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ISOLATION, CHARACTERIZATION AND DETERMINATION OF TRACE ORGANIC IMPURITIES IN FD&C RED NO. 40[a]

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SUMMARY

The unsulfonated aromatic amine 4-nitro-*p*-cresidine (2-methoxy-5-methyl-4-nitrobenzenamine) was identified as an impurity in the regulated color additive FD&C Red. No. 40. The compound was isolated from the water-soluble color by extraction with chloroform, followed by transfer of the free amines to acid solution and subsequent separation by reversed-phase high-performance liquid chromatography. The 4-nitro-*p*-cresidine was collected and then identified by gas chromatography–mass spectrometry. The levels of 4-nitro-*p*-cresidine as well as *p*-cresidine and aniline were determined in commercial batches of FD&C Red. No. 40.

INTRODUCTION

FD&C Red No. 40 is a synthetic water-soluble color that is permitted in the U.S.A. for coloring foods, drugs and cosmetics[1]. A sample from each manufactured batch of the color additive must be submitted to the Food and Drug Administration (FDA) (Washington, DC, U.S.A.) for chemical analysis to certify that the composition of the batch conforms with the chemical specifications published in the *Code of Federal Regulations*[1].

FD&C Red No. 40 is synthesized by coupling diazotized *p*-cresidine sulfonic acid (4-amino-5-methoxy-2-methylbenzenesulfonic acid) with Schaeffer's salt (the sodium salt of 6-hydroxy-2-naphthalenesulfonic acid) as shown in Fig. 1A. Because of impurities in the reactants and side reactions which occur during the manufacture of FD&C Red No. 40, the commercially prepared color is rarely pure. Included in the possible impurities that may contaminate color additives such as FD&C Red No. 40 are unsulfonated aromatic amines arising as unreacted compounds from the manufacturing intermediates. Many unsulfonated aromatic amines, including *p*-cresidine, the most likely amine contaminant of FD&C Red No. 40, have been associated with carcinogenic effects in man and/or animal[2].

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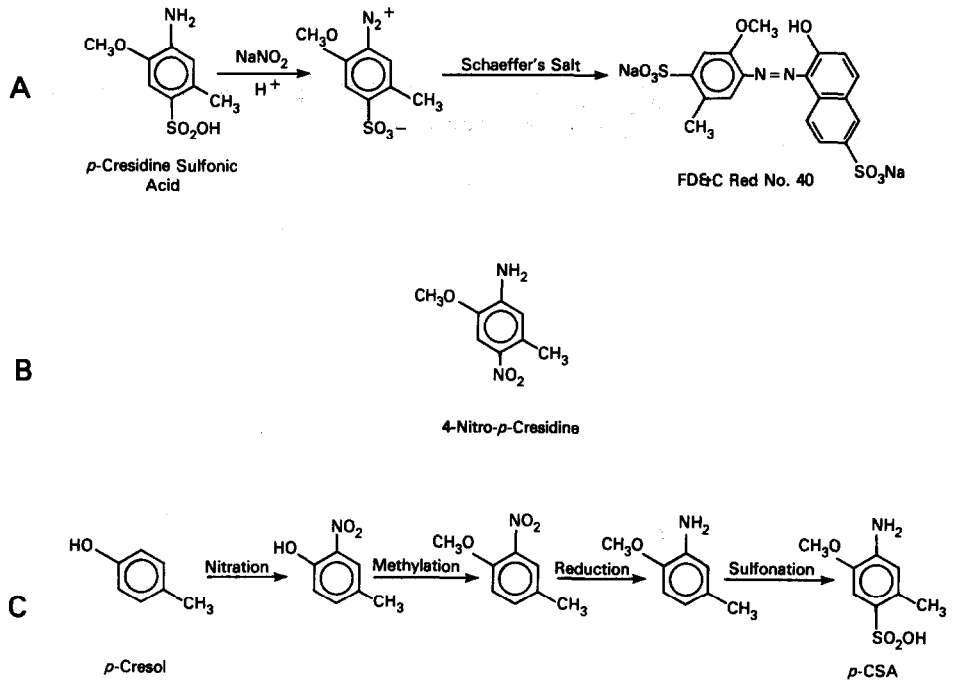

Fig. 1. (A) Manufacturing procedure for synthesis of FD&C Red No. 40. (B) 4-Nitro-*p*-cresidine. (C) Manufacturing procedure for synthesis of *p*-cresidine sulfonic acid.

