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Separation of synthetic food colourants in the mixed micellar system Application to pharmaceutical analysis

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Abstract

The paper presents a rapid method for the determination of commonly used synthetic food dyes by micellar electrokinetic capillary chromatography. Detection and separation conditions allowing complete resolution of 15 synthetic food colourants were investigated. The effect of different surfactants on the analytes mobility in relation to their structure was tested. After optimization procedure a dual micellar system was selected. All food dyes were separated in less then 20 min using a fused silica capillary in the borate/dodecylsulfate/deoxycholate buffer containing acetonitrile as organic modifier. The detection wavelength was set at 210 nm. The method was successfully validated by determination of linearity ranges, detection limits, precision and repeatability for all colourants tested. In order to apply the method for pharmaceutical analysis a sample pretreatment procedures were found. Liquid pharmaceuticals were used as it or just after dilution with water. From tablets or capsules the colourants were isolated by adsorption on acidic aluminium oxide. The method was used for identification and if possible for quantification the synthetic food dyes in pharmaceuticals. The analytes are detectable at a concentration level 0.3–0.8 $\mu g\,ml^{-1}$.

Keywords: Food dyes; Micellar electrokinetic capillary chromatography; Mixed micelles; Deoxycholate; Multiwavelength detection

1. Introduction

Colourants have been used for many years in the pharmaceutical industry in order to add colour to many medicinal products, as well as to ensure the same colour for all the batches of a given product [1,2]. Adding a colour makes the medicinal product more attractive, easier to recognise, and in some cases, by forming an opaque layer, it stabilizes the ingredients of the medicine which are light sensitive [3].

Synthetic food dyes are more long-lasting and often cheaper than natural ones, but nowadays, using many of them gives rise to serious reservations concerning health [4–7]. Some of them, such as, e.g. tartrazine (E 102), cochineal red (E 124), and sunset yellow (E 110), belonging to the group of azo dyes, can themselves, or in combination with other colourants, provoke allergic or pseudo-allergic reactions (PARs), particularly in people allergic to aspirin and other non-steroidal anti-inflammatory agents, or those suffering from urticaria or asthma [8,9].

In Europe, the use of colourants in food and pharmaceutical products is regulated by the "Food Colour Directive" 94/36/EC and 95/45/EC dated 26 July 1995. Due to regulatory restrictions regarding the use of food dyes, it seems significant that the control of medicinal products should encompass not only qualitative and quantitative studies of the active ingredients, but also of the inactive ingredients including colourants, to a greater extent. New methods are required for rapid detection and identification colourants, in so doing, confirming the correctness of the product composition.

Until now, studies of colourants in medicinal products have mainly been carried out using chromatographic methods [10–15] and spectrophotometric methods [16–18].

The ionic character of the colourants means that they can be separated and assayed using an alternative to HPLC, viz. CE [19–25]. This method is simpler, more economical, and, a fact nowadays greatly emphasised, less time- and labour-consuming than HPLC. For this reason we have attempted to develop a quick screening test for colourants,

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to identify and assay them in selected medicinal products, which differ both in their ingredients and their pharmaceutical form.

2. Experimental

2.1. Instruments

The studies were carried out on BioFocus 2000 (Bio-Rad, Hercules, CA, USA) apparatus, equipped with a UV detector (200–365 nm) and a fused-silica 50 cm (45.4 cm to detector), 50 μm I.D. capillary (Beckman, Fullerton, CA, USA) thermostated at a temperature of 20 °C. Separations were carried out at a voltage of 20 kV (positive polarity) and detected at various wavelengths: 210, 254 and 280 nm. Samples were applied by hydrodynamic method for 5 psi s.

New capillaries were prepared for electrophoresis by successive rinsing with isopropanol (2 min), 0.5 M NaOH (5 min), deionized water (2 min), separating buffer (2 min). Before each separation, conditioning was carried out with 0.1 M NaOH (40 s) and separating buffer (40 s).

Spectrophotometric studies performed using the Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) in 1 cm quartz cuvets. The spectra of the colourants tested were recorded for aqueous solutions with concentrations of approximately 20–50 $\mu g\,ml^{-1}$.

