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DEVELOPMENT OF AN UPLC METHOD FOR SIMULTANEOUS DETERMINATION OF TARTRAZINE, CONGO RED AND METHYL ORANGE

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ABSTRACT. An UPLC method was developed to determine the concentrations of tartrazine (TAR), Congo red (CR), and methyl orange (MO) in aqueous mixtures. The chromatographic method was optimized considering the temperature of the column, the composition of mobile phases and the gradient program. The chosen method has allowed the separation and identification of TAR, CR, and MO from aqueous samples in 2 min. The calibration plots (R^2> 0.991) were linear over the ranges 0.5 – 50 µg/mL. Accuracy of the method was investigated, by applying recovery tests, with average recovery higher than 99%. The precision analysis included an intra-day variation, for which RSD (%) had values lower than 0.94, and an inter-day variation with RSD (%) values lower than 1.07. The developed method was successfully tested on water samples collected from a river nearby a textile industry plant.

Keywords: *azo dyes; Congo red; methyl orange; tartrazine; UPLC.*

INTRODUCTION

Industries such as textiles, paper, leather, rubber, cosmetics, plastics, automotive and other consumer goods discharge large amounts of colored wastewater containing various dyes [1], some of which influence food chains, aquatic ecosystems and are even mutagenic and carcinogenic to humans [2, 3]. Contamination of water with dyes is especially objectionable because of their acute toxicity. However, color in aqueous environments is also unacceptable due to the limitation of the reoxygenation capacity of the affected water [4, 5],

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reduction of sunlight penetration and not in the least because of natural aesthetic reasons. Therefore, the removal of color from process or waste effluents becomes environmentally important.

Tartrazine, Congo red, and methyl orange (Figure 1) are azo dyes used commercially in foods, textiles, paper and drugs in order to make them more attractive for consumers. Congo red, 3,3′-[(1,1′-diphenyl)-4,4′-diylbis(azo)] bis-(4-amino-1-naphthalene acid) disodium salt, is a potentially toxic dye, if orally ingested due to the fact that it can be metabolized to benzidine, a highly carcinogenic compound. Additionally, it can decrease the concentration of proteins in serum and cause thrombocytopenia, platelet aggregation, and disseminated microembolism [6, 7]. CR mainly occurs in the effluents discharged from textile, paper, printing, leather industries etc. During dyeing operations, up to 15% of CR can end up in wastewaters [8].

Tartrazine, trisodium 5-hydroxy-1-(4-sulfonatophenyl)-4-(4-sulfonato-phenylazo)-H-pyrazol-3-carboxylate, is one of the most frequently used food additives. It is being used abundantly in cosmetics, foodstuffs, medicines and textile materials [9]. Out of all the azo dyes, tartrazine appears to cause the most allergic and/or intolerance reactions, asthmatics and aspirin intolerant persons being particularly affected by tartrazine. Tartrazine sensitivity is mainly manifested by urticaria, but common symptoms can also include migraines, itching and blurred vision [10, 11].

Methyl orange, sodium [[(*p*-dimethylamino)phenyl]-azo] benzene sulphonate, is a water-soluble azo dye, which is widely used in textile, printing, paper manufacturing, pharmaceutical, food industries and in research laboratories as an acid base indicator. MO can inadvertently enter the body through oral ingestion and metabolize into aromatic amines, which can ultimately lead to intestinal cancer [12]. The toxic nature of the dye has not been yet properly quantified, but its presence in living organisms can prove to be harmful [13].

Ultra performance liquid chromatography (UPLC) is a chromatographic separation technique in which the use of high operating pressures (up to 1000 bar as opposed to the 400 bar maximum of HPLC) enables columns packed with particles having a diameter under 2 μm to be operated at high linear velocities. UPLC means that high peak capacities and high resolving powers can be generated along with short separation times [14]. The UPLC method is an accurate and rapid method used increasingly frequently [15, 16, 17] for the quantification of the amount of many compounds, including azo dyes [18, 19].