Our laboratory has reported the development of methodology for the determination of trace levels of unsulfonated aromatic amines in several water-soluble color additives, including FD&C Red No. 40[3–6]. The previous report on FD&C Red No. 40 described the determination of *p*-cresidine and aniline at the ppb[a] level and included the results of a survey that measured the amounts of these impurities in color samples from certified batches. We observed that all the samples analyzed in the survey contained an extractable aromatic amine of unknown identity which, on the basis of instrument response, appeared to be present at much higher levels than the expected impurity (*p*-cresidine).

The work reported here covers the isolation and characterization of this impurity and its identification as 4-nitro-*p*-cresidine (2-methoxy-5-methyl-4-nitrobenzenamine). The impurity was extracted from FD&C Red No. 40, the extract was fractionated by high-performance liquid chromatography (HPLC), and the isolated material was identified by gas chromatography–mass spectrometry (GC–MS). Also described is the determination of 4-nitro-*p*-cresidine, *p*-cresidine and aniline in certified batches of FD&C Red No. 40, by the methodology described in ref. 6.

[a] Throughout this article the American billion (10^9) is meant.

EXPERIMENTAL

Apparatus

HPLC chromatographic system. A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 liquid chromatograph was used with a Perkin-Elmer ISS-100 Intelligent Sampling System and a Waters Assoc. (Milford, MA, U.S.A.) Model 440 dual-channel absorbance detector with 254- and 365-nm filters. A Perkin-Elmer Chromatographics 2 data system (including a Model 3600 data station and a Model 660 graphics printer) was connected to the 2-V output of both detector channels. The output signal corresponded to actual absorbance. Both the 254- and 365-nm chromatograms were recorded at 32 mV (0.032 a.u.f.s.). The data system was programmed to simultaneously plot both detector signals.

HPLC column. A Bio-Sil (5 μm) C_{18} column, 250 mm × 4 mm I.D., was obtained from Bio-Rad (Richmond, CA, U.S.A.).

Extraction columns. Extrelut QE disposable columns, 15 cm × 4 cm I.D. (EM Science, Gibbstown, NJ, U.S.A.), were used for liquid–liquid extractions.

Gas chromatograph–mass spectrometer. A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 5970 mass-selective detector with a Model 236 work station was coupled to a Hewlett-Packard Model 5880 gas chromatograph.

GC column. An HP-5 (Hewlett-Packard) capillary column coated with a cross-linked 5% phenyl methyl silicone stationary phase (25 m × 0.31 mm I.D., 0.52-μm film thickness) was employed.

Reagents

All organic solvents were glass distilled (J. T. Baker, Phillipsburg, NJ, U.S.A., or EM Science). All other chemicals were reagent grade. Water was purified by using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

HPLC eluents. Eluent A was Milli-Q-purified water and eluent B was methanol.

Standard stock solutions. (i) A 102.1-mg portion of analytical-reagent-grade aniline (Mallinkrodt, St. Louis, MO, U.S.A.) was dissolved in 100 ml of methanol. The purity was established from the absorbance of diluted aliquots in water (1-cm cell) at 229 nm (molar absorptivity of aniline in water = 7900[7]). (ii) A 17.0-mg portion of 4-nitro-*p*-cresidine (Pfaltz and Bauer, Westbury, CT, U.S.A.) was dissolved in 100 ml of methanol. (iii) A 21.7-mg portion of *p*-cresidine (Eastman, Rochester, NY, U.S.A.) was dissolved in 100 ml of 95% ethanol. The purities of (ii) and (iii) were established by elemental analysis. Fresh dilutions in water were prepared from the stock solutions before calibration analyses were performed.

