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The structure and purity of a reference dye standard used for quantification of C.I. Solvent Red 164 in fuels

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ABSTRACT

This study characterizes a primary reference dye standard, certified as being 99% C.I. Solvent Red 26, that is used in the United States to identify tax-free fuel, using nuclear magnetic resonance spectroscopy, thin-layer chromatography, gas chromatography/mass spectrometry and high-performance liquid chromatography/mass spectrometry. It was found that 72% of the primary reference dye is equivalent to National Institute of Standards and Technology Standard Reference Material 2037 (C.I. Solvent Red 24). Several of the impurities were tentatively identified and a number of additional impurities were partially characterized. Possible explanations and implications are briefly discussed.

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1. Introduction

The industrial preparation of azo dyes dates back to the 1870s [1], well before the advent of analytical tools for determining product composition. Commercial azo dyes often consist of a mixture of synthetic products, by-products, intermediates, and unreacted starting materials. Components in these mixtures may vary widely in their volatility, polarity, and molecular weights [2]. Even dyes that are purportedly pure are occasionally found to contain substantial amounts of impurities [3]. Dye compositions are difficult to completely characterize due to the broad range of possible products and, therefore, require several different analytical techniques, each with their unique capabilities, to obtain complete characterization. The work described herein applies to a number of diverse analytical approaches to characterize the composition of *Morton Red 26*, hereafter referred to as *Red 26*, a reference dye used in the United States to quantify the amount of dye added to tax-free fuel.

C.I. Solvent Red 164 is a commercial dye that is added to off-road diesel fuel in the United States to identify its tax-free status. Federal regulations dictate the amount of C.I. Solvent Red 164 added to off-road diesel be spectrally equivalent to 11.1 mg/L of C.I. Solvent Red 26 (Fig. 1a) [4]. Fortification at this concentration is verified by a visible absorbance spectroscopic method that references a standard curve prepared with *Red 26*, a dye that is certified by the manufacturer as 99% C.I. Solvent Red 26. Researchers have assumed the equivalency of *Red 26* and C.I. Solvent Red 26 based on this product certification; however, this identification has not been experimentally verified. Recently, a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) (#2037, Chemical Abstract Service [CAS] #85-83-6) became available that has the structure shown in Fig. 1b (also commonly known as C.I. Solvent Red 24). C.I. Solvent Red 164 diesel fuel dye is a mixture of dyes with similar structures to those shown in Fig. 1 except they contain additional alkyl chains that enhance solubility in diesel fuel [4].

Quantification of C.I. Solvent Red 164 in fuel is performed by second derivative visible absorbance spectroscopy [4–6] referencing a standard curve prepared with *Red 26*. This determination assumes similar spectral properties between *Red 26* and C.I. Solvent Red 164 (both wavelength maximum and molar extinction coefficient), as well as use of pure *Red 26* for preparation of calibration standards. During the course of various studies at Pacific Northwest National Laboratory (PNNL), it was observed that equivalent gravimetric concentrations of *Red 26* absorbed approximately 25% less than NIST SRM 2037. These studies are described in more detail

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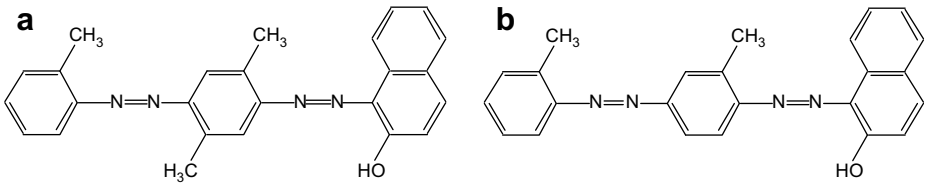


Fig. 1. Reported chemical structures for a) C.I. Solvent Red 26 (MW = 394 amu) and b) NIST SRM 2037 (MW = 380 amu) also known as C.I. Solvent Red 24.

below. A previous technical report also noted similar absorbance discrepancies [7]. The most likely explanation for this discrepancy was that the *Red 26* was not pure, despite its certified 99% purity.

The objective of this study was to compare the chemical structures and purity of NIST SRM 2037 [8,9] and *Red 26* [4]. This work documents that *Red 26* is actually C.I. Solvent Red 24, not C.I. Solvent Red 26 as expected from the certification. Once the dye chemical identity was firmly established, a range of analytical techniques was applied to determine the purity of the dyes with the objective of resolving the absorbance discrepancy noted above. The analytical techniques applied ranged from widely available traditional methods to state-of-the-art instrumental approaches. Multiple analytical techniques were necessary since purity determinations were subject to analytical biases inherent to each technique and this resulted in varying apparent purities. Although techniques giving outlying purity values can provide important information about the impurities contained in the sample, confidence in the actual bulk composition can only be gained by obtaining converging values obtained from diverse methodologies. A minor objective of these studies was to characterize and identify impurities in the dyes.

2. Experimental

2.1. Dye materials

Red 26, lot number A1006-2, was supplied by Morton International Specialty Products (Paterson, NJ, USA). This dye was certified in 1994 by the manufacturer as 99% active C.I. Solvent Red 26. Upon receipt of *Red 26* at PNNL in 2003, the dry material was stored in a desiccator in the dark. Prior to arrival at PNNL, the *Red 26* powder was stored in a glass vial contained within a cardboard mailing tube that was further enclosed by a sealed plastic bag, as originally supplied by the manufacturer. C.I. Solvent Red 24 was obtained from NIST as SRM #2037. NIST certified this product in 2005 as 98% C.I. Solvent Red 24, with a 95% uncertainty interval of 92–100%.

2.2. Visible absorbance spectrophotometry

Dye quantification was performed according to the American Society for Testing and Materials (ASTM) Method D6258 [4]. This method uses the difference between the maximum and minimum second derivative peaks found in the ranges of 538 ± 20 and 561 ± 20 nm. An Agilent (Santa Clara, CA, USA) Model 8453 diode array spectrophotometer interfaced with an Agilent Model G1811A XY sampler was used for these studies. Spectra were smoothed using a filter length of fifteen and a polynomial degree of two.

2.3. Insoluble matter

Insoluble matter was removed from concentrated dye stock solutions by processing through 0.5-μm Millex-LCR filter units (Millipore, Billerica, MA, USA).