2.2. Reagents and solutions

Food dyes standards were used: tartrazine (E 102) and Patent Blue V (E 131) from Fluka (Sigma–Aldrich, Poznań, Poland); Sunset Yellow (E 110) and Cochineal Red (E 124) from Mucos Pharma (Geretsried, Germany); azorubine (E 122) from Pliva (Zagreb, Croatia); Quinoline Yellow (E 104), carminic acid (E 120), amaranth (E 123), erythrosine (E 127), Food Red 2G (E 128), Allura Red (E 129), indigotine (E 132), Brillant Blue FCF (Erioglaucine) (E 133), Brillant Green (E 142) and Brillant Black PN (E 151) from Sigma–Aldrich (Poznań, Poland).

Initial solutions of each of the food dyes standard were prepared at a concentration of approximately 2 $mg\,ml^{-1}$. All colourants were dissolved in deionized water, only Quinoline Yellow (E 104) and Patent Blue V (E 131) were dissolved in 5% (v/v) methanol.

All reagents used for buffers, capillary conditioning and sample pretreatment were of analytical-reagent grade. Sodium dodecylsulfate (SDS), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), sodium deoxycholate (DC) were purchased from Sigma–Aldrich (Poznań, Poland); boric acid, sodium hydroxide (NaOH) and aluminium oxide active acidic (sorbent for column chromatography) from Merck (Darmstadt, Germany); isopropanol, methanol and acetonitrile (ACN) from Lab-Scan (Dublin, Ireland); 25% ammonia solution from POCH (Gliwice, Poland). Highly-purified deionized water was prepared using EASY Pure RF deionizer (Barnstead-Thermolyne, Dubuque, IA, USA).

Electrolytes for electrophoresis were prepared from 200 mM boric acid stock solution made up to an appropriate pH (7.5–9.5) with 4 M NaOH. The stock borate buffer was filtered through Glass Microfiber GD/X 0.45 μm filters (Whatman, Maidstone, UK) to a volumetric flask in an amount corresponding to desired buffer concentration after filling up to the mark. Acetonitrile was added to the buffer at 10–20% of final volume.

Micellar electrokinetic chromatography was performed in buffers containing sodium dodecylsulfate, CHAPS, and sodium deoxycholate. SDS, CHAPS and DC were prepared as stock solutions at concentrations of 500, 100 and 200 mM, respectively. All additives were mixed with stock borate buffer in proportions appropriate to reach the final concentration of the modifier after filling up in the volumetric flask.

2.3. Samples

A number of commercial medicinal products of various dosage forms, listed in Table 1, were tested during the study.

Samples of coated and film-coated tablets (five of each) were prepared for testing by elution a colour layer with a 50%

Table 1
Medicinal products tested during the study

Product	Dosage form	Active substance	Colourant used
A	Coated tablet	Herbal extracts	E 132
B	Coated tablet	Multi-vitamin formula	E 124
C	Film-coated tablet	Naproxene sodium	E 132
D	Film-coated tablet	Paracetamol, Pseudoephedrine HCl, Dextrometorphan HBr	E 129, E 110
E	Film-coated tablet	Paracetamol, Pseudoephedrine HCl, Dextrometorphan HBr, Chlorpheniramine maleate	E 132, E 133
F	Film-coated tablet	Amino acids and ketoanalogues	E 104
G	Gargle	Benzydamine HCl	E 104, E 131
H	Hard capsule	L-arginine HCl	E 104
I	Hard capsule	Chondroitin sulfate sodium	E 104, E 132
J	Hard capsule	Pancreatin	E 104, E 132
K	Orodispersible tablet	Benzalkonium chloride, Mentha oil, Eucalyptus oil	No data
L	Soft capsule	Chlorpheniramine maleate, Paracetamol, Pseudoephedrine HCl	E 129, E 133
M	Syrup	Triprolidine HCl, Pseudoephedrine HCl, Dextrometorphan HBr	E 122
N	Syrup	*S*-Carboxymethylcysteine	E 124

(v/v) methanol (3–5 ml). The coloured solutions obtained were filtered through GD/X Cellulose 0.2 μm (Whatman) filters. A significant quantity of ballast substances was detected in samples prepared in this manner, which often made the detection and identification of the colourants impossible. In certain cases, it proved advantageous for electrophoretic separation to dilute the eluate with deionized water (5–10×) thus, lessening the overload of the system and increasing the stacking effect.

Gelatin capsules (5 units) were initially dissolved in heat in a 50% (v/v) methanol (3–5 ml). The gelatin was then precipitated with ACN (2–4 ml). Samples prepared in this way, however, still contained significant quantities of gelatin, which hindered the electrophoresis because of deactivation of the capillary wall and loss of the system efficiency. In order to prevent large quantities of gelatin from the capsules passing into the sample solution, elution of the colourants was carried out with a 50% (v/v) methanol, unheated. Unfortunately, the concentration of colourants in samples prepared by this method was often at a level of LOD.

