DEVELOPMENT OF A NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD WITH DIODE ARRAY DETECTOR (HPLC-DAD) FOR THE DETECTION OF CONGO RED AND METHYL ORANGE DYES FROM SYNTHETIC WATER SOLUTIONS

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A sensitive method using liquid chromatography with diode array detector for the simultaneous determination of Congo Red (CR) and Methyl Orange (MO) synthetics dyes was developed and applied to synthetic water solutions. Chromatographic separation was achieved using an Acclaim Surfactant Plus chromatographic column (150 x 3.0 mm, 3 µm) in gradient elution using different proportions between aqueous and organic solvents (20/80, 50/50, 60/40, 65/35, 70/30, v/v). As the HPLC mobile phase 100 mM ammonium acetate in ultrapure water with a pH value of 5.0 (solvent A) and acetonitrile (solvent B) in gradient elution was used to separate the analytes. Injection volume was 10 µL and the chromatogram run time was only 12 minutes (MO retention time approx. 6.7 min. and CR retention time approx. 12.6 min). Detection of compounds of interest was carried out at the optimal wavelengths identified after the maximum absorption in UV-VIS spectra: λ= 506 nm for CR and λ= 428 nm for MO. The method developed (HPLC-DAD) was successfully applied for the determination of dyes from 25 synthetic water solutions samples.

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1. Introduction

In recent years, the use of synthetic chemical dyes in various industrial processes has increased considerably. Some areas where these chemicals are commonly used are paper and cellulose manufacturing, canvas painting, plastics, skin treatment, printing, etc. Because many of the dyes are toxic in nature, their removal from industrial effluents is a major environmental problem.

These pollutants have been found to be carcinogenic, toxic and harmful causing numerous health problems such as allergies, skin irritation, difficulty breathing, sweating, vomiting, confusion, headache and mutations [1]. Colorants are classified as anionic (acid and reagents dyes), cationic (basic dyes) and non-ionic (dispersion dyes). Of these, azo dyes make up more than 50% of all textile dyes used in the industry and are characterized by the presence of double azo-nitrogen bonds in the molecule. Several methods are used for the detection and analysis of azo dyes in food and water samples. These instruments include: HPLC with electrochemical detection [2], tandem mass spectrometry coupled with isotopic dilution [3], electrochemical detection based on multi-wall carbon nanotube [4] and capillary pressure electro-chromatography (CEC) with amperometric detection [5]. By separating the dyes from mixture solutions and the sample environment, a higher sensitivity should be achieved and reliable quantification should be allowed. This has been shown to have been successful with liquid chromatography in other cases where it was necessary to detect several dyes simultaneously. In addition to the separation techniques mentioned above, combined with different detectors, visible ultraviolet spectro-photometry (UV-VIS) is still widely used, mainly for wastewater analyses, or due to its simplicity and low costs. The main disadvantage of the spectrophotometry is its low sensitivity compared to chromatographic methods [6]. Depending on the complexity of the sample analysed, a different sample preparation protocol should be applied before instrumental detection of the target analytes. The purpose of the preparation of the sample is to extract the analyte from a matrix, the cleaning of the extract obtained and the preconcentrate of the analytes [7]. For the preparation of aquatic samples, hydrographic sediments or wastewaters for the determination of dyes, various techniques have been used: dispersive liquid-liquid micro-extraction (DLLME) [8], molecularly imprinted solid phase extraction (MISPE) [9], supramolecular liquid–liquid microextraction [10], or solid phase extraction (SPE) [11].

Thus, the goal of this study was to development of a sensitive and selective HPLC-DAD method for the quantification of Congo Red (CR) and Methyl Orange (MO) synthetics dyes from aqueous synthetic solutions.

2. Materials and Methods

2.1. Chemicals and working standard solution

Dyes used, Congo Red (purity > 97%) and Methyl Orange (purity > 95%), were purchased from Sigma-Aldrich (Germany), acetonitrile, methanol and ammonium acetate were purchased from Merck (Germany). CHROMAFIL XTRA PTFE type filters, with a pore size of 0.45 μm and a diameter of 25 mm used for samples filtration were produced at Macherey-Nagel. The basic standard solutions (CR and MO), with a concentration of 1000 mg/L, were prepared in methanol. Individual dilutions and mixed standard solutions of analytes were prepared in ultrapure water. All solutions have been refrigerated at 4°C and were protected against light in amber flask. Preparation of individual stock standard solutions was prepared in methanol, the concentrations being presented in Table 1.