The objective of this work was to develop a simple, accurate, sensitive, economical, reproducible, and rapid UPLC method for the analysis of ternary mixtures of azo-dyes. The desired method should be suitable for routine quality control of wastewaters from the textile industry, and therefore it would be used successfully on river samples acquired from nearby a textile producing plant.

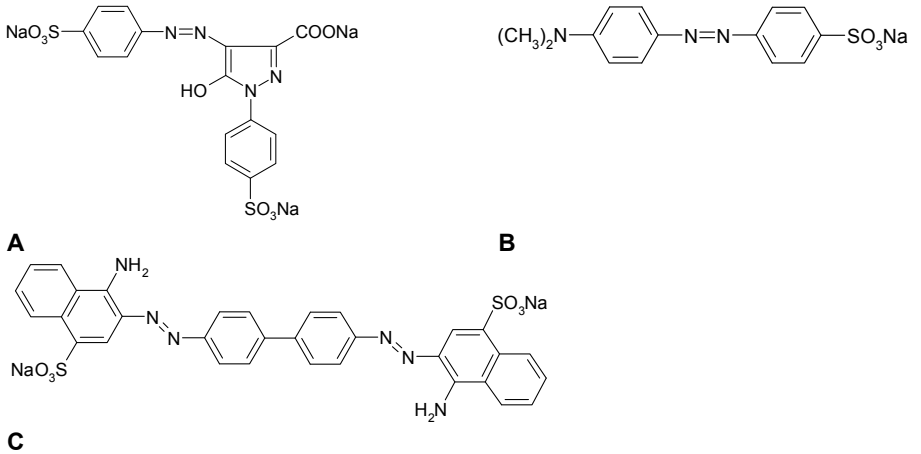

Figure 1. The chemical structures of industrial dyes:
(a) tartrazine; (b) methyl orange; (c) Congo red.

RESULTS AND DISCUSSION

Chromatographic Method Development

Different mobile phases containing various proportions of methanol and formic acid, as well as a few different buffer solutions (*p*H between 5 and 7) introduced in mobile phase B, were examined (data not shown). Finally, 10 % methanol in acetate buffer, *p*H = 5.6 was selected, as appropriate resolution was achieved. The optimum run time was 2 min. The MaxPlot chromatogram obtained from a mixed standard solution of TAR, CR, and MO is shown in Figure 2. At a flow rate of 0.3 mL/min, the retention time was 0.417 min for TAR, 0.903 min for CR, and 1.11 min for MO.

The left part of Figure 3 presents the spectra of the dyes recorded in the range 210 – 500 nm. In the right part, the extracted chromatograms at the wavelength, where the maximum absorption occurs, are presented for each dye. These chromatograms were used, in comparison with MaxPlot chromatograms for quantification of the analytes. As there were no significant differences between the two sets of results, quantification based on MaxPlots was used further. As each dye has two maximum absorptions in the recorded spectra range, the extracted chromatograms at these maximum absorption wavelengths were also used for confirmation that a peak belonged to a certain dye. The specificity of the method was tested for solutions containing only one dye at a time and the mixture of all three dyes, and no interferences were noticed when the detection was checked at two maximum absorption wavelengths. These results were also corroborated with the results obtained by *Peak Purity Check* and *Library Match* subroutines.

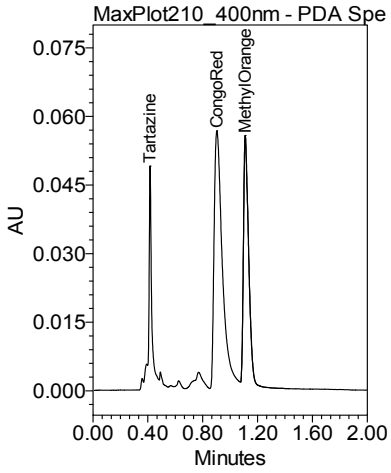

Figure 2. UPLC chromatogram of the separation of TAR, CR and MO using gradient elution program.

