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Separation and quantitation of components in FD&C Red No. 3 using capillary electrophoresis

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Abstract

The use of capillary electrophoresis as a technique to separate and quantitate components of FD&C Red No. 3 (erythrosine, color index No. 45430) is described. The fluorescein isomers, 2′,4′,5′-triiodofluorescein (2,4,5-I_3F) and 2′,4′,7′-triiodofluorescein (2,4,7-I_3F), the most abundant by-products formed during the preparation of the dye, were selected for quantitation studies. The separation of other lower halogenated impurities was also demonstrated. Electrophoretic mobility of the compounds was achieved in a 50 m*M* borate, 25 m*M* sodium dodecyl sulfate buffer at pH 9.3. The limits of quantitation were found to be 0.15% (w/w) (2,4,5-I_3F) and 0.14% (w/w) (2,4,7-I_3F) (relative to the mass of FD&C Red No. 3). The method is linear from 0.08 to 20.0% (w/w) for 2,4,5-I_3F and between 0.06 and 17.0% (w/w) for 2,4,7-I_3F. In addition, relative standard deviations of 2.03 and 5.11% were determined from precision studies in the repeat analysis of FD&C Red No. 3 for 2,4,5-I_3F and 2,4,7-I_3F, respectively. Overall, the CE method produced data in excellent agreement with the reference HPLC method, used considerably less solvent and sample, generated less waste and was found to be considerably more cost efficient.
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Keywords: FD&C Red No. 3; Dyes; Erythrosine; Color additives

1. Introduction

During the 2001 fiscal year, the U.S. Food and Drug Administration (FDA) certified over 6.8 million kilograms of synthetic color additives (dyes) intended for use in foods, drugs or cosmetics in the USA [1]. The analyses were performed using validated HPLC, spectrophotometric, and gravimetric methods. However, new confirmatory methods based on alternative methodology are needed to avoid dependency on a single method and to provide a means of cross checking method accuracy. As a result, capillary electrophoresis (CE) has been identified as a viable technique for this task.

Capillary electrophoresis is a basic instrumental technique. Requiring only a capillary, a high power voltage supply, two electrodes and buffer reservoirs and a detector, high efficiency separations can be achieved in a short period of time. In addition, minimal amounts of sample and solvent are needed, thus generating little waste and making the technique cost effective. Also, the ease of automation for CE affords great efficiency and productivity.

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The use of CE, initially for the analysis of DNA and proteins, has spread to the separation of metal chelates [2], chiral drugs [3], dyes [4] and a host of other analytes [5,6]. While the technique has been extensively used to investigate synthetic dyes, few studies have focused on quantitation of starting materials, and impurities remaining in the final dye product. However, CE has been used significantly as a qualitative technique. Methods have been developed for the qualitative analysis of water-soluble reactive and non-reactive triazine dyes [7] as well as for the separation of extremely polar dye degradation products [8]. Other qualitative CE methods have been used for the analysis of dyes in the presence of artificial sweeteners and preservatives found in soft drinks [9], the simultaneous determination of xanthene and azo dyes [10] and to monitor the formation of carcinogenic aryl amines in azo dyes [11]. CE has also been employed in the qualitative analysis of neutral and ionic water soluble and insoluble intermediates formed during dye synthesis [12]. One of the few studies reporting quantitative data was performed using a group of fluorescein dyes. The emphasis of that work was solely quantification of the major dye component and not the colored impurities or intermediates [13]. A similar study was also conducted on vinylsulfone reactive dyes found in wastewater after being discharged from textile plants [14,15].

FD&C No. Red 3 (erythrosine, color index No. 45430, Fig. 1) is a synthetic color additive permitted for use in food and drugs. The tetraiodinated fluorescein dye is prepared by iodination of fluorescein, followed by alkaline hydrolysis. In addition to the tetraiodinated fluorescein product, mono-, di- and triiodinated fluoresceins are sometimes formed from either incomplete halogenation or from degradation of tetraiodofluorescein. Of the three lower iodinated fluoresceins, the triiodinated fluoresceins are most prevalent while the mono- and diiodinated species are rarely present. Therefore, the triiodofluorescein positional isomers, 2′,4′,5′-triiodofluorescein (2,4,5-I_3F) and 2′,4′,7′-triiodofluorescein (2,4,7-I_3F) were selected as model compounds to evaluate the suitability of CE as a technique capable of separating and quantifying components found in the final dye product. Specifications for the two isomers are given in the Code of Federal Regulations (CFR), where they are grouped with mono- and diiodinated fluoresceins as ''other lower iodinated fluoresceins''. The CFR gives a maximum allowable amount of 9% for the entire group [16].

