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TLC-DENSITOMETRIC DETERMINATION OF SYNTHETIC FOOD COLORANTS FROM PHARMACEUTICAL POWDERS

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ABSTRACT. A thin-layer chromatographic method combined with a sample preparation procedure has been developed for determination of Sunset Yellow (E110) and Amaranth (E123) synthetic dyes in different pharmaceutical powders. For an accurate separation of investigated dyes, different mobile phases were tested using silica gel G chromatographic plates. The dyes were analyzed using slit scanning densitometry with specific wavelength selection (λ=485 nm for E110 and λ=520nm for E123). Experimental results showed a very good linear correlation (R^2 > 0.9940) between area of colored spots and concentration of dyes in range of 4-20 µg/mL. In addition, low values of LOQ (0.29 µg/mL for E123 and 0.46 µg/mL for E110) and LOD (0.58 µg/mL for E123 and 0.92 µg/mL for E110) were obtained. The sample preparation step was focused on the quantitative desorption of dyes from starch based matrix and sugars removing. In the case of non soluble matrix, different extraction solvents and techniques were tested. The best results were obtained using ultrasounds assisted extraction (UAE) using the mixture of MeOH-NH_3 (9:1 v/v) as extraction agent. In the case of water soluble samples, a purification step by ion-pair – solid phase extraction (IP-SPE) on C18 cartridges using hexadecyltrimethyl-ammonium bromide (CTAB) was developed. High recovery values (R%) and a good reproducibility (RSD) was obtained for E110 and E123 (99.27±3.73% and 98.84±1.17% respectively). The applicability of the developed method was assessed for Coldrex and Daleron Junior pharmaceutical powders. The obtained results (42.64±3.29mg/kg E110 and 218.86±10.73mg/kg E123 in Coldrex and 54.21±3.95mg/kg E110 in Daleron) showed that the proposed method is suitable for rapid routine analysis of synthetic dyes in pharmaceutical powders.

Keywords: *pharmaceutical powders, food synthetic colorants, sample preparation, TLC-densitometry*

INTRODUCTION

The first notable characteristic of foods is its colour and often predetermines our expectation of flavour and taste. Natural colorants (pigments) were the first compounds used for colouring. Synthetic colorants (dyes) were discovered in 1856 by William Henry and are organic compounds derived from coal tar. Since then, more and more substances of every colour and tint

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of the rainbow were synthesized, many of them being used in food industry after few testing of their safety. Despite their risk upon the human health, synthetic dyes remain the most popular type of food colors, as they are brighter, more uniform, better characterized, higher tinctorial strength, and less expensive than colors derived from natural sources. The currently used dyes are classified into four chemical groups: azo, xanthene, triphenylmethane, and indigoid [1].

Some of this dyes belonging to the group of azo dyes, such as tartrazine (E 102), cochineal red A (E 124), and sunset yellow (E 110), can cause allergic or pseudo-allergic reactions (PARs), particularly to the people allergic to aspirin and other non-steroidal anti-inflammatory agents, or those affected by urticaria or asthma [2]. The presence of azo dyes, even at low doses, is also correlated with body-weight gain, changes in lipidic profile, blood glucose content, and biomarkers of oxidative stress in tissue and different toxic effects on renal and hepatic function [3].

In European Union, according to “Food Colour Directive” 94/36/EC, 30 June 1994 and EC Regulation No 1333/2008 of the European Parliament the following synthetic food dyes are allowed to be used (if the purity criteria are fulfill): Tartrazine (E102); Quinoline Yellow (E104); Sunset Yellow (E110); Carmoisine (E122); Amaranth (E123); Ponceau 4R (E124); Erythrosine (E127); Allura Red (E129); Patent Blue V (E131); Indigo Carmine (E132); Brilliant Blue FCF (E133); Brown HT (E155) [4, 5]. Due to regulatory restrictions regarding the use of dyes in foodstuff, the control of colorants in pharmaceuticals by their qualitative and quantitative analysis seems to be also necessary. According to the US regulations, FDA has classified the dyes in three categories: FD&C, D&C and Ext. D&C. The acronym FD&C indicates that the dyes are approved to be used for coloring of foods, drugs, and cosmetics. Colors in a more-limited category, D&C, are considered safe to use in drugs and c.osmetics. Colors in a third category, Ext. D&C, are certified only for external use in drugs and cosmetics.