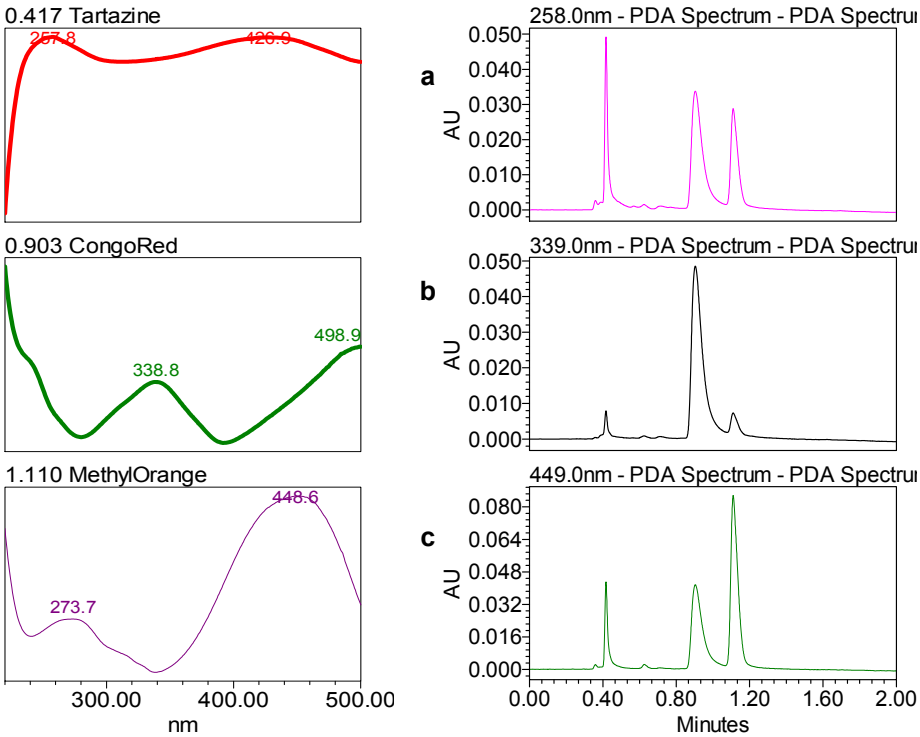

Figure 3. DAD-UV-Vis spectra and chromatograms corresponding to mixtures of the three dyes at different wavelengths (a) 258 nm, (b) 339 nm and (c) 449 nm.

The calculated parameters corresponding to the standard curves, as well as a few statistical parameters are shown in Table 1. The regression equation coefficients were higher than 0.991. At the MaxPlot, the calibration equations gave good linearity and successful results for TAR, CR, and MO.

Method Validation

Standard calibration plots were linear over the range 0.5 – 50 *μ*g/mL, with regression coefficients higher than 0.991, obtained for all three dyes. The LOD and LOQ were 0.06 and 0.3 *μ*g/mL for TAR, 0.12 and 0.9 *μ*g/mL for CR, and 0.05 and 0.3 *μ*g/mL for MO, respectively. The validation data are summarized in Table 1.

Table 1. Calculated standard curve parameters, LOD, and LOQ values

Compound	Standard curve equation	R^2	LOD (*μ*g/mL)	LOQ (*μ*g/mL)
Tartrazine	$2.49\times10^4x + 3.40\times10^4$	0.993	0.06	0.3
Congo red	$4.16\times10^4x + 9.96\times10^4$	0.991	0.12	0.9
Methyl orange	$1.10\times10^4x + 1.18\times10^4$	0.994	0.05	0.3

Note: R^2: regression coefficient; LOD: limit of detection; LOQ: limit of quantification

To study the accuracy and precision of the method, recovery was determined for three different mixtures containing known concentrations of dyes. Results from recovery studies, as well as linear regression analysis and other statistical results based on the relationship between added and measured concentrations are reported in Table 2.