2.4. Inorganic content

A *Red 26* sample in kerosene (121 mg/L) was diluted by a factor of ten with xylenes (semiconductor grade, Alfa Aesar, Ward Hill, MA, USA). Inductive coupled plasma/mass spectroscopy (ICP/MS) analysis was performed by directly aspirating the diluted solution into the spray chamber of an Agilent Model 7500C ICP/MS. To cover a broad range of elements, the Agilent semiquantitative multiple element scan was performed, an approach that typically gives concentration values accurate to within ±20%. This analysis involved bracketing a sample between calibration standards that contained 19 elements.

2.5. Water content

Dyes were subjected to rigorous drying conditions by placing them under vacuum in the presence of phosphorus pentoxide.

2.6. Liquid chromatography/mass spectrometry (LC/MS) analysis

LC/MS was performed using an Agilent 1100 series LC/mass selective detector (MSD). Dye samples (10-μL aliquots in ethyl acetate) were analyzed by normal-phase high-performance liquid chromatography (HPLC) on a Zorbax amino column (150×2.1 mm i.d., $d_p = 5$ μm, obtained from Agilent). Separations were accomplished using an isocratic hexane:methylene chloride (80:20, v:v) mobile phase delivered at 0.5 mL/min. Detection of the separated components was either by photodiode array or atmospheric pressure chemical ionization (APCI) mass spectrometry (MS). APCI conditions used a 60 psi nebulizer pressure with a drying gas flow of 4 L/min at 325 °C, and a 350 °C vaporizer temperature. The capillary was adjusted to a 4 kV potential which resulted in a 4-μA current.

2.7. Proton nuclear magnetic resonance (NMR) analysis

Samples were gravimetrically prepared by weighing either 11.0 mg NIST SRM 2037 (C.I. Solvent Red 24) or 10.7 mg *Red 26* dye and bringing to 10.0 mL with deuterated chloroform. This resulted in 2.89 mM and 2.82 mM concentrations for the NIST SRM and *Red 26*, respectively (based on the assumption that *Red 26* is pure and has the same molecular weight as the NIST SRM). Methanol was added as an internal standard to 5 mL of each dye solution at a concentration of 2.47 mM. Estimated dye concentrations were based on integrated proton resonances referenced to the methanol internal standard signal. Approximately 600 μL of each dye, with and without the methanol reference, was transferred into silylated, 5-mm o.d. × 178 mm high precision NMR tubes (542-PP-7, Wilmad, Buena, NJ, USA) and sealed with Teflon caps. Spectra were collected at 20 °C at a ^{1}H resonance frequency of 800 MHz on a Varian Inova spectrometer (Palo Alto, CA, USA) equipped with a triple resonance cryoprobe and pulse-field gradients. All spectra contained 16,000 data points collected with a 15 s delay to allow full relaxation between transients and a more accurate estimation of product

concentrations using resonance peak area integration methods [10]. Such a protocol is a well-established, highly reproducible method of estimating the concentration of compounds in solution with a typical standard error of approximately ±3% [11]. Data were processed and analyzed with Felix software (1998 version, MSI, San Diego, CA, USA). All chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS, $\delta = 0$ ppm) using indirect methods [12].

2.8. GC and GC/MS

Dyes were analyzed by gas chromatography (GC) using cool on-column injection for sample introduction with detection by either flame ionization or MS. A 15 m × 320-μm i.d. fused silica capillary coated with a 0.1-μm film of DB-5 HT was used with helium carrier gas. Separations were accomplished by a temperature program that ramped from 200 °C to 375 °C at 20 °C/min with a 5 min hold at 375 °C. An Agilent 6890N gas chromatograph coupled with a 5973 inert source MSD was used for detection of the separated components.

2.9. Thin-layer chromatography (TLC)

Dyes were separated by TLC [13,14] on silica gel GHLF plates (Analtech, Newark, DE, USA) using toluene as a mobile phase. Initial experiments were performed on standard analytical plates that contained a 250-μm layer of silica mixed with a fluorophore that allowed compound visualization by fluorescence quenching. An electronic image of the TLC plate was generated using a flatbed scanner. The resulting grayscale image was then integrated using un-scan-it gel software (Silk Scientific, Orem, UT, USA) to provide an estimate of the material contained in each spot.

High-performance TLC (HPTLC) used 10 × 10 cm HPTLC plates (HLF type with pre-adsorbent zone, Analtech) that contained a 150-μm silica layer of narrow distribution 8-μm silica particles. Separation used two sequential developments with toluene or a single development with toluene:ethyl acetate (95:5, v:v).

Silica GF plates (Analtech) with a pre-adsorbent zone and a 1000-μm layer of silica were used for preparative-scale separations. Samples were streaked on the plate and produced colored zones after development. Zones were then scraped from the plate, extracted with ethanol, the extract filtered through a plug of silanized glass wool followed by a Millex-LCR 0.5-μm filter, and then reduced to dryness with a stream of dry nitrogen. Fractions were reconstituted in either 2.0 mL of ethyl acetate [for analysis by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS)] or 0.75 mL of deuterated chloroform (for analysis by NMR). For analysis of TLC fractions by NMR, the extracted material was dissolved in the same $CDCl_3$ that was used for characterization of NIST SRM and *Red 26* dyes. NMR spectra of the isolated fractions were collected under a similar set of instrumental conditions at a ^1H resonance frequency of 800 MHz as previously discussed.

3. Results and discussion

3.1. Visible absorption spectrophotometry

The ASTM second derivative absorbance method for analysis of C.I. Solvent Red 164 in diesel fuel is based on referencing a standard curve prepared with C.I. Solvent Red 26 [4]. *Red 26* is used as the source of C.I. Solvent Red 26 based on the product certification that states the dyes are equivalent. All three dyes have absorbance maxima near 512 nm and quantitative information is based on the maximum and minimum second derivative peak differences found in the 538 ± 20 and 561 ± 20 nm ranges. Derivative methods are commonly used for samples that have a broad absorbance background [5,6].

Numerous studies conducted at PNNL show gravimetrically equivalent solutions of *Red 26* absorb approximately 25% less than corresponding solutions of NIST SRM. In an initial study, dyes were analyzed in duplicate and quantification performed by reference to a standard curve prepared with *Red 26*. From the second derivative peak differences, a response factor was calculated for each dye by dividing the difference of the second derivative absorbance minima and maxima by the gravimetric concentration. Results indicated that *Red 26* had a response factor ($5.511 \times 10^{-5} \pm 0.1\%$ L/mg) that was 26% lower than that calculated for NIST SRM ($6.928 \times 10^{-5} \pm 7\%$ L/mg), implying a purity of only 74%. Further studies that involved larger sample sets produced similar results showing that equal gravimetric concentrations of *Red 26* absorbed only 72.9% of the NIST SRM. In additional studies, dyes ranging in concentration from 7.7 to 13.0 mg/L were analyzed and yielded response factors of $(5.6 \pm 0.06) \times 10^{-5}$ L/mg and $(7.2 \pm 0.24) \times 10^{-5}$ L/mg for *Red 26* and NIST SRM, respectively. Stated differently, the response factor for *Red 26* dye was 78 ± 3% of the NIST SRM. In yet another study, 16 dye samples were prepared at gravimetric concentrations between 0.499 and 42.1 ppm and compared to concentration values calculated by referencing a *Red 26* dye standard curve. The *Red 26* samples gave an average relative deviation from the *Red 26* gravimetric value of +2.2% ($n = 16$), whereas the NIST SRM samples gave a relative deviation of +30.1%. These results again indicated the *Red 26* dye purity was approximately 72.1%. These observations are consistent with a previous technical report that found the absorbance of *Red 26* was 24–34% lower than a NIST-purified C.I. Solvent Red 24 standard (a precursor material to the NIST SRM 2037) [7].

The most straightforward explanation for these results was that the *Red 26* was not pure. These studies indicated the likely purity of *Red 26* was in the range of 72–78%, not the expected 99%.

3.2. Insoluble material, inorganics, and water content

To investigate the absorbance discrepancy the dye samples were analyzed for insoluble material, inorganics, and water content. Concentrated stock solutions were passed through a 0.5-μm filter prior to dilution and analyzed by second derivative visible spectrophotometry. It was observed that filtration had no effect on the calculated concentration values and, therefore, particulate matter greater than 0.5-μm was not present. *Red 26* was also analyzed by ICP/MS to determine inorganic content. ICP/MS analysis did not identify metal concentrations above the PNNL threshold criterion (10 times the kerosene background) for any of the 19 tested elements. Rigorous drying under vacuum in the presence of phosphorous pentoxide did not reduce the dye weights indicating the dyes were anhydrous. In conclusion, the presence of insoluble material, inorganics, or water can be ruled out as causative factors for the absorbance disparity.

3.3. HPLC/MS studies

An HPLC separation (512 nm absorbance) of a NIST SRM 2037 (C.I. Solvent Red 24) sample is shown in the top of Fig. 2. This chromatogram features one symmetrical peak eluting with a retention time of 4.84 min. The visible spectrum of this component confirms an absorbance maximum of 512 nm. The APCI mass spectrum of the peak at 4.84 min features an ion at 381 atomic mass units (amu). Since APCI MS generally yields mass spectra that contain M + 1 ions, this study confirms the expected molecular weight (MW) of 380 amu [15] (see Fig. 1b).

HPLC analysis of the *Red 26* dye is shown in the center of Fig. 2. The principal component has an identical retention time (4.84 min)

as observed for NIST SRM 2037. This material accounted for about 93% of the sample (based on the integrated area of the peak at 4.84 min compared to the sum of the peak areas at 2.69, 3.59, and 4.84 min in the 520-nm trace) and had a visible absorbance spectrum with a maximum at 512 nm. Fig. 2 (bottom) shows the APCI mass spectrum of the principal component (MW of 380 amu). This result was unexpected since, according to the structure listed in the ASTM procedure [4] the *Red 26* should have an additional ring methyl group, and hence a larger molecular weight (394 amu). Extracted ion current chromatograms of $m/z = 395$ gave no indication of a 394 amu component. The retention time match, along with identical mass and visible absorbance spectra between the NIST SRM 2037 and the *Red 26* major components, indicates the principal constituent of these dyes are identical and that the major component in *Red 26* is actually C.I. Solvent Red 24.

The HPLC chromatogram of *Red 26* also contained two minor hydrophobic impurities with retention times of 2.69 ($\lambda_{max} =$ 560 nm, blue colored) and 3.59 ($\lambda_{max} = 482$ nm, orange colored) min. Based on absorbance detection at 520 nm, the component eluting at 2.69 min consisted of roughly 5% of the material and had a mass spectrum that indicated a MW of 429 amu. The material eluting at 3.59 min made up about 2% of the sample, however, the quantity was insufficient to obtain a mass spectrum.

HPLC with detection at 520 nm likely overestimates the *Red 26* purity due to molar extinction coefficient differences between the impurities and the principal dye component combined with impurity wavelength maxima that are far removed from the detection wavelength. A consequence of a poor detection wavelength match is low absorbance for the impurities and a corresponding higher calculated purity value for *Red 26*. An additional contributing factor is that highly polar impurities will be strongly retained on the amino column and, therefore, not eluted under isocratic HPLC conditions.

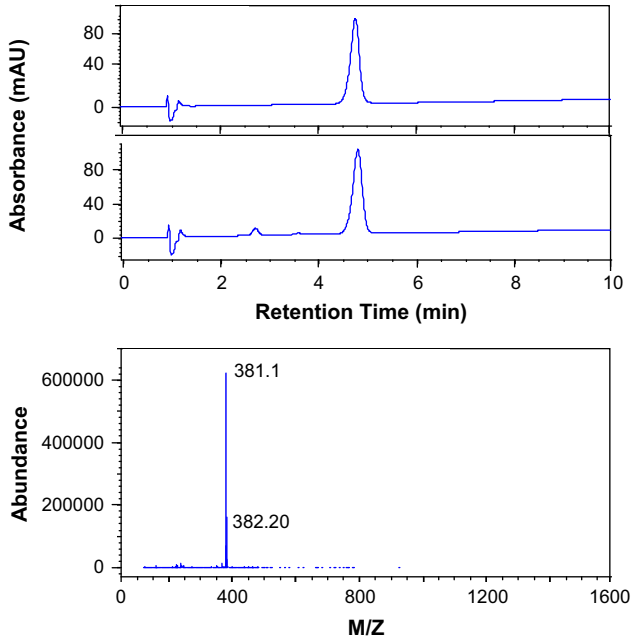


Fig. 2. Results from the HPLC/APCI MS studies. Chromatograms that compare the NIST SRM standard to *Red 26* are shown in the top and middle panels, respectively. The bottom panel is the APCI MS of the principal component in *Red 26* that elutes with a retention time of 4.84 min. The spectrum is identical to that obtained from the NIST SRM.

3.4. Proton NMR

The upfield (6.0–0.0 ppm) and downfield (17.0–6.0 ppm) regions of the ^{1}H spectrum of NIST SRM 2037 and *Red 26* are compared in Figs. 3 and 4, respectively. The two resonances between 2.6 and 2.9 ppm in Fig. 3 are due to the ring methyl groups. Importantly, the spectra for *Red 26* and NIST SRM show the same methyl substitution. The expected structure for *Red 26* (Fig. 1a) would give three distinct resonances in this spectral region, not the observed two. Another important observation is that the spectrum for NIST SRM 2037 is due to one chemical species. Spectral assignments arrived at by deuterium exchange, ^{1}H–^{1}H correlation spectroscopy (COSY) [16] and ^{1}H–^{1}H total correlated spectroscopy (TOCSY) [17,18] experiments corroborate the chemical structure of NIST SRM as shown in Fig. 1b. The proton chemical shifts and assignments for NIST SRM are summarized in Table 1. The bulk chemical purity of NIST SRM 2037 is established by the correct assignment of all resonances and the near absence of additional impurity signals.

To further study the purity of NIST SRM 2037 the intensities of the impurity resonances were compared to the one-bond ^{1}H–^{13}C *J*-couplings (~160 Hz) due to naturally abundant carbon-13 in the 6.8–9.3 ppm region [19,20]. Since the natural abundance of ^{13}C is ~1.1% and the concentration of NIST SRM is 2.89 mM, the integrated intensity of the ^{13}C-satellite resonances corresponds to a concentration of 0.015 mM. Smaller impurity resonances are present in this region that reflect less than ~0.5% of the sample.

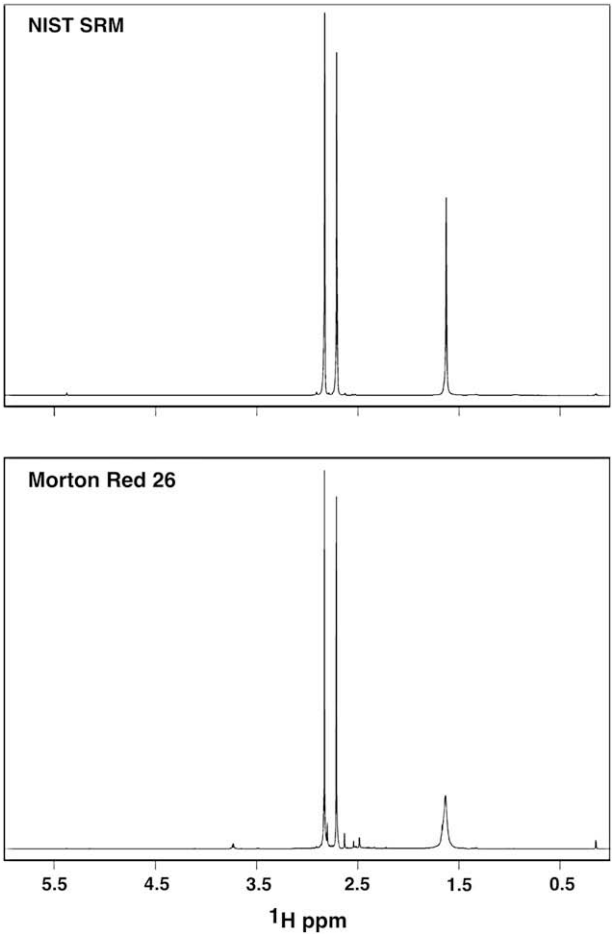


Fig. 3. Comparison of the upfield ^{1}H NMR spectrum of NIST SRM (top) and *Red 26* (bottom).

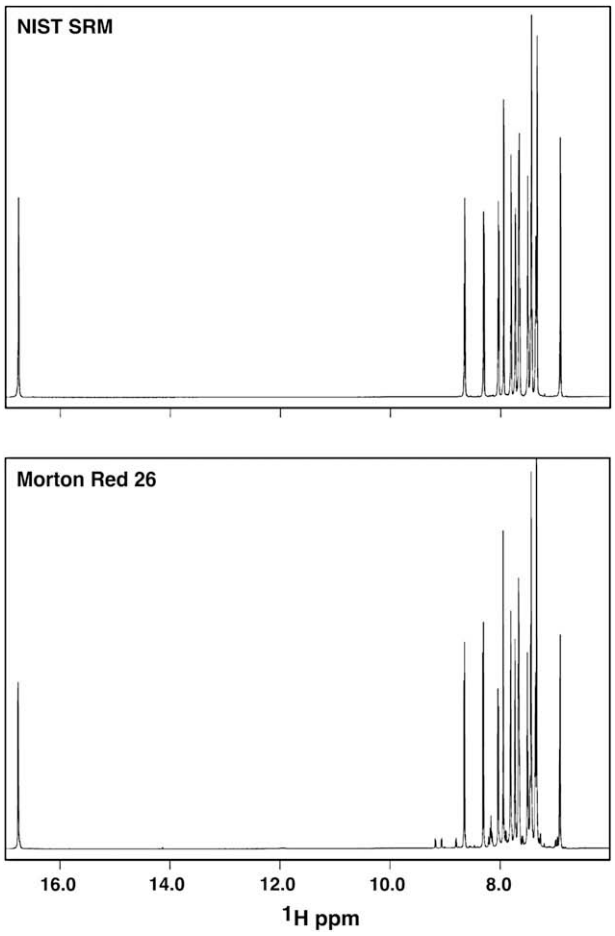


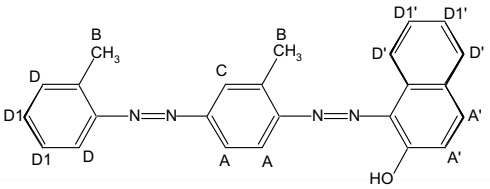
Fig. 4. Comparison of the downfield ^{1}H NMR spectrum of NIST SRM (top) and *Red 26* (bottom).

NMR comparison of *Red 26* and NIST SRM 2037 (Figs. 3 and 4) show the major resonances of *Red 26* are identical to those of the pure NIST SRM. This observation is in strong agreement with other spectral data that indicate the major component in *Red 26* is NIST SRM 2037 (or C.I. Solvent Red 24). Examination of the expanded aromatic region in the *Red 26* spectrum shows numerous non-NIST SRM resonances that likely represent chemically modified aromatic dye impurities.

The furthest downfield resonance in the NIST SRM 2037 spectrum at 16.75 ppm is due to the azo-phenol/quinone–hydrozone tautomeric proton [8,9,21]. Assignment of this resonance was assisted by a deuterium chase (exchange) experiment [22] where 100-μL D_2O was added directly to the NMR tube containing 600 μL of ~6.0 mM *Red 26* dissolved in $CDCl_3$. The tautomeric proton resonance almost completely disappeared within an hour after the addition of the D_2O, indicating that the proton is readily exchangeable. A new resonance at 4.75 ppm appeared during the exchange process that was due to the formation of HDO following exchange with D_2O [23]. Detailed analysis of the 17.0–16.0 ppm region revealed that *Red 26* contained a shoulder on the upfield side of the major tautomeric proton resonance (16.72 ppm) in addition to a few very small resonances (*e.g.*, 16.56 ppm) suggesting that the chemical structures of these impurities also contain similar tautomeric protons.

Further evidence of impurities in *Red 26* was present in the upfield aliphatic region (Fig. 3). A number of small resonances were observed in *Red 26* and likely reflected either low-molecular-weight aliphatic impurities or aromatic compounds with aliphatic groups attached to them.

Table 1
Proton chemical shifts for NIST SRM at 20 °C in $CDCl_3$. Proton assignments are listed on the dye chemical structure.



Chemical shift (ppm)	Coupling pattern	Assignment
1.63	Singlet	Water, H_2O
2.71	Singlet	Methyl group B
2.83	Singlet	Methyl group B
6.91	Doublet	Aromatic A′
7.33	Singlet	Chloroform, $CHCl_3$
7.36	Doublet	Aromatic D
7.43	Triplet	Aromatic D1′
7.44	Triplet	Aromatic D1′
7.50	Doublet	Aromatic D
7.66	Triplet	Aromatic D1
7.67	Triplet	Aromatic D1
7.73	Doublet	Aromatic D′
7.81	Doublet	Aromatic A′
7.94	Singlet	Aromatic C
8.04	Doublet	Aromatic A
8.30	Doublet	Aromatic A
8.65	Doublet	Aromatic D
16.75	Singlet	Tautomeric proton

To estimate relative purity, dye samples were prepared with a 2.47 mM methanol spike. Silylated glassware was used for volumetric transfers, sample storage, and NMR analysis as a precaution against methanol loss. The methanol methyl resonance was compared to the proton resonance from residual $CHCl_3$ (7.33 ppm) present in the $CDCl_3$ to verify equal spike concentrations between *Red 26* and NIST SRM samples. The 5% relative variance in the calculated concentration of $CHCl_3$ in the $CDCl_3$ (0.53 ± 0.03 mM) confirmed equimolar methanol spikes. Given that NMR, as well as other spectral studies, indicated that NIST SRM 2037 was greater than 99% pure, the purity of *Red 26* based on reference to the methanol spike was only 62%, a value that was lower than previously seen with visible absorbance spectrophotometry, HPLC analysis, or TLC determinations (see below). Purity estimates based on the proton NMR analysis of individual TLC fractions separated from *Red 26* (TLC separation and NMR analysis are described below) were calculated to provide further clarification. The methanol reference method described above could not be used to estimate concentrations in TLC fractions since methanol was not added to these samples. However, by referencing the residual $CHCl_3$ as described below, it was possible to estimate the concentrations of the major components in each TLC fraction.

3.5. GC/MS studies

A total ion current chromatogram of NIST SRM resulted in a single peak with a retention time of 19.64 min. The mass spectrum of this compound matched a NIST library reference spectrum for C.I. Solvent Red 24. Injection of *Red 26* gave a principal peak with the same GC retention time (19.64 min) and identical mass spectrum as observed for the NIST SRM. This match provides further validation that both dyes have the same principal components. GC analysis of *Red 26* with flame ionization detection (FID) also

revealed three minor impurities that eluted at 8.03, 12.97, and 19.75 min. Integration of the impurity peaks gave a purity (by area percent) for *Red 26* of 97%; however, this value is misleading since low volatility of the NIST SRM makes this dye borderline applicable for GC analysis. Synthetic product impurities having lower volatility (for example, compounds with high polarity or molecular weight) or lower thermal stability will not be observed during GC analysis. Therefore, the high purity value indicated by GC analysis is more a consequence of the impurities in the mixture being incompatible with GC analysis, and cannot be used as an indication of product purity. It should be noted that cool on-column injection was necessary to avoid thermal decomposition of NIST SRM on the hot surfaces of the injector port, an observation that illustrates these dyes are thermally labile and difficult to successfully analyze by GC [24]. A similar experiment determined *Red 26* purity from examining total ion current chromatograms. These experiments found an area percent purity of 93% and a 75% purity based on the response factor relative to a NIST SRM standard. The area percent purity is similar to the value obtained using FID detection. Purity determined based on relative response was comparable to values obtained using other analytical approaches.

Identification of the first two eluting impurity peaks was possible by obtaining high quality mass spectral matches with reference library spectra. These identifications were tentative since authentic reference standards were not available to verify retention times and mass spectra. The first compound to elute (retention time of 8.03 min) was tentatively identified as *m*,*m*′-dimethylazobenzene (MW = 210 amu). The structure of *m*,*m*′-dimethylazobenzene is shown in Fig. 5a. The second compound (retention time of 12.97 min) was 1-[(2-methylphenyl)azo]-2-naphthalenol (MW = 262 amu) with the chemical structure shown in Fig. 5b. Both components (or related isomers) are structurally related to the principal dye product and logically could be present in the synthetic starting materials, or arise as by-products from dye synthesis. The third impurity did not give a spectral library match; however, the mass spectrum indicated a molecular weight of 434 amu and featured loss of 91 amu, a fragment loss that was also observed in the spectrum of NIST SRM (diagnostic for methylazobenzene), suggesting the unknown structure is structurally related to the SRM.

3.6. TLC studies

3.6.1. Standard analytical TLC

Analytical TLC separations of NIST SRM 2037 gave a single spot, whereas separation of *Red 26* yielded five visible spots (three major components). The principal component in *Red 26* gave an identical migration as the NIST SRM 2037. The R_f values for the five spots, along with observations on spot color and intensity are summarized in Table 2. This table also contains corresponding identifications for preparative-scale TLC zones that are discussed below in a later section. The R_f calculations were based on migration distance measurements starting at the pre-adsorbent/sorbent interface since the pre-adsorbent zone was not retentive. Spot integration of the *Red 26* TLC plate indicated the principal component ($R_f = 0.09$) made up 73% of the sample, a value that closely agrees with the purity determined by visible absorbance spectrophotometry. The other major components in this mixture, an orange component ($R_f = 0.14$) that was not completely resolved from the principal red spot and a highly mobile blue spot ($R_f = 0.60$), each accounted for 12% of the sample. The blue component was likely responsible for the darker coloration of equal gravimetric concentrations of *Red 26* compared to the NIST SRM. The remaining 3% of the material was located between the application point and the principal component. It is likely that the major blue and orange impurities correspond to the compounds previously observed during HPLC analysis. Quantitative measurements were based on several assumptions; paramount among these was the assumption that integration of the grayscale spot intensity was directly proportional to the amount of material present.

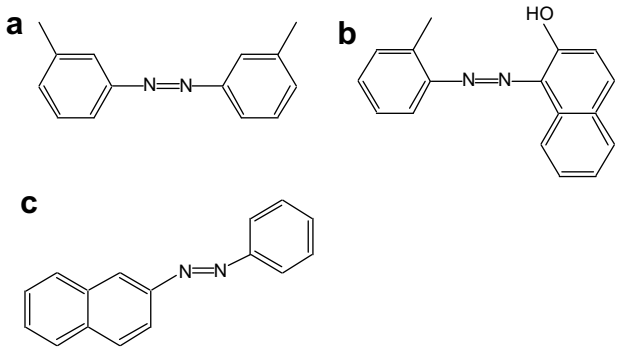


Fig. 5. Structures of tentatively identified impurities in *Red 26*. Compounds are: a) *m*,*m*′-dimethylazobenzene (MW = 210 amu); b) 1-[(2-methylphenyl)azo]-2-naphthalenol (MW = 262 amu); and c) 2-naphthalenylphenyldiazene (MW = 232 amu).

3.6.2. HPTLC studies

Further TLC development was undertaken to provide a rapid high-resolution separation for components in *Red 26*. Initial separations used two sequential developments with toluene (5 min each) with an interim drying period. This resulted in complete resolution of the red ($R_f = 0.29$) and orange dye ($R_f = 0.36$) components, with the blue spot migrating with an R_f of 0.84. Quantification using this method gave a purity value for *Red 26* of 78%. The orange and blue components accounted for 3 and 16% of the sample with 3% of the material appearing between the origin and the red spot.

Further experiments investigated a mobile phase (toluene:ethyl acetate, 95:5, v:v) that provided complete component resolution with a single solvent development. The R_f values for this development solvent system were 0.40, 0.51, and 0.64 for the principal red, orange, and the blue components, respectively. Although quantification was not performed with this solvent system, the results are presented here since compound resolution was adequate and a substantial savings in time was achieved by using a single solvent development.

3.6.3. Preparative-scale TLC

A photograph of the preparative-scale TLC separation of *Red 26* is presented in Fig. 6. The thick silica layer facilitated application of large samples on these plates without losing resolution due to sorbent overloading. Dye components appeared as bands rather than spots since the material was streaked rather than spotted. TLC

Table 2
TLC spot mobility and appearance. The R_f values reported are for standard analytical plates developed with toluene. The preparative-scale TLC zone identifications are slightly different from the analytical TLC spot numbers (see text for further clarification).

TLC spot (prep zone)/characteristic attribute	R_f value	Color	Intensity
(Prep zone 1)	–	Red	Faint
Spot 1 (prep zone 2)	0.09	Red	Intense
Spot 2 (prep zone 3)	0.14	Orange	Moderate
Spot 3 (prep zone 4)	0.20	Red	Faint
Spot 4 (prep zone 4)	0.26	Red	Faint
Spot 5 (prep zone 5)	0.60	Blue	Intense

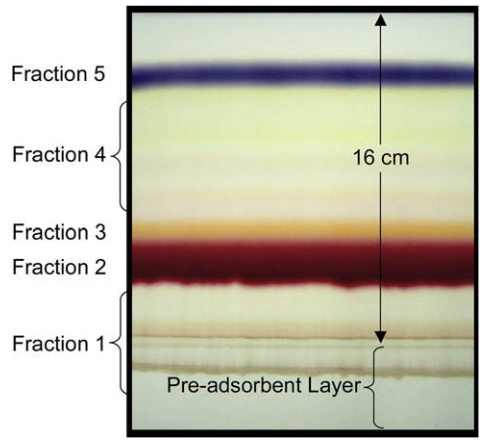


Fig. 6. Photograph of a preparative-scale TLC separation of *Red 26*. Separation was obtained using toluene as a mobile phase. The TLC separation shows the dye has numerous impurities. Fraction 2 was shown to be identical to the NIST SRM using a variety of analytical techniques. The major impurities are present in TLC fractions 3 and 5.

fraction analysis was advantageous because the isolated fractions had a simplified composition with concentrations that could exceed those in the *Red 26* mixture. TLC fraction 1 was a composite of compounds located from the sample origin up to the major red component. Fraction 2 contained the principal red dye component and was the most intense band on the TLC plate. TLC fraction 3 was an orange band that displayed higher migration than the principal material. Fraction 4 was the composite material migrating between fractions 3 and 5. Fraction 5 (the blue band) was the least polar material in the dye mixture.

3.7. Analysis of TLC fractions

Chromatographic separation of individual TLC fractions yielded some components that matched retention times and mass spectra of compounds previously observed during *Red 26* separations, as described further below. Although retention time matches are close, they are usually not exact since studies were performed several months apart under conditions that may have been slightly different (*e.g.*, columns, mobile phase solvents, and even instruments). The observed variances in retention time matches are well within the acceptable range commonly encountered during chromatographic studies.

3.7.1. HPLC/APCI MS of TLC fractions

HPLC/APCI MS studies performed on separated *Red 26* TLC fractions allowed additional information to be obtained on components contained in fractions 2 and 5. HPLC/MS of fraction 2 confirmed the presence of a single peak that gave a retention time match (4.89 min) with the NIST SRM (4.84 min), as well as an absorbance maximum of 512 nm, and molecular weight of 380 amu as seen for the NIST SRM peak in previous HPLC/MS and GC/MS studies. TLC fraction 5 contained an HPLC peak (2.65 min, absorbance maximum of 562 nm) that contained two compounds with molecular weights of 429 (intense) and 399 amu (moderate). The 429 amu compound was seen previously (retention time of 2.69 min) during HPLC analysis of *Red 26*. These components were different from those observed by GC/MS of *Red 26* (these studies observed compounds with molecular weights of 210, 262, and 434 amu), a result that is not surprising given the ability of LC/MS to analyze more polar and thermally labile components.

3.7.2. NMR of TLC fractions

The TLC fractions were reconstituted in 0.75 mL of $CDCl_3$. The impurity concentrations in these fractions were determined by referencing the residual 0.53 mM $CHCl_3$ resonance (value determined earlier by comparison to a methanol internal standard). The proton NMR spectrum of fraction 1 displayed a mixture of aliphatic, few aromatic, and no tautomeric protons. Impurity concentrations in the reconstituted fraction were less than 0.013 mM for each of the estimated three impurities. Fraction 2 gave a spectrum that was identical to the NIST SRM at a concentration (based on the residual $CHCl_3$ resonance) of ~0.25 mM. The NMR spectrum of fraction 3 contained aromatic protons plus three tautomeric proton resonances at 16.73, 16.53, and 16.41 ppm at concentrations of approximately 0.015, 0.007, and 0.003 mM, respectively. The NMR spectrum of fraction 4 indicated a number of components, all at very low concentrations (<0.009 mM). The downfield resonance region had at least two different levels of intensities, with the larger set having six resonances. This suggested that one of the impurities may have, at maximum, only six aromatic resonances, and may represent a truncated form of NIST SRM (*e.g.*, synthetic by-product or decomposition product). The NMR spectrum of fraction 5 contained aromatic resonances, suggesting a mixture of dye-related components. Tautomeric protons were absent. The estimated concentrations of the impurities in fraction 5 were all small, with the most concentrated impurity estimated at roughly 0.03 mM.

Table 3 lists concentration estimates for the major impurities in each of the TLC fractions as based on NMR analysis. The purity estimate of 64% was based on numerous assumptions including quantitative extraction of polar dye components from the TLC silica. However, this purity value was surprisingly close to the 62% purity estimated from the one dimensional proton spectrum of the original *Red 26* sample.

3.7.3. GC/MS studies of TLC fractions

GC/MS studies of TLC-purified zones were performed to further identify impurities in the *Red 26*. Analysis of fraction 1 yielded a variety of unknown peaks with major components eluting with retention times of 12.28 min (apparent molecular weight of 236 amu) and 20.16 min (molecular weight of 378 amu and a base fragment at 223 amu). Fraction 2 consisted almost entirely of NIST SRM eluting with a retention time of 19.75 min (19.64 min in *Red 26* studies). Fraction 3 contained a major component that eluted with a retention time of 12.95 min (12.97 min in *Red 26* studies) that had a mass spectrum that matched the NIST library spectrum of 1-[(2-methylphenyl)azo]-2-naphthalenol (see Fig. 5b for structure). This result was consistent with NMR studies that showed several tautomeric proton environments, one of which could arise from 1-[(2-methylphenyl)azo]-2-naphthalenol. A second synthetic by-product resulting from diazonium coupling at the 3 position of beta-naphthol could be responsible for another of the tautomeric protons. Fraction 4 contained several compounds, the first of which eluted at 8.58 min, had an apparent molecular weight of 223, and did not yield a high quality spectral library match. Another peak in fraction 4 eluted at 19.75 min and gave a mass spectrum with an apparent molecular weight of 434 amu. The mass spectrum and retention time of the 434 amu compound matched the unknown impurity previously seen eluting on the shoulder of the principal dye during the GC/MS analysis of *Red 26*. Fraction 5 contained four major components. The first peak in fraction 5 was medium intensity, eluted with a retention of 8.00 min (8.03 min in *Red 26* studies), and gave a mass spectral library match with *m,m′*-dimethylazobenzene, an impurity also seen during GC/MS analysis of *Red 26* (see Fig. 5a for structure). A second peak in fraction 5 was high intensity, eluted with a retention time of 8.66 min, and had a complex mass spectrum that suggested a molecular weight of

Table 3
NMR estimation of the number and concentration of components along with the estimated percentage of total material in each TLC fraction.

TLC fraction	Approximate number of components	Approximate concentration (mM)[a]	Percentage of the *Red 26* mixture
TLC-1	2–3	All <0.013	10.0
TLC-2	1	0.25	64.3
TLC-3	3 at least	0.015; 0.007; and 0.003	6.4
TLC-4	2–3	<0.009	6.9
TLC-5	3 at least	0.03; 0.011; and 0.007	12.3

[a] Concentrations are determined by reference to the residual 0.53 mM $CHCl_3$ resonance in the reconstituted TLC fractions.