Extraction

A 5-g (±0.01 g) test portion of color (Sample E in Table I) was weighed into a beaker and dissolved in approximately 35 ml of warm water containing three drops of 1 *M* sodium hydroxide. The dissolved color was poured onto a dry Extrelut QE column that had been tamped to remove any void spaces. The beaker was rinsed several times with water and the rinsings were poured onto the column. No more than a total of 50 ml of aqueous solution was applied to the column.

After the aqueous solution was allowed to drain into the column for 5 min, a 25-ml portion of chloroform was poured onto the top of the column and a 200-ml

TABLE I

DETERMINATION OF *p*-CRESIDINE, 4-NITRO-*p*-CRESIDINE AND ANILINE IN COMMERCIAL FD&C RED NO. 40 WITH QUANTITATION AT TWO WAVELENGTHS

Sample	*p-Cresidine (ppb)*		*Nitro-p-cresidine (ppb)*		*Aniline*[a] *(ppb)*	
	254 nm	*546 nm*	*254 nm*	*546 nm*	*254 nm*	*436 nm*
A[b,c]	203	203	675	678	125	123
B[c]	24	24	163	166	381	384
C	109	113	626	622	–	–
D	444	445	236	235	7	12
E	146	146	7487	7564	5	8
F	917	923	614	610	27	28
G	60	66	2215	2212	–	–
H	20	13	2192	2195	–	–
I	15	10	960	962	10	12
J	153	153	320	320	22	24
K	13	8	263	263	–	–
L	8	6	1129	1128	–	–
M	7	5	511	512	–	–
N	7	7	773	763	–	–
O	6	6	677	680	–	–
P	14	14	794	790	–	–
Q	260	265	515	518	–	–
R	19	19	871	870	–	–
S	15	16	1159	1164	2	4
T	144	146	387	389	–	–
U	14	12	263	265	–	–
V	4	4	840	838	–	–
W	110	109	228	228	57	62
X	12	12	165	166	5	7
Y	67	71	463	465	–	–
Z	12	9	1860	1850	–	–
AA	8	8	2034	2028	6	6
BB	121	121	523	526	83	84
Average	105	105	1034	1036	26	27

[a] Aniline was determined by using pyrazolone-T as the coupling agent instead of R-salt. Only FD&C Red No. 40 samples which had significant responses for aniline coupled to R-salt at both detection wavelengths were reanalyzed using pyrazolone-T. For each dash, a value of zero was used in the calculation of the average.

[b] The *p*-cresidine and 4-nitro-*p*-cresidine results for samples A–G are averages of multiple determinations. See Table III for details.

[c] Pharmacology samples.

round-bottom flask was placed under the column. A second 25-ml portion of chloroform was added after the first portion had drained into the column. After a 3- to 5-min wait, two more 25-ml portions were added to the column to make a total of 100 ml of chloroform. A 5-ml portion of 0.005 *M* sulfuric acid was added to the chloroform extract collected in the 200-ml round-bottom flask, and the chloroform was removed under aspirator vacuum (*ca.* 11 mmHg) on a rotary evaporator (45°C). Care was taken to ensure that no chloroform droplets remained in the aqueous layer and that the flask was removed from the vacuum as soon as all of the chloroform was gone. Residual chloroform vapors were gently driven from the flask with a 2-min nitrogen purge.

HPLC analysis and isolation of 4-nitro-p-cresidine

The HPLC column was equilibrated with 100% eluent A for 10 min at a flow-rate of 1 ml/min. The pH of the aqueous solution containing the extracted amines was adjusted to between 2 and 3 by adding 1 ml of a 1.5% (w/v) solution of sodium dihydrogenphosphate. A 1- to 2-ml portion of the aqueous solution was transferred to an autosampler vial by using a Pasteur pipet. At the end of the equilibration the autosampler was programmed to inject 200 μl onto the column and to initiate a linear gradient from 0 to 50% eluent B in 2 min, followed by 50–100% eluent B in 10 min. At the completion of the gradient, the column was flushed with 100% eluent B for 5 min, and the column was equilibrated with 100% eluent A to begin the next analysis.