Table 2. Recovery results obtained by applying the proposed method to the synthetic mixtures of dyes

Added (μg/mL)			Recovery (%)		
TAR	CR	MO	TAR	CR	MO
15	45	15	97.3	98.8	101.6
9	27	9	101.2	99.4	101.3
3	9	3	98.4	98.7	100.1
Average			99.0	99.0	101.0
RSD			2.03	0.38	0.79

Note: RSD: Relative standard deviation between the recovery percentages obtained for each dye

The precision of the method was also evaluated by assay of dye mixture samples containing TAR, CR, and MO. Six replicate analyses were performed on the same day on accurately weighed amounts of the dyes and the experiments were repeated on three different days. Intra-day precision RSD

(n = 6) was 0.94% for TAR, 0.48% for CR, and 0.63% for MO, respectively. For inter-day precision experiments, the calculated RSD (n = 3) was 1.07% for TAR, 0.55% for CR, and 0.81% for MO, respectively.

Chromatographic method application

The previously developed method was applied to river samples, acquired from downstream a textile producing plant. In order to assure the applicability of the developed method, river samples were spiked with reference standard solutions of TAR (3 *μg*/mL), CR (9 *μg*/mL) and MO (3 *μg*/mL), proving that the method is suitable for real wastewater samples. Based on the two chromatograms presented in Figure 4 the following concentrations were quantified in the river sample: 6.08 *μ*g/mL TAR, 15.05 *μ*g/mL CR, and 4.17 *μ*g/mL MO.

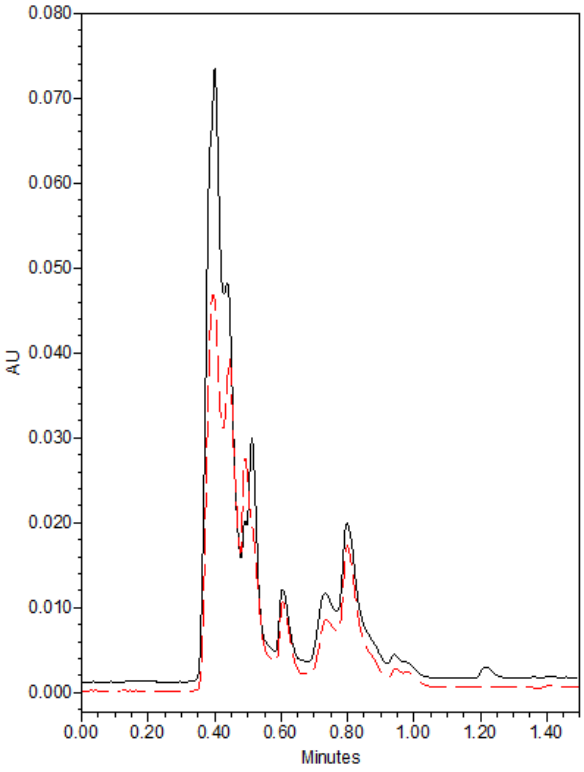

Figure 4. Comparative chromatograms of an aqueous mixture of standard solutions (dashed red line) and a downstream river sample spiked with reference standard solutions (solid black line).

CONCLUSIONS

The validated UPLC method proved to be simple, fast, accurate, precise, and robust and could, thus, be used for routine analysis of TAR, CR, and MO in combined dye samples. The UPLC method developed proved to be efficient in the separation of the three dyes in less than 2 min.

The method was validated, by studying its accuracy and specificity. Accuracy was investigated, using recovery and precision tests. The average recovery value was higher than 99%. The intra-day and inter-day precision assessments had values of RSD (%) lower than 0.94 and 1.07, respectively. Good correlation ($R^2 > 0.991$) of individual plots of all three dyes, using data obtained by applying the UPLC method, demonstrated that the developed method was efficient for the separation of TAR, CR, and MO.

EXPERIMENTAL SECTION

Materials and methods

TAR, CR, MO, sodium chloride, acetic acid and sodium hydroxide were purchased from Sigma Aldrich Chemie GmbH (Germany). Standards were analytical-reagent grade. Methanol was bought from Carl Roth (Switzerland) HPLC grade. All other chemicals were of analytical grade. Mobile phases and all injected samples were filtered on 0.2 µm filter before use.