The focus of this study was to develop a CE method capable of performing the routine quantitative analysis of two components of FD&C Red No. 3 that are normally analyzed by reversed-phase high-performance liquid chromatography (HPLC). Capillary electrophoresis will allow for more cost effective analyses that generate significantly less waste than HPLC. In this paper, several analytical parameters, including method accuracy, precision, linearity and sensitivity are reported and used to validate the separation and quantification of the two positional isomers, 2′,4′,5′-triiodofluorescein and 2′,4′,7′-triiodofluorescein, in FD&C Red No. 3.

2. Experimental

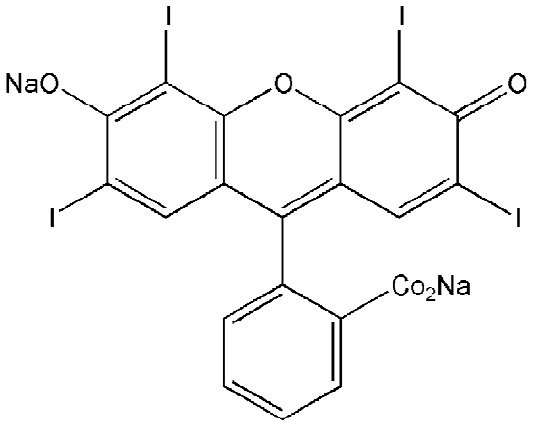
2.1. Instrumentation

Capillary electrophoresis experiments were performed on a Beckman P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA, USA) equipped with a fused-silica capillary (43 cm effective length×75 μm I.D.). Instrument control and data processing was accomplished using 32 Karat software, version 4.01.

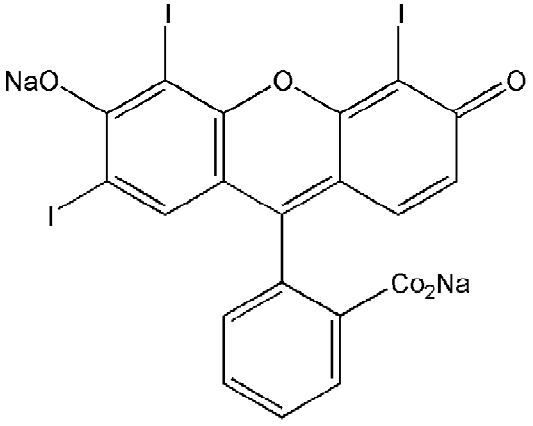
HPLC analysis was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Wilmington, DE, USA). The system was equipped with an Eclipse XDB C_8 analytical column (150 mm×4.6 mm, 5 μm), an Eclipse XDB C_8 guard column (12.5 mm×4.6 mm, 5 μm) and a 1100 diode array UV–Vis detector. Chemstation software (Revision A.08.03) controlled all components and carried out the data collection and processing.

2.2. Reagents and chemicals

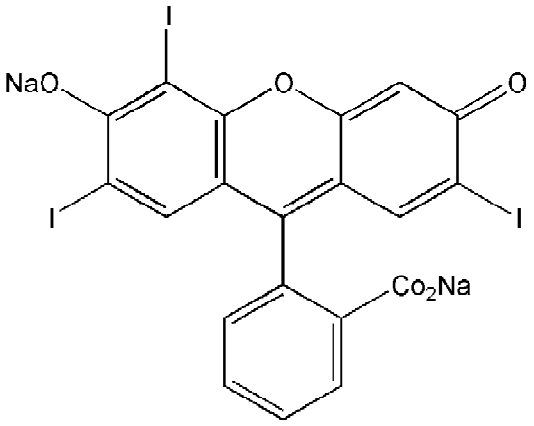
Capillary regeneration solution A (0.1 *M* NaOH) was purchased from Beckman Coulter. Sodium borate was purchased from EM Science (Gibbstown, NJ, USA), sodium dodecyl sulfate (SDS) from Aldrich (Milwaukee, WI, USA), ammonium hydroxide and ammonium acetate from Fisher (Fair Lawn,



2',4',5',7'-Tetraiodofluorescein



2',4',5'-Triiodofluorescein



2',4',7'-Triiodofluorescein

Fig. 1. Structures of 2′,4′,5′-triiodofluorescein, 2′,4′,7′-triiodofluorescein, and 2′,4′,5′,7′-tetraiiodofluorescein (major components FD&C Red No. 3).

NJ, USA) and high-purity methanol and water from Burdick and Jackson (Muskegon, MI, USA).

Standard 2,4,5-I_3F, 2,4,7-I_3F and, 2′,4′,5′,7′-tetraiodofluorescein (2,4,5,7-I_4F, FD&C Red No. 3), had been isolated and collected earlier by other FDA researchers using preparative pH-zone-refining counter-current chromatography by fractionating a commercial lot of FD&C Red No. 3 submitted to the FDA for certification [17].

2.3. Sample preparation

Stock solutions of 2,4,5-I_3F, 2,4,7-I_3F, and 2,4,5,7-I_4F were prepared in water containing two

drops of ammonium hydroxide. After dilution with water, spectrophotometric analysis was then used to determine the concentration of each (2,4,5-I_3F, ε= 0.116 l/mg cm, 516 nm; 2,4,7-I_3F, ε=0.140 l/mg cm, 518 nm; 2,4,5,7-I_4F ε=0.110 l/mg cm, 530 nm).

Calibration solutions were prepared by placing the appropriate volume of stock 2,4,5-I_3F and 2,4,7-I_3F into a 100-ml volumetric flask and diluting with water to yield between 0.75 and 7.5% (w/w) (n=5 levels) and 0.3–3.0% (w/w) (n=5 levels). Samples used to compare HPLC and CE were prepared by weighing out a 50 mg quantity of each commercial lot of FD&C Red No. 3 sample into a beaker. A drop of concentrated ammonium hydroxide was added to the beaker followed by the addition of 30 ml of water. After mixing, the solution was transferred to a 100-ml volumetric flask, filled to the mark with water and mixed thoroughly.

2.4. CE procedure

A fused-silica capillary was used for electrophoresis. Prior to each run, the capillary was filled with the separation buffer (50 m*M* sodium tetraborate, 25 m*M* SDS, pH 9.3) for 2 min. Samples were hydrostatically injected into the capillary for 5 s, after which a water plug was injected for 5 s. A +15 kV potential was applied to achieve separation. Detection was accomplished at the cathodic end with a photodiode array detector with data being acquired at 516 nm. At the completion of each run, the capillary was rinsed with capillary regeneration solution A for 1 min, followed by a 30 s rinse with water.

2.5. HPLC procedure

A 20-μl volume of the sample was injected onto the C_8 column, maintained at 35 °C. The mobile phase included solvent A, consisting of 0.1 *M* ammonium acetate in water and solvent B, methanol. Separation was performed using the gradient elution program: 0 min, A–B (55:45); 20 min, A–B (35:65); 21 min, 100% B; 25 min, 100% B; 26 min, A–B (55:45); 30 min, A–B (55:45) at a flow-rate of 1 ml/min. Column equilibration was accomplished over a 10 min span between injections.

2.6. Method validation

Method precision was evaluated using the relative standard deviation (RSD) for the repeat analyses of eight different test portions of a commercial sample of FD&C Red No. 3. To determine the accuracy of the method, test portions of FD&C Red No. 3 were spiked with 3.20% (w/w) 2,4,5-I_3F and 2.46% (w/w) 2,4,7-I_3F. Method linearity was characterized using the correlation between concentration and the corresponding peak area. A commercial sample of FD&C Red No. 3 absent of 2,4,5-I_3F and 2,4,7-I_3F was not available; therefore a commercial sample containing low amounts of 2,4,5-I_3F and 2,4,7-I_3F was spiked at six levels with each of the isomers. The limits of detection (LODs) were determined at a signal-to-noise ratio of 3:1. The limits of quantitation (LOQs) were established at the minimum concentration at which each of the isomers could be determined with accuracy and precision.

3. Results and discussion

3.1. Method validation

Optimal separation of the components of FD&C Red No. 3 was achieved in a 50 m*M* borate, 25 m*M* SDS buffer at pH 9.3. Fig. 2 shows an electropherogram of a standard mixture of the components of FD&C Red No. 3, which includes the seldom-detected mono- and diiodinated fluoresceins, separated under these conditions. Control of the SDS concentration and buffer pH was critical to maintain optimal separation. Increasing the SDS concentration improved migration time but as a result, decreased peak resolution. Varying the buffer pH by more than 1.0 pH unit decreased the method precision.

The precision of the method was investigated with respect to repeatability or repeat analysis. This was achieved by analyzing eight (50 mg) test portions of a commercial sample of FD&C Red No. 3. Relative standard deviations of 2.03 and 5.11% were obtained for 2,4,5-I_3F and 2,4,7-I_3F, respectively (Table 1).

Method accuracy describes the agreement between experimental and reference values. Spiking was used to evaluate method accuracy by determining percent recovery. The amounts of 2,4,5-I_3F and 2,4,7-I_3F

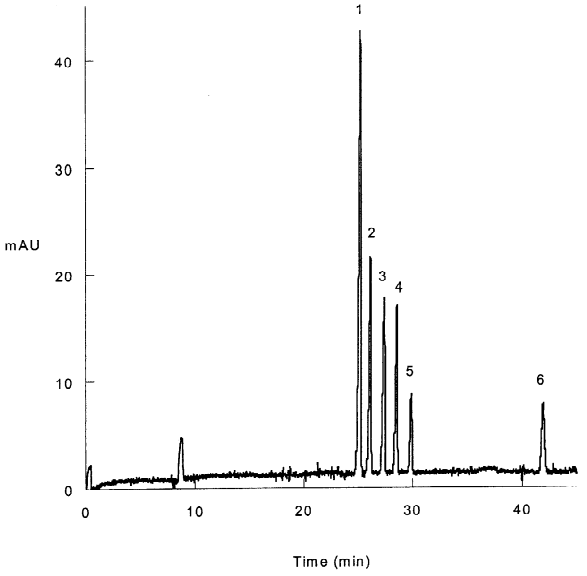


Fig. 2. Electropherogram of a standard mixture of (1) 2′,7′-diiodofluorescein, (2) 2′-monoiodofluorescein, (3) 4′,5′-diiodofluorescein, (4) 2′,4′,7′-triiodofluorescein, (5) 2′,4′,5′-triiodofluorescein, and (6) 2′,4′,5′,7′-tetraiiodofluorescein. CE run conditions: +15 kV, 50 m*M* sodium tetraborate, 25 m*M* SDS, pH 9.3.

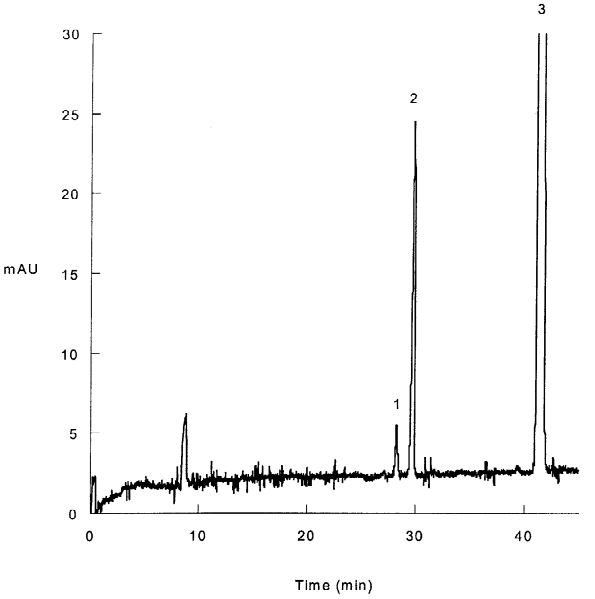


Fig. 3. Electropherogram of a FD&C Red No. 3 sample submitted for certification; (1) 2′,4′,7′-triiodofluorescein, (2) 2′,4′,5′-triiodofluorescein, and (3) 2′,4′,5′,7′-tetraiiodofluorescein. CE run conditions: +15 kV, 50 m*M* sodium tetraborate, 25 m*M* SDS, pH 9.3.

found were 3.34 and 2.30% (w/w), respectively. This translated to recoveries of 105% for 2,4,5-I_3F and 92.7% for 2,4,7-I_3F (Table 1).

A linear response was observed for both triiodofluorescein isomers over a 100-fold range using peak area (2,4,5-I_3F, $r^2 \geq 0.9998$; 2,4,7-I_3F, $r^2 \geq 0.9978$). The linear ranges represented were between 0.08 and 20.0% (w/w) ($n=6$ levels) for 2,4,5-I_3F and from 0.06 to 17.0% (w/w) ($n=6$ levels) for 2,4,7-I_3F. When both isomers were present at amounts ≥10.0% (w/w), significant band broadening was apparent, thus diminishing peak resolution. The limits of detection were 0.06% (w/w) for 2,4,5-I_3F and 0.05% (w/w) for 2,4,7-I_3F, which suggest the ability of the method to function as a qualitative technique at levels below the limits of quantitation. Limits of quantitation of 0.15% (w/w) (2,4,5-I_3F) and 0.14% (w/w) (2,4,7-I_3F) were determined and are considered acceptable for the analysis of FD&C Red No. 3.

Table 1
Precision and accuracy data for 2′,4′,5′-triiodofluorescein and 2′,4′,7′-triiodofluorescein

	RSD (%)	Recovery (%)
2′,4′,5′-Triiodofluorescein	2.03	105±1.74
2′,4′,7′-Triiodofluorescein	5.11	92.7±1.54

3.2. Application to commercial samples

The method was applied to the analysis of 10 lots of FD&C Red No. 3 samples submitted for certification by both domestic and foreign manufacturers. Fig. 3 illustrates a typical electropherogram of a commercial lot of FD&C Red No. 3. For comparative purposes, analysis was also performed by the standard HPLC method. The data given in Table 2 suggests the CE method to be in good agreement with the reference HPLC method.

4. Conclusions

A CE method for the analysis of 2′,4′,5′-triiodofluorescein and 2′,4′,7′-triiodofluorescein was developed and validated with regard to precision, linearity, and accuracy as well as limits of quantita-

Table 2
Triiodofluoresceins found in certified batches of FD&C Red No. 3 by HPLC and CE

Sample	2′,4′,5′-Triiodofluorescein found (%)		2′,4′,7′-Triiodofluorescein found (%)	
	HPLC	CE	HPLC	CE
A1	0.65	0.61	0.02	NPO
A2	3.03	3.13	0.14	0.14
A3	4.69	4.68	0.30	0.28
B1	5.46	5.31	NPO	NPO
B2	6.01	5.91	NPO	NPO
B3	6.72	6.56	NPO	NPO
B4	4.26	4.06	NPO	NPO
C1	4.30	4.41	NPO	NPO
D1	0.52	0.48	0.21	0.21
E1	4.28	4.18	0.01	NPO

NPO=No peak observed.

tion and detection. Other lower iodinated impurities found in FD&C Red No. 3 were separated as well. The method was found to be precise, accurate and sufficiently sensitive to allow for the quantitation of compounds at levels specified in the Code of Federal Regulations. In addition, the results obtained by CE for several FD&C Red No. 3 samples submitted for certification were in excellent agreement with those obtained using HPLC. As a result, capillary electrophoresis has proven itself as an effective technique capable of performing routine analysis of certifiable colors.

Acknowledgements

I would like to thank Dr. Adrian Weisz for all of the standards used in this work. Each standard was isolated and collected by preparative pH-zone-refining counter-current chromatography, a technique he has successfully developed for color additives [17].

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