



High Performance Liquid Chromatography-Diode Array Detector Method for the Determination of Tartrazine and Indigotine

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A quantification method was developed for indigotine and tartrazine by high performance liquid chromatography and diode array (DAD) detector. Mobile phase consisted of 6 mM tetrabutylammonium hydrogensulphate solution (TBAHS)/ACN (57:43) with 1 mL min⁻¹ flow rate, A Phenomenex, Luna, ODS-2 RP- C18(2) (5 µm, 4.6 mm × 250 mm) column and Agilent 1100 Model DAD detector was used. The wavelength used was 610 nm for indigotine and 427 nm for tartrazine (band width: 4 nm). Efficient separation was obtained by using 0.3 mol L⁻¹ acetate buffer (pH: 4.70) for the dilution of the standard stock solutions to obtain the working solutions. For indigotine, linearity was obtained in the concentration range of 1.096-16.4 µg mL⁻¹, $y = 561.01x + 5.978$ ($r^2 = 0.9999$); limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.25 and 0.82 µg mL⁻¹, respectively. For tartrazine, linearity was obtained in the concentration range of 3.79-38 µg mL⁻¹, $y = 568.25x - 49.029$ ($r^2 = 0.9997$); LOD and LOQ were determined as 1.095 and 3.644 µg mL⁻¹, respectively. System suitability parameters theoretical plates (N), tailing factor (T), resolution (R) and retention factor (k') were reported. The possible impurities, counterfeits and degradation products (totally related substances) were assessed for indigotine and tartrazine which were supplied from the pharmaceutical and food companies. The results of the over loadings and the forced degradation studies showed that indigotine from the food company was containing different kinds of related substances. Indigotine and tartrazine from the pharmaceutical company were containing these substances in insignificant amounts, which were used as the standards in this study. Indigotine was stable in 0.5 M HCl solutions, but degradable in a small scale in neutral solutions; tartrazine was stable in neutral solutions, not stable in 0.5 M HCl solutions at 80 °C. While the tartrazine peak lost its symmetry and gave a front; the indigotine peak totally disappeared with 0.5 N NaOH and H₂O₂ 3 % solutions at 80 °C. The developed method was applied to the samples of a local soft drink and a medicine in the dragee form. The recovery was 81.49 % for indigotine; 84.83 % for tartrazine for the soft drink; 90.15 % for indigotine for the dragee. Tartrazine couldn't be determined in the dragee due to the double peaks. The amounts of indigotine and tartrazine were found in the soft drink and dragee under the regulatory limits. While the founded amounts of indigotine and all related substances differed in the batches of the soft drink, there was no significant difference in the batches of dragees.

Key Words: Indigotine, Tartrazine, Tetrabutylammonium hydrogen sulphate, Reversed phase liquid chromatography.

INTRODUCTION

Colorants are added to drugs to improve their aspect and to avoid confusion during the manufacturing phase or in administration. They are added to foods to make them attractive, to replace their natural colour that can be lost during the industrial process or to avoid variations in the colour of the final product.

The usage of synthetic colorants has become more popular if compared to the natural ones, due to their brightness, uniformity, stability, inexpensiveness and potency. Although they have many advantages over the natural dyes; their toxic properties are being reported¹. Human toxicity data do not yet exist for many chemicals including most of the colorants. Thus,

even the amounts of a colorant is to be found under the regulatory limits, one can not extrapolate that the chemical is not definitely hazardous without considering the factors such as light, heat and pH, which might effect the substance's properties. Impurities and degradation products that might arise during the synthesis of raw materials and production process of the medicines and foods, have also to be considered¹. Additionally, counterfeiting of the dyes is a big problem especially in food industry.

Colorants are regarded as excipients of drugs according to the Annex of Directive 2001/83/EC². Generally, any component with a recognized action on effect should be mentioned on the labelling. However, giving the name of the excipient is necessary, referring of its amount isn't. According to this

directive, tartrazine is a substance that may cause allergic reactions and has to be informed on the package leaflet.

Efforts have been made to control and limit the amount of synthetic colorants that are added in foods; the more toxic ones are banned³. In 1994, the European Community formulated the Directive 94/36/EC for unifying the legislation on food colorants in the member countries. There are two kind of lists which one indicates the permitted food colorants, the other two indicate the permitted amounts for certain foodstuffs. Tartrazine and indigotine are in the permitted food colorants in these lists. However, tartrazine amount is restricted to 100 mg L⁻¹ in certain liquid food and to 100 mg kg⁻¹ in processed peas. There is no information about the permitted limits of indigotine according to this directive. The regulation about colorants for use in foodstuffs in Turkey is exactly the same as the Directive 94/36/EC⁴.

Nevertheless, there are some regulations in Europe and USA; there is a need for internationally agreed regulations and legal infrastructures about the usage of colorants. Less attention has been devoted to the safety of synthetic colorants, due to their usage in extremely small amounts. Whereas, a small amount of impurity, degradation product or counterfeit might have a high potency of toxicity. Therefore precise, accurate and robust quantification methods have to be developed and the products on the market have to be checked.

Tartrazine is one of the widely used colours in the world⁵. It is used in soft drinks, foods, drugs and cosmetics and generally in combination with other colorants⁶; derived from coal tar and approved by the US FDA as FD and C Yellow No. 5 and also known as E 102. It has an ADI (Acceptable Daily Intake) of 7.5 mg/kg body weight⁵. The content of tartrazine in one tablet may vary from 0.02 to 2.5 mg, while 250 mL of soft drink may contain from 0.8 to 8.0 mg⁷. Urticaria⁶, hyperactivity, concentration difficulties and learning difficulties in children⁸ were reported for tartrazine. It is also known to be potentially dangerous in aspirin intolerant individuals^{9,10}. Contradictory results were reported on the carcinogenic effect of tartrazine on animal studies¹¹⁻¹³. It is prohibited in Austria, Finland and Norway and restricted in Sweden and Germany.