During the gradient analysis, the absorbance display on the 365-nm detector was monitored as the retention time of the compound of interest approached. When the absorbance value began to increase, a 4-ml vial was placed at the detector outlet port tube, and the eluting solvent was collected until the absorbance value was back to the baseline reading. Fourteen injections were made and the 4-nitro-*p*-cresidine was collected each time in the same manner.

The combined isolated amine solution was transferred to a 50-ml round-bottom flask. The methanol was removed under aspirator vacuum (*ca.* 11 mmHg) at 45°C until only about 1 ml of solution remained. A 5-ml portion of water and one drop of 1 *M* sodium hydroxide were added to the flask. The contents of the flask were transferred to a 30-ml separatory funnel for extraction with three 5-ml portions of chloroform. The chloroform extract was saved for GC–MS analysis.

GC–MS analysis

A multilevel program was used to control the oven temperature of the gas chromatograph initially set at 30°C for 1 min; the oven was heated to 120°C at 30°/min and then to a final temperature of 265°C at 8°/min. The injector temperature was 220°C. Helium was used as the carrier gas with a linear velocity of 40 cm/s (measured at final temperature). The column head pressure was 13 p.s.i. The injection volume was 1 μl in the splitless mode. The mass detector was operated in the electron ionization mode at an electron energy of 70 eV, scanning over a mass range of 40–550 daltons.

Determination of aromatic amines in FD&C Red No. 40

The experimental conditions for the determination of aromatic amines in FD&C Red No. 40 were the same as described previously[6]. The standard stock solutions prepared for 4-nitro-*p*-cresidine, *p*-cresidine and aniline calibration are described above.

Samples for the analytical survey described here were selected to reflect the volume of color produced by the various manufacturers. Almost 70% of the samples represented two major producers of FD&C Red No. 40. The other manufacturers were represented by at least one batch of color. Included in the survey were two FD&C Red No. 40 pharmacology samples. These batches were used in conducting the animal feeding studies used by FDA to establish the safety of the color additive. Excluding the pharmacology samples, all the survey samples were certified between 1983 and 1987.

RESULTS AND DISCUSSION

Isolation and identification of 4-nitro-p-cresidine

p-Cresidine, an impurity in the FD&C Red No. 40 intermediate *p*-cresidine sulfonic acid (*p*-CSA), had been considered the most likely unsulfonated aromatic amine in commercial batches of the color additive. However, the determination of *p*-cresidine in a previous survey of FD&C Red No. 40 samples showed that *p*-cresidine was in most cases a minor impurity and that other unsulfonated aromatic amines were present at much higher levels[6]. A major impurity present in nearly all samples analyzed was isolated and identified as 4-nitro-*p*-cresidine (Fig. 1B). The manufacturing procedure for the synthesis of *p*-CSA is presented in Fig. 1C. The presence of 4-nitro-*p*-cresidine in the intermediate may occur if reaction conditions allow nitration at both the 1- and 4-positions on the ring, thereby blocking sulfonation in the 4-position.

An FD&C Red No. 40 sample containing the highest amount of the unidentified material was selected for the preparative work. This batch (sample E in Table I) was estimated to contain 7 ppm of the material (calculated as *p*-cresidine). The chloroform extract of this sample was yellow, whereas the extracts of the majority of FD&C Red No. 40 samples were colorless. It was speculated that the unidentified amine imparted the yellow color; therefore, a 365-nm filter was used for HPLC detection.

An HPLC chromatogram of the acid extract of sample E (Fig. 2) shows one major component eluting at about 11 min. In order to isolate sufficient quantities of this material for identification, repeated injections of the extract were made onto the HPLC column, and the fractions corresponding to the impurity were collected. The material thus isolated was extracted back into chloroform for identification by GC–MS.

The total-ion current profile and the mass spectrum obtained from GC–MS analysis of the isolated material are shown in Fig. 3B. The evaluation of this mass spectrum and the molecular weight of the isolate suggested that the material was 4-nitro-*p*-cresidine. GC–MS analysis of authentic 4-nitro-*p*-cresidine (Fig 3A) yielded an ion fragmentation pattern identical to that obtained for the isolated compound, thus confirming the identification.

To confirm that the 4-nitro-*p*-cresidine collected from sample E corresponded to the unidentified amine coupling product for that sample, a portion of the isolated

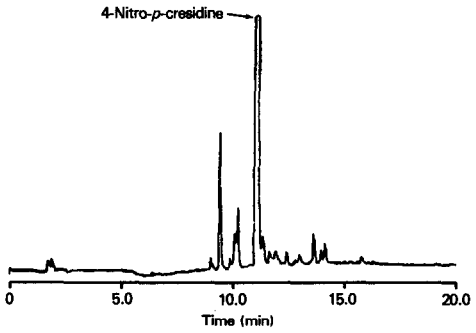

Fig. 2. HPLC chromatogram of an FD&C Red No. 40 acid extract. Detection at 365 nm (0.032 a.u.f.s.).

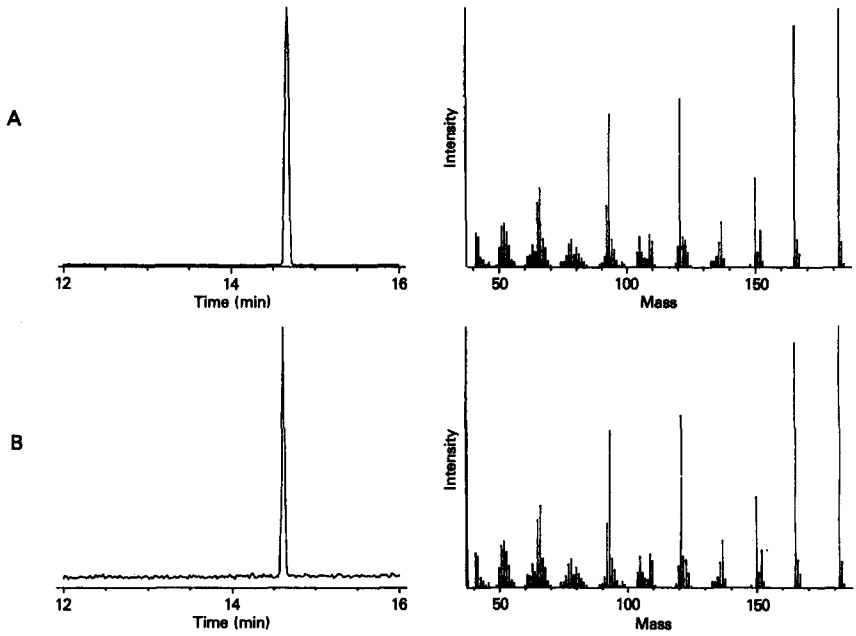

Fig. 3. (A) Total-ion current profile and mass spectrum of 4-nitro-*p*-cresidine standard. (B) Total-ion current profile and mass spectrum of 4-nitro-*p*-cresidine isolated from FD&C Red No. 40.

material was diazotized and coupled to the disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid (R-salt) and analyzed by HPLC under the conditions described in ref. 6. The retention times and peak area ratios of the isolated 4-nitro-*p*-cresidine coupled to R-salt and the unknown amine coupling product matched exactly. In addition, a portion of the 4-nitro-*p*-cresidine standard was diazotized, coupled to R-salt and chromatographed under the conditions described in ref. 6. The retention times and peak area ratios for the derivatives of the authentic material were identical.

Determination of amines

The levels of 4-nitro-*p*-cresidine as well as *p*-cresidine and aniline in certified FD&C Red No. 40 samples were quantitated by using the procedure described in ref. 6. HPLC calibration data were obtained by using the external standard method. The liquid chromatograph was calibrated in the presence of the color to compensate for any matrix effects that might alter the extraction of the aromatic amines from a test solution. For the calibration analyses, separate weighings of color were spiked at several levels with the aromatic amines of interest. The FD&C Red No. 40 used for calibration contained small amounts of the analytes. The peak areas obtained for the unfortified color sample were subtracted from the calibration responses.

