Structural Determination of Unknown Subsidiary Colors in Food Yellow No. 5 (Sunset Yellow FCF)

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Major unknown subsidiary colors A (Sub A) and B (Sub B) in commercial Sunset Yellow FCF (Food Yellow No. 5 in Japan) have been isolated by preparative HPLC. Spectroscopic analyses of Sub A and Sub B revealed that their structures are trisodium salt of 6-hydroxy-7-(4-sulfophenyl)-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid, and disodium salt of 3-hydroxy-4-(4-sulfophenylazo)-2-naphthalenesulfonic acid, respectively.

Key words Sunset Yellow FCF; Food Yellow No. 5; FD&C Yellow No. 6; subsidiary color; coal-tar dye

Twelve coal-tar dyes are presently approved in Japan as food colors and coloring agents for pharmaceutics and cosmetics. In a series of fundamental studies to develop specifications for coal-tar dyes, we have reported on the survey, identification and quantification of impurities such as raw materials, intermediates and subsidiary colors in these dyes.[1)] Food Yellow No. 5 (Y5; C.I. 15985, Sunset Yellow FCF, FD&C Yellow No. 6) is classified as a monoazo color obtained by diazotizing sulfanilic acid (SA, sodium salt of 4-aminobenzenesulfonic acid) and salting out after coupling with Schaeffer's salt (SS, sodium salt of 6-hydroxy-2-naphthalenesulfonic acid). Based on findings in our previous paper[1a)] and the research by Cox *et al.*,[2)] Bailey,[3)] and Uematsu *et al.*,[4,5)] the azo-coupling between the raw materials (SA or SS) and the impurities in them gives several subsidiary colors shown in Fig. 1.

From the results of HPLC analyses of Y5, we recognized the existence of other unknown subsidiary colors than those listed in Fig. 1. Since the chemical structures were not known, the authors investigated the structure of the two major contents in some commercial products.

Experimental

Chemicals Authentic Y5 for mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses was a standard product distributed by NIHS. Trisodium salt of 3-hydroxy-4-(4-sulfophenylazo)-2,7-naphthalenedisulfonic acid (RS-SA), sodium salt of 6-hydroxy-5-phenylazo-2-naphthalenesulfonic acid (SS-AN, orange RN) and sodium salt of 4-(2-hydroxy-1-naphthylazo)benzenesulfonic acid (2N-SA, orange II) were used in the previous study.[1a)] Trisodium salt of 7-hydroxy-8-(4-sulfophenylazo)-1,3-naphthalenedisulfonic acid (GS-SA) was synthesized according to Bailey and Calvey.[6)] Commercially available Y5 products (27 samples from 6 companies) were analysed by HPLC. Other reagents used were HPLC grade or the highest grade available from commercial sources.

Instruments HPLC was performed with a JASCO 900 system with a Sic system instrument Chromatocoder 21. MS was taken on a VG Biotech Platform spectrometer. NMR spectra were determined with a JEOL A600 spectrometer. A Shimadzu UV-260 was used for UV–Vis spectroscopy.

Analytical HPLC Analytical HPLC was performed on an L-column ODS (4.6 mm i.d. × 250 mm; Chemicals Inspection and Testing Institute, Tokyo) at 30 °C with detection at 482 nm (the λ max of Y5). The solvent flow rate of 1.0 ml/min was used with a 50 min linear gradient progressing from 100% 0.02 M aqueous ammonium acetate to 40% acetonitrile in 0.02 M ammonium acetate. Y5 was dissolved in 0.02 M ammonium acetate (pH 8.0) at a concentration of 5.0 mg/ml and a 20 μl aliquot of the solution was injected.