Indigotine (Saxon Blue) is approved by US FDA as Indigo Carmine and as FD and C Blue No. 2 and also known as E132. It is widely used in foods, cosmetics and drugs; as a biological stain in chromoendoscopy for early diagnose in quite high concentrations (0.1-1.0 % aqueous solution)¹⁴ and pH indicator as well. It has an ADI of 0-5 mg/kg¹⁵. FAO and WHO report that it is considered biologically inert and extremely safe. However, there is not enough study on its genotoxicity, mutagenesis and metabolism. Severe hypertension¹⁶ or hypotension¹⁷ and damaging on DNA activity on strains H17 and M 45¹⁸ had been reported. It is also reported that indigotine is reduced P450 reductase¹⁹.

There are many reported spectrophotometric-chemometric/derivative methods for the determination of the mixtures of tartrazine and the other colorants²⁰⁻²³; of which some are include in indigotine²⁴⁻²⁶ in the literature.

The chromatographic methods are superior if compared to the spectrophotometric methods which couldn't solve the interference problems, even if the chemometric or derivative

approaches are included. HPLC-RP methods are being widely used for ionizable colorants due to their advantages and inexpensiveness if compared to ion-exchange methods^{27,28}. In most of the previously reported HPLC-RP methods for the simultaneous determination of tartrazine and the other azo-dyes²⁹⁻³⁴; indigotine, tartrazine, azo and/or non-azo dyes^{35,36} the elutions were gradient. Despite the difficulties in the efficiency of the sulphonated azo dyes and considerable improvement in the efficiency under the gradient conditions is due to so called "gradient band compression effect"³⁷, we didn't use the gradient elution considering the disadvantages. While acetate buffers were used as a component of the mobile phases in some methods^{29,30,32,34,36}; ion pairing agents (IPA's) were used in other ones^{31,33,35}.

On the other hand, obtaining a primary standard of any colorant is still standing as a major problem. Tsuji *et al.* prepared the indigotine standard for Japan Dye Standard of the National Institute of Hygienic Sciences^{38,39}, which is not available on the market. Presently, a stability-indicating study for the possible impurities and degradation products of indigotine and tartrazine was performed. Possible counterfeits were also emphasized. Finally, the colorants obtained from the pharmaceutical company were used as the standards in this study.

An isocratic HPLC-DAD method was developed and validated by using TBAHS as IPA for the simultaneous determination of a mono-azo dye tartrazine and a non-azo dye indigotine whose mixtures are used for yielding the various shades of green. The method was applied to a local soft drink and a medicine in dragee form.

EXPERIMENTAL

Tartrazine and indigotine standards were kindly obtained from Ilsan Pharmaceutical Company (Turkey). Both dyes are originally from Acros. The dye samples were kindly obtained from a local food company. Different batches of the soft drink were obtained from the local market and the medicine in dragee form was obtained from the local pharmacies. Acetic acid (Merck), tetrabutylammonium hydrogen sulphate (Merck), sodium acetate (Sigma) were reagent grade. Acetonitrile (Lab Scan) was HPLC grade. Bidistilled deionized water was milli Q quality. All of the stock solutions were stored in the dark in glass-stoppered bottles at 4 °C.

The HPLC (Agilent Technologies) was a combination of a Model G1311A quaternary pump, a Model G1322A vacuum degasser and a Model G1315A diode array detector. Sample solutions were injected with a syringe through a Rheodyne Model 7725i loop type injector. Loop volume was 50 mL. The separation was performed on Phenomenex, Luna, 5 µm, C₁₈ (2) 250 mm × 4.6 mm column. Data was processed through the Agilent ChemStation. Genex beta (50 mL) and Eppendorf (1000 mL) automatic pipettes, IKA Labortechnik KS 125 basic shaker were used. All glassware was calibrated with distilled water.

Chromatographic conditions: The following mobile phases were used: Mobile phase was consisted of 6 mM TBAHS solution/ACN; (57:43). The flow rate was 1.0 mL min⁻¹. Detection was performed with an Agilent 1100 Model diode array detector at 1:427 and 610 nm, band width: 4 nm.

Ambient temperature was used. Injection volume was 50 mL. 0.3 M acetic acid/ sodium acetate buffer was used for the void volume (2.15 min) at 258 nm.

Coating procedure: A commercial RP-column from Phenomenex was dynamically coated by a solution of 6 mM TBAHS/ACN; (57:43) at 0.4 mL min⁻¹. The analytical column was equilibrated at 1 mL/min for 2 h until the baseline was stabilized.

System suitability parameters: The capacity factor (k') was calculated according to the expression: $k' = (T_R - T_o)/T_o$ where T_R is retention time of peak (min). The tailing factor (T) was calculated according to the expression: $T = W_{5.0}/2.t_w$, where $W_{5.0}$ is the width of the peak at 5 % of the peak height (min); t_w is the distance in min between the peak front and the T_R , measured at 5 % of the peak height. The resolution (R) was calculated according to the expression: (pertaining to peaks a and b, T_R of a peak a < T_R of a peak b; T_R in min).