In order to preconcentrate the food dyes and separate them from the remaining matrix, isolation of colourants from eluates was attempted using acidic aluminium oxide. Due to the lack of SPE columns with this type of sorbent, adsorption of the colourants was carried out by shaking out a colour eluate with 1 g of aluminium oxide in a test-tube. The solution of the sorbent was transferred to an empty SPE column, the ballast substances were washed out with a few portions (3 × 2 ml) of deionized water or 50% (v/v) methanol, then the colourant was displaced with 1 ml of 1% ammonia solution. The columns were washed with deionized water until the dye had been completely removed from the sorbent. The resulting eluates were subjected to electrophoretic analysis. This method of preparing samples turned out to be the most appropriate for carrying out further analysis.

Samples of the medicines in liquid form (syrups, liquids) were analyzed without further preparation, or with only double dilution with water.

3. Results and discussion

3.1. Optimization of detection

In order to choose optimal detection conditions for the colourants being tested, their spectra were analyzed in the UV–vis range (200–800 nm). Characteristic maxima of absorption were seen in the visible spectrum: for yellow dyes at 400–490 nm, for red ones at 500–550 nm; for blue and green at 570–640 nm. Despite the presence of characteristic wavelengths in the vis range of spectra, UV detection is more often used especially when simultaneous food dyes determination is required [20–22,24,25]. Thus, on the basis of spectra of the tested substances in the UV range, three wavelengths: 210, 254 and 280 nm were chosen to compare the height of the peaks obtained as a result of electrophoresis (data not shown). The best results were obtained at a wavelength of 210 nm.

In the case of ambiguous interpretation of the signal at a wavelength of 210 nm, or the testing of samples where the dyes present had not been specified, detection was carried out at a wavelength corresponding to the maximum of UV spectrum for the colourant most probably present in the sample.

3.2. Method development

The optimization of the electrophoretic conditions of investigated dyes was initially tested for a mixture of standard dyes. All tested food dyes contain either sulfon or carboxy groups, thus, in basic media anions of different charge are formed. For the reason the experiments were conducted in borate buffer solutions adjusted to pH ranging from 8.0 to 9.5. The initial tests have shown that for approximately one-half of tested compounds the peaks recorded were either tailed or not well developed, and the migration times were often irreproducible.

Despite relatively good solubility of the colourants in water, the hydrophobic regions of the molecules hinder the migration in the water base electrolyte. Application of acetonitrile as a separation buffer modifier resulted in an increasing solutes solubility and a reduction of the unfavourable interactions with the capillary wall, which in turn gave a significant improvement in the peak shape as well as an increasing of resolution and system efficiency. In further studies the separation buffer used contained a minimum quantity of 10% (v/v) ACN.

The changes in mobility and migration of the compounds tested, observed in electrolytes in the pH range of 8.0–9.5 did not show the same tendencies but were a result of various overlapping events. For some colourants, a decreasing in migration time was observed with an increase in electrolyte pH, since the increasing electroosmotic flow (EOF) exceeded the anodic migration of the analytes. On the contrary, the migration time of dyes E 122, E 123, E 124, E 151 and E 102 increased in these conditions. These compounds contain three or more sulfon and/or carboxyl groups, therefore, their anodic migration velocity rise with increasing pH values more than for the other dyes, and it has a significant effect on the effective mobility despite an increasing EOF. In addition, the dissociation of phenolic groups (–OH) occuring at a pH of about 9.0, increases the net negative charge of the molecules. In the case of E 123 and E 124, which are structural isomers, an increase in pH value above 8.8 caused comigration of peaks. A similar effect was observed in the case of dye E 104. Quinoline yellow (E 104) is a mixture of di- and trisulfonic acids. At a pH of below 8.5 the dye is visible as two closely migrating peaks but at a higher pH both compounds migrate as a single peak. In the pH range tested, only dye E 142 showed a migration velocity higher than EOF. The cathodic migration of this compound is related to the presence of a quaternary amino group.