Colorants are used in the pharmaceutical practice in order to ensure the same colour for all the batches of a given product. Adding a colour makes the medicinal product more attractive, easier to recognise and, in some cases, is stabilizing the sensitive ingredients by deposition as a continuous film [6].

First step of dyes analysis is sample preparation, which sometimes can be difficult. Usually, two important steps, namely the extraction from the matrix and the purification of the extract are involved. The used methodology depends on the nature and complexity of the matrix and the presence of other compound that may interfere during the analyses. The sample preparation is strongly linked with the analytical technique that will be used for compounds determination [7]. The solid samples insoluble in water are subjected to extraction with aqueous solution of acetone, methanol, or alkalized alcohols [8]. The composition of the extraction system decisively influences the dyes recovery. Before chromatographic analysis the extracts must be purified of sugars, fats and other substances that may disturb the dyes separation. The purification step is

carried out by colorant adsorption on wool fibre, polyamide [9], cellulose, alumina [6], and on anion resins exchangers or by liquid-liquid extraction of the ion pair compounds formed with different reagents [10, 11]. The solid phase extraction (SPE) on C18 sorbents is being used lately on a large scale [12]. The retention of the anionic colorant is carried out either as unionised species (low pH value), or as ion pairs (IP). Improved solid phase extraction systems which can prevent the development of preferential flowing channels have been also used [13].

The analysis of food dyes involves their identification as well as their quantitative determination. The UV-Vis spectrophotometric absorption methods can be used for the analysis of liquid matrices, which contains one, maximum two food dyes from different colour classes. Complex spectrophotometric methods combined with chemometric processing and interpretation of data must be applied in the case of the sample containing more colorants [14-17]. Currently, both the qualitative and the quantitative analysis are achieved using mainly chromatographic techniques. The dyes have low retention time in RP-HPLC due to the presence of sulphonic and carboxylic groups in their structure [18]. In order to solve this inconvenient the gradient elution [19], the adjustment of the pH value with different buffers [20] or the adding of ion pairs reagents (IPR) [21, 22] can be used. Also, the use of triethylamine as a mobile phase component has been studied [23]. The ion chromatography on an anion exchanger with low hydrophobicity as stationary phase and gradient elution can be applied for food dyes analysis due to their ionic character. [24]. Capillary electrophoresis (CE) was allso used as an alternative technique to HPLC [25-28]. The dyes from pharmaceutical products were determined by micellar electrokinetic capillary chromatography (MEKC) [6].

The dyes began to be analysed since the 1950s, using paper chromatography [29]. Currently, normal phase TLC on silica gel plates developed with a mixture of isopropyl alcohol-aqueous ammonia [30] and reversed phase TLC and HPTLC on C18 and CN [31, 32] are used. HPTLC - densitometry has proven to be an accurate quantitative method but, digital processing of plates image obtained by scanning using special software were also used [33, 34].

In view of the aforementioned considerations, the aim of the present work is to develop a simple, fast, precise and economical method for simultaneous determination of food dyes from pharmaceutical powders using TLC-densitometry. Special attention is paid to sample preparation, which is a quite difficult step due to the requirements that must to be fulfilled.

RESULTS AND DISCUSSION

Coldrex HotRem Blackcurrant pharmaceutical powder analysis

Coldrex powder contains paracetamol, phenilephrine chlorhydrate, ascorbic acid, sucrose (4 g), flawors and Amarant (E123), Sunset Yellow (E110) and Black PN (E151) as colorants. The high sucrose content will disturb

the TLC analysis leading to tailing spots and lower R_f values. A simple dissolution of the powder in water followed by IP-SPE should be sufficient for obtaining an extract suitable for TLC analysis.