Preparative HPLC Subsidiary Color A (Sub A): Preparative HPLC (Prep. HPLC) was performed on an L-column ODS (10 mm i.d. × 250 mm) at ambient temperature with detection at 482 nm. The solvent flow rate of 4.0 ml/min was used with a 25 min linear gradient progressing from 8% to 24% acetonitrile in 0.01 M ammonium acetate. Y5 manufactured by A company was dissolved in water at a concentration of 50 mg/ml and a 50 μl aliquot of the solution was injected for each

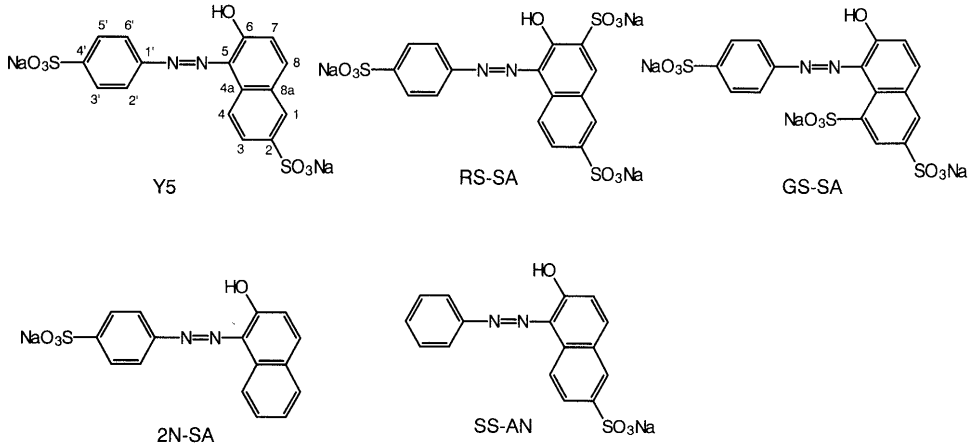

Fig. 1. Structures of Sunset Yellow FCF (Y5) and Known Subsidiary Colors in Commercial Y5

Y5, disodium salt of 6-hydroxy-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid; RS-SA, trisodium salt of 3-hydroxy-4-(4-sulfophenylazo)-2,7-naphthalenedisulfonic acid; GS-SA, trisodium salt of 7-hydroxy-8-(4-sulfophenylazo)-1,3-naphthalenedisulfonic acid; 2N-SA, sodium salt of 4-(2-hydroxy-1-naphthylazo)benzenesulfonic acid; SS-AN, sodium salt of 6-hydroxy-5-phenylazo-2-naphthalenesulfonic acid.

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HPLC run. A small peak eluted between 12.0 and 13.5 min, just before the main peak, was collected by a prep. HPLC to obtain Sub A. After 150 runs the collected solutions were combined, evaporated to dryness, redissolved in water and rechromatographed using the same conditions to give Sub A fraction. After evaporation to dryness, the Sub A fraction was dissolved in a small amount of water and charged on a Sephadex LH-20 column (1.0 cm i.d. × 35 cm) to remove ammonium acetate. Following column purification, the eluate was evaporated to dryness and dried in a vacuum oven at 40 °C for 48 h to give orange powders of Sub A (5.8 mg). The organic impurities of Sub A were less than 5% by analytical HPLC.

Subsidiary Color B (Sub B): Preparative HPLC (Prep. HPLC) was performed on Deverosil ODS-5 (20 mm i.d. × 250 mm; Nomura Chemical Co.) with detection at 482 nm. The mobile phase of 0.01 M ammonium acetate–acetonitrile (94 : 6) was used at a flow rate of 8.0 ml/min. Y5 manufactured by B company was dissolved in water at a concentration of 20 mg/ml and a 100 μl of the solution was injected for each HPLC run. A small peak eluted between 25.0 and 27.5 min, just after the main peak, was collected by a prep. HPLC to obtain Sub B. After 200 runs the collected solutions were combined, evaporated to dryness, redissolved in water and rechromatographed using the same conditions to give pure Sub B fraction. After evaporation to dryness, the Sub B fraction was treated as described for Sub A, to give orange powders of Sub B (1.8 mg). The organic impurities of Sub B were less than 3% by analytical HPLC.

Spectroscopic Analyses MS analyses were performed under the following conditions : the negative mode of the electrospray ionisation, a scan speed of 100—600 m/z for 2 s, and the cone voltage of 30 V. Samples dissolved in water were directly injected.