$R = 2(T_{R(b)} - T_{R(a)})/W_{B(b)} + W_{B(a)}$ where $W_{B(x)}$ is the base width for peak x (min). Theoretical plates (N) was calculated according to the half-width method. All the above mentioned parameters were reported as the results of 5 measurements.

Stock standard solutions: 11.0 mg of tartrazine and indigotine were dissolved separately in 100 mL calibrated flasks with deionized water and prepared daily. The vessels were coated with aluminium folia.

Calibration curves: 350, 1000, 1500, 2000, 2500, 3500 mL of tartrazine standard stock solutions were transferred separately to a 10 mL volumetric flasks and each solution diluted to volume with 0.3 M CH₃COOH/CH₃COONa buffer (pH:4.7). 50 mL of each diluted solution was injected into the column three times. 100, 250, 500, 750, 1000, 1250, 1500 mL of indigotine stock solutions were transferred separately to a 10 mL volumetric flasks. The same procedure was followed as for indigotine.

Overloading and forced degradation studies: Standard stock solutions of tartrazine and indigotine were overloaded to the column to investigate the unknown impurity peaks. The procedure was followed according to ICH-Q 2B Validation of Analytical Procedures. Standard stock solutions of 5 mL indigotine and tartrazine were taken. 5 mL of 0.5 N HCl, 0.5 N NaOH, distilled water and 3 % H₂O₂ were added separately. Each sample was heated up to 80 °C for 4 h. Finally the samples were neutralized if needed and diluted to appropriate concentration.

Recovery procedure: Certain amounts of tartrazine and indigotine were added to appropriate amounts of soft drink blank matrix and dragee powder, properly mixed in a porcelain vessel for 10 min. Appropriate amount of powdered mixture was solved in 0.3 M CH₃COOH/CH₃COONa buffer and shaken for 0.5 h. This solution was filtered two times with a 5893

blue band Ø 125 mm filter. 50.0 mL of the filtered solution was injected into the HPLC column three times. This procedure was applied to the three samples of soft drink and dragee blank matrixes. The same procedure was applied to the blank matrixes. No interfering substances were investigated in the chromatograms of the blank matrixes at the wavelengths used.

Preparation and analysis of samples

Soft drink samples: 1.375 g product was weighted and an appropriate amount of powdered mixture transferred to a 10.0 mL volumetric flask and dissolved in 0.3 M CH₃COOH/CH₃COONa buffer. The same procedure was followed as in the recovery. Three different batches of product were studied. Three samples were studied from each batch.

Medicine samples: Ten dragees were weighed and mixed thoroughly in a porcelain vessel. The amount equal to two dragees was weighted and dissolved in the 0.3 M CH₃COOH/CH₃COONa buffer. The procedure was followed as in the soft drink samples.

RESULTS AND DISCUSSION

Mobile phase composition: The IPA's had been used due to their resolving efficiency in the previous chromatographic studies of the ionic dyes^{3,31}. TBAHS and cetyltrimethyl ammoniumbromide (CTMAB) were used in the preliminary experiments of this study. Moderate retention times and efficient resolutions were obtained with TBAHS if compared to CTMAB. All the possible related substances of the indigotine and tartrazine efficiently resolved by using TBAHS. After a series of experiments the mobile phase ratio was fixed to 57:43 (6 mM TBAHS solution in distilled water/ACN). Only two of the all mobile phase compositions studied were given in the Table-1. Two solutions were used to dilute the stock solutions for obtaining the working solutions: a. 0.1 mol L⁻¹ pH: 4.7 CH₃COOH/CH₃COONa buffer b. 6 mM TBAHS solution in distilled water/ACN (57:43) the tailing factors of the peaks (T) were better with the diluting solution a, compared to b. However tailing were not efficient enough with the values of 1.233 for indigotine and 1.390 for tartrazine (Table-1) in these conditions.

Evaluation of the system suitability parameters: The stock solutions of the standards of tartrazine and indigotine (supplied from the pharmaceutical company) were diluted with 0.3 mol L⁻¹ CH₃COOH/CH₃COONa buffer (pH: 4.7) instead of 0.1 mol L⁻¹ tailing CH₃COOH/CH₃COONa buffer. The chromatograms of the dye mixtures from the local food company were given in Fig. 2a (at 610 nm) and Fig. 2b (at 427 nm); the chromatograms of the standard dye mixtures from the pharmaceutical company were given in Fig. 5a (at 610 nm) and Fig. 5b (at 427 nm) in these conditions. The tailing values drastically

TABLE-1
CAPACITY FACTORS (k'), RESOLUTIONS (R) TAILING FACTORS (T) AND RETENTION TIMES (T_R) OF INDIGOTINE AND TARTRAZINE STANDARD SOLUTIONS SUPPLIED FROM THE PHARMACEUTICAL COMPANY. THE IMPROVED VALUES OBTAINED WITH 0.3 M Ac/NaAc BUFFER (pH: 4.7) AS THE DILUTING SOLUTION FOR INJECTION

Mobile phase	SIn for injection	Mobile phase ratio	Impurity 1			Indigotine			R (Imp l/IN)	Tartrazine		
			k'	T_R	T	k'	T_R	T		k'	T_R	T
6 mM TBAHS/ACN	0.1 M AcOH/AcONa	60:40	2.016	6.487	1.436	2.503	7.581	1.287	1.603	5.430	13.826	1.827
6 mM TBAHS/ACN	0.1 M AcOH/AcONa	57:43	1.532	5.444	1.352	2.080	6.626	1.233	1.611	4.142	11.127	1.390
6 mM TBAHS/ACN	0.3 M AcOH/AcONa	57:43	1.375	5.074	1.163	2.076	6.571	1.154	2.127	4.184	11.074	1.240

