Cervantes C., H. Loza-Tavera H., Avudainayagam S., **2005**. Chromium toxicity in plants. *Environment International* 31, 739-753.
52. Shanker A.K., Djanaguiraman M., Venkateswarlu B., **2009**. Chromium interactions in plants: current status and future strategies. *Metallomics* 1, 375-383.
53. Soran M.L., Opriş. O., **Copaciu F.**, Varodi C., **2012**. Determination of flavonoids in Triticum aestivum L. treated with ampicillin, in: Lazar, M.D. (Ed.), Processes in Isotopes and Molecules (PIM 2011). American Institute of Physics, AIP Conf. Proc., Melville, New York, 1425, p. 47-49.
54. Subrahmanyam D., **2008**. Effects of chromium toxicity on leaf photosynthetic characteristics and oxidative changes in wheat (*Triticum aestivum* L.). *Photosynthetica* 46, 339-345.
55. Sur R., Dunemann L., **2004**. Method for the determination of five toxicologically relevant arsenic species in human urine by liquid chromatography–hydride generation atomic absorption spectrometry. *Journal of Chromatography B* 807, 169-176.
56. Şahin S., Demir C., Güçer Ş., **2007**. Simultaneous UV-Vis spectrophotometric determination of disperse dyes in textile wastewater by partial least squares and principal component regression. *Dyes and Pigments* 73, 368-376.
57. Umbuzeiro G.A., Freeman H.S. Warren S.H., 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* 60, 55-64.
58. Vernay P., Gauthier-Moussard C., Hitmi A., **2007**. Interaction of bioaccumulation of heavy metal chromium with water relation, mineral nutrition and photosynthesis in developed leaves of *Lolium perenne* L. *Chemosphere* 68, 1563-1575.
59. von Caemmerer S., Farquhar G.D., **1981**. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153, 376-387.
60. www.phenomenex.com
61. www.textile.clariant.com
62. Yoshioka N., Ichihashi K., **2008**. Determination of 40 synthetic food colors in drinks and candies by high-performance liquid chromatography using a short column with photodiode array detection. *Talanta* 74, 1408-1413.
63. Zadoks J.C., Chang T.T., Konzak D.F., **1974**. A decimal code for the growth stages of cereals. *Weed Research* 14, 415-421.
64. Zengin F.K., Munzuroglu O., **2005**. Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. *Acta Biologica Cracoviensia Series Botanica* 47, 157-164.

ANNEX I

ABREVIATION LIST

LR	**L**anasyn **R**ed
NR	**N**ylosan **R**ed
NDB	**N**ylosan **D**ark **B**rown
LDB	**L**anasyn **D**ark **B**rown
OB	**O**ptilan **B**lue
LB	**L**anasyn **B**lue
UV-Vis	**U**ltra**V**iolet-**V**isibil
IR	**I**nfra**R**ed
LLE	**L**iquid **L**iquid **E**xtraction
SPE	**S**olid **P**hase **E**xtraction
TLC	**T**hin **L**ayer **C**hromatography
HPTLC	**H**igh **P**erformance **T**hin **L**ayer **C**hromatography
SPE-HPTLC	**S**olid **P**hase **E**xtraction and **H**igh **P**erformance **T**hin **L**ayer **C**hromatography
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
DAD	**D**iode **A**rray **D**etection
ESI	**E**lectro**S**pray **I**onization
MS	**M**ass **S**pectrometry
SPE-LC/ESI(-)-MS/MS	**S**olid **P**hase **E**xtraction and **L**iquid **C**hromatography **E**lectro**S**pray **I**onization tandem **M**ass **S**pectrometry
CCO	Chemical Oxigen Demand
CBO	Biochemical Oxigen Demand
TSS	**T**otal **S**uspended **S**olids

TN	**T**otal **N**itrogen
TP	**T**otal **P**hosphorus
Strata NH_2	Cartridges filled with silica gel chemically modified amino
Strata SAX	Cartridges filled with silica gel and quaternary amino ligand
Strata C18-U	Cartridges filled with uncapping *n*-octadecyl silica gel (with free silanol groups)
Strata C18-E	Cartridges filled with endcapping *n*-octadecyl silica gel (silanol groups hydrofobically bonded)
SD	**S**tandard **D**eviation
RSD	**R**elative **S**tandard **D**eviation
LOD	**L**imit **O**f **D**etection
LOQ	**L**imit **O**f **Q**uantification
MRM	**M**ultiple **R**eaction **M**onitoring
FIA	**F**low **I**njection **A**nalysis
g_s	Stomatal Conductance to Water Vapour
A	Net Asimilation Rate
VOC	**V**olatile **O**rganic **C**ompounds
LOX	Lipoxygenase Pathway Products

ANNEX II

LIST OF ISI PAPERS ELABORATED UP TO PRESENT AND INCLUDED IN THE TOPIC OF THIS PhD THESIS

1. **Florina Copaciu**, Virginia Coman*****, Dorina Simedru, Simion Beldean-Galea, Ocsana Opriş, Dumitru Ristoiu, Determination of two textile dyes in wastewater by solid phase extraction and liquid chromatography/electrospray ionization tandem mass spectrometry analysis, *Journal of Liquid Chromatography & Related Technologies*, ID: 695312 DOI:10.1080/10826076.2012.695312, in press. *(Impact Factor 0.706 / 2011)*
2. **Florina Copaciu**, Virginia Coman*****, Mihaela Vlassa, Ocsana Opriş, Determination of some textile dyes in wastewater by solid phase extraction followed by high performance thin layer chromatography, *Journal of Planar Chromatography-Modern TLC*, DOI: 10.1556/JPC.25.2012.6.0, in press. (*Impact factor 0.767 / 2011*)
3. Ocsana Opriş, **Florina Copaciu***, Virginia Coman, Dumitru Ristoiu, UV-VIS Study regarding the influence of two potential environmental pollutants on the total flavonoid content in *Triticum aestivum* L. and *Secale cereale* L., *Studia Universitatis Babeş-Bolyai Seria Chemia*, Volum 56 (4) 2011, 17-25. *(Impact factor 0.129 / 2011)*
4. Loredana Soran, Ocsana Opriş, **Florina Copaciu**, Codruța Varodi, **2012**. Determination of flavonoids in *Triticum aestivum* L. treated with ampicillin, in: Lazar, M.D. (Ed.), Proceedings of Conference Processes in Isotopes and Molecules (PIM 2011). American Institute of Physics, AIP Conf. Proc., Melville, New York, 1425, p. 47-49.
5. **Florina Copaciu**, Ocsana Opriş, Virginia Coman, Dumitru Ristoiu, Ülo Niinemets, Lucian Copolovici*, Diffuse pollution by anthraquinone and azo dyes in environment importantly alters foliage volatiles, carotenoids and physiology in wheat (*Triticum aestivum*), *Water, Air, & Soil Pollution*, under revision. (*Impact Factor 1.625 / 2011*)
6. **Florina Copaciu,** Carmen Andreea Roba*, Ocsana Opriş, Vioara Mireşan, Physicochemical analysis of effluents generated by local industries from Cluj county - Romania and their possible impact on the surface water quality, *Water and Environment Journal*, under revision. (*Impact Factor 0,792 / 2011*)