276 amu (base fragment was 205 amu). The third peak in fraction 5 was of medium intensity with a retention time of 10.38 min that gave a close spectral match with the library reference spectrum of 2-naphthalenylphenyldiazene (molecular ion at 232 with base fragment at 127 amu). The structure of 2-naphthalenylphenyldiazene is shown in Fig. 5c. This impurity was not previously seen in the analysis of *Red 26*, and its identification here likely reflects a higher concentration in the isolated TLC fraction. The last medium intensity peak in fraction 5 eluted with a retention time of 19.76 min and had an identical mass spectrum as the 434 amu compound seen previously in Fraction 4, an indication that TLC did not provide a clean separation of this compound between fractions 4 and 5. GC/MS results that tentatively identified *m*,*m*′-dimethylazobenzene and 2-naphthalenylphenyldiazene in fraction 5 were consistent with the ^{1}H NMR results that indicated the presence of aromatic compounds devoid of tautomeric protons.

Overall, GC comparison of NIST SRM and *Red 26* TLC fractions provided further validation that the principal component in *Red 26* is C.I. Solvent Red 24. This conclusion was based on mass spectral and retention time matches with an authentic NIST SRM standard. Analysis of *Red 26* identified three impurities eluting at 8.03 (MW = 210 amu), 12.97 (MW = 262 amu), and 19.75 (MW = 434 amu) min. GC analysis of the individual TLC fractions provided more detailed information regarding the impurities contained in *Red 26*. Analysis of individual TLC fractions located the same impurities observed in the composite mixture in TLC fractions 3 (MW = 262 amu), 4 (MW = 434), and 5 (MW = 210 and 434 amu). Several tentative impurity identifications, *m*,*m*′-dimethylazobenzene and 1-[(2-methylphenyl)azo]-2-naphthalenol, were possible based on high quality mass spectral library matches. The polarity of the impurities identified in the TLC fractions seems consistent with decreasing compound polarity being found in the more mobile TLC fractions. GC analysis of the fractions also allowed an additional tentative identification of 2-naphthalenylphenyldiazene as an impurity in TLC fraction 5. A variety of other unknown components were observed in the fractions and partially characterized by chromatographic retention time and MS.

Table 4
Compilation of purity values for *Red 26* dye determined using a variety of different analytical techniques.

Analytical technique/ determined purity	Comments	Purity
Visible absorbance	Study 1	74%
	Study 2	72.9%
	Study 3	78%
	Study 4	72.1%
HPLC[a]	Normal-phase amino column	93%
NMR	Methanol spike	62%
	Analysis of TLC fractions	64%
TLC	Standard analytical	73%
	HPTLC 2× toluene development	78%
GC analysis[a]	FID (area percent)	97%
	Total ion current (area percent)	93%
GC analysis	Total ion current (relative response)	75%

[a] For reasons described in the text, these analytical methods result in overestimating the purity of *Red 26*. The average of the remaining values (± the standard deviation) gives an estimated 72 ± 6% purity.

3.8. Synopsis of purity studies

The purity determinations arrived at during this study are summarized in Table 4. Purity values ranged between 62 and 97% depending on the analytical method. HPLC (absorbance at 520 nm) and GC (area percent) studies overestimated the purity for reasons detailed earlier. By averaging the remaining values, a purity estimate of 72 ± 6% was obtained for *Red 26*. In general, the discrepancy observed during visible absorbance derivative spectrophotometry agreed with the chemical purity values obtained using other analytical techniques. Purity based on ^{1}H NMR studies yielded lower values than other analytical techniques investigated.

4. Conclusions

The goal of this study was to explain the lower than expected visible absorption of *Red 26*. Accordingly, studies focused on verifying the structure and purity of this dye. Initial research verified that NIST SRM 2037 was a pure compound that had the expected chemical structure [8,9]. The chemical structure of the principal component in *Red 26* was shown to be identical to NIST SRM 2037 by HPLC/APCI MS, GC/MS, and ^{1}H NMR studies that referenced an authentic standard of the NIST SRM. Additional verification was provided by TLC studies. These results stand in contrast to the product certification that indicates *Red 26* is equivalent to C.I. Solvent Red 26. This leads to the assumption that *Red 26* has an additional ring methyl group that, in actuality, is not present in the molecule (see Fig. 1). A previous technical report indicated that NIST suspected *Red 26* was primarily C.I. Solvent Red 24 [7]. Studies presented here verify these suspicions.

The initial hypothesis was that the second derivative visible absorbance discrepancy between *Red 26* and NIST SRM 2037 (C.I. Solvent Red 24) was due to an impure *Red 26* material. Preliminary experiments showed that *Red 26* did not contain associated water, insoluble particulate matter, or inorganic impurities. A number of analytical techniques were then applied to verify the purity of *Red 26*. After excluding techniques known to exhibit analytical bias, these studies indicated a purity of 72 ± 6% for *Red 26*. In general, this purity agrees with the purity determined by visible absorbance spectrophotometry.

A number of possibilities exist for the lower than the expected purity value for *Red 26*. First, it is possible that the analytical methodologies used yielded artificially high purity values due to analytical bias (*e.g.*, purity determined by area percent GC analysis). Second, the dye may not be stable in prolonged storage. What originally may have been 99% pure dye at the time of certification (1994) may have degraded over the 13-year interim period to the observed 72% purity. However, the limited data available for standards prepared from *Red 26* indicate this dye is stable provided it is stored in the dry state and protected from light exposure. In addition, a report prepared in 2001 indicated that *Red 26* displayed 66–76% the absorbance of a NIST-purified C.I. Solvent Red 24 standard [7]. Hence, the purity of this material was essentially the same in 2001 as in 2007.

Several studies aimed at identifying impurities in *Red 26* were initiated. GC/MS studies allowed tentative identification of three

impurities: 1) *m,m′*-dimethylazobenzene; 2) 1-[(2-methylphenyl)-azo]-2-naphthalenol; and 3) 2-naphthalenylphenyldiazene. In addition, a number of impurities were partially characterized by GC/MS, HPLC/MS, and NMR.

In summary, these studies reached two principal conclusions: 1) the *Red 26* does not have the expected C.I. Solvent Red 26 chemical structure but rather was found to be structurally equivalent to NIST SRM (C.I. Solvent Red 24); and 2) the purity of the *Red 26* was substantially lower (~72%) than the expected value of 99%. Because of the impure status of *Red 26*, this dye may not be the best choice for calibrating C.I. Solvent Red 164 dilutions in middle distillates. Based on these studies, NIST SRM 2037 (C.I. Solvent Red 24) would be a more appropriate calibration standard.

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