Stock standard solutions of TAR (1004.8 *µ*g/mL), CR (1008 *µ*g/mL), and MO (1001 *µ*g/mL) were made in 0.1 M NaCl solution. Diluted standard solutions were prepared from stock solutions with ultrapure water by serial dilutions.

Chromatographic Conditions

The UPLC system (Milford, USA) consisted of Acquity Binary Solvent Manager, Acquity Sample Manager and Acquity PDA Detector. The detector was set to collect data between 210 and 500 nm. The UPLC column was Acquity UPLC BEH C18 2.1 mm x 50 mm (1.7 µm particle diameter). Column temperature was kept at 30 ºC. The autosampler temperature was set at 4 ºC. The injection volume was varied between 2 and 10 µL (partial loop method) depending on standard or sample concentration. UV signal was detected as spectra in the range 210 – 500 nm (sampling rate: 20 pts/s).

The gradient elution (0.3 mL/min) program was prepared with 100% MeOH as mobile phase A and 0.1 M acetate buffer in 10% MeOH as mobile phase B. The integration was performed with Empower software, using the

MaxPlot extracted chromatogram (a special chromatogram that plots the maximum spectral absorbance measured at each time point in the data file). The subroutine Peak Purity Check was used for the evaluation of the purity of the eluted peaks and the subroutine Library Match for the identification of the eluted peaks based on comparison of their spectra with the spectra of standards stored in the spectra library [20].

Procedure for Analysis of Dye Mixtures

Different volumes of TAR, CR, and MO stock solutions were mixed in vials and afterwards diluted with ultrapure water. Previous to the injection, the solutions were filtered on 0.2 μm cellulose Millipore syringe filters and 10 μL of the filtrate was injected in the UPLC system. After the chromatographic run, quantification was performed either using MaxPlot subroutine or at channels extracted at the wavelength where each dye had its maximum absorption in the range of the recorded spectra, e.g. 258, 339 and 449 nm, for TAR, CR, and MO, respectively. The amount of each dye in every mixture was determined based on the respective calibration plot.

Preparation of Calibration Plots

Six diluted standard solutions of all three dyes in the concentration range 0.5 – 50 μg/mL were injected in the UPLC system, under the conditions described above. Each amount was analyzed five times and peak areas were recorded.

Method Validation

From the calibration plot of each dye, the Limits of Detection (LOD) and Quantification (LOQ) parameters were calculated from the regression equation of TAR, CR, and MO, using Equation (1) and Equation (2), respectively:

$$LOD = 3.3\frac{\sigma}{S} \tag{1}$$

$$LOQ = 10\frac{\sigma}{S} \tag{2}$$

where σ is the standard deviation of the response and S is the slope of the calibration plot.

Accuracy of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to estimate the repeatability of the experiments, for the same concentration of each dye six

samples were analyzed during the same day. To study inter-day variation, an analysis of three mixed standard solutions of the same concentration was performed on three different days [21]. To confirm the specificity of the method, solutions of each single dye and a mixture of all three dyes, having the same concentrations, were injected into the UPLC system and the concentrations of individual dye and of the mixture were compared.

Recovery Studies

To check the accuracy of the method, recovery studies were conducted after addition of standard dye solution for three different mixtures containing known concentrations of dyes, at three different levels on the linear part of the standard curves. Three samples were prepared for each recovery level. The solutions were analyzed and the percentage of recoveries was calculated from the calibration curves.

Method application

In order to prove that the developed method is relevant for real samples, river water samples (the spiking experiment was done in triplicate) were obtained from downstream a textile plant in Hunedoara county (Romania). Prior to the analysis, the river water samples were filtered on 0.2 μm filter and kept well sealed, at 4 °C. For the spiked samples, standard dyes solutions mixture was added to downstream river samples (3 *μ*g/mL TAR, 9 *μ*g/mL CR, and 3 *μ*g/mL MO).

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