^{1}H- and ^{13}C-NMR spectra were measured in methanol-d_4 with tetramethylsilane as the internal standard. The signals of the ^{13}C-NMR spectra of Y5 and Sub A were assigned on the basis of chemical shifts and the data of heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC) and differential nuclear Overhauser effect (dif-NOE) spectra. The signals of the ^{13}C-NMR spectra of Sub B were assigned on the basis of chemical shifts, HMQC and dif-NOE spectra.

Results and Discussion

Chromatograms of commercial samples A and B are shown in Fig. 2. The chromatogram of sample A showed the existence of RS-SA (retention time (t_R): 11.3 min), SS-AN + 2N-SA (t_R: 47.6 min), and 8 unidentified subsidiary colors (t_R: 18.1, 20.0, 20.4, 21.7, 21.9, 22.4, 27.2, 27.5 min) having areas equal to 0.01% or larger of the main color. Similarly, analytical HPLC of sample B showed the existence of RS-SA, GS-SA (t_R: 13.7 min), SS-AN + 2N-SA, and 9 unidentified subsidiary colors (t_R: 20.0, 20.4, 21.7, 25.2, 27.2, 27.5, 35.8, 42.7, 48.6 min) with areas of 0.01% or greater of the main color. Of these unidentified subsidiary colors, Sub A (t_R: 20.4 min) and Sub B (t_R: 27.2 min) are the main subsidiary colors of samples A and B, respectively. Therefore, chromatographic purification was performed to isolate them.

The UV–Vis spectra for Sub A, Sub B, and Y5 are shown in Fig. 3. The absorption maxima for Sub A and Sub B were 486 and 492 nm, respectively, and they were longer than that of Y5.

MS analyses of Sub A, Sub B, and Y5 were performed under the same conditions. In the spectrum of Sub A, three quasi-molecular ion peaks at m/z 563 $[M-3Na+2H]^-$, 281 $[M-3Na+H]^{2-}/2$ and 187 $[M-3Na]^{3-}/3$ were observed. These ion peaks indicate that the structure of Sub A possesses three sodium sulfonato groups, and the formula of Sub A as $C_{22}H_{13}N_2Na_3O_{10}S_3$. In the spectrum of Sub B, three quasi-molecular ion peaks were observed at m/z 429 $[M-Na]^-$, 407 $[M-2Na+H]^-$ and 203 $[M-2Na]^{2-}/2$, which were similar to those in the spectrum of Y5. These data suggest Sub B was a structural isomer of Y5 with the formula of $C_{16}H_{10}N_2Na_2O_7S_2$.