Table 2
Micellar electrolyte systems tested during the method development

Electrolyte	1	2	3	4	5	6	7
Borate buffer concentration (mM)	80	50–100	50–100	50–100	50–100	50	50
pH	7.5	7.8	8.0	8.3	8.5	8.7	9.0
ACN (v/v) (%)	10–15	10–20	10–15	10–15	10–20	10	10
Micellar systems							
1 SDS (mM)	10–20	10–25	10–25	10–20	10–50	10–20	10–15
2 CHAPS[a] (mM)	–	–	5–10	–	5–10	–	–
3 DC[a] (mM)	–	–	2–8	–	2–10	–	–
4 SDS (mM)	10–20	10–25	10–25	10–20	10–50	10–20	10–15
CHAPS[a] (mM)	5–15	5–15	5–15	5–15	5–15	5–15	5–15
5 SDS (mM)	10–20	10–25	10–25	10–20	10–50	10–20	10–15
DC[a] (mM)	2–8	2–8	2–8	2–8	2–8	2–8	2–8

[a] Cholic acid derivative concentrations were selected based on ref. [29].

The most efficient CZE separation of a food dyes standards was obtained using a 50 mM borate buffer (pH 8.5) with 10% (v/v) ACN (data not shown). However, these conditions did not allow for a complete separation of peaks from E 128, E 129, E 132 or E 124, E 151. The average efficiency of the system was only 55,000 m^{-1} theoretical plates.

Further optimization of separation conditions was carried out with micellar electrokinetic chromatography (MEKC) due to the expected higher selectivity of the method as regards compounds of similar structure. Therefore, a number of various electrolytes based on borate buffer were tested, that differed with buffer concentration, pH, ACN content, micelle type and content. Table 2 shows data of the studied electrolyte systems.

On the basis of the studies performed, it was found that properties of micelles formed by SDS do not allow satisfactory separation of the compounds tested. Since the physical properties of micelles (size, net charge and geometry) have a significant impact on the system selectivity, attempts were made to find surfactants of properties different than SDS. Analysis of the structure of the colourants tested revealed that most of them contain a naphthalene group of planar geometry. For this reason, it appeared beneficial to use cholic acid derivatives as surfactants, since their micelles interfere well with planar regions of molecules of the separated compounds [26–28].

A zwitterionic surfactant – CHAPS – and an anionic one – DC – were chosen for the following studies. The presence of cholic acid derivatives in the separating buffer exerted an advantageous effect on the separation of the compounds tested. A change of the migration order and an increase of efficiency for certain peaks was observed (E 122, E 120, E 110) when compared with separations in buffer containing SDS. DC turned out to be more efficient than CHAPS were. Using the DC micelles, we observed an increase in resolution between peaks (E 128, E 129, E 132, E 110) that could not be resolved in SDS buffer. Unfortunately, for other peaks (E 123, E 124, E 142, E 151) the same electrophoretic mobility and lack of separation were observed in DC buffer.

Optimal separation was obtained using the system of mixed micelles – SDS and DC, which was the result of the interaction of the analytes tested with both types of micelles, effecting on the greatest difference in their electrophoretic mobility. The optimal separating electrolyte was 80 mM borate buffer pH of 7.8, containing 15% (v/v) ACN, 20 mM SDS and 4 mM DC. The separation time of all colourants tested in these conditions was less then 20 min (Fig. 1).

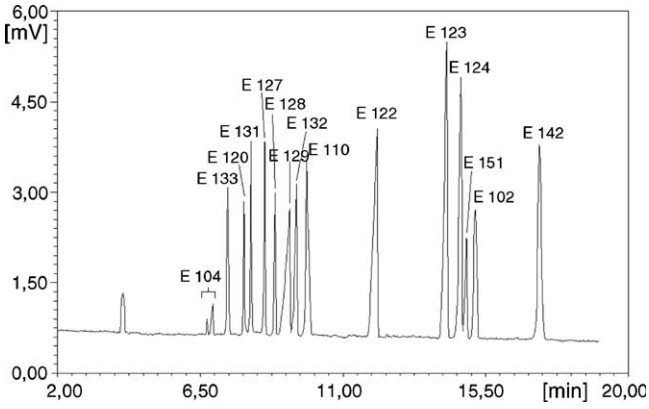

Fig. 1. Optimized MEKC separation conditions: 80 mM borate buffer pH 7.8 containing 15% (v/v) ACN, 20 mM SDS, 4 mM deoxycholate; 50 cm (45.4 cm to detector), 50 μm I.D., fused silica capillary, 20 kV, 210 nm. Each food dye at a concentration approx. 30 μg ml^{-1}.