TLC determinations were performed on silica plates. A new mobile phase isopropyl alcohol–NH_3conc–CTAB 0.1M, (7:3:2, v/v) was developed for separation of the dyes E110 and E123. The determined values of the retention factor R_f (±RSD) for E123 and E110 were 0.653(±0.014%) and 0.871(±0.016%) respectively. Good separation was achieved with this system obtaining an R_s value of 4.59. Both dyes declared on the label: Sunset Yellow (E110) and Amaranth (E123) were identified by comparing their R_f values with those of standards. Also, trace amounts of Black PN (E151,) were observed as a pail spot with intermediate R_f value (fig 1a).

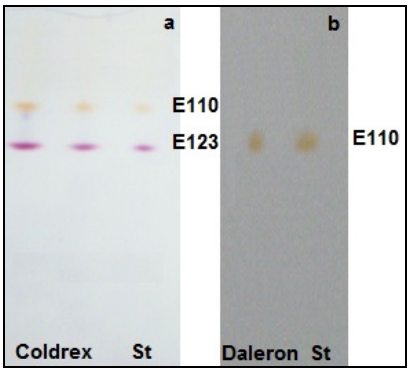

Figure 1. Dyes Identification in pharmaceutical samples.
(a) Coldrex powder using isopropyl alcohol–NH_3conc–CTAB 0.1M;
(b) Daleron powder using isopropyl alcohol–NH_3conc as mobile phase

Quantitative determinations for E110 and E123 were performed by scanning the developed plate at specific wavelengths 485 nm for E110 and 520 nm for E123 (fig 2).

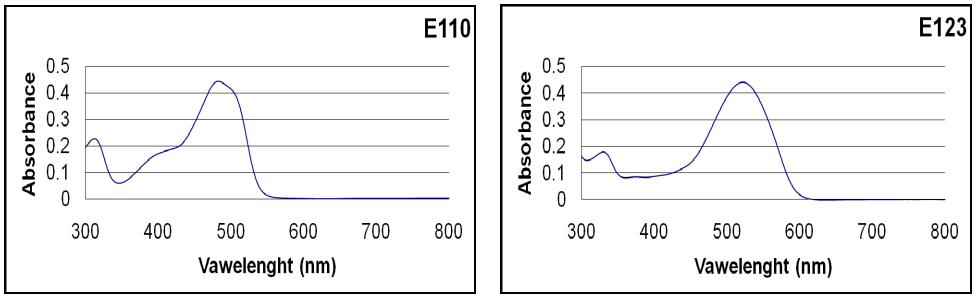

Figure 2. UV-Vis spectra of the studied dyes.

The linear domain of quantitative determination was determined by using seven different concentrations of standards. The standards were applied in duplicate and analyzed using the previously mentioned TLC conditions. In all cases linear relationships (Y=bX+a) were observed by plotting peak areas against dye concentrations. The correlation coefficients (R^2) corresponding to linear regression equations were at least 0.9940. The limit of detection (LOD) and limit of quantification (LOQ), calculated on the basis of the confidence bands of calibration curve using ordinary least squares method, were found to be between 0.29-0.45µg/mL and 0.58-0.88µg/mL respectively (Table 1).

Table 1. Linearity range and linear regression parameters for the investigated dyes (probability of error 0.05, t_s=2.57)

Dye	Linearity range (µg/mL)	b (±SD)	a (±SD)	R^2	LOD (µg/mL)	LOQ (µg/mL)
E110	4 - 20	723449 ±1501.8	7631.8 ±758.4	0.9940	0.45	0.88
E123		558341 ±6907.2	1003.6 ±388.9	0.9975	0.29	0.58

The recovery of the dyes after sample preparation by IP-SPE was calculated as ratio between the peak area of the processed sample and the peak area of the aqueous dye solution (E110+E123, 0.1mg/mL each). High values of recovery were obtained 99.27% for E110 and 98.84% for E123. Moreover, the proposed method shows good precision, the RSD value being 3.73% and 1.17% respectively.