Table 1

Concentrations of individual stock standard solutions

Analyte	Quantity (g)	Volume of the volumetric flask (mL)	Concentration of stock solution (mg/L)
Congo Red	0.0100	10	1000
Methyl Orange	0.0100	10	1000

2.2. Equipment and chromatographic method

The purpose of this work was to simultaneously determine the Congo Red and Methyl Orange dyes from synthetic water solutions samples, using a simple and rapid method using the HPLC-DAD method. Experiments to establish optimal conditions for chromatographic separation and detection were carried out using an Agilent 1200 HPLC system consisting of: solvent cabinet and membrane degasser; quaternary pump, capable of providing in the system a mobile phase with up to 4 components with variable flow; autosampler with a capacity of 100 positions and variable injection volume (0.1-100 μL); thermostat for constant temperature maintenance in the chromatographic column; the Acclaim Surfactant Plus chromatographic column with a length of 15 cm, an inner diameter of 3 mm and the diameter of 3 μm stationary phase particles; variable wavelength DAD detector and ability to record up to 8 different wavelengths simultaneously; Agilent ChemStation software for data acquisition, processing and reporting. In order to simultaneously determine the two compounds, it was attempted to establish the conditions of chromatographic separation using gradient elution. Different compositions of mobile phase were tested using different proportions of acetonitrile and ammonium acetate. The composition of mobile phase is 100 mM

ammonium acetate was ultrapure water with a pH value of 5.0 (solvent A) and acetonitrile (solvent B). Experiments were performed in gragient elution using different proportions between aqueous and organic solvents (20/80, 50/50, 60/40, 65/35, 70/30, v/v). Injection volume was 10 µL and the chromatogram run time was only 12 minutes. Detection of compounds was carried out at optimal wavelengths identified after the maximum absorption in UV-VIS spectra: 506 nm for CR and 428 nm for MO. Fig. 1 shows the absorption spectra and Fig. 2 shows the structural formulas for each dyes: CR and MO.

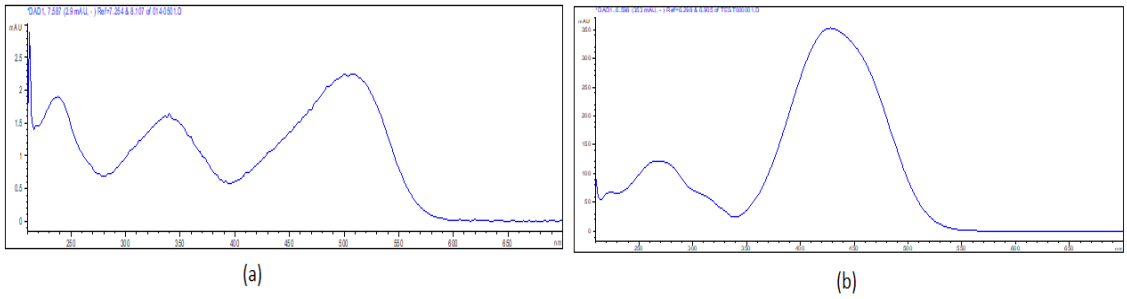

Fig. 1. UV-VIS absorption spectra of Congo Red (a) and Methyl Orange (b) obtained by HPLC-DAD (200-700 nm)

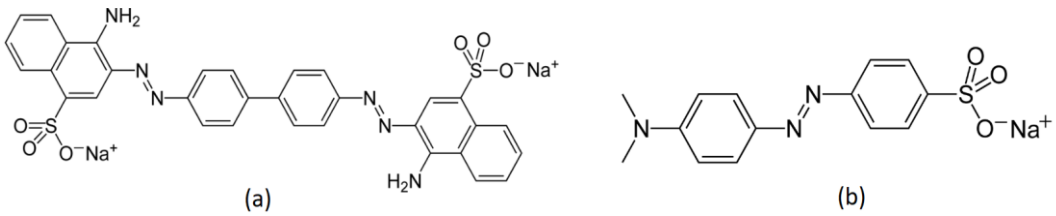

Fig. 2. Chemical structure of Congo Red (a) and Methyl Orange (b)