3.3. Method validation

The method developed was validated by determining the linearity range, limit of detection, "run to run" and "day to day" repeatability. The system efficiency and the resolution of the worst separated peaks were calculated as well.

Signal dependent linearity range relative to the dye concentration was determined at a range of 1–150 μg ml^{-1}. The limit of detection was defined as a concentration resulting signal-to-noise ratio = 3 (S/N). LODs for particular colourants were found at a level of 0.35–0.85 μg ml^{-1}. The correlation coefficients (R^2) for linearity range tested and detection limits established for particular compounds are shown in Table 3. "Run to run" and "day to day" repeatability of normalized peak area was determined for a mixture of food dye standards, and the results are shown in the same table as RSD

Table 3
Validation data for all colourants studied. Correlation coefficients were determined for the concentration range of 1–150 μg ml^{-1} from seven measurement points. RSD values of relative migration times (RMT) and normalized peak areas (A_{norm}) were calculated with $n = 5$

Colourant	Linearity corelation coefficient (R^2)	LOD (μg ml^{-1})	RMT (run to run) RSD (%)	A_{norm} RSD (%)	
				Run to run	Day to day
E 102	0.9991	0.68	2.89	2.09	3.76
E 104	0.9977	0.69	0.60	1.56	1.52
E 110	0.9992	0.68	1.61	1.24	2.98
E 120	0.9987	0.67	1.42	2.39	3.65
E 122	0.9990	0.41	1.89	1.53	3.83
E 123	0.9992	0.38	2.83	2.85	4.01
E 124	0.9989	0.35	2.86	2.61	3.58
E 127	0.9939	0.42	0.64	0.81	3.36
E 128	0.9991	0.35	1.24	2.65	3.95
E 129	0.9992	0.36	1.24	1.18	3.83
E 131	0.9992	0.71	1.38	1.48	2.93
E 132	0.9990	0.85	1.25	1.66	2.87
E 133	0.9977	0.68	0.54	2.30	4.32
E 142	0.9926	0.66	2.54	1.57	3.09
E 151	0.9990	0.75	2.85	2.51	3.76

from five records. “Run to run” test comprised consecutive separations on the same day, while “day to day” test concerned runs performed within five successive days. “Run to run” and “day to day” repeatability for all food dyes tested did not exceed 3% and 4.5% respectively.

For the reason to confirm a stability of separation conditions relative migration time (RMT) repeatability was determined and presented as RSD of five consecutive runs. RMT was calculated in relation to EOF “migration time”. RMT repeatability was better then 3%.

Average efficiency of the electrophoretic system was 240,000 m^{-1} theoretical plates, and the resolution between the closest migrating peaks (E 124 and E 151) was 1.3.

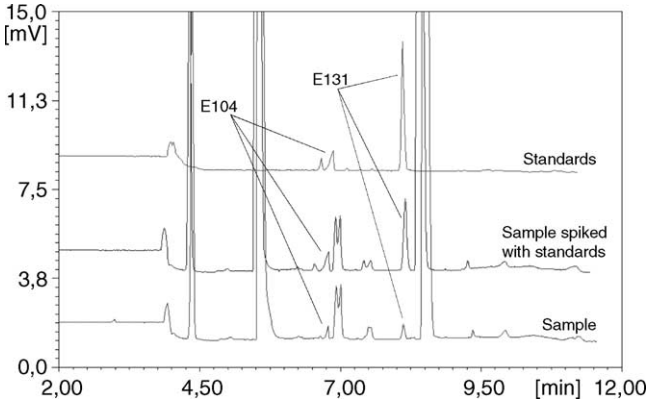

Fig. 2. Electrophoregram of medicinal product G (gargle). Sample without pretreatment. Separation conditions as in Fig. 1.

3.4. Commercial sample studies

In medicinal products, food dyes were identified by comparison of their migration times with standards, as well as by sample spiking. In studies of liquid samples (products G, M, N) the content of cochineal red (E 124), azorubine (E 122) and Quinoline Yellow (E 104) was determined as well as patent blue (E 131). The quantification was carried out using the standard curve method. The results obtained were in accordance with the manufacturer’s specification. Examples of electrophoregrams are shown in Fig. 2.

In capsules, coated and film-coated tablets the procedure of the sample pretreatment did not allow for quantitative isolation of the colourant, therefore, only the presence of colouring substances specified for the given product was confirmed. Nevertheless flat electrophoregram baseline and absence of interfering peaks show effective isolation from sample matrix. Examples of electrophoregrams are shown in Fig. 3.