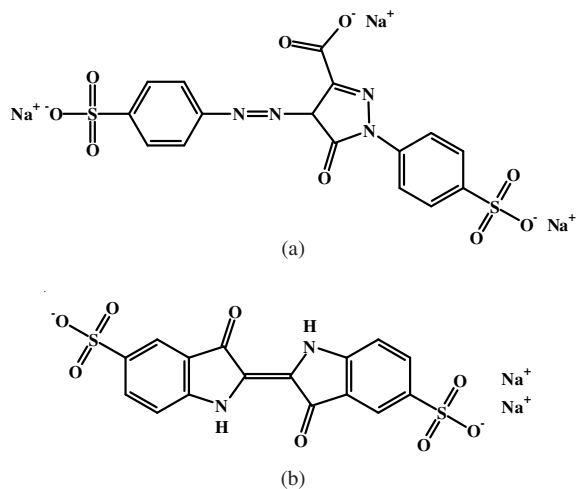


Fig. 1. (a) Chemical structure of tartrazine. (b) chemical structure of indigotine

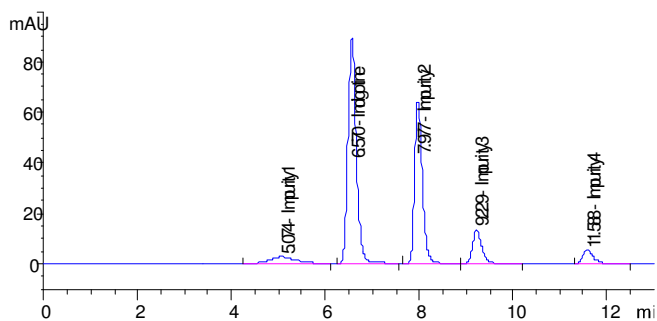


Fig. 2. (a) Chromatogram of the dye mixture of indigotine and tartrazine supplied from the food company at 610 nm. Column: Luna C₁₈ (2) (25 cm × 0.46 cm, 5 μm). Mobile phase: 6 mM TBAHS/ACN (57:43) with isocratic elution. Flow rate: 1.0 mL min⁻¹. Detector Agilent 1100 Model DAD; band width: 4 nm, sample volume 50 mL. Indigotine: 10.96 μg mL⁻¹, tartrazine: 10.95 μg mL⁻¹

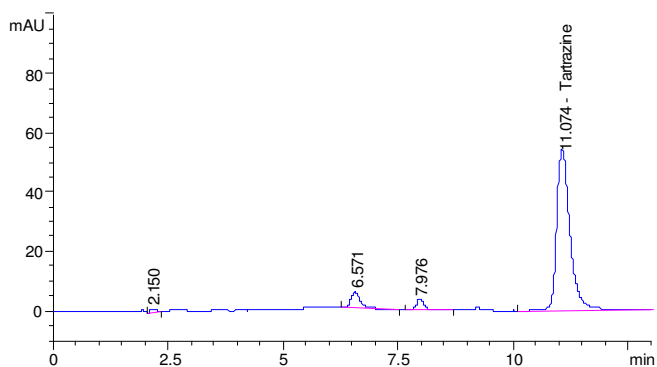


Fig. 2. (b) Chromatogram of the indigotine and tartrazine (supplied from the food company) at 427 nm. Chromatographic conditions are the same as in Fig. 2a

decreased for tartrazine (from 1.390 to 1.243) and for indigotine (from 1.233 to 1.154); the R value of the closest peak “impurity1” and the major compound indigotine increased from 1.611 to 2.127 in these conditions (Table-1). The T_R of tartrazine and indigotine and the R of the closest peak and indigotine was acceptable for a precise quantification.

The system suitability parameters were shown in Table-2. We found good agreement for R, k' and especially tailing (T) and plate number (N) values.

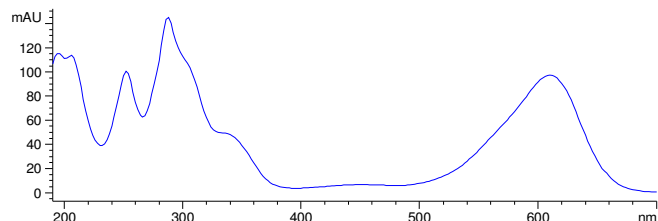


Fig. 3. Spectrum of indigotine in 0.3 M CH₃COOH/CH₃COONa buffer (pH: 4.7) 10.96 μg mL⁻¹

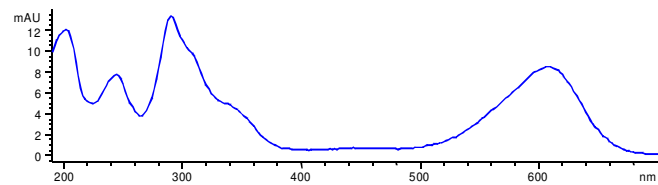
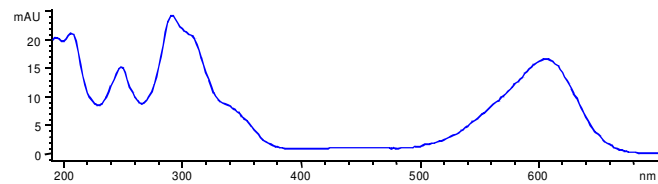
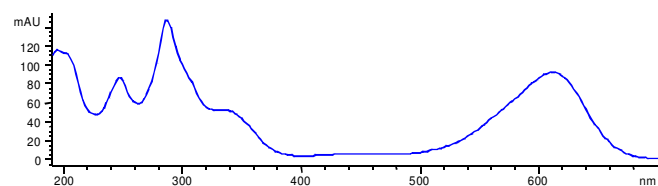
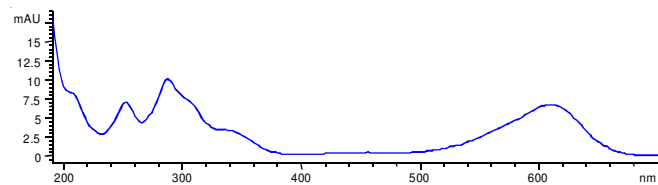