3. Results and discussion

3.1. Optimization of the detection method to increase the sensitivity of the determination of the two analytes

The column temperature range between 20 and 40°C was tested for better separation of the two analytes, and a range of injection volumes of 2 to 10 µL was tested for the sensitivity of the method. The temperature of 30°C and the injection volume of 10 µL were found to be the optimal values for the highest-resolution separation, the efficiency and the maximum sensitivity of the method. Different compositions of the mobile phase were tested for gradient elution using different proportions between aqueous and organic solvents (20/80, 50/50, 60/40, 65/35, 70/30, v/v). To achieve an optimal result and achieve narrow peaks with high

chromatographic efficiency, a mobile phase and flow gradient, were presented in Table 2.

Table 2

Mobile phase and flow gradient

Time (min)	Solvent A (%)	Solvent B (%)	Mobile phase flow (mL/min)
0	30	70	0.5
3	30	70	0.5
5	95	5	0.9
15	95	5	0.9

The optimized conditions of the liquid-chromatographic parameters allowed the separation of the two analytes into a chromatographic run-time of only 12 minutes. Taking into account the very low concentrations at which the two dyes are found in synthetic water solutions (at mg/L level), an HPLC method has been developed to allow their simultaneous detection at a concentration level located in 0.1-5 mg/L by direct injections, without the need for any stage of extraction and concentration of the analytes, applying only the filtration of the synthetic water solutions samples using 0.45 μm filters. To optimize these parameters, a mixture solution of CR and MO was used, with a concentration of 5 mg/L, 10 mg/L and 20 mg/L. UV detection: λ= 506 nm for Congo Red and λ= 428 nm for Methyl Orange, separation time: 12 minutes (Methyl Orange retention time approx. 6.6 min. and Congo Red retention time approx. 12.6 min.) (Fig. 3).

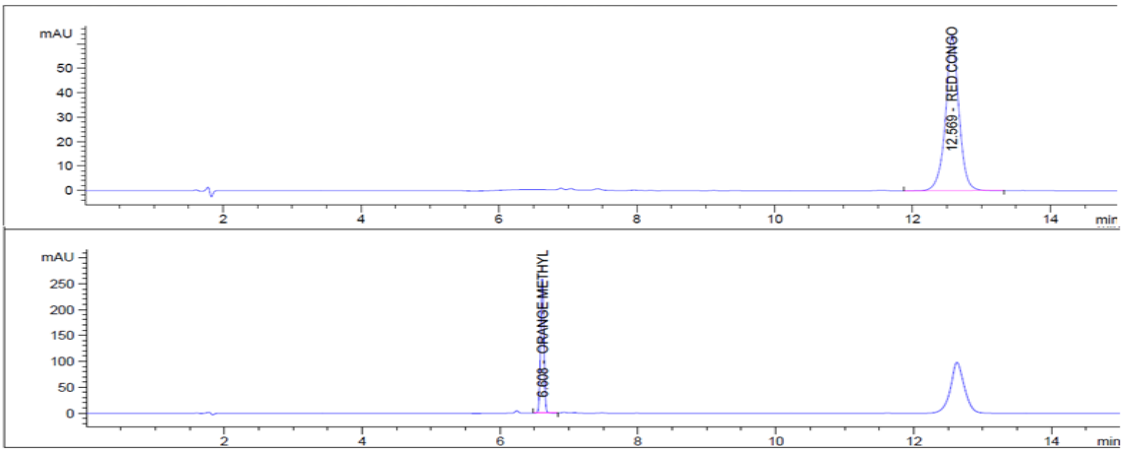

Fig. 3. Chromatograms obtained from the analysis of a mixed solution of CR and MO

3.2. Performance parameters of the analytical method developed in the laboratory

For the internal validation of the analytical method, the following performance parameters were taken into account: linearity, selectivity/specificity, limit of detection, limit of quantification, repeatability and reproducibility.