The ^{1}H- and ^{13}C-NMR data of Sub A, Sub B and Y5

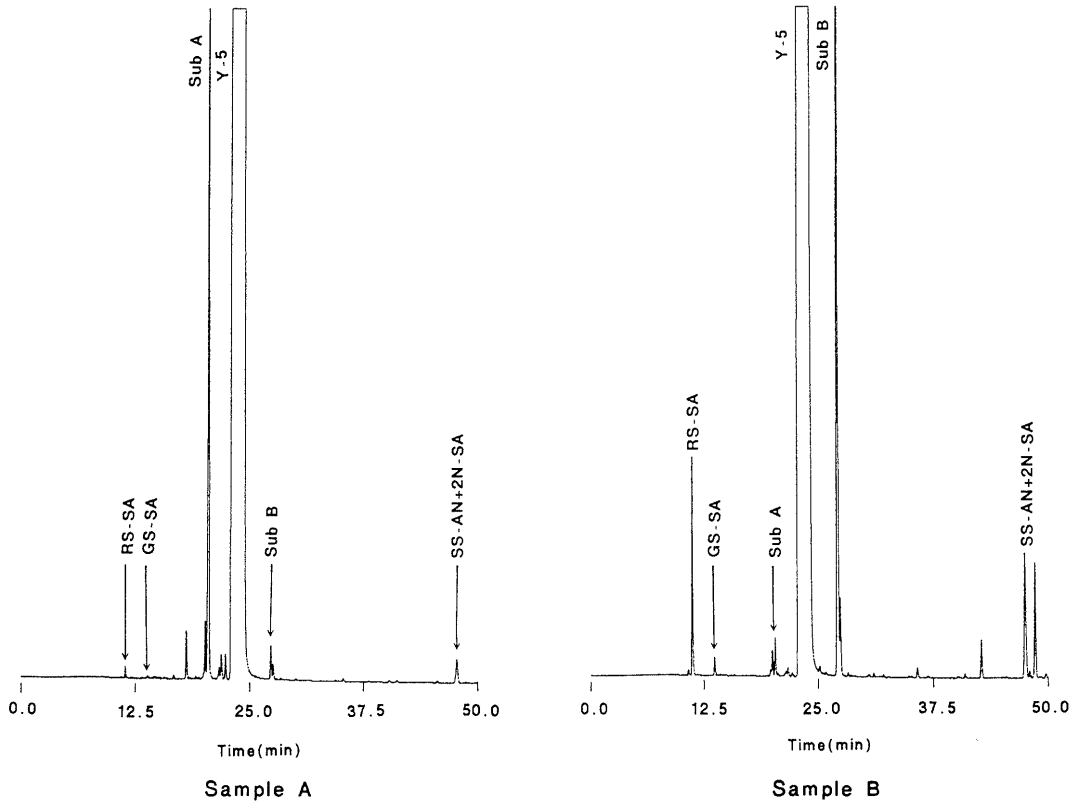

Fig. 2. Analytical HPLC of Commercial Y5

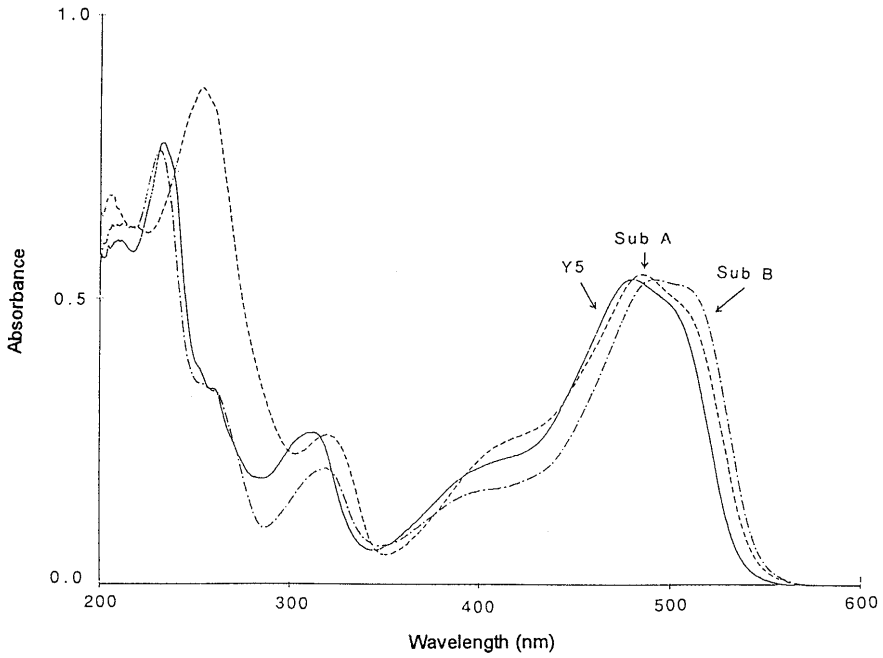

Fig. 3. UV–VIS Spectra of Y5, Sub A, and Sub B (20 μM) in 0.02 M Ammonium Acetate

Table 1. ^{1}H-NMR Data (δ Value, 600 MHz/TMS) of Y5, Sub A, and Sub B in CD_3OD[a]

