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Synthesis and Purification of Trisulphoindigo and Reversed-Phase High Performance Liquid Chromatographic Determination of Trisulphoindigo in FD & C Blue No. 2

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SUMMARY

The effect of temperature on product formation during the sulphonation of indigo with concentrated sulphuric acid and 30% fuming sulphuric acid was studied with the aid of high performance liquid chromatographic (HPLC) analysis. Trisulphoindigo was prepared by the sulphonation of indigo with concentrated sulphuric acid at 160°C. The di- and tetrasulphoindigo side products formed during the reaction were removed by preparative HPLC to yield a product of high chromatographic purity. The trisulphoindigo content of the food dye FD & C Blue No. 2 and the purity of commercially available trisulphoindigo were determined by HPLC analysis.

ABBREVIATIONS

5,5′-diSI, 2-(1,3-dihydro-3-oxo-5-sulpho-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5-sulphonic acid, disodium salt (CAS 860-22-0);

5,7′-diSI, 2-(1,3-dihydro-3-oxo-7-sulpho-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5-sulphonic acid (CAS 54947-75-0);

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5,5′,7-triSI, 2-(1,3-dihydro-3-oxo-5-sulpho-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5,7-disulphonic acid, tripotassium salt (CAS 67627-18-3);

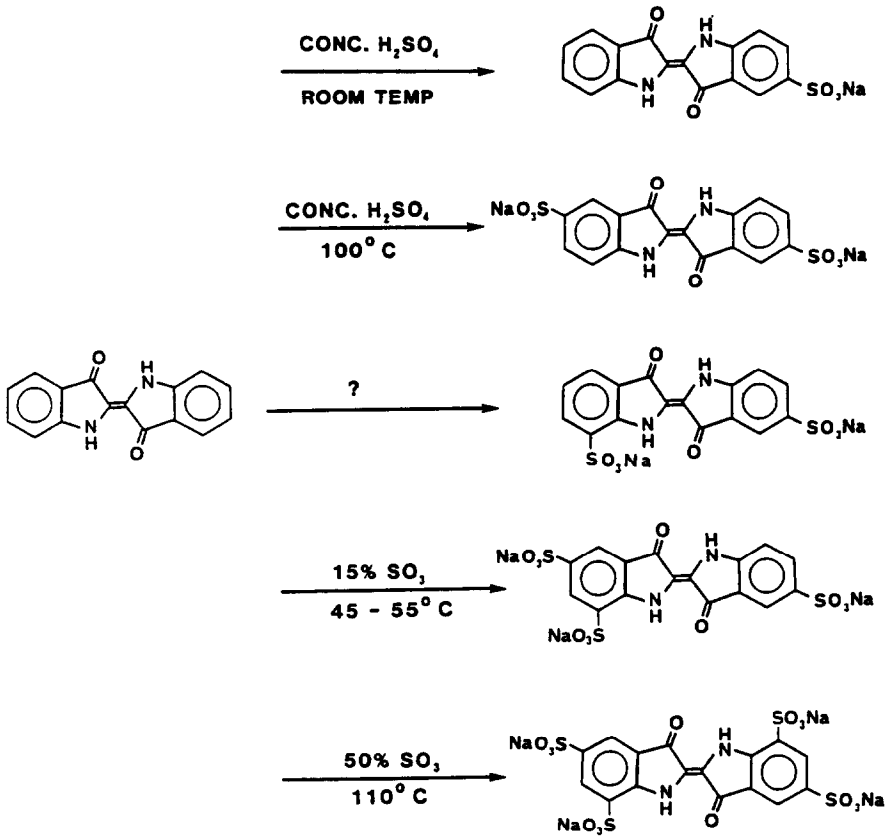
5,5′,7,7′-tetraSI, 2-(1,3-dihydro-3-oxo-5,7-disulpho-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5,7-disulphonic acid, tetrasodium salt (CAS 6371-42-2);

5-monoSI, 2-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5-sulphonic acid (CAS 605-18-5).

1. INTRODUCTION

Sulphonated indigo dyes are compounds of some commercial importance and chemical and biological utility. Commercially, 5,5′-diSI is a widely used food and drug colorant in the United States that has been regulated since 1907.[1] Primary uses include the colouring of candy, confections, beverages, dessert powders, bakery goods, pet foods and pharmaceuticals.[2] Chemical applications of sulphonated indigo dyes include use as analytical reagents for nitrate,[3] nitrite,[4] bismuth,[5] zirconium,[6] arsenic,[7] cobalt[8] and copper.[8] These dyes are also used to determine ozone dissolved in water[9] and in air[10] as well as oxygen in water[11] and in blood.[12] Biological applications include use in kidney function tests[13,14] and in pepsin assay (as Fibrin-blue)[15] as well as use as a tissue stain[16] and as a counter-ion in fluorescent immunological diagnostic reagents.[17]

Sulphonation of indigo with sulphuric acid or sulphur trioxide/sulphuric acid yields a variety of products depending on the conditions, i.e. acid employed, temperature and length of reaction. Very little work has been published that defines the relationship between reaction conditions and the sulphonation products formed. Investigations by Sullivan *et al.*[18] suggest that the reaction of indigo with concentrated sulphuric acid at room temperature favours the formation of a monosulphonated product, whereas the same reagents at 100 °C yield a disulphonated product (5,5′-diSI). The preparation of tri- and tetrasulphonated indigo requires the use of fuming sulphuric acid at elevated temperatures. Control over the products was achieved by adjusting the temperature and the ratio of indigo to acid. In the preparation of 5,5′,7-triSI, sulphonation was performed by using 50g indigo (0·19 mol) and 500 ml 15% fuming sulphuric acid ($\sim$1·8 mol free SO_3) at 45–55 °C. Preisler *et al.*[19] found that these conditions yielded primarily 5,5′,7,7′-



Scheme 1

tetraSI and reported that better results were obtained by using one-half the amount of 15% fuming sulphuric acid (~0·9 mol free SO_3). Jones *et al.*,[20] in a study of the composition of FD & C Blue No. 2 (principally 5,5′-diSI), detected the isomeric 5,7′-diSI as an additional product formed in the sulphonation of indigo. The known products of the sulphonation of indigo from studies by Sullivan *et al.*[18] and Jones *et al.*[20] are summarized in Scheme 1.