Fig. 4. Spectrum of the impurities 1, 2, 3 and 4 of indigotine (supplied from food company) in 0.3 M CH₃COOH/CH₃COONa buffer (pH: 4.7) respectively

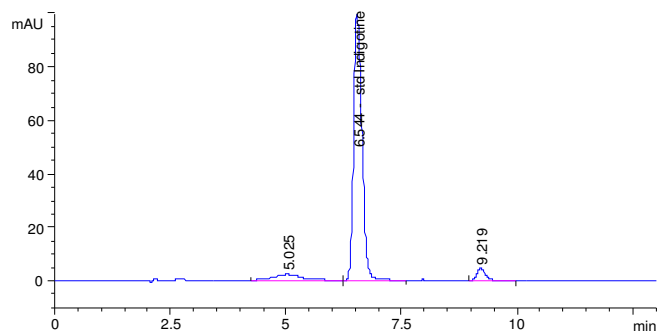


Fig. 5. (a) Chromatogram of the standard dye mixture of indigotine and tartrazine (supplied from the pharmaceutical company) at 610 nm. Chromatographic conditions are the same as in Fig. 2a. Indigotine: 10.96 μg mL⁻¹

Results of the forced degradation and over loadings:

The results of the over loadings were given in Fig. 6a-b. The resulting chromatogram after the degradation with water was shown in Fig. 7a-b. The chromatogram after the degradation with 0.5 N HCl was shown in Fig. 8a-b. The chromatograms

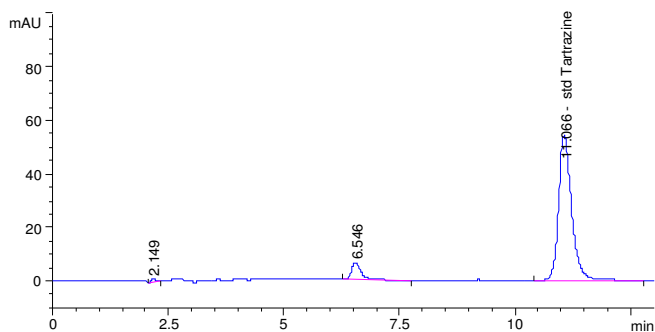


Fig. 5. (b) Chromatogram of the standard dye mixture of indigotine and tartrazine at 427 nm. Chromatographic conditions are the same as in Fig. 2a. Tartrazine: $10.95 \mu\text{g mL}^{-1}$

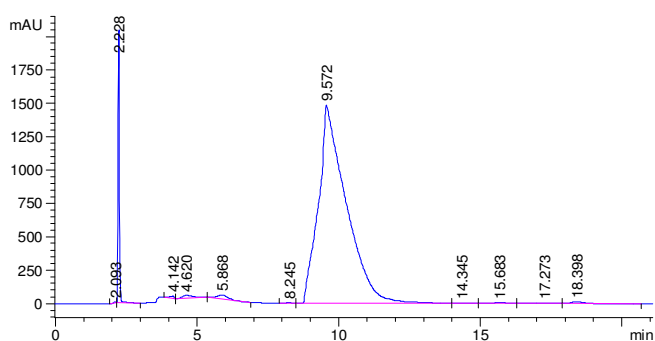


Fig. 6. (b) Chromatogram of the overloaded tartrazine standard. Chromatographic conditions are as in the Fig. 2a. $\lambda = 427 \text{ nm}$ (band width: 4 nm) tartrazine: $10.95 \mu\text{g mL}^{-1}$

TABLE-2 SYSTEM SUITABILITY PARAMETERS					
Number	Test name	IN	Result	TAR	Results
1	$k' > 2$	SST 1	2.029	SST 1	4.122
		SST 2	4.118	SST 2	2.036
		SST 3	4.115	SST 3	2.038
		SST 4	4.146	SST 4	2.043
		SST 5	2.044	SST 5	4.150
2	$RSD < 1$	SST5	0.294	SST5	0.399
3	$R_s \geq 2$	SST 1	2.271	SST 1	10.641
		SST 2	2.350	SST 2	10.886
		SST 3	2.288	SST 3	10.964
		SST 4	2.053	SST 4	10.766
4	$T \leq 2$	SST 1	1.269	SST 1	1.204
		SST 2	1.202	SST 2	1.280
		SST 3	1.251	SST 3	1.263
		SST 4	1.228	SST 4	1.299
		SST 5	1.215	SST 5	1.246
5	Theoretical plates (USP) ≥ 2000	SST 1	5685	SST 1	8571
		SST 2	6105	SST 2	8163
		SST 3	5684	SST 3	8349
		SST 4	6135	SST 4	8255
		SST 5	5741	SST 5	8077

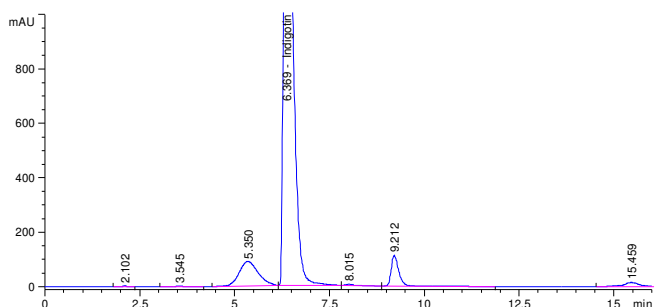


Fig. 7. (a) Chromatogram of indigotine after accelerated degradation with water at $80 \text{ }^\circ\text{C}$ for 4 h. Final concentration of indigotine: $5.46 \times 10^{-1} \text{ mg/mL}^{-1}$

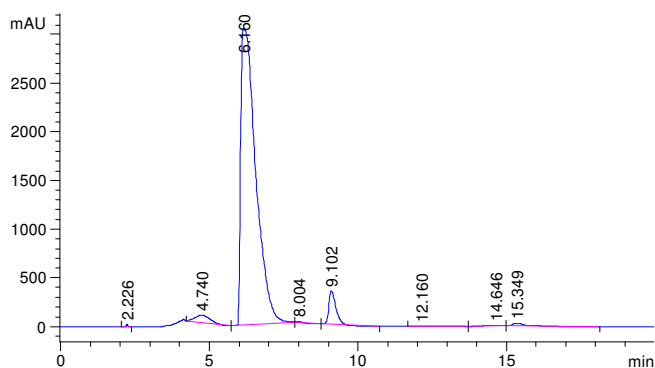


Fig. 6. (a) Chromatogram of the overloaded indigotine standard. Chromatographic conditions are as in the Fig. 2a. $\lambda = 610 \text{ nm}$; band width: 4 nm. Indigotine: $10.96 \mu\text{g mL}^{-1}$

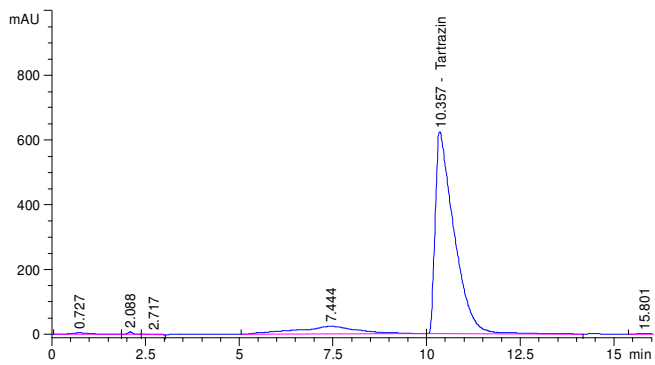


Fig. 7. (b) Chromatogram of tartrazine after accelerated degradation with water at $80 \text{ }^\circ\text{C}$ for 4 h. Final concentration of tartrazine: $5.45 \times 10^{-1} \text{ mg/mL}^{-1}$

after the degradation with 0.5 N NaOH and H_2O_2 3 % were not shown in the text. While the tartrazine peak lost its symmetry and gave a front; the indigotine peak totally disappeared with 0.5 N NaOH and H_2O_2 3 % solutions. If the chromatograms in Figs. 7a-b and 8a-b were compared to the over loadings (Fig. 6a-b); we could say that the indigotine was stable in 0.5 M HCl solutions (Fig. 8a), but degradable in a small scale in neutral solutions (Fig. 7a). Tartrazine was stable in neutral solutions (Fig. 7b), not stable in 0.5 M HCl solutions (Fig. 8b).

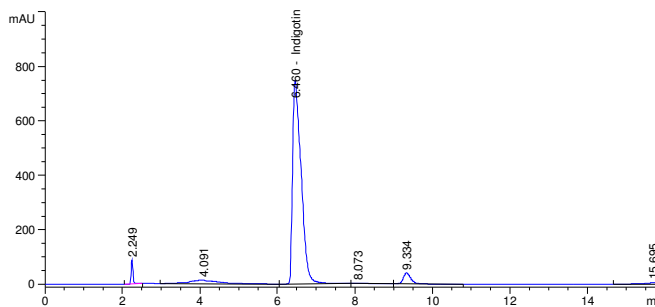


Fig. 8. (a) Chromatogram of indigotine after accelerated degradation with 0.5 N HCl at $80 \text{ }^\circ\text{C}$ for 4 h. Final concentration of indigotine: $5.46 \times 10^{-1} \text{ mg/mL}^{-1}$

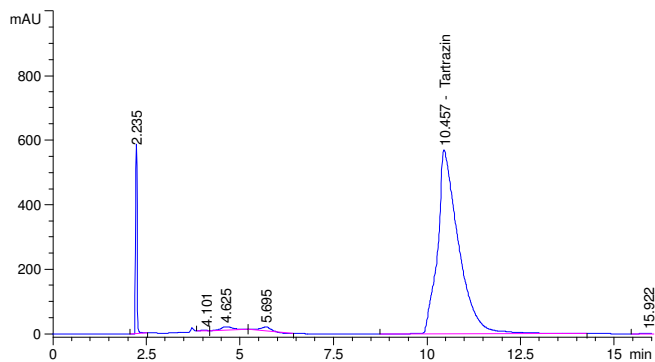


Fig. 8. (b) Chromatogram of tartrazine after accelerated degradation with 0.5 N HCl at 80 °C for 4 h. Final concentration of tartrazine: 5.45×10^{-1} mg/mL⁻¹

Specificity: The spectra of the chromatograms of indigotine and its related substances 1, 2, 3 and 4 (indicated in Fig. 2a) were given in Figs. 3 and 4, respectively. The spectra of indigotine and impurities were quite similar to each other. The two related substances of indigotine were observed in the chromatogram of dye mixture from the local food company (Fig. 2a), but not in the chromatogram of the dye mixture from the pharmaceutical company (5a). We called them as impurity 2 and impurity 4 (Fig. 2a). The retention time (T_R) of the impurity 2 was 7.977 min and T_R of the impurity 4 was 11.588 min. These peaks were not observed in the resulting chromatograms of the over loadings and the degradations as well, therefore these were attributed to existence of the counterfeits in indigotine from the local food company. However, their peaks were investigated in some batches of the soft drink sample but not in other ones. This case strengthened the existence of counterfeits in the indigotine from the local food company. Conversely, the peaks named as impurity 1 and 3 (Fig. 2a) were investigated in the chromatograms of the over loadings and forced degradations (6a, 7a, 8a). These results indicated that the peaks of impurity 1 and 3 could be attributed to the substances of indigotine related to its production process.

Due to the efficient resolving of all these related substances, indigotine and tartrazine could be precisely quantified without interfering of any substances.

Purity factor: The peak purity was based on the comparison of spectra recorded during the elution of the peak. Five spectra per peak were used to assess the purity. Two spectra on each of the up and down the slopes and one at the top. The five spectra were averaged and compared with all spectra recorded in the peak. Similarity curve technique was used to judge the peak's purity. This technique calculates a purity factor representing the degree of similarity between the spectra. Values above the 990 indicate the spectra were similar. The peak purity curves obtained for the peaks corresponding to tartrazine and indigotine standards.

The purity factors were above 990 with the values of 999.996 and 999.998 for tartrazine and indigotine, respectively. There was no significant difference between the spectra ($n = 3$). These values indicated that the purities of the peaks in the different standard samples were satisfactory.

Linearity: For indigotine, linearity was obtained in the concentration range of 1.096-16.4 $\mu\text{g mL}^{-1}$, $y = 561,01x + 5.978$

($r^2 = 0.9999$); limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.25 and 0.82 $\mu\text{g mL}^{-1}$, respectively. Detection and quantification and limits were calculated corresponding to the analyte concentrations equivalent to a signal-to-noise ratio of three and ten, respectively. For tartrazine, linearity was obtained in the concentration range of 3.79-38 $\mu\text{g mL}^{-1}$, $y = 568.25x - 49.029$ ($r^2 = 0.9997$); LOD and LOQ were determined as 1.095 and 3.644 $\mu\text{g mL}^{-1}$, respectively. The related statistics were given in Table-3.

TABLE-3
STATISTICS OF THE CALIBRATION CURVE
OF TARTRAZINE AND INDIGOTINE

	Sample 1	Sample 2	Sample 3	SD	RSD
Tartrazine	a	-49.152	-45.153	-59.855	
	b	551.63	569.02	585.49	7.601
	r^2	1	0.9997	0.9991	16.93
Indigotine	a	6.4982	5.9183	6.003	0.3132
	b	561.25	550.54	567.56	8.60
	r^2	0.9996	0.9999	0.9998	-

a: Intercept, b: slope, square root of regression.

Accuracy

Soft drink: The mean recovery was 84.83 % for tartrazine with the RSD of 1.56 (Table-4); the mean recovery was 81.49 % for indigotine with the RSD of 3.21 % (Table-4).

Medicine in dragee form: The mean recovery was 90.16 % for indigotine with the RSD of 0.27 (Table-4). The recovery was not reported for the tartrazine, because of the splitting of its peak.

TABLE-4
STATISTICAL EVALUATION OF THE RESULTS
OBTAINED FROM THE RECOVERY PERCENTAGES FOR
TARTRAZINE AND INDIGOTINE FOR THE SOFT DRINK AND
THE MEDICINE OF DRAGEE FORM. THE RECOVERY OF
TARTRAZINE COULDN'T BE OBTAINED FOR THE
DRAGEE DUE TO THE SPLITTED PEAKS

Statistical values	TAR	IN
Recovery % for soft drink	84.83	81.49
S	1.32	2.62
RSD	1.56	3.21
n	3	3
$\pm t \times S/(n)^{1/2}$	3.28	6.51
Confidence interval (95 %)	81.55-88.81	74.98-88.00
Recovery % for dragee		90.16
S		0.251
RSD		0.27
n		3
$\pm t \times S/(n)^{1/2}$		4.44
Confidence interval (95 %)		85.72-94.60

Determination of tartrazine and indigotine in soft drink and medicine: The results for the soft drink were shown in Table-5 and for the dragee were shown in Table-6. No interference of the ingredients was investigated. The amounts of tartrazine and indigotine were found in the soft drink and the dragee reasonably lower than the permitted limits (ADI: 7.5 mg/kg body weight for tartrazine, 0-5 mg/kg body weight