Position[b]	Y5	Sub A	Sub B
1	8.14 (d, J=1.9 Hz)	8.17 (d, J=2.0 Hz)	8.38 (s)
3	8.01 (dd, J=9.1, 1.9 Hz)	8.01 (dd, J=8.4, 2.0 Hz)	—
4	8.63 (d, J=9.1 Hz)	8.58 (d, J=8.4 Hz)	—
5	—	—	8.45 (br d, J=8.1 Hz)
6	—	—	7.63 (td, J=8.1, 1.2 Hz)
7	6.91 (d, J=9.4 Hz)	—	7.45 (td, J=8.1, 1.2 Hz)
8	7.91 (d, J=9.4 Hz)	8.03 (s)	7.68 (br d, J=8.1 Hz)
2′,6′	7.86 (d, J=8.8 Hz)	7.83 (d, J=9.0 Hz)	7.77 (d, J=9.0 Hz)
3′,5′	7.98 (d, J=8.8 Hz)	7.97 (d, J=9.0 Hz)	7.93 (d, J=9.0 Hz)
2″,6″	—	7.78 (d, J=8.8 Hz)	—
3″,5″	—	7.93 (d, J=8.8 Hz)	—

a) Multiplicities and coupling constants are given in parentheses. *b*) Position on azobenzene moiety and benzene moiety is indicated by prime and double prime, respectively.

Table 2. ^{13}C-NMR Chemical Shifts (δ Value, 150 MHz/TMS) of Y5, Sub A and Sub B in CD_3OD

Position[a]	Y5	Sub A	Sub B	Position[a]	Y5	Sub A	Sub B
1	127.6	127.9	142.7	2′	119.4	118.7	118.2
2	144.2	144.6	139.6[d]	3′	128.8	128.9	128.8
3	127.5	127.7	173.9	4′	145.4	144.8	145.3[e]
4	123.1	123.0	135.9[d]	5′	128.8	128.9	128.8
4a	135.9	135.9	131.7[d]	6′	119.4	118.7	118.2
5	131.3[b]	131.5[c]	123.1	1″		139.5	
6	173.3	175.6	132.0	2″		130.4	
7	126.4	137.9	128.1	3″		126.8	
8	141.9	141.5	131.6	4″		145.9	
8a	129.0[b]	129.0[c]	127.8	5″		126.8	
1′	147.4	146.2	145.5[e]	6″		130.4	

a) Position on azobenzene moiety and benzene moiety is indicated by prime and double prime, respectively. *b*—*e*) Signal may be interchanged within each column.

are shown in Tables 1 and 2, respectively. The ^{1}H- and ^{13}C-NMR data for Sub A were very similar to those of Y5 except for the additional aromatic ring signals and the 7- and 8-position signals. The coupling pattern in ^{1}H-NMR data of Sub A indicates the additional aromatic ring was substituted at the 1″- and 4″-positions. The formula defined by MS suggests the existence of one additional sulfonatophenyl moiety, compared with the structure of Y5. On the basis of the combined data described, the structure of Sub A is estimated as 4-sulfonatophenylated Y5 at the 7- or 8-position in the naphthalene ring. Dif-NOE experiments were performed

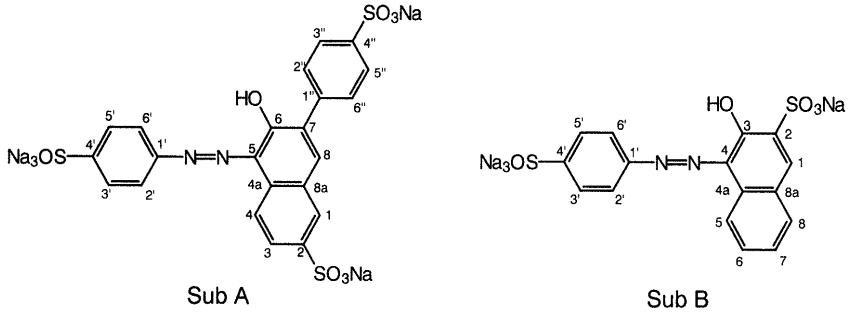

Fig. 4. Structures of Sub A and Sub B

to confirm the substituted position. When the singlet proton signal at δ 8.03 was irradiated, NOE was observed at the signals of δ 8.17 (assigned to the 1-position) and δ 7.78 (assigned to the 2″- and 6″-positions). These results clearly indicate that the new sulfonatophenyl group is attached to the 7-position of Y5. The HMBC analyses also suggest this linkage. In the HMBC spectrum, the singlet proton signal at δ 8.03 and the doublet proton signals at δ 7.78 (assigned to 2″- and 6″-positions) show the correlation to the carbon signals at δ 175.6 (assigned to the 6-position) and at δ 139.5 (assigned to the 1″-position), and the carbon signal at δ 137.9 (assigned to the 7-position), respectively. Therefore, the structure of Sub A was found to be trisodium salt of 6-hydroxy-7-(4-sulfophenyl)-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid.

The ^{1}H- and ^{13}C-NMR data of Sub B and Y5 indicate that structural differences between the two compounds are ascribable to the locations of three substituents (azo, hydroxy, sulfonato) on the naphthalene moiety. The coupling pattern of aromatic protons of the naphthalene moiety of Sub B indicates the substituents exist on the same ring. Irradiation of the singlet signal of the naphthalene ring (δ 8.38) caused NOE to the signal of the broad doublet signal at δ 7.68 assigned to 8-position. This indicates the substituents locate at 2-, 3- and 4-positions. The similarity of UV-Vis spectrum of Sub B to that of Y5 (Fig. 3) suggests that both compounds have the same conjugated system. Considering the origin, the hydroxy group should be located at β-position of naphthalene ring because Sub B should be an azo-coupled product between SA and a sulfonated β-naphthol, which is an impurity of SS. On the basis of these data, the structure of Sub B was estimated as disodium salt of 3-hydroxy-4-(4-sulfophenylazo)-2-naphthalenesulfonic acid.

Sub B was eluted after Y5 on reversed phase HPLC and showed a little longer absorption maximum than that of Y5. Based on the estimated structure, it is possible to form an intramolecular chelate ring between sulfonato group at 2-position and hydroxyl group at 1-position. Therefore, the chelating formation may offer a plausible explanation for these phenomena because it makes the compound less polar and gives bathochromic effect on its spectrum.

From technical experience as a manufacturer of Y5, Sub A seems to be produced when Y5 is synthesized at temperature a little higher than usual. Y5 is formed by coupling reaction between diazotized SA and SS. Under high temperature the elimination of N_2 from diazotized compound would occur, to produce a cationic intermediate which reacts as electrophile with Y5. It is important to clarify the mechanism of the side reaction to reduce subsidiary colors and obtain good commercial Y5. Further studies are needed for this.

The quantitative HPLC analyses of Sub A, Sub B, and other known subsidiary colors were performed on commercial Y5 (27 samples of 6 makers). The average contents $\pm$ standard deviation of Sub A and Sub B were $0.257 \pm 0.241\%$ and $0.132 \pm 0.232\%$ as Y5, respectively, and the average content of Sub A was greater than those of any other known subsidiary colors of Y5. Detailed results of the analyses will be reported elsewhere.

References

1) *a*) Yamada M., Inoue T., Kato Y., Nakamura M., Shibata T., Kimura M., Tsuji S., Ito Y., *Shokuhin Eiseigaku Zasshi*, **34**, 239—247 (1993); *b*) Takeda Y., Goda Y., Noguchi H., Yamada T., Yoshihira K., Takeda M., *Food Additives and Contaminants*, **11**, 97—104 (1994); *c*) Yamada M., Kato Y., Nakamura M., Ishimitsu S., Shibata T., Ito Y., *Shokuhin Eiseigaku Zasshi*, **36**, 417—422 (1995).
2) Cox E. A., Richfield-Fratz N., Bailey C. J., Albert R. H., *J. Assoc. Off. Anal. Chem.*, **67**, 240—249 (1984).
3) Bailey J. E., Jr., *J. Chromatogