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Gel-Electrophoretic Separation of a Number of Synthetic Food Dyes with Following Determination by Spectrophotometric and Visual Method: Simply and Economically

Olga Yu. Konovalova*†, Nataliia O. Nikitina†, Valentyna Yu. Nesterenko‡, Valeriia S. Savchenko†, and Yelyzaveta G. Kobzar†

† Department of Chemical Metrology, Kharkiv V.N. Karazin National University, 4 Svobody Sq., Kharkiv 61022, Ukraine; *e-mail: o.yu.konovalova@karazin.ua, o.konovalova@hotmail.com
‡ Department of Economics and Entrepreneurship, Kharkiv National Automobile and Highway University, 25 Yaroslava Mudrogo St., Kharkiv 61002, Ukraine

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An aim of investigation was separation of synthetic food dyes E 102, E 110, E 122, E 124, E 129, E 132 and E 133 by method of planar gel-electrophoresis with following detection and determination of analytes directly on gel plate. Agar-agar gel or polyacrylamide gel was used as carrier. The influence of electrophoretic buffer pH, amperage, voltage as well as time of electrophoresis on dyes separation in agar-agar gel was investigated. Changing of dyes mobility with pH changing was explained by analysis of their ionization constants. The results of dyes electrophoretic separation were evaluated directly on gel plate by spectrophotometric and visual methods. The metrological characteristics of dyes spectrophotometric quantification and visual semi-quantification after analytes separation were evaluated. The metrological characteristics of visual detection and semi-quantification of dyes were evaluated on the basis of statistical approach and investigation of analyte detection probability distribution in the range of reaction unreliability. The suggested technique of electrophoretic separation and following spectrophotometric or visual determination of dyes was successfully checked in analysis of pharmaceuticals.

Keywords: synthetic food dye, agar-agar, gel-electrophoresis, metrological characteristic, VIS spectrophotometry, visual method

Individual synthetic food dyes and their mixtures are widely used in the food and pharmaceutical industries to give an attractive color to foods and drugs. However, for more than a decade, hazards of using such dyes that can cause allergic reactions, damage human lymphocytes, bind directly to DNA and many other negative effects have been pointed out [1, 2]. The most dangerous are azo-dyes with their potential carcinogenicity, which is caused by dyes reduction to carcinogenic metabolites in the intestine [2, 3]. Respectively, the development of simple, reliable and cheap methods of the content control of synthetic food dyes in different objects is a critical task.

The most prevalent are chromatographic methods of analysis that provide efficient separation, identification and determination of synthetic food dyes [4-9]. In the papers [4, 5] dyes were separated using the method of reversed phase high-performance liquid chromatography (HPLC) with preliminary sample preparation by the method of dispersive solid-phase extraction with the polymeric sorbent Oasis HLB [4] or using traditional solid-phase extraction [5]. All dyes were eluted from the sorbrent by solutions of methanol and ammonium hydroxide (95:5) [4] or *i*-propanol (18 %) [5], respectively. Gradient elution mode with mobile phase on the base aqueous ammonium acetate solution (pH around 6 [5] or 7 [4] (eluent A)) and acetonitrile and methanol mixture, in the work [5] with addition of water, (eluent B) were used in investigations. Analytes were detected with ultraviolet-visible [4] or diode-array (DAD) [5] detectors. The liquid-phase extraction (LPE) is lesser used in sample preparation for HPLC [6, 7]. For the simultaneous separation and determination of 27 dyes the reversed phase HPLC method was proposed with the preliminary extraction of dyes by a mixture of methanol with an ammonium acetate solution [6]. For the enzymatic treatment of starch-containing products at the initial stage of sample preparation the α-amylase solution was added. The separation was carried out in a gradient mode: mobile phases A and B consisted of a mixture of a 50 mmol L^{-1} ammonium acetate solution, methanol, and acetonitrile, acidified by 0.1 % (v/v) of glacial acetic acid, with respective ratios of 97:3:0 (v/v) and 10:3:7 (v/v/v), while mobile phase C consisted of a 1 mol/l ammonium acetate solution, methanol, and acetone in a ratio of 1:5:4 (v/v/v) [6]. Ammonium acetate was chosen as the ion-pair reagent due to its low cost and optimal chromatography. Color compounds were detected with DAD. Determination of 20 synthetic dyes in chili powders and syrup-preserved fruits by liquid chromatography with tandem mass spectrometry using C 16 chromatographic column has been reported [7]. Acetonitrile was used