A. Linearity

The scope of work specific to each analyte of interest was established to confirm that the analytical procedure developed provides an acceptable degree of linearity, accuracy and precision when applying to samples containing the analyte of interest within or at the extremities of the specified domain. The linearity of a quantitative analytical method is its ability to obtain results commensurate with the concentration of the analyte in the samples. After estabilish the chromatographic separation and UV-VIS detection program, the six standard solutions prepared in ultrapure water with 0.1 ppm, 0.2 ppm, 0.5 ppm, 1.0 ppm, 2.5 ppm and 5.0 ppm concentrations were injected in duplicate. As shown in Fig. 4 (a and b) and Table 3, for all compounds the correlation coefficient were higher than 0.99.

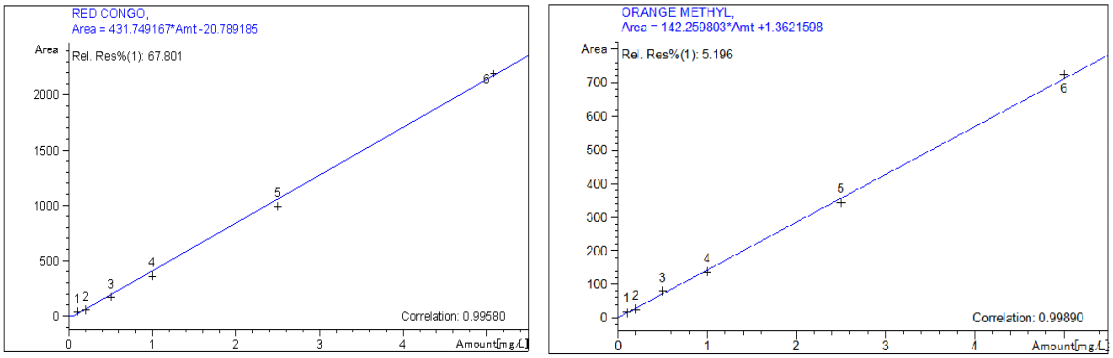

Fig. 4. Calibration solutions curve for Congo Red (a) and Methyl Orange (b)

Table 3

Linear regression parameters obtained for the CR and MO

Analyte	Concentration range	Regression equation	R^2
Congo Red	0.1-5 mg/L	y= 431.749x-20.789	0.9958
Methyl Orange	0.1-5 mg/L	y=142.260x+1.362	0.9989

B. Selectivity/Specificity

The selectivity (specificity) of the method was assessed by injecting a standard with the two dyes, a synthetic water solutions sample containing no analyses of interest and a sample of ultrapure water (blank). Since no interference peaks have occurred at the wavelengths of interest at the retention times corresponding to the two dyes, the method may be considered selective (Fig. 5).

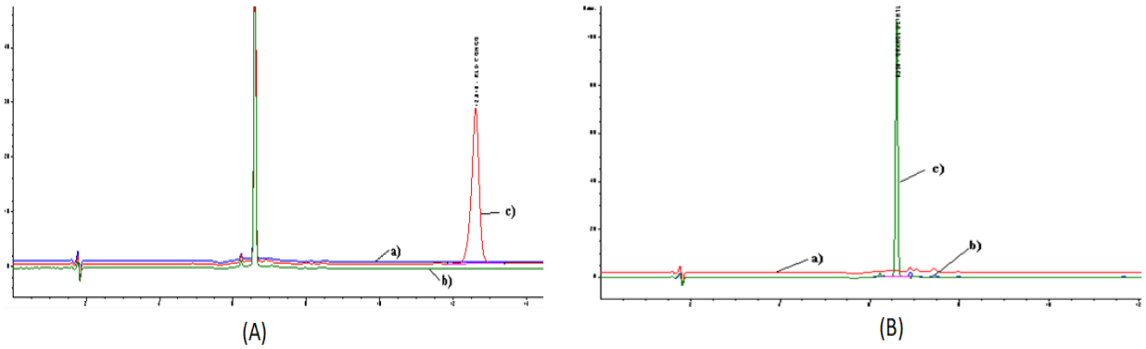

Fig. 5. Overlaid chromatograms corresponding to injection of: blank (a), synthetic water solution (b) and standard (c) at λ= 506 nm Congo Red (A) and at λ= 428 nm Methyl Orange (B)

C. Limit of detection and quantification

The detection (LOD) and instrumental quantification (LOQ) limits were determined by injecting solutions with lower analyt concentrations until the experimentally determined signal-to-noise ratio was equal to 3 (LOD) and 10 (LOQ) respectively. The values of the detection and quantification limits thus determined are given in Table 4.