The analytes content in Coldrex powder was calculated on the basis of the regression equation and spot area. The amounts of the dyes determined in Coldrex powder were 42.64±3.29 mg/kg for E110 and 218.86±10.73 mg/kg for E123 (SD, for 95%).

Daleron Junior powder analysis

Daleron Junior pharmaceutical product is a powder containing paracetamol, ascorbic and citric acid, maize starch, orange flavor, sucrose and Sunset Yellow (E110) as coloring agent. Based on these informations (provided by the label), sample preparation must to be focused on two problems: colorant desorption from starch matrix and sucrose removing. The synthetic dyes are strongly adsorbed on starch due to the sulphonic moieties, so when desorption is desired the active adsorption sites must be deactivated or the sulphonic groups blocked by reaction with an ion-pair reagent. The problems posed by sucrose presence can be over passed by using extraction solvents with low water content or by performing SPE on C18. When sample preparation fulfills its objectives the determination step do not presents difficulties especially in this particular case when only one component must be determined.

TLC analysis was performed on silica plates using isopropyl alcohol–NH_3conc (7:3, v/v) as mobile phase [13]. The Sunset Yellow spot was identified in the upper part of the developed plate (fig.1b), at the R_f value of 0.667 (RSD=0.012%). The chromatogram obtained for Daleron confirms the presence of E110.

The quantitative determination was performed at the optimum wavelength of λ_{opt}=485nm as indicated by the adsorption spectra (fig. 2b).

The linear regression equation Y=127005(±1396.9)X+3087.3(±139.5) was obtained on the working range 4-20 µg/mL and the correlation coefficient (R^2) was 0.9981. The limit of detection (LOD) and limit of quantification (LOQ), calculated on the basis of the confidence bands of calibration curve using ordinary least squares method, were 0.466 µg/mL and 0.92 µg/mL respectively.

Daleron Junior product required a complex sample preparation due to its matrix. Three procedures for sample preparation were applied. The first extraction method was focussed on E110 desorption from starch by optimizing the composition of the extraction system. In a previous paper [35] it was find out that NH_3 can block the active adsorption sites of starch. Different extraction solvent systems: H_2O (S1), MeOH (S2); MeOH-H_2O (9:1, v/v) (S3); MeOH-NH_3conc. (9:1, v/v) (S4); MeOH-NH_3conc-H_2O (8:1:1, v/v) (S5); MeOH-NH_3conc (1:1, v/v) (S6); and MeOH-NH_3conc (2:1, v/v) (S7) were tested. The obtained extracts were directly analyzed by TLC. Tailed spots and lower R_f values were observed for extracts obtained with systems S1, S6 and S7 and in consequence they were eliminated from further determinations. These anomalies were correlated with a high content of extracted sucrose. The systems S2, S3, S4, and S5 were found to be appropriate for extraction, but their compositions have a major influence upon the desorption process of dye from starch (Table 2). The S4 and S5 systems show best extraction efficiency providing extracts with high amounts of dye.

Table 2. TLC determination of E110 from Daleron pharmaceutical powder using different sample preparation procedures

Sample preparation technique	Solvent system	E110 content (mg/kg)	
		Average	SD
Magnetic stirring	H_2O (S1)	-	-
	MeOH (S2)	29.415	4.509
	MeOH-H_2O(9:1, v/v) (S3)	40.847	4.639
	MeOH-NH_3conc. (9:1, v/v) (S4)	45.276	3.044
	MeOH-NH_3conc-H_2O (8:1:1, v/v) (S5)	45.635	2.633
	MeOH-NH_3conc (1:1, v/v) (S6)	-	-
	MeOH-NH_3conc (2:1, v/v) (S7)	-	-
Ultrasound assisted extraction	MeOH-NH_3conc. (9:1, v/v) S4	54.187	3.949
IP-SPE		53.999	3.563