The sulphonation of indigo with sulphuric acid and sulphur trioxide/sulphuric acid would be expected to be relatively nonspecific and to yield a mixture of sulphonation products.[21] This would be particularly true of the preparation of 5,5′,7-triSI by the sulphonation of indigo, for which substantial amounts of the di- and/or tetrasulphonated products would be expected as reaction products. Very careful control would have

to be maintained over the reaction in order to obtain 5,5′,7-triSI as the primary reaction product, and adequate methods for the analysis of the reaction products would have to be employed to establish the purity of the isolated material.

The development of a high performance liquid chromatographic (HPLC) procedure for the analysis of FD & C Blue No. 2[22] provides a powerful tool for the study of the sulphonated indigo compounds. The purpose of this work was to perform a preliminary study of the sulphonation of indigo with concentrated sulphuric acid and 30% fuming sulphuric acid and to use preparative HPLC to prepare a very pure sample of 5,5′,7-triSI. FD & C Blue No. 2 was then analyzed by HPLC to determine 5,5′,7-triSI content. In addition, commercially available 5,5′,7-triSI was examined to assess its purity.

2. EXPERIMENTAL

2.1. Materials

Samples of 5,5′,7,7′-tetraSI, 5,5′-diSI, 5,7′-diSI and 5-monoSI were available from previous investigations and their purity was determined by elemental analysis and HPLC examination. Indigo was obtained from Buffalo Color Corp. (Buffalo, NY 14240, USA); its purity was determined by elemental analysis and it was used without further purification. The sulphuric acid and 30% fuming sulphuric acid were reagent grade and were used as received. Commercial 5,5′,7-triSI, potassium salt, was purchased from Aldrich Chemical Co. (Milwaukee, WI 53233, USA) and Crescent Chemical Co. (Hauppauge, NY 11788, USA). Methanol and acetonitrile were HPLC grade, and Milli-Q water (Millipore Corp., Bedford, MA 01730, USA) was used in all analytical HPLC mobile phases. Distilled water was used in all preparative HPLC mobile phases. All other chemicals employed were reagent grade.

2.2 Sulphonation of indigo with concentrated sulphuric acid

Approximately 10 ml (18 g) concentrated sulphuric acid, added from a tared graduated cylinder, was placed in a 23 × 200 mm test tube. The weight of the acid was accurately determined by difference, and a small magnetic stirrer was added. The tube was placed in an oil bath preheated

to the desired temperature (±5 °C) and stirring commenced. An accurately weighed sample of indigo powder (~1 g) was added, and the test tube was capped with a watch glass. The products of the reaction were monitored as a function of time by taking aliquots as described below. The diluted aliquot was then analyzed by HPLC, also as described below.

2.3. Sulphonation of indigo with 30% fuming sulphuric acid

An accurately weighed sample of indigo powder (~10 g) was placed in a 250 ml two-neck flask fitted with a mechanical stirrer and mounted in a temperature bath preheated to the desired temperature (±5 °C). A 90–95 ml portion of 30% fuming sulphuric acid (175–185 g) was added from a tared graduated cylinder and the weight to the nearest 0·01 g was determined by difference. The reaction vessel was loosely stoppered, and the reaction products were monitored as a function of time by taking aliquots as described below. The diluted aliquots were analyzed by HPLC as described below.

2.4. Sampling reaction mixtures

The sulphonation reaction mixtures were sampled by drawing an aliquot of the mixture (~1 g) into an accurately tared 9 in Pasteur capillary pipette/graduated cylinder combination. The weight of the aliquot was determined, and the sample was transferred to a 1-litre volumetric flask with the aid of a water wash and diluted to volume with water. The solution was analyzed by HPLC and the yield of each component, expressed as micromoles, was determined. The amount of indigo (μmol) taken in the aliquot was calculated, and the yields of the components were calculated as mole percentages. At the end of the reaction, a mass accounting was made by summing the weights of the aliquots and the weight remaining in the reaction mixture and comparing the result with the starting weight. In general, material accountability was 101–102%.

2.5. Preparation of 5,5′,7-triSI

Sulphonation

Concentrated sulphuric acid (75 ml) was placed in a 250 ml flask and indigo powder (10 g) was added in one portion. An air-cooled condenser was installed, and the mixture was swirled and then heated to 160 °C. The

reaction was monitored by HPLC analysis and terminated at ~140 min when the HPLC response for 5,5',7-triSI began to decrease in relation to the other components. The reaction was terminated by carefully quenching the solution in 300 g ice. The resulting solution was neutralized by the addition of 50% NaOH solution (with cooling), refrigerated overnight and then filtered to remove the precipitated solids. The precipitate (mostly 5,5'-diSI) was discarded. The mother liquor was diluted to 900 ml and retained for purification by preparative HPLC.

Preparative HPLC purification of 5,5',7-triSI

Preparative HPLC purification of the reaction solution was performed by using a Jobin–Yvon Chromatospac Prep-100 liquid chromatograph (Instruments SA Inc., Metuchen, NJ 08840, USA) with an 80 mm × 1 m column packed with 2 kg Lichroprep RP-18 (25–40 μm particle size) reversed-phase support (MC/B Manufacturing Chemists, Norwood, OH 45212, USA). The effluent from the column was monitored by using an Altex Model 153 UV detector (Altex Scientific Inc., Berkeley, CA 94710, USA) with a preparative flow cell of 0·5 mm pathlength. The column was first equilibrated with 4 litres of a solution containing 1 g ammonium acetate/100 ml water (void volume = 2 litres); next, 450 ml diluted reaction solution was applied to the column and the ammonium acetate eluent was continued. Fractions were collected when the detector response indicated that solute was eluting. After eleven 500 ml fractions were collected (total of 7·5 litres of ammonium acetate eluent), the solvent was changed to a solution containing 1 g ammonium acetate/100 ml 10% (v/v) methanol in water, and nine additional 500 ml fractions were collected. The eluent was then changed to 50% (v/v) methanol in water to flush the column. Each fraction was analyzed by HPLC and, on the basis of the results, the solvent was removed from fractions 6–15 and the syrupy residues were combined. Ethanol (500 ml, 95%) was added to the residue and the mixture was heated on a steam bath. The mixture was filtered while hot and the insoluble matter was collected. HPLC analysis showed that the dried product still contained 5,5'-diSI and 5,5',7,7'-tetraSI. A salting out procedure with sodium chloride was used several times to recrystallize the product from aqueous solution. However, the residual side reaction products were not completely removed.

Semi-preparative HPLC purification of 5,5',7-triSI

Semi-preparative HPLC purification of the product was performed on an

Altex Model 322 MP gradient liquid chromatograph with two Model 110A pumps fitted with preparative pump heads (flow rates up to 28 ml min^{-1}). A Whatman Magnum 20 preparative HPLC column (22 mm ID × 50 cm) packed with Partisil-10 ODS-3 support (10 μm particle size) (Whatman Inc., Clifton, NJ 07014, USA) was used with an Altex Model 153 UV detector (254 nm) fitted with a flow cell of 0·5 mm pathlength. A Rheodyne Model 7125 injector with a 5 ml loop was used (Rheodyne Inc., Cotati, CA 94928, USA). Solvent A was 1 g ammonium acetate/100 ml 0·5 % (v/v) methanol in water and solvent B was methanol. The column was equilibrated with 300 ml solvent A at a flow rate of 10 ml min^{-1}. The sample was dissolved in 50 ml water and ~4 ml was injected. Solvent A was continued for 4 min and then a gradient of 0–50 % B in A (linear) in 60 min was programmed. The main dye component, which eluted at 34 to 43 min, was resolved from several other components and collected. The process was continued until all of the sample was consumed (13 runs); the dye fractions were combined and the solvent was removed under vacuum. The residue was dissolved in water (75 ml) and sodium chloride was added (15 g). The mixture was heated and then filtered by suction while hot. Refrigeration overnight yielded precipitate which was collected and dried to yield 1·5 g product. Approximately 1 g of the product was recrystallized three times from aqueous solution by salting out with potassium chloride to yield 0·8 g of the potassium salt of 5,5',7-triSI. $C_{16}H_7N_2S_3O_{11}K_3$ requires C = 31·16 %, N = 4·54 %; found, C = 31·37 %, N = 4·45 % (corrected for moisture and KCl content). The ^{1}H NMR spectrum in DMSO-d_6 is shown in Fig. 1(A). The wavelengths at which significant electronic absorption occurs and the corresponding molar absorptivities (extinction coefficients) in 0·1N HCl are 253 nm (22 750), 290 nm (29 585), 310 nm (32 160) and 602 nm (22 300).

2.6. Analysis of sulphonated indigos by HPLC

The HPLC procedure for the quantitative analysis of mixtures of sulphonated indigo compounds has been described previously.[22] The analyses for the sulphonation studies were performed with the liquid chromatograph calibrated by the external standard method for the various sulphonated indigos. Calibration solutions were prepared for each of the sulphonated indigo compounds at three concentration levels and each of these was analyzed three times for a total of nine calibration points. The calibration data were evaluated statistically,[23] and the

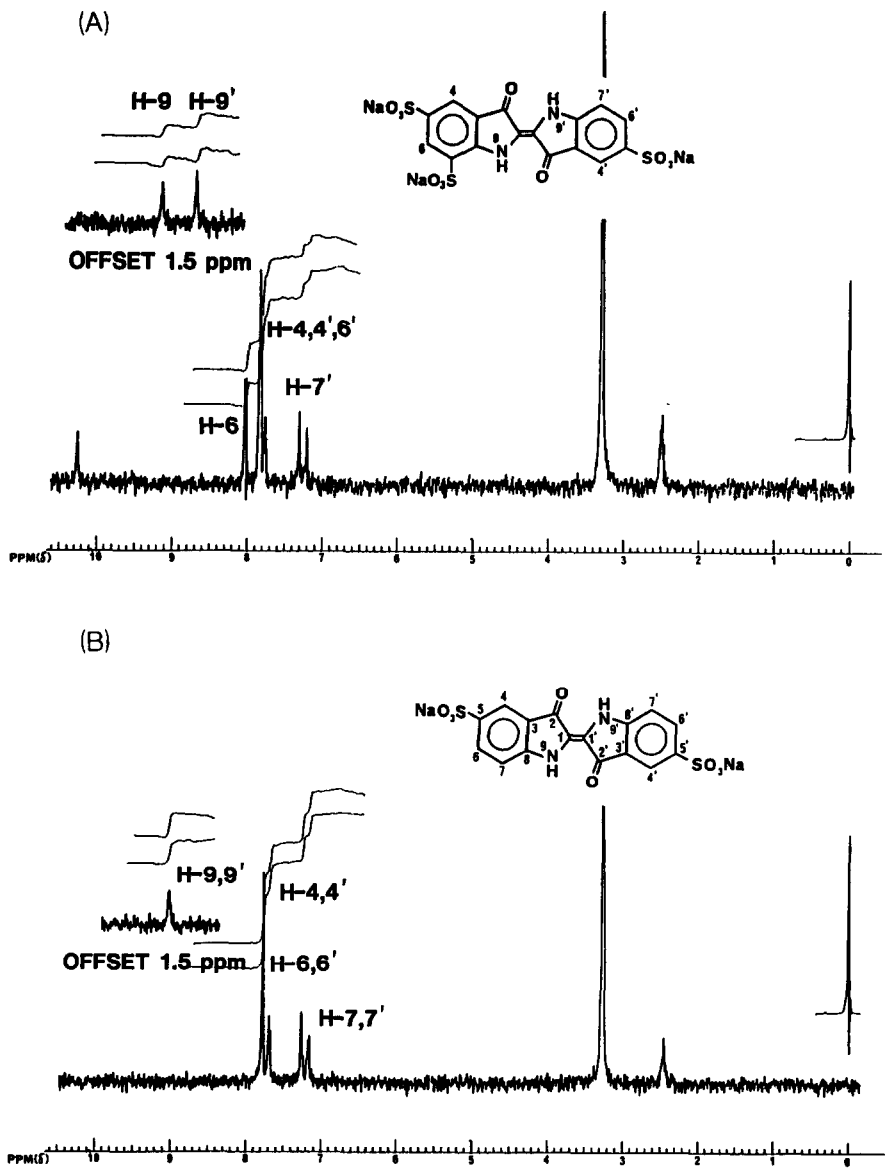


Fig. 1. ^{1}H NMR spectrum of 5,5',7-triSI in DMSO-d_6 (A) and ^{1}H NMR spectrum of 5,5'-diSI in DMSO-d_6 (B).

resulting regression equation was used to quantify the yields of sulphonation products. The analyses of FD & C Blue No. 2 samples for 5,5′,7-triSI were performed by using a Supelcosil LC-18 column, 25 cm × 4·6 mm ID, 5 μm particle size (Supelco Inc., Bellefonte, PA 16823, USA). The analyses of FD & C Blue No. 2 and commercial 5,5′,7-triSI were performed on a Varian Model 5060 gradient liquid chromatograph with a dual channel Vista 401 data system (Varian Associates, Palo Alto, CA 94303, USA). The eluate was monitored at 254 nm by using a Varian Model 5000 fixed wavelength detector and at 610 nm by using an Altex Model 155-10 UV/VIS variable wavelength detector. The detector signals were plotted simultaneously by using the data system. The HPLC eluants, gradient program and analysis parameters were the same as described previously.[22] The instrument was calibrated for 5,5′,7-triSI by preparing calibration solutions at nine different concentration levels and analyzing as described. The regression equation was calculated as described above[23] from the responses obtained at 254 and 610 nm. FD & C Blue No. 2 samples were analyzed by injecting 20 μl of an aqueous solution containing 0·5 g dye/100 ml. The amount of 5,5′,7-triSI present was determined from the regression equation by the response obtained at 254 and 610 nm. The instrument was then calibrated for 5,5′-diSI (nine concentration levels) and 5,5′,7,7′-tetraSI (five concentration levels). Commercial 5,5′,7-triSI samples were analyzed by first preparing an aqueous solution containing 0·1–0·2 g 5,5′,7-triSI/100 ml and then diluting (3:100) and chromatographing as for FD & C Blue No. 2; the levels of di-, tri- and tetra-SI were determined from the regression equation.

2.7. UV/VIS spectra of eluting HPLC solutes

UV/VIS absorption spectra of some of the components of FD & C Blue No. 2 were obtained during HPLC analysis by using a rapid-scan diode array spectrophotometer. A flow-through cell (Model 178.32, $Z = 15$, 10 mm pathlength, 8 μl cell volume; Hellma Cells Inc., Jamaica, NY 11424, USA), was installed in the cuvette holder of a Hewlett-Packard Model 8450A spectrophotometer and connected to the outlet of the UV detector. The wavelength range to be measured was selected, and a reference baseline (balance) of the HPLC eluent, measured at the end of the equilibration time, was stored in the memory of the spectrophotometer. As the solutes were eluted, as determined by the HPLC detector

response, the spectrophotometer was instructed to measure and store a 1s spectrum every 2s until five spectra of the eluting component were collected. After the chromatographic analysis was complete, each spectrum was examined and the best representative spectrum was selected and plotted.

2.8. Measurement of physical constants

Elemental analysis was performed in this laboratory on a Carlo–Erba Model 1106 elemental analyzer.

^{1}H NMR spectra were obtained on a Varian Model EM-390 spectrometer in DMSO-d_6 with tetramethylsilane in $CDCl_3$ used as an external reference.

The electronic absorption spectra of 5,5′,7-triSI were obtained on a Hewlett-Packard Model 8450A spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. Sulphonation of indigo

The sulphonation of indigo with concentrated sulphuric acid and 30% fuming sulphuric acid was studied at a variety of temperatures to assess the manner in which the products vary with reaction temperature. The study described here does not constitute a rigorous investigation of the sulphonation of indigo but is adequate to detect trends and to assess the nature of product distribution under various reaction conditions. A more rigorous study is planned for future investigations.

The calibration of the liquid chromatograph for the sulphonated indigo compounds during the study of the sulphonation of indigo yielded calibration equations with correlation coefficients of 0·999 or better. The concentrations of the products ($\mu g\,ml^{-1}$) in the diluted aliquot were determined and converted to micromoles in the sample aliquot. The number of micromoles of indigo in each aliquot was calculated on the basis of the weight of the aliquot, and these values were used to determine the yields of the various products in mole percentages, which were plotted as a function of time.

The sulphonation of indigo with concentrated sulphuric acid was studied at temperatures ranging from 0 to 125°C. The product

distributions as a function of time, calculated as mole percentage yields, are shown in Figs 2–6. At 0 °C (not shown), only 5-monoSI was observed in low yield. At 26 °C (Fig. 2), 5-monoSI, 5,5′-diSI and 5,7′-diSI are the observed products. At 62 and 75 °C (Figs 3 and 4), 5,5′-diSI and 5,7′-diSI quickly reach and maintain fairly constant yields of 75 and 25%, respectively, throughout the course of the reaction; 5-monoSI is observed as a rapidly disappearing product of the reaction. At 100 °C (Fig. 5), 5,5′,7-triSI is observed as a product gradually increasing in yield over the course of the reaction. At the same time, the yield of 5,7′-diSI gradually decreases, whereas that of 5,5′-diSI remains constant at 75%, suggesting that 5,5′,7-triSI is formed primarily by the sulphonation of 5,7′-diSI. Sulphonation of indigo at 125 °C with sulphuric acid (Fig. 6) shows a similar increase in the yield of 5,5′,7-triSI, but at a faster rate. However, at 125 °C, the level of 5,7′-diSI observed during the reaction remains fairly constant at 10–15%, whereas the yield of 5,5′-diSI gradually decreases, suggesting that 5,5′,7-triSI is formed by the sulphonation of 5,5′-diSI.