for LPE, while chromatography was carried out with mobile phase consisting of acetonitrile (eluent A) and 20 mmol L^{-1} ammonia acetate buffer with 1 % acetic acid (eluent B). The separation of food dyes by planar chromatography was carried out in the investigations [8, 9]: paper chromatography on normal-phase paper Whatman № 43 [8] and thin layer chromatography on silica gel 60 plates [9]. The dyes were extracted from sample solutions in acetic acid by sorption into white commercial wool yarn, and then the dyes were recovered by mixing the yarns with 2 mol L^{-1} ammonia solution and heating for 10 min at 90 °C [8]. The mixture of sodium citrate (2.4 % w/v) and pure ethanol (1:1; v/v) as mobile phase and image-analysis method using digital images of chromatograms, obtained with a flatbed scanner, have been used. Synthetic food dyes were extracted from liquid preparations by SPE on aminopropyl-bonded silica, which was used as an anion exchanger [9]. The 0.01 mol L^{-1} solution of sodium hydroxide was used for the elution of the anionic analytes from the sorbent. The complete TLC separation of all dyes with a "universal" mobile phase was not achieved; consequently, a different mobile phase was used depending on the combination of dyes in a particular formulation [9]. All these mobile phases were the mixtures of chloroform, *i*-propanol, ethyl acetate, methanol, ethanol or toluene in different ratios. TLC-chromatograms were analyzed with the densitometer. Thus, chromatographic methods are associated with the use of complex and expensive equipment (HPLC), as well as mixtures of volatile and toxic organic solvents.

The authors of [10, 11] described the methods for the spectrophotometric determination of synthetic food dyes after their extraction and concentration by the SPE method on a cartridge filled with silica chemically modified with C16 groups [10] or aminopropyl groups [11] respectively. The dyes were eluted from the sorbent with an aqueous solution of sodium hydroxide or, in the case of Ponceau 4R, with triethanolamine [11]. However, this technique allows to analyze objects containing only one dye. While the technique [10] makes it possible to estimate the content of Sunset Yellow and Tartrazine after their separation during elution with water/acetonitrile mixture at pH 3 with a step gradient of acetonitrile content from 2.5 % to 10 % after Tartrazine release. This separation is possible due to providing difference between dyes' effective charge and hydrophobicity in eluent containing 2 mmol L^{-1} potassium dihydrogen phosphate at pH 3.

Piezosensors based on polymers with molecular imprints were proposed for the selective determination of a specific dye of the triarylmethane series in soft drinks [12, 13]. Synthesis of polymers was carried out from a mixture of 1,2,4,5-benzenetracarboxylic acid and 4,4'-diaminodiphenyl oxide in the presence of the target dye-template, which was then removed.

Due to the complexity and restrictions of the considered methods, it seems to be interesting to separate dyes by electrophoresis in agar-agar gel, which, along with separation in polyacrylamide gel, is classically used to separate proteins, DNA and RNA molecules [14, 15].

The aim of this work was to develop a simple, cheap, and corresponding to the "green chemistry" concept [16–19] technique for the electrophoretic separation of synthetic food dyes in agar–agar gel followed by spectrophotometric quantitative or visual semi-quantitative determination of analytes.

Materials and methods

Chemicals and reagents

The synthetic dyes: trisodium (4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate (Tartrazine, E102), disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate (Sunset Yellow, E110), disodium 4-hydroxy-2-[(E)-(4-sulfonato-1-naphthyl)diazenyl]naphthalene-1-sulfonate (Carmoisine, E122), trisodium 7-hydroxy-8-[(4-sulfonatonaphthalen-1-yl)diazenyl] naphthalene-1,3-disulfonate (Ponceau 4R, E124), disodium 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfonatophenyl)diazenyl]naphthalene-2-sulfonate (Alure Red, E129), disodium [2(2′)E]-3,3′-dioxo-1,1′,3,3′-tetrahydro[2,2′-biindolylidene]-5,5′-disulfonate (Indigo carmine, E132), disodium;2-[[4-[ethyl-[(3-sulfonatophenyl)methyl]amino]phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]cyclohexa-2,5-dien-1-ylidene]methyl]benzenesulfonate (Brilliant Blue, E133) were from "Tayna Exports" (India). All standards were over 85 % purity level and used without further purification.

Agar-agar for microbiology was from "Roko" (Spain) and was of analytical grade. Acrylamide, N,N'-methyllenbis(acrylamide), N,N,N',N'-tetramethylethylethylenediamine were from "Reanal" (Hungary). All reactants were over 99 % and used without further purification. Potassium persulphate was from "Reachim" (Russia) and was of analytical grade.

Glycerol (85 % m/m) was from "Halychfarm" (Ukraine), methanol (99 % v/v) was from "Reachim" (Russia). Potassium dihydrophosphate, disodium hydrophosphate, tris(hydroxymethyl)aminomethane, glycine, hydrochloric acid, sodium hydroxide, sodium tetraborate were of analytical grade and were used to regulate the solution acidity. Buffer solutions with pH 5.0 – 10.0 were prepared by the techniques in [20, 21].

Distilled water was used to prepare reagents solution.

Equipment

The separation of synthetic dyes was carried out with horizontal electrophoresis cell "SE-2" from "Helicon" (Russia) and "Elf-4" from "DNA-TECHNOLOGY" (Russia), which was used as power supply. Gels were prepared using a pouring table, gel frame and combs.

The absorption spectra of synthetic dyes in the

solution and in agar-agar gel were measured with a photocolorimeter KFK-3 (Russia).

The pH value was determined using a pH-meter ion meter "Expert-001" (Russia) with a combined glass electrode ESK-10603, calibrated beforehand with standard buffers.

The parameters of calibration curves were calculated using the STATISTICA 8.0 program packet (2007 StatSoft, Inc.).

Procedures

Preparation of the agar-agar gel plate

A plate of agar-agar gel was prepared from 1 % (by weight) agar-agar solution. For this, 1.5 g of agar-agar was dissolved in 150 mL of a phosphate buffer solution (pH 6) by heating in a water bath for 35 min. The dissolution was carried out with slow stirring, avoiding boiling. Then the solution was cooled at room temperature to 55-60 ^{0}C and poured into a gel frame installed in the pouring table, preventing the formation of air bubbles [14]. The combs were placed at a distance of 4 cm from each other without touching the bottom of the mold. After complete solidification of the gel, the combs were removed, trying not to damage the wells and gel.

Preparation of the polyacrylamide gel plate

To make a polyacrylamide gel, solutions A, B, C, and D were prepared [22]. Solution A was prepared by addition of 4.5 g of tris-(hydroxymethyl)-aminomethane to a 25-mL volumetric flask, 2 mL of distilled water, 6 mL of a 1 mol L^{-1} HCl solution, 0.05 mL of N,N,N',N'-tetramethylethylethylenediamine and adjusted to the mark with distilled water. To prepare solution B, 0.2 g of N,N'-methylene-bis-acrylamide was added to a 25-mL volumetric flask, a small amount of water was added, and the mixture was stirred until complete dissolution. Then 7 g of acrylamide was added to the flask and adjusted to the mark with distilled water. The solution was filtered. To prepare solution C, 0.15 g of potassium persulfate was added to a 50-mL volumetric flask and brought to the mark with distilled water. Solution C was prepared immediately before the studies to prevent decomposition of the main component. To prepare working solution D, solutions A, B, and C were mixed in the ratio 1:1:2.

Preparation of dyes stock solutions

Stock solutions of dyes E110, E122, E124, E129, E132, and E133 with a concentration of 0.01 mol L^{-1} and dye E102 with a concentration of 0.1 mol L^{-1} were prepared by dissolving a precisely weighed quantity of the dye sample in distilled water. Working solutions of dyes were prepared by diluting stock solutions with an appropriate buffer solution. Glycerol was added to working dye solutions up to a volume fraction of 25 %.

Technique for the separation of dyes in agar-agar gel

After filling the cells with electrodes with electrophoretic buffer, 10 μL of the working dye solution was added to the well in agar-agar gel. Contact in the system was provided by filter paper moistened with the same buffer. Gel electrophoresis was carried out at a limiting current (*I*) of 400 mA and voltage (*U*) of 100, 200, 300, or 400 V for 1, 2, or 3 h. The analytical effect was observed within 60 min after electrophoretic separation completion.

Technique of spectrophotometric studies in agar-agar gel

To study the absorption spectra of dyes in an agar-agar gel medium, a section of the gel with a dye after electrophoretic separation was cut out according to a stencil-plate using a scalpel and placed with a spatula into a glass cuvette 1 cm thick so that the spectrophotometer beam passed through the center of the spot. As a reference sample, a section of the gel treated under the same conditions as the testing sample and containing all the components of the solution, except for the dye, was used.

Research results and discussion

The choice of a medium for dyes separation by gel-electrophoresis

The possibility of electrophoretic separation of synthetic food dyes was investigated in agar-agar and polyacrylamide gel media. Electrophoresis in agar-agar gel was performed using a phosphate buffer with pH 6. Tris buffer solution with pH 8.6 was used as an electrophoretic buffer for separation in polyacrylamide gel. All the investigated dyes moved to a positively charged anode during electrophoresis and, therefore, had a negative charge. The stained areas of the dyes were clearly visible against the light yellow background of the agar-agar gel and the colorless background of the polyacrylamide gel. The dye zones and the gels were transparent, which was convenient for spectrophotometric studies and visual observation.

The rate of food dyes separation in the medium of polyacrylamide gel was higher, and the blurring of dyes stains was slightly less than in agar-agar gel. However, due to the difficulty of obtaining a gel plate with a satisfactorily even surface, the laboriousness of preparing a polyacrylamide gel, and the costliness of reagents, agar-agar gel was chosen for electrophoresis.

It was found in our previous studies that the optimal gels for analysis purposes are obtained from a 1 % agar-agar solution [23]. It is known that the pores of gels obtained from polysaccharide solutions with a concentration below 2 % are quite large and the effect of a molecular sieve is negligible during the electrophoretic separation of proteins [14]. It is obvious that the effect of this phenomenon on the separation of much smaller than proteins dyes is absent.

The study of the agar-agar gel medium effect

The absorption spectra of dyes in solution and gel at pH 6 were compared (Fig. 1) to evaluate the effect of the agar-agar gel medium. In all cases, the absorption maxima of dyes in solution and gel were observed at the same wavelength: 425 nm (E102), 485 nm (E110), 515 nm (E122), 505 nm (E124), 505 nm (E129), 615 nm (E132) and 630 nm (E133). This fact

indicates the absence of the agar-agar gel medium effects. These analytical wavelengths were used for further spectrophotometric analysis of solutions and gels containing synthetic food dyes.

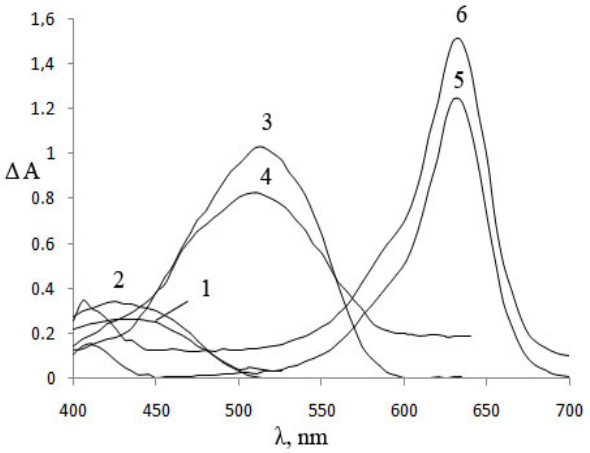

Fig. 1. Absorption spectra of dyes in solution (1,3,5) and agar-agar gel (2,4,6) for E102, E124 and E133 respectively.

Optimization of conditions of synthetic dyes electrophoretic separation

The choice of optimal pH of medium convenient for separation

Agar-agar is a hydrocolloid, which is a mixture of polysaccharides: agarose and agaropectin. The length of agarose polymer chains is much longer than that of agaropectin [24]. Gel formation of agar occurs only due to agarose [24, 25]. Polymer molecules of agarose connect only by hydrogen bonds and form a three-dimensional gel structure [24]. Agarose gels and, consequently, gels obtained from agar are stable in the pH range from 4 to 9 [26]. Therefore, the dependency of the food dyes mobility and efficiency of their separation from the pH of electrophoretic buffer in the range from 5 to 10 was studied. Fig. 2 shows the dependences passed by the dyes during electrophoresis (*l*) from the pH of the electrophoretic buffer. The *l* value was measured from the center of the well where the dye solution was injected to the center of the analyte spot.

Some of the physicochemical characteristics of the synthetic food dyes under study, which are necessary to explain the observed effects, are given in Table 1. The dyes had a negative charge in the studied pH range except for the dye E133, which was present in the form of a bipolar ion up to pH 8 and in a more alkaline medium it turned into a singly charged anion (Table 1).

Respectively, the analytes moved towards the positively charged anode during electrophoresis in the gel plate, while the E133 dye had the least mobility in the group (Fig. 2). Apart from that, E133 has a molar weight significantly higher than that of the other studied dyes (Table 1).

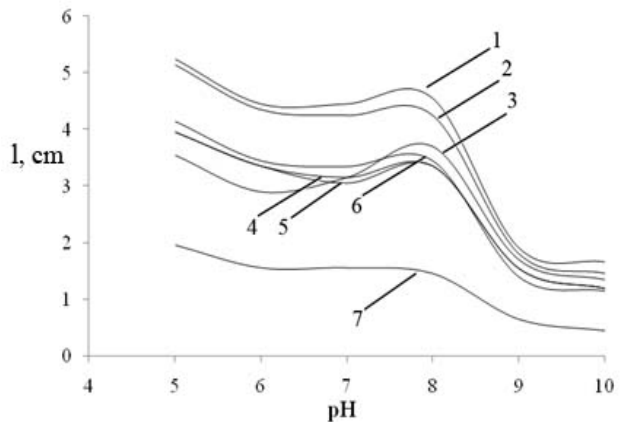

Fig. 2. The dependency of the distance, passed by the dyes E102 (1), E124 (2), E122 (3), E129 (4), E132 (5), E110 (6) and E133 (7) during electrophoretic separation on the pH of the electrophoretic buffer.

Table 1. Physicochemical properties of studied synthetic food dyes.

Name	Molar mass, g/mol	Index of ionization constant [27-29]		
		pK_1	pK_2	pK_3
Azo-dyes				
Tartrazine, E102	534.37	2.42	4.42	10.12
Sunset Yellow, E110	452.37	2.90	10.60	—
Carmoisine, E122	502.43	3.88	8.33	—
Ponceau 4R, E124	604.48	12.16	—	—
Alure Red, E129	496.42	Not found		
Pyrrole-based dyes				
Indigo carmine, E132	466.36	12.69	—	—
Triphenylmethan dyes				
Brilliant Blue, E133	792.85	1.93	7.58	9.87

According to the discussed food dyes mobility in the pH range from 5 to 8, the analytes can be divided into groups: dyes E102 and E124, possessing the highest mobility; dyes E110, E122, E129 and E132 with intermediate mobility; dye E133, which has the lowest mobility, Fig. 2. This is correspond to the ionization constants of the analytes (Table 1): dyes E102 and E124 were present in the form of a three-charged anion throughout the studied pH range; the dyes of the second group were present in the form of a doubly charged anion (for dye E122 up to pH 8); dye E133, as was mentioned earlier, up to pH 8 was present in the form of a bipolar ion. Ionization constants for dye E129 could not be found. However, the structures of the dyes E129 and E110 differ only in the methyl and methoxy groups in the benzene ring, so we can talk about the closeness of the ionization constants values of these analytes and the presence of dye E129 in the form of a doubly charged anion in the studied pH range.

The factor determining the mobility of the studied synthetic food dyes is precisely the charge of the ion, and its molar mass has a much smaller effect: the most mobile dyes E102 and E124 have a larger molar weight in comparison with that of intermediate mobility dyes (Table 1).

It is interesting that the mobility of the E122 dye changes with an increase in the pH of the electrophoretic buffer, especially in the pH range from 6 to 8 (curve 3, Fig. 2). The increase of its mobility is obviously associated with the transition of a doubly charged anion to a triply charged anion (pK = 8.33, Table 1). As a result, the value of its mobility turned from the lowest in the group of dyes with intermediate mobility to the highest (curve 3, Fig. 2).

A sharp decrease in the mobility of all the dyes being studied in media with pH above 8 is probably associated with the increase of the electroosmotic flow. It is known that agar contains a significant amount of negatively charged sulfate and carboxyl groups (agaropectin fraction). The charged groups of agar cannot move in an electric field, since they are immobilized by intertwining gel threads [14]. Mobile positively charged counterions under the action of an electric field begin to move towards the negatively charged cathode, carrying with them almost all the liquid in the narrow channels of the gel. The resulting electroosmotic flow slows up the movement of sufficiently small analytes-anions to the anode. Moreover, agarose is not stable at pH above 9 [26], which influences the properties of the gel carrier.

In further studies, gel electrophoresis was carried out with phosphate buffer with pH 6: in such a medium the maximum possible difference in the mobility of the synthetic food dyes under study was provided (Fig. 2).

The choice of optimal voltage and amperage for electrophoretic separation

To optimize the conditions of electrophoretic separation the limiting current for the device was set to 400 mA, and the voltage varied from 100 V to 400 V. The completeness of the electrophoretic separation of dyes was estimated according to the distance passed by the dyes and the width of their spots, measured along the direction of the analytes moving in the electric field. The electrophoretic mobility of the dyes was low and their separation was insignificant at the voltage of 100 V. The increase in the electric field voltage to 300 V and 400 V led to a sharp increase in power and overheating of the electrophoretic system. Under these conditions melting of the agar gel was observed, which made it difficult to obtain reproducible analysis results. Moreover, at 400 V overdrying of the filter paper on the anode side and even its charring was observed. This may be connected with caused by significant electroosmosis. The latter causes the appearance of hydrostatic pressure and due to the inability of the filter paper to pass a relatively large amount of water moving towards the cathode, loss of water and drying of the paper on the anode leads to serious problems [14]. The optimal separation of the selected dyes was observed at the voltage of 200 V. Further studies were carried out at the current of 400 mA and voltage of 200 V.

The choice of optimal time of electrophoresis and of analytical effect observed after separation

The choice of the optimal time of electrophoresis and that of observation of the analytical effect was carried out according to the distances passed by the dyes from the line of injection of the sample and the parameters of their zones after electrophoresis. Processing of the results of analytes separation in planar gel electrophoresis and thin layer chromatography are similar. Therefore, the resolution parameter (R_S) was used as a parameter characterizing the degree of electrophoretic separation of dyes; it is widely used in chromatography [30–32]. To R_S calculation the distances passed by the dyes and the diameters of the dye spots, measured from the vector of the electrophoretic force, was used. The position and parameters of the dye spots were measured directly on the gel electrophoregram.

One-hour electrophoresis showed no satisfactory separation of dyes. However, with the increase in the electrophoresis time to three hours, overdrying of the gel and charring of the filter paper closing the electrophoretic circuit were observed. Two hours was chosen as the optimal electrophoresis time that corresponded to a satisfactory separation of dyes and preservation of gel properties. The R_S resolution values calculated, obtained from electrophoresis under optimal conditions, are presented in Table 2.

Table 2. The values of the R_S parameter for food dyes separated in agar-agar gel. (U = 200 V, I = 400 mA, *time of electrophoresis* = 2 h, pH = 6).

Synthetic food dye	E 102	E 110	E 122	E 124	E 129	E 132	E 133
E 102	–	0.93	1.41	0.09	0.96	1.00	2.32
E 110	0.93	–	0.71	1.12	0.12	0.13	2.05
E 122	1.41	0.71	–	1.76	0.53	0.56	1.42
E 124	0.09	1.12	1.76	–	1.14	1.21	2.87
E 129	0.96	0.12	0.53	1.14	–	0	1.80
E 132	1.00	0.13	0.56	1.21	0	–	1.90
E 133	2.32	2.05	1.42	2.87	1.80	1.90	–

The dyes E102 and E122, E102 and E132, E110 and E124, E122 and E133, E124 and E129, E124 and E132 were satisfactorily separated in the agar-agar medium (Table 2). The dyes E102 and E133, E110 and E133, E122 and E124, E124 and E133, E129 and E133, E132 and E133 were decently separated in such conditions (Table 2). All the studied synthetic food dyes were separated with dye E133.

The optimal observation time for the analytical effect was found spectrophotometrically. The value of light absorption of the dye in the gel changed insignificantly within one hour after electrophoretic separation, although stains of the dyes got blurred over time as a result of diffusion in the gel. This is due to the fact that during the measurement time the blurring spots were completely "covered" by the non-point beam of the spectrophotometer. The analytical effect was further recorded during the first 60 min after electrophoresis. After an hour strong blurring of dye spots was observed, this prevented visual and spectrophotometric detection.

The quantitative analysis after gel-electrophoresis

The quantitative analysis was carried out by direct treating a gel plate with stained areas of electrophoretically separated dyes. The metrological characteristics of the technique of quantitative spectrophotometric determination of dyes were evaluated in the work. The metrological characteristics of the technique of semi-quantitative visual determination of analytes were found using the evaluation apparatus used in the visual test analysis [33-37].

Evaluation of the metrological characteristics of semi-quantitative determination of E102, E124, E132 and E133

The detection limit (L_D) and quantification limit (L_Q) are the most important metrological characteristics [38]. Evaluation of the value L_D in the semi-quantitative analysis is based on investigations in the interval of unreliability, where the probability of analyte detection varies from 0 to 1 [33-35, 37]. To compare systems with different concentration ranges, it was proposed to use the value of relative width of unreliability interval. It can be calculated as a difference between upper and lower concentration limits of unreliability interval divided into the lower concentration limit [34, 36, 37]. To estimate the detection limit, frequencies of the analyte detection in the unreliability interval were found and the type of distribution experimental frequencies function was determined by calculation method.

The algorithm for L_D determination, described in [33, 35, 37], was applied in our work. The interval of unreliability was identified by a group of 15-20 independent observers as the area of concentrations, in which there were positive and negative results of the analyte detection. 7-12 equidistant concentration values were chosen in this interval, which differed from each other by at least a threefold value of the absolute error of the solutions preparation. The test was repeated 3 times for each concentration of the dye, the results of each test were visually evaluated by 15 people. The frequency of analyte detection was calculated as the ratio of the number of positive responses to the presence of color to the total number of observations. According to the detection frequencies values, obtained in parallel tests, the average value and dispersion of the detection frequency of the analyte for each concentration were found.

The experimental dependency of the detection frequency on analyte concentration was described by the well-known probability distribution functions: the Weibull distribution, normal, lognormal and exponential distribution [33, 35, 37, 39]. The quality of the experimental data description was assessed using the χ^2 and Kolmogorov-Smirnov (λ) criteria [40]. If the calculated values did not exceed the values of the parameters at the significance level 0.05, the hypothesis about the correspondence of the experimental distribution to the theoretical distribution function was accepted. If these conditions were correct for several types of distribution, we chose the one for which the values of the calculated criteria χ^2 and λ were the smallest. For the selected distribution, the dye visual detection limit L_D was calculated [33-35, 37].

The length of the intervals of detection unreliability of dyes E102, E124, E132 and E133, as well as their relative width, are indicated in Table 3. The distribution of the experimental detection frequencies of the dye E102 in the unreliability interval was best described by the Weibull distribution, the E124 and E132 dyes – by the normal distribution, and the E133 dye – by the normal and the Weibull distributions in the same manner. The values of the dyes L_D were calculated for the selected theoretical function. The calculated detection limits for synthetic food dyes are presented in Table 3.

Table 3. The metrological characteristics of visual detection and determination of E102, E124, E132 and E133 after separation by gel-electrophoresis.

Metrological characteristic	Food dye			
	E102	E124	E132	E133
Unreliability interval, 10^{-5} mol L^{-1}	4 – 9	1-7	4-12	0.9-2.0
Relative width of unreliability interval	1.3	6	2	1.2
Detection limit L_D, 10^{-5} mol L^{-1}	8	6.6	11	1.8
Standard deviation of concentration s_C, 10^{-5} mol L^{-1}	5.3	3.3	4	0.9
Quantification limit L_Q, 10^{-5} mol L^{-1}	15.9	9.9	12	2.7

Visual semi-quantitative determinations are based on the comparison of the control spot color of the analyte with the color comparison scale. To create a color scale, it is necessary to estimate the value of the quantification limit. For this purpose, the concentration of a dye solution, which limits the unreliability interval from above, was chosen as the approximate quantification limit. Dye solutions with different concentrations were injected into the wells on the gel plate. The dye concentration changed from one solution to another exponentially with factor 2. Simultaneously, a control dye solution was added. After gel electrophoresis the observers evaluated the dye content in the control sample using the color scale. The L_Q was taken to be the minimum content that can be determined by this technique with a relative standard deviation of 33 %, hence $L_Q = 3 \cdot s_C$ [33-35,37], where s_C is the standard deviation of determining the concentration of a dye experimentally estimated using a color scale in accordance with the technique [33-35].

The values of the experimentally estimated s_C and L_Q of food dyes E102, E124, E132 and E133 after their electrophoretic separation are presented in Table 3. The largest values of the relative width of the unreliability interval of dyes E124 and E132 indicate a lower resistance of such systems to the influence of external factors. The largest value of quantification limit in the group of the examined dyes was obtained for the synthetic food dye E102. This is due to the low contrast of observing the lemon color of the dye on a light-yellow background of the agar-agar gel plate.

Evaluation of the metrological characteristics of E102, E124, E132 and E133 spectrophotometric determination

To describe the spectrophotometric determination of synthetic food dyes in agar-agar gel after electrophoresis, the region of the linear dependence of the analyte light absorption in gel on its concentration in solution was found (Table 4). The parameters of calibration dependences were used to calculate the limits of the spectrophotometric determination of dyes (Table 4) in accordance with the IUPAC recommendations [38].

Using of suggested technique of separation and determination of dyes in analysis of pharmaceuticals

The developed technique was used for separation and further determination of the content of E102, E133 dyes in the mouthwash from “Polissya” (Ukraine) and E124, E132 dyes in the lozenges “Neo-Angin cherry” from “Divapharma” (Germany).

The mouthwash was boiled down tenfold (for the following visual analysis) or twenty-fivefold (for the following spectrophotometric analysis) for electrophoretic separation and determination of dyes E102 and E133. 9 mL of the obtained solution, components of a phosphate buffer (pH 6) were added in a volumetric flask with the capacity of 10 mL and adjusted to the mark with distilled water. The mouthwash initially contained glycerin, so it was not added to the test solution.

For electrophoretic separation and determination of dyes E124 and E132, twenty lozenges of “Neo-Angin cherry” were dissolved in 200 mL of distilled water and boiled down fivefold. 6.5 mL of the obtained solution, 2.5 mL of glycerol, components of a phosphate buffer to make pH 6 were added in a volumetric flask with the capacity of 10 mL and adjusted to the mark with distilled water.

10 μL of the prepared samples were added to the wells in an agar-agar gel plate using a microsyringe. The electrophoretic separation of the dye mixtures was carried out for 2 h at the current of 400 mA and voltage of 200 V. The analytical effect was observed visually or spectrophotometrically within 60 min after the end of electrophoresis.

The visual determination was carried out according to the color scale obtained simultaneously and under the same conditions with the analyzed sample. According to the authors’ recommendations [41, 42], the results of visual semi-quantitative determination of the dyes content were presented as an average value and its scatter range of determination results.

Table 4. Characteristics of dyes spectrophotometric determination in agar-agar gel after their electrophoretic separation.

Dye	Concentration range of linear calibration, mmol L^{-1}	Parameters of calibration equation $A = a + bc$		Correlation coefficient, R^2	L_Q, mmol L^{-1}
		$a \pm t_{(95\,\%,3)}\, s_a$	$b \pm t_{(95\,\%,3)}\, s_b$		
E102	1 − 8	-0.03±0.02	0.133±0.009	0.9828	1
E124	1.8 − 10	0.05±0.04	0.100±0.006	0.9930	1.8
E132	0.5 − 5	-0.22±0.04	0.184±0.011	0.9890	0.5
E133	0.25 − 1	-0.18±0.06	1.12±0.09	0.9808	0.25

The spectrophotometric determination was carried out by measuring the absorbance of the dye in the gel at 425 nm (dye E102), 505 nm (dye E124), 615 nm (dye E132) or 630 nm (dye E133) against the gel sample, which was treated under the same conditions as the analyzed sample. The dye content was determined according to the calibration curve.

The accuracy of the proposed technique of electrophoretic dyes separation with the following visual or spectrophotometric determination of their content was checked by the method ‘‘added - found’’ (“spiked probes analysis”). To do this, a certain quantity of a food dye solution at a certain concentration was added to a pharmaceutical sample and treated like the samples described above in this section. As can be seen from Table 5, the found amount of the dyes satisfactorily agrees with their added amount. This indicates the absence of a systematic error in the results of the analysis of pharmaceuticals according to the proposed technique.

The results of visual and spectrophotometric determination of synthetic food dyes E102, E124, E 132 and E133 in pharmaceuticals after electrophoretic separation of analytes in agar-agar gel are presented in Table 6.

Table 5. Results of “spiked probes analysis’’.

Dye	Medicine	Quantification method				
		Spectrophotometry (P=0.95, n=3)		Visual method (number of observations = 10)		
					Found content	
		Added content, mmol L^{-1}	Found content, mmol L^{-1}	Added content, mmol L^{-1}	Average result, mmol L^{-1}	Results scatter range, mmol L^{-1}
E102	Mouthwash from “Polissya” (Ukraine)	1.5	1.3±0.3	0.4	0.43	0.08 – 0.48
E133		0.3	0.25±0.05	0.05	0.09	0.04 – 0.20
E124	Lozenges “Neo-Angin cherry” from “Divapharma” (Germany)	Dyes determination was impossible, because their content in sample was less then spectrophotometric L_Q 0.15		0.4	0.54	0.24 – 0.72
E132				0.14	0.08 – 0.15	

Table 6. Spectrophotometric and visual determination of dyes in medicines after electrophoretic separation of analytes.

Dye	Medicine	Content was determined by spectrophotometric method (P=0.95, n=3), mg mL^{-1}	Content was determined by visual method (number of observations = 10)	
			Average result	Results scatter range
E102	Mouthwash from “Polissya” (Ukraine)	(0.030±0.005)	0.017 mg mL^{-1}	0.009 – 0.034 mg mL^{-1}
E133		(0.010±0.003)	0.005 mg mL^{-1}	0.003 – 0.014 mg mL^{-1}
E124	Lozenges “Neo-Angin cherry” from “Divapharma” (Germany)	Dyes determination was impossible, because their content in sample was less then spectrophotometric L_Q	0.7 mg per 1 candy	0.3 – 1.3 mg per 1 candy
E132			0.2 mg per 1 candy	0.1 – 0.3 mg per 1 candy

Conclusions

The technique of synthetic food dyes E102, E110, E124, E129, E132 and E133 separation by gel-electrophoretic method with following spectrophotometric and visual analysis of results was suggested. Agar-agar or polyacrylamide gels were investigated as carrier. Agar-agar gel was simple, cheap and stable in preparation as well as it provided spectrophotometric and visual observation of analytical effect direct on gel plate. The absence of agar-agar gel medium influence was shown because the dyes absorbance maximums in solution and gel were observed at the same values.

The influence of medium pH (in the range from 5 to 10) on synthetic food dyes separation was studied. Observed dependencies of separation were explained by using values of dyes ionization constants. The maximum possible difference of investigated dyes moving was provided with phosphate buffer with pH 6.

The influence of current and voltage values as well as electrophoresis time on separation process was investigated. It was shown that amperage of 400 mA, voltage of 200 V and 2 hours of electrophoresis were optimal. It is recommended to observe the analytical signal during one hour after finishing of electrophoresis.

The metrological characteristics of techniques of spectrophotometric quantity and visual semi-quantity E102, E124, E132 and E133 dyes determination after their electrophoretic separation were evaluated. The metrological characteristics of dyes visual detection and semi-quantification were evaluated on the bases of statistical approach. The evaluation of visual detection limit was done on the basis of investigation of analyte detection probability distribution in the range of reaction unreliability.

The absence of systematic component of error in results of pharmaceuticals analysis with suggested technique was shown by ‘‘added - found’’ method. Developed technique was used successfully for spectrophotometric quantification of E102 and E133 dyes as well as visual semi-quantitative determination of E102 and E133, E124 and E132 in pharmaceuticals after their separation by electrophoresis method. Spectrophotometric quantification of E124 and E132 dyes in Lozenges “Neo-Angin cherry” did not manage to do because content of analytes even after limit possible evaporation was less then spectrophotometric quantification limit.

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