TABLE-5
AMOUNTS OF TARTRAZINE AND INDIGOTINE IN THE SOFT DRINK SAMPLES FOR DIFFERENT BATCHES

IN (soft drink)	Sample 1 (mg/kg)	Sample 2 (mg/kg)	Sample 3 (mg/kg)	RSD	TAR (soft drink)	Sample 1 (mg/kg)	Sample 2 (mg/kg)	Sample 3 (mg/kg)	RSD
Batch 1	100.49	98.88	98.06	1.24	Batch 1	135.51	133.65	136.14	0.95
Batch 2	109.04	112.95	110.30	1.80	Batch 2	129.28	128.82	129.49	0.26
Batch 3	58.09	59.09	58.51	0.51	Batch 3	81.31	84.75	85.96	2.86

TABLE-6
AMOUNTS OF INDIGOTIN IN THE DRAGEE SAMPLES FOR DIFFERENT BATCHES.
TARTRAZINE COULDN'T BE QUANTIFIED DUE TO THE SPLITTED PEAKS

IN (in dragee)	Sample 1 (mg/2 in dragee)	Sample 2 (mg/2 in dragee)	Sample 3 (mg/2 in dragee)	RSD	TAR (in dragee)
Batch 1	0.0592	0.0554	0.0596	3.98	The splitted peaks investigated
Batch 2	0.0539	0.0511	0.0541	3.14	
Batch 3	0.0559	0.0552	0.0591	3.65	

for indigotine). The impurity 2 and the impurity 4 (Fig. 2a) were observed in just one batch of the soft drink sample (Table-5, batch 3), but neither in the other batches of the soft drink and nor the dragee. The peak areas of impurity 1 and 3 were drastically lower in the chromatograms of the dragee if compared to chromatograms of the soft drinks. We couldn't determine the amount of tartrazine in the dragee, because tartrazine peak doubled in all batches studied. The related chromatogram was shown in Fig. 9. However, we studied in the same conditions when constructing the calibration curve, the same peak belong to tartrazine didn't double. It could be drawn a conclusion that the method is robust, but splitting of the tartrazine peak was occurring during the application. We found that tartrazine is not stable in strong acid solutions when heated to 80 °C (Fig. 8b). It was reported that the electrochemical reduction of tartrazine results in the formation of hydrazone derivative in a two electron pathway⁴⁰. However, more study has to be done if the doubled peak is belonging to hydrazone derivative or any other degradation product of tartrazine.

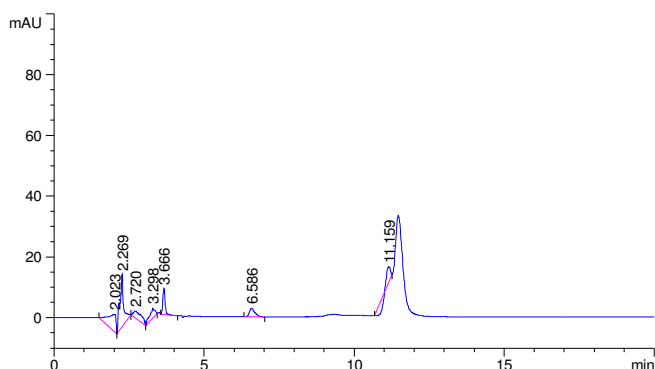


Fig. 9. Chromatogram which shows the splitted peak of tartrazine for the medicine sample in the dragee form

Conclusion

The analysis of the sulphonated dyes are still being a challenge in some aspects. The most important matter is the fully ionization over a broad pH range of the anionic polysulphonate groups of the dyes. Their dissociation can not be suppressed in buffered mobile phases even at low pH's. Studying at very low pH's give harm to the RP columns. Ammonium acetate buffer as the mobile phase component have

been used in most of the analysis of sulphonated dyes in the previous studies^{29,30,32,36}. System suitability parameters like tailing and theoretical plates were not reported in none of these studies. The efficiency of the RP-HPLC analysis might have been effected by the reported conditions of these analysis. Various alkylammonium compounds in low concentrations (pH adjusted to 6.2, 4.0, 5.5, 7.2 with acetic acid or orthophosphoric acid) were used in the mobile phases to overcome this problem^{31,33,35,37,41}. While there was no report on tailing and theoretical plates in these studies as well. Fuh *et al.*³¹ reported that severe tailing had been observed in some sulphonated azo dyes when pH was below 6.00. Furthermore, they observed double peak for tartrazine when the triethylamine (as ion pairing agent) concentration was below 3 mM in the aqueous part of the mobile phase. Vanerková *et al.* investigated the behaviour of sulphonated azo dyes in ion pairing RP-HPLC and they found the more long the alkyl chain is, the more improved the separation selectivity along with an increase in the retention.

Considering the above mentioned issues, we used 6 mM TBAHS in the mobile phase without adjusting the pH. While severe tailing was observed in this condition, it was significantly improved by using 0,3 M acetate buffer as the working solution instead of the mobile phase as usual. We also obtained efficient peaks with high plate number values (Table-2).

Another challenge along with the efficiency, is the matter of the selectivity in the analysis of sulphonated dyes, considering the related compounds. Few studies were reported on the related compounds of tartrazine and indigotine up to date^{38,39,28,42,43}. Presently, a stability-indicating study for the possible impurities and degradation products of indigotine and tartrazine was reported.

As a conclusion, a validated, robust isocratic HPLC method was developed for the determination of an azo and non-azo sulphonated dye and applied to the products in the market. However the amounts of tartrazine and indigotine were found lower than the regular limits in the soft drink and the medicine, it is difficult to draw firm conclusions without considering the presently observed degradation products, impurities and counterfeits. Although, the authorities in many countries still do not prescribe regulations on the validated analysis of the dyes used in food and medicine; we propose using validated methods for controlling the synthetic dyes in medicine and food. Our method can be used safely for this purpose.

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