Table 4

Limits of detection and quantification of analytes by the two methods

Analyte	LOD (mg/L)	LOQ (mg/L)
Congo Red	0.05	0.45
Methyl Orange	0.06	0.55

D. Repeatability and reproducibility

The method repeatability was evaluated by analyzing the 6 samples with addition of 1 mg/L standard solutions and reproducibility was measurement by analyzing the 12 samples with addition of 1 mg/L standard solutions, prepared on different days by several analysts. The precision of the method was expressed as relative percentage standard deviation (RSD %), as was presented in table 5. The precision of method varied for repeated measurements below 10%. The precision values showed that method was reproducibility and robustness.

Table 5

RSD% values obtained for method precision

Analyte	Concentration (mg/L)	Repeatability (RSD%) (n=6)	Reproducibility (RSD%) (n=12)
Congo Red	1.0	5.2	9.6
Methyl Orange	1.0	5.5	9.2

3.3. Application of the method developed for the analysis of Congo Red and Methyl Orange from samples of synthetic water solutions

For the analysis of CR and MO dyes by the HPLC-DAD method, samples of synthetic water solutions were tested. The samples were filtered through syringe filters CHROMAFIL XTRA PTFE filters with pore size of 0.45 μm and diameter of 25 mm. An injection volume of 10 μL was used. The values determined by this method are present in Table 6.

Table 6

Concentrations determined in samples of synthetic water solutions [12]

Samples	Conc. CR (mg/L)	Conc. MO (mg/L)
1	4.99±0.05	5.01±0.13
2	9.98±0.09	9.99±0.18
3	25.01±0.15	24.98±0.21
4	50.02±0.21	50.03±0.22
5	75±0.17	75.05±0.18
6	99.97±0.18	100.01±0.19
7	3.62±0.07	4.25±0.16
8	4.99±0.11	5.00±0.19
9	3.56±0.08	7.56±0.11
10	3.27±015	<LOQ
11	12.34±0.21	13.35±0.22
12	3.34±0.06	6.17±0.18
13	2.80±0.07	<LOQ
14	<LOQ	9.99±0.22
15	2.82±0.11	8.91±0.19
16	2.89±0.15	<LOQ
17	<LOQ	7.41±0.17
18	2.93±0.17	8.85±0.19
19	3.93±0.11	<LOQ
20	<LOQ	9.62±0.11
21	3.93±0.16	9.72±0.17
22	15.36±0.24	16.21±0.19
23	2.22±0.04	3.45±0.08
24	45.67±0.18	46.78±0.22
25	32.11±0.14	31.02±0.19

The applicability of the method was tested on 25 synthetic water solution samples [12]. The dyes concentration in synthetic water solutions has been performed in triplicate. The maximum experimental error is 5%.

These water samples have been used to determine the removal efficiency and adsorption capacity of different adsorbent materials [12].

4. Conclusions

In this study a newly developed HPLC-DAD method for the simultaneous detection Congo Red and Methyl Orange synthetic dyes was validated and applied to synthetic water solution samples having separation duration of only 12 minutes. All liquid-chromatographic conditions (nature and composition of the mobile phase, injection volume, detection wavelength, column temperature, etc.) have been optimized for the rapid separation of the two analytes with a high sensitivity in order to determine these synthetic dyes at concentration levels located of the order of parts per million (mg/L) of complex matrices of synthetic water solutions. The quantification limits of the method were 0.45 mg/L in the case of CR and 0.55 mg/L for MO. These extremely low values for an HPLC-type method with DAD detection are mainly due to chromophore-rich structures of the analytes that show intense absorption bands (high molar absorption coefficients in the visible field (wavelengths between 506 and 428 nm) which is generally protected from the interferences of most organic compounds present in water solutions. The method developed was successfully applied for the determination of dyes from 25 synthetic water solutions samples.

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