Further improving of extraction efficiency can be achieved by using ultrasound assisted extraction (UAE) instead magnetic stirring to break analyte-starch bonds. At the first sight S5 is the best system that can be used, but there are two considerations that lead to S4 selecting: (i) the water content of extraction system must to be as lower as possible in order to obtain an extract with low content of sucrose; (ii) comparing the results obtained with S4 and S5 using Student's t test no statistical significant differences were revealed $t_{exp}(0.218816)<t_{crit}(2.23)$. A higher content of dye 54.216mg/kg with a good precision (RSD=±3.949) was determined when UAE and S4 as extraction system were used (table 2).

The third method based on an advanced desorption of the dye from the starch followed by SPE purification. The advanced desorption was achieved by using CTAB as extraction agent. The ion-pair reagent interacts with the dye destroying its charge and forming a compound which weakly interacts with the starch. On the other hand the surfactant inactivates the adsorption sites. The content of E110 determined by this method was 53.999±3.563) mg/kg and do not differ from that obtained by UAE (statisticaly proofed by Student's t test: $t_{exp}(0.093854)<t_{crit}(2.23)$. Based on these results we can assume that both methods are accurate and precise.

CONCLUSIONS

TLC analysis of the synthetic food colorants can be performed in good conditions on silica gel plates using isopropyl alcohol–NH_3conc–CTAB 0.1M (7:3:2, v/v) or isopropyl alcohol–NH_3conc (7:3, v/v) as mobile phases. Linear calibration curve for both dyes (R^2>0.9940) on the range 4-20 µg/mL and low values for LOD (0.29 µg/mL for E123 and 0.46 µg/mL for E110) and LOQ (0.58 µg/mL for E123 and 0.92 µg/mL for E110) were obtained. Similar values of LOD (2.7 µg/mL) and LOQ (8.1 µg/mL) for Sunset Yellow were obtained by TLC [12], LOD 0.68 µg/mL for E110 and 0.38 µg/mL for E123 by micellar electrokinetic capillary chromatography [6].

Sample preparation method of the pharmaceutical powders must to be selected according with the sample composition. Water soluble matrices can be processed by IP-SPE for removing the interferences such as sucrose. The procedure using CATB as ion-pair reagent provide good recoveries (R>98%) and high precision (RSD<3.73%). Complex matrices containing soluble interferences as well as insoluble compounds which adsorb the analytes need a more laborious sample preparation. Two methods were found to be efficient UAE using MeOH-NH_3conc (9:1, v/v) and solid-liquid extraction with CTAB solution followed by IP-SPE. The content of dye E110 determined by these methods do not present significant statistical differences.

The Daleron pharmaceutical powder contains only Sunset Yellow (54mg/kg) while the Coldrex product contains along Sunset Yellow (42mg/kg) also Amaranth (218mg/kg) in the context of a maximum level of dyes in foods is 100-150 mg/kg E110 and 30-100 mg/kg E123 as indicated by EC Regulation No 1333/2008 of the European Parliament.

EXPERIMENTAL SECTION

Chemicals

The solvents used for sample preparation were purchased from Chemical Company (Iasi, Romania). The mobile phases for TLC analysis were prepared using methanol, isopropyl alcohol (HPLC grade), ammonia (concentrated solution), and hexadecyltrimethylammonium-bromide (CTAB) and were purchased from Merck (Darmstad, Germany). The TLC Sil G 60 plates (20x10cm) and LiChrolut® RP-18 SPE cartridges (200 mg) were also purchased from Merck (Germany). The Sunset Yellow (E110) and Amaranth (E123) of analytical purity were purchased from Fluka (Switzerland). The Daleron Junior and Coldrex Hotrem Blackurrant pharmaceutical powders were purchased from local pharmacy. Aqueous solution of, CTAB (0.01M), sucrose (5%), E110 (0.1mg/mL) and E110+E123 (0.1mg/mL each) were prepared. Methanolic dyes standard solution for quantitative analysis were prepared by appropriate dilution of aqueous solution in the concentration range 2.0 - 40.0 μg/mL) were also prepared.

Instrumentation

Sample preparation was carried out by using an ultrasound bath Transsonic TS3100 (900W, 35 kHz), an Eba 20 centrifuge, and a VacMaster SPE system. The samples were applied with an applicator device - TLC Camag Linomat 5 and the dyes were quantified by scanning densitometry using the Shimadzu CS-9000 dual wavelength flying-spot scanner.

Experimental

Dyes determination was carried out in two steps: sample preparation and TLC-densitometric analysis. Different approaches were tested for sample preparation due to different composition of the pharmaceutical powders.

Coldrex pharmaceutical powder (0.5g) was dissolved in 45 mL water and 5 mL CTAB solution and purified by IP-SPE on C18. The cartridge was conditioned with 5 mL MeOH and washed with 5 mL CTAB. The dyes retention was performed at a low flow rate (5 mL/min) of the sample solution. After that the sorbent was washed with 5 mL CTAB solution and then dried for 10 min in air current. A mixture of MeOH-conc.NH_3 (9:1, v/v) was used for elution of dyes from cartridge. The final volume was brought up to 10 mL in a volumetric flask. The SPE recovery was assessed by processing 50 mL sample that contains 5 mL aqueous dyes solution (E110+E123 0.1mg/mL each), 5 mL CTAB (0.01 M) and 2 mL sucrose solution (5%) in the same manner as described above but, the final volume was 5 mL instead of 10 mL. TLC analysis was performed on Sil G 60 plates developed with the mixture of isopropyl alcohol-NH_3conc.-CTAB 0.01 M (7:3:2, v/v) as mobile phase, in normal chromatographic chamber previously saturated for 30 min. The

methanolic standards of E110+E123 and the samples were applied as 5 µL spots with 1 cm distance between spots, at 1.5 cm from lower edge of the plate. The speed application was 50 nL/s. The developed plates (8 cm migration distance) were scanned in reflection mode at λ_{max} = 485nm for E110 and λ_{max}=520nm for E123.

Daleron Junior pharmaceutical powder was processed in three different ways. The first one involves the dyes extraction from 3g powder with 5 mL extraction solvent, 3 times for 5 min under magnetic stirring at room temperature. Between the extraction steps the samples were separated by 5 min centrifugation at 3500 rpm. Due to the presence of starch, which strongly retains the dyes, seven different extraction systems: H_2O (S1); MeOH (S2); MeOH-H_2O (9:1, v/v) (S3); MeOH-NH_3conc.(9:1, v/v) (S4); MeOH-NH_3conc-H_2O (8:1:1, v/v) (S5); MeOH-NH_3conc (1:1, v/v) (S6); and MeOH-NH_3conc (2:1, v/v) (S7) were tested. The extracts were evaporated to dryness and the residues were dissolved in 10 mL MeOH-H_2O (9:1, v/v). The second procedure used ultrasounds instead magnetic stirring. The extraction was carried out in the same condition as mentioned above with the mixture MeOH-NH_3conc (9:1, v/v). In the third procedure the powder (3g) was mixed with 45 mL H_2O and 5 mL CTAB (0.01 M). The suspension was centrifugated and the supernatant was further processed by IP-SPE on C18 cartridge. The sorbent was then washed with 5 mL CTAB solution, dried for 10 min, and then the compounds were eluted with MeOH-NH_3conc (9:1, v/v). The final volume was adjusted to 10 mL with MeOH in a volumetric flask. TLC analyse was performed on on Sil G 60 plates using a mixture of isopropyl alcohol-NH_3conc (7:3, v/v) as mobile phase in normal saturated (30 min) chromatographic chamber. The methanolic standards of E110 and the samples were applied as 5 µL spots, 1 cm distance between spots, at 1.5 cm from the lower edge. The application speed was 50 nL/s. The photodensitograms were obtained by scanning the plates in reflection mode at λ_{max}=485nm.

Each determination was performed on six replicates.

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