This apparent change in the behaviour of the reaction in going from 100 to 125 °C may be explained by the possible isomerization of 5,5′-diSI

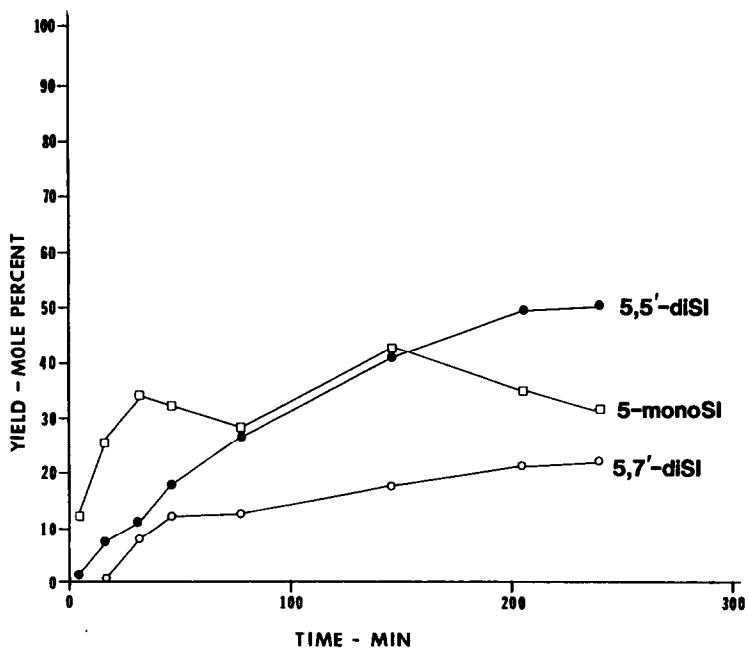


Fig. 2. Sulphonation of indigo with concentrated sulphuric acid at 26 °C.

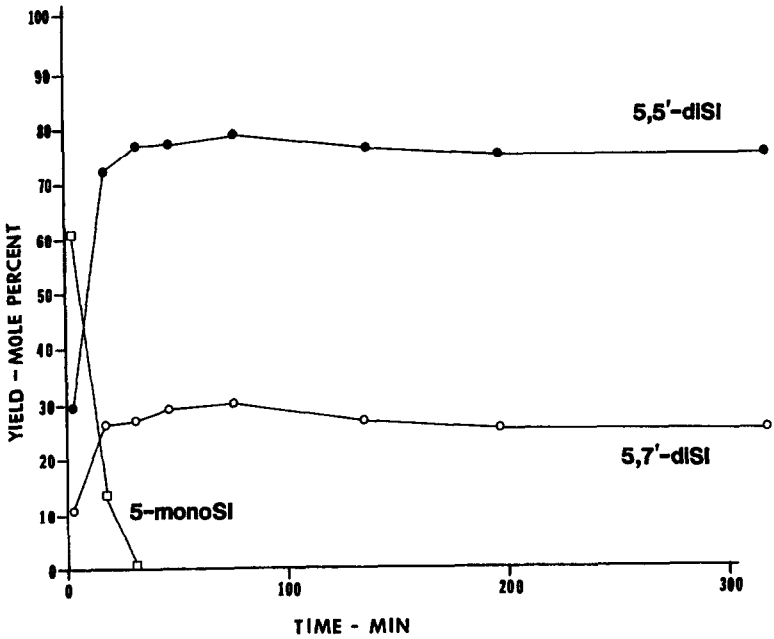


Fig. 3. Sulphonation of indigo with concentrated sulphuric acid at 62 °C.

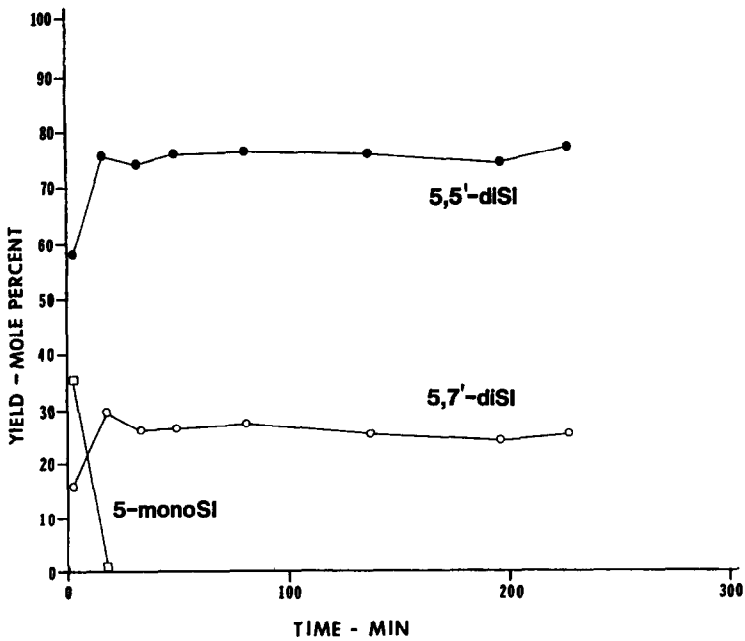


Fig. 4. Sulphonation of indigo with concentrated sulphuric acid at 75 °C.

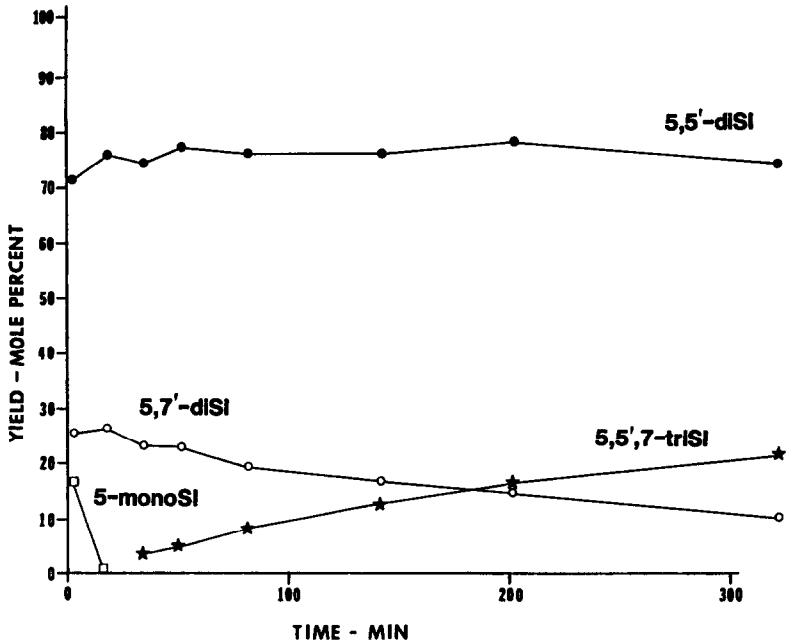


Fig. 5. Sulphonation of indigo with concentrated sulphuric acid at 100 °C.

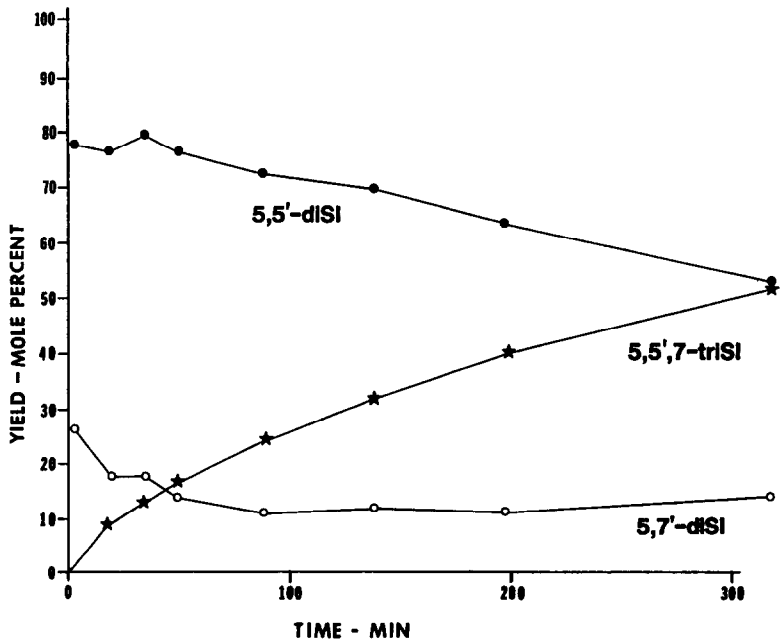


Fig. 6. Sulphonation of indigo with concentrated sulphuric acid at 125 °C.

to 5,7′-diSI at the higher temperature, followed by sulphonation of 5,7′-diSI to yield 5,5′,7-triSI. By analogy, 4,4′-disulphobiphenyl was observed to rearrange to 3,4′-disulphobiphenyl when heated at 140 and 180 °C in 75% sulphuric acid.[24]

In order to test the possibility of the rearrangement of 5,5′-diSI to 5,7′-diSI, a portion of highly purified 5,5′-diSI, free of 5,7′-diSI, was heated at 125 °C in the presence of concentrated sulphuric acid. Analysis of the reaction solution revealed that 5,7′-diSI was one of the products formed and that it maintained a relatively constant level. Similar treatment of purified 5,5′-diSI with sulphuric acid–water (3:1) at 125 °C gave a higher relative yield of 5,7′-diSI as well as the formation of some 5-monoSI as a desulphonation product. 5,5′,7-triSI was still observed as a product. Treatment of purified 5,5′-diSI with sulphuric acid–water (1:1) at 125 °C failed to produce either 5,7′-diSI or 5,5′,7-triSI with only low levels of 5-monoSI observed as a desulphonation product.

In the studies of the sulphonation of indigo with 30% fuming sulphuric acid, none of the mono- or disulphonated products were observed. At 0 °C, 5,5′,7-triSI and 5,5′,7,7′-tetraSI were formed instantaneously, with the 5,5′,7-triSI predominating. Over the course of the reaction, the yield of 5,5′,7-triSI gradually diminished while the yield of 5,5′,7,7′-tetraSI increased. At 26 °C, 5,5′,7-triSI and 5,5′,7,7′-tetraSI were initially formed in approximately equal amounts with the yield of 5,5′,7-triSI falling to zero 200 min after the start of the reaction. At 68 and 100 °C, the yield of 5,5′,7-triSI dropped to zero at 32 and 20 min, respectively, with a concomitant rise in the yield of 5,5′,7,7′-tetraSI to 100%.

3.2. Preparation of 5,5′,7-triSI

The preparation of 5,5′,7-triSI by the sulphonation of indigo with concentrated sulphuric acid at 160 °C yielded a reaction solution (after filtration) containing a mixture of sulphonation products which, according to HPLC analysis, consisted of 12·1% 5,5′,7,7′-tetraSI, 69·5% 5,5′,7-triSI, 11·5% 5,5′-diSI and 6·9% 5,7′-diSI in terms of area percentages. Preparative chromatography of this product and recrystallization yielded 5,5′,7-triSI that was much purer but which still contained 6·4% 5,5′,7,7′-tetraSI and 4·4% 5,5′-diSI. These components were not removed by recrystallization. However, semi-preparative chromatography and recrystallization of the product removed these contaminants to yield 5,5′,7-triSI which was 98·6% pure (by area

percentage). The elemental analysis was appropriate for the expected product. The ^{1}H NMR spectrum taken in DMSO-d_6, Fig. 1(A), also confirmed the structure for 5,5′,7-triSI. (The ^{1}H NMR spectrum of 5,5′-diSI in DMSO-d_6 is shown in Fig. 1(B) for comparison.) An upfield doublet (d, δ 7·2, 1H) corresponds to the single aromatic proton located *ortho* to the nitrogen atom (H-7′) and *ortho*-coupled to H-6′. The proton flanked by two sulphonic acid groups (H-6) is observed as a downfield doublet (d, δ 8·0, 1H) exhibiting *meta*-coupling to H-4. The three remaining aromatic protons (H-4, H-4′, and H-6′) are observed as overlapping signals (m, δ 7·8, 3H). In addition, the two N—H protons (H-9 and H-9′) are observed downfield as two well-resolved singlets (s, δ 10·2 and s, δ 10·6) whose integration was somewhat low because of deuterium exchange.

The electronic absorption spectrum obtained for 5,5′,7-triSI was similar to that reported by Preisler *et al.*[19] with the slight difference attributed to the different media employed (pH 7 buffer vs. 0·1N HCl). It should be noted that the sulphonated indigo compounds rapidly decompose in strong base. For example, heating 5,5′-diSI at 50 °C in the presence of 0·1M sodium carbonate resulted in complete degradation of the dye in 300 min.[25] Therefore, care must be exercised when obtaining or using spectral data for the sulphonated indigo compounds in alkaline media.

5,5′,7-triSI in FD & C Blue No. 2

The liquid chromatograph was calibrated by using 5,5′,7-triSI as an external standard. Nine calibration levels were prepared at concentrations ranging from 0·053 to 0·53% by weight of 5,5′,7-triSI (2·66–26·6 μg ml^{-1}), based on a 0·5 g sample size. The peak areas for the nine calibration points were measured at 254 and 610 nm, and the data were treated statistically to yield regression equations. The correlation coefficient was greater than 0·999 at both wavelengths of detection. After the analysis of FD & C Blue No. 2 samples for 5,5′,7-triSI, the instrument was calibrated for 5,5′-diSI (nine levels, 1·71–17·1 μg ml^{-1}) and for 5,5′,7,7′-tetraSI (five levels, 3·44–17·2 μg ml^{-1}) at 254 and 610 nm. Statistical analysis yielded correlation coefficients greater than 0·999. The calibrated instrument was then used to analyze commercial samples of 5,5′,7-triSI. Attempts to determine 5,5′,7-triSI in FD & C Blue No. 2 by using the published method[22] yielded discrepancies between the results

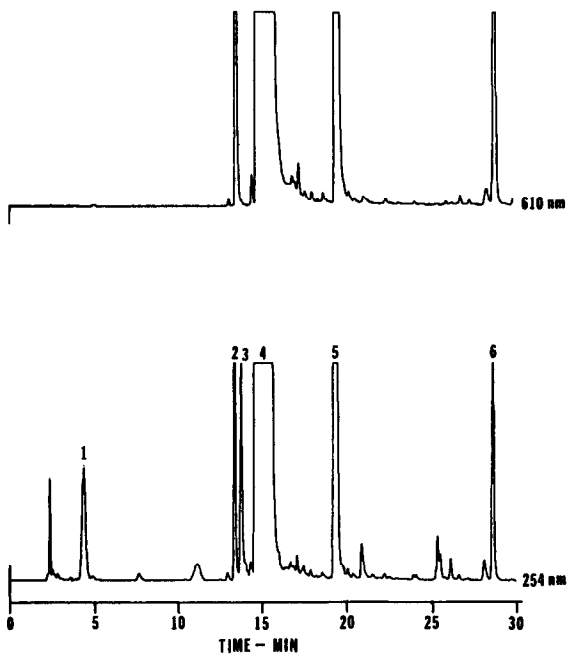


Fig. 7. HPLC chromatogram of commercial FD & C Blue No. 2 sample: 1, isatin-5-sulphonic acid; 2, 5,5′,7-triSI; 3, unknown; 4, 5,5′-diSI; 5, 5,7′-diSI; 6, 5-monoSI. Top tracing from 610 nm detector; bottom tracing from 254 nm detector. Peak numbers in bottom tracing apply to corresponding peaks in top tracing.

obtained at 610 and 254 nm because of incomplete resolution of two components. The use of the Supelcosil LC-18 column, with the identical HPLC parameters, resolved 5,5′,7-triSI from the interfering substance. A typical chromatogram for a sample of commercial FD & C Blue No. 2 is shown in Fig. 7. The interfering component, which can be seen as a peak corresponding to the component eluting just after 5,5′,7-triSI, does not show a response on the 610 nm detector.

The results of the analysis of 28 FD & C Blue No. 2 samples for 5,5′,7-triSI are presented in Table 1. Values were calculated by using the regression equation at both 254 and 610 nm and from the peak areas reported by the integrator. The results at 254 and 610 nm generally agree well with each other, suggesting little interference from co-eluting components. 5,5′,7-triSI consistently appears as a contaminant of FD & C Blue No. 2, generally at levels below 1 %. However, the samples of manufacturer E showed no trace of the constituent and were also free of the unknown component eluting just after 5,5′,7-triSI. This was true of

TABLE 1
Results for the HPLC Determination of 5,5',7-TriSI in FD & C Blue No. 2

Manufacturer	*Sample*	*5,5',7-triSI* (%)	
		254 nm	*610 nm*
A	1	0·85	0·90
A	2	0·49	0·49
A	3	0·70	0·72
A	4	0·48	0·48
A	5	0·51	0·51
B	1	0·66	0·68
B	2	0·82	0·86
B	3	0·58	0·58
B	4	0·67	0·69
B	5	0·52	0·52
C	1	0·78	0·80
C	2	0·47	0·46
C	3	0·50	0·49
D	1	0·01	—
D	2	0·49	0·49
D	3	0·02	—
E	1	—	—
E	2	—	—
E	3	—	—
E	4	—	—
E	5	—	—
F	1	0·04	0·04
F	2	0·49	0·48
F	3	0·03	—
F	4	0·58	0·58
G	1	0·09	0·09
H	1	0·53	0·53
I	1	0·80	0·85

other samples containing low levels of 5,5',7-triSI and suggests a relationship between these components.