In the case of medicinal product K, orodispensible tablets, the information was not available which colourants had been added. A sample for testing was prepared by the dye adsorption on aluminium oxide. The yellow-green colour of the test

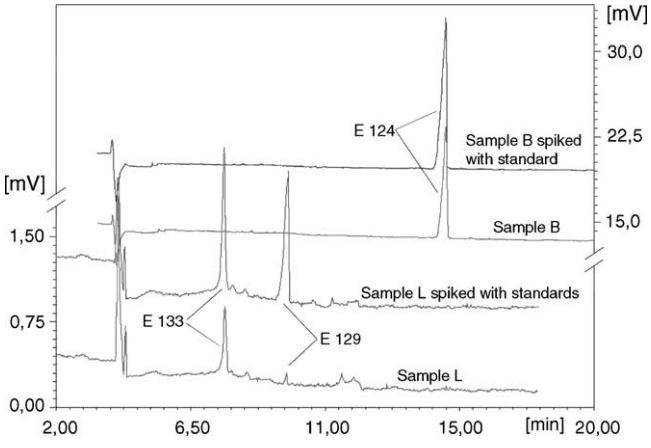

Fig. 3. Electrophoregarms of medicinal products B (coated tablet) and L (soft capsule). Samples prepared by elution from dosage form followed by adsorption on aluminium oxide (see Section 2). Separation conditions as in Fig. 1.

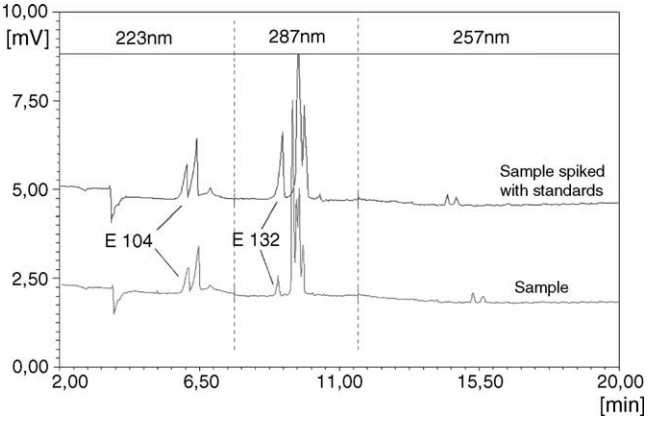

Fig. 4. Electrophoregram of medicinal product K (orodispensible tablet). Sample prepared by elution from dosage form followed by adsorption on aluminium oxide (see Section 2). Unknown colourants identified by multi-wavelength detection (changes programmed during the run) and confirmed by sample spiking. Separation conditions as in Fig. 1.

solution suggested the presence of a yellow colourant (E 102 or E 104) with the addition of a blue one (E 131, E 132 or E 133). Analysis of the electrophoregram obtained with detection at 210 nm did not permit definite identification of the dyes. For the reason, electrophoresis was performed with detection at three wavelengths 223, 287 and 257 nm, which were successively changed during the run, with the time of the expected appearance of the colourant peaks mentioned above. The wavelengths for the detection were selected on the basis of UV spectra recorded for the dyes to assure the highest signal-to-noise ratio. Based on the electrophoregram obtained in this manner, E 104 and E 132 were detected in the sample. Additionally, the identity of the colourants was confirmed by analysis of the spiked sample. The electrophoregrams are shown in Fig. 4.

4. Conclusions

The method developed allows for the separation and identification of a mixture of 15 synthetic food colourants used in medicinal products. The electrophoretic system used (80 mM borate buffer pH 7.8 containing 15% (v/v) ACN, 20 mM SDS and 4 mM DC) allowed for the separation of the compounds being tested in less then 20 min at a system efficiency of 240,000 m^{-1} theoretical plates and resolution not less than 1.3 (E 124 and E 151).

Based on the validation carried out, the suitability of the method was confirmed for quantitative and qualitative testing for colourants in medicinal products. Appropriate sample pretreatment of the medicines from various dosage forms permitted full identification of the colourants they contained, and in case of liquid samples also the quantification. Using specific wavelengths for detection allowed for the identification of colourants in unknown samples.

Applying this method in routine testing can be of use not only in pharmaceutical analysis, but also in testing of food products, where rapid identification of forbidden additives is important in detecting falsifications in food.

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