The calibration data were evaluated statistically to calculate the regression equation and to evaluate the performance of the method[8]. Summaries of the statistical evaluations of the calibration data for *p*-cresidine and 4-nitro-*p*-cresidine collected over a 2.5-month period are presented in Table II. The calibration data for 4-nitro-*p*-cresidine are excellent and reflect the relatively high concentration levels

TABLE II

STATISTICAL EVALUATION OF CALIBRATION DATA FOR DETERMINATION OF *p*-CRESIDINE, 4-NITRO-*p*-CRESIDINE AND ANILINE IN FD&C RED NO. 40

Amine	n^a	*Calibration range (ppb)*	*Wavelength (nm)*	r^b	$X_{LD}{}^c$ *(ppb)*	*C.V.*[d] *(%)*
p-Cresidine[e]	4	4–42	546	0.9929	4	13
			254	0.9892	5	17
	5	104–417	546	0.9886	42	13
			254	0.9874	45	13
4-Nitro-*p*-cresidine	10	102–5100	546	0.9985	74	7
			254	0.9986	71	6
Aniline[f]	4	5–163	436	0.9995	4	4
			254	0.9994	5	4

[a] Number of calibration points.

[b] Correlation coefficient (serves as a crude indicator of possible non-linearity but is not the parameter of choice for measuring linearity).

[c] Limit of determination at the 95% confidence level.

[d] Coefficient of variation.

[e] The best statistical fit for *p*-cresidine was obtained when the data were divided into sets of high and low range.

[f] Pyrazolone-T was used as the coupling agent instead of R-salt.

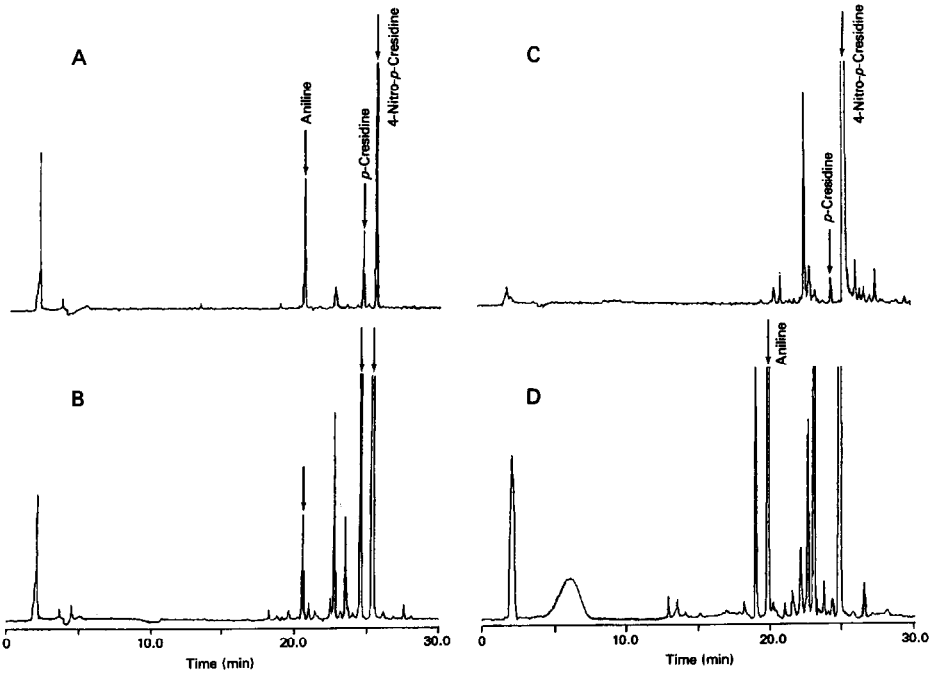

Fig. 4. HPLC chromatograms from analysis of samples of commercial FD&C Red No. 40 by the procedure described in ref. 6. (A) sample B; (B) sample A; (C) sample Z; (D) sample A. Chromatograms A, B and C represent R-salt coupling products. Chromatogram D represents pyrazolone-T coupling products. Detection (A, B, C) at 546 nm; (D) at 436 nm (0.016 a.u.f.s.).

used for 4-nitro-*p*-cresidine. For *p*-cresidine the best statistical fit was obtained when the data were divided into sets of high and low range. Depending on the peak area, the levels of *p*-cresidine in commercial FD&C Red No. 40 were calculated by using either the high or low calibration sets. The calibration data for aniline, collected in one day, were obtained by using pyrazolone-T as the coupling agent instead of R-salt. The wavelengths of measurement were 254 and 436 nm. The 546-nm filter was replaced

TABLE III

MULTIPLE ANALYSES OF COMMERCIAL FD&C RED NO. 40 FOR *p*-CRESIDINE AND 4-NITRO-*p*-CRESIDINE WITH QUANTITATION AT 254 AND 546 nm

Sample	*p-Cresidine (ppb)*		*4-Nitro-p-cresidine (ppb)*	
	254 nm	*546 nm*	*254 nm*	*546 nm*
A[a]	217	220	708	713
	188	187	641	644
Average	203	203	675	678
C.V. (%)	10	12	7	7
B[b]	24	24	178	182
	20	22	153	156
	27	25	158	160
Average	24	24	163	166
C.V. (%)	13	8	8	9
C[c]	112	114	654	645
	106	111	597	598
Average	109	113	626	622
C.V. (%)	4	2	6	5
D[a]	460	458	250	252
	452	454	242	236
	428	430	229	227
	437	436	223	225
Average	444	445	236	235
C.V. (%)	3	3	5	5
E[b]	145	142	7448	7534
	148	150	7526	7595
Average	146	146	7487	7564
C.V. (%)	1	4	1	1
F[b]	1086	1095	657	656
	1042	1053	621	626
	622	621	564	547
Average	917	923	614	610
C.V. (%)	28	28	8	9
G[b]	69	76	2199	2192
	50	53	2202	2201
	61	67	2244	2244
Average	60	66	2215	2212
C.V. (%)	16	18	1	1

[a] Analyzed over a 9-week period.
[b] Analyzed over a 6-week period.
[c] Analyzed over a 4-week period.

because 436 nm is closer to the absorption maximum for the aniline–pyrazolone-T coupling product.

A total of 28 certified samples of FD&C Red No. 40 were surveyed for 4-nitro-*p*-cresidine, *p*-cresidine and aniline. The results of the analyses of the color additive are presented in Table I. Representative chromatograms obtained from the analysis of four survey samples are shown in Fig. 4.

4-Nitro-*p*-cresidine was found in all 28 of the samples surveyed at an average level of 1035 ppb with a range of 165 to 7526 ppb. *p*-Cresidine was found in all the samples at an average level of 105 ppb with a range of 4 to 920 ppb.

HPLC responses for aniline were observed for many samples; however, there usually was poor agreement between the results for the two different wavelengths, suggesting an interfering coeluting compound. In order to resolve the interference, pyrazolone-T was used as an alternative coupling agent in place of R-salt to change the chromatographic properties of the coupling products. The samples that had measurable aniline responses with R-salt coupling were reanalyzed after pyrazolone-T was used as the coupling agent. Aniline was found at an average level of 26 ppb with a range of 0 to 383 ppb. The FD&C Red No. 40 batches that were not reanalyzed after pyrazolone-T coupling were reported as having no aniline present.

The repeatability of the amines determination was tested by performing multiple analyses (R-salt coupling) on several samples of FD&C Red No. 40 (Table III). The calculated % coefficient of variation (C.V.) values for 4-nitro-*p*-cresidine were all below 10%, indicating good repeatability at the levels tested. Although the C.V. values for *p*-cresidine were higher than those for 4-nitro-*p*-cresidine, they were comparable with values found previously[6].

CONCLUSIONS

4-Nitro-*p*-cresidine has been isolated and identified as a trace-level impurity in FD&C Red No. 40 by using preparative HPLC and GC–MS. The levels of 4-nitro-*p*-cresidine as well as those of *p*-cresidine and aniline were determined in commercial batches of the color additive. The method employed allows quantitation of these aromatic amines at levels greater than or equal to 5 ppb. Good repeatability of the method has been demonstrated.

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