Characterization of 5,5',7-triSI in FD & C Blue No. 2 was obtained by using the UV/VIS spectrophotometer connected in-line with the effluent from the HPLC column. First, a solution of a sample of FD & C Blue No. 2 containing 5,5',7-triSI was chromatographed and the electronic absorption spectrum of the eluting constituent was obtained. Then the

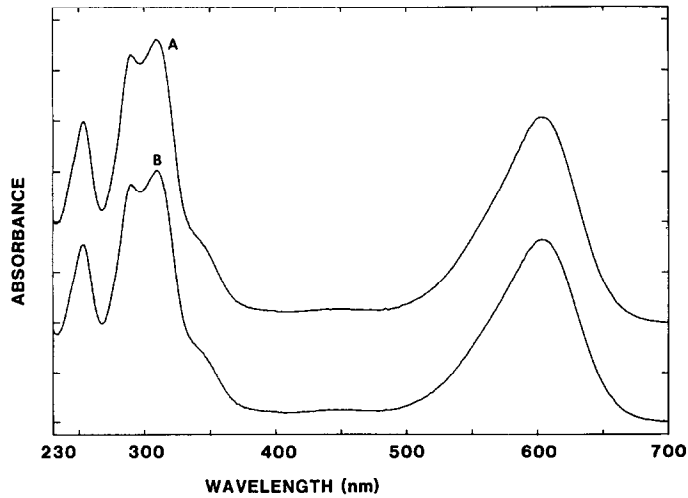


Fig. 8. UV/VIS spectra 5,5',7-triSI: (A) in commercial sample of FD & C Blue No. 2 and (B) the reference compound.

spectrum of a solution of the 5,5',7-triSI reference compound was obtained during HPLC analysis in the same manner. As shown in Fig. 8, the spectra, which are identical, confirm the identity of 5,5',7-triSI as a constituent of FD&C Blue No. 2.

Figure 9 is the absorption spectrum of the component which elutes just after 5,5',7-triSI in the chromatogram of FD&C Blue No. 2. The spectrum of this component is similar in some respects to those reported for the sulphonic acid derivatives of isatin.[20] The lack of absorption above 400 nm suggests the possibility that this material may be a degradation product. This seems likely since 5,5',7-triSI is formed only when indigo is sulphonated with concentrated sulphuric acid at higher temperatures. These conditions subject the dye to greater thermal stress during the reaction.

Analysis of commercial 5,5',7-triSI

Samples of 5,5',7-triSI obtained from two commercial suppliers were analyzed by HPLC. Analysis of relatively concentrated solutions (0·1–0·2%) showed that these samples were impure and that they produced many responses in addition to those for 5,5',7,7'-tetraSI, 5,5'-diSI, 5,7'-diSI and 5,5',7-triSI. The sample solutions were diluted and rerun to quantify the levels of 5,5',7,7'-tetraSI, 5,5',7-triSI and 5,5'-diSI,

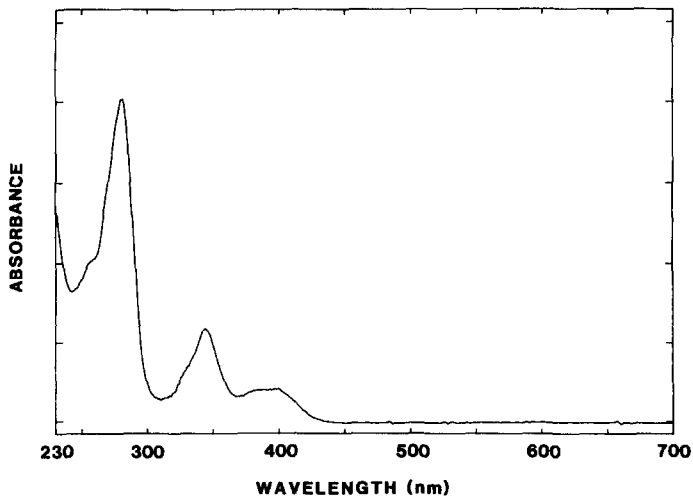


Fig. 9. UV/VIS spectrum of the unknown constituent (peak 3 in Fig. 7) in commercial FD & C Blue No. 2.

which were the major constituents. The results of these analyses, expressed in weight percentages and normalized mole percentages, are shown in Table 2. As can be seen, both samples are of comparable purity with approximately equal amounts of higher and lower sulphonated products. Thus, a sample with this composition would yield an elemental analysis appropriate for 5,5',7-triSI. The presence of 5,5',7,7'-tetraSI suggests that the sulphonation reaction used commercially to prepare 5,5',7-triSI employs fuming sulphuric acid, probably in the manner prescribed by Preisler *et al.*[19] On the basis of the sulphonation studies described above, it appears that a better procedure might be 30% fuming sulphuric acid at 0 °C.

TABLE 2
Composition of Commercial 5,5',7-TriSI in Weight Percentages and Normalized Mole Percentages

Manufacturer	*5,5',7,7'-tetraSI*		*5,5',7-triSI*		*5,5'-diSI*	
	(*wt* %)	(*mol* %)	(*wt* %)	(*mol* %)	(*wt* %)	(*mol* %)
A	9·72	11·33	55·5	77·1	6·74	11·58
B	6·77	7·1	71·0	80·9	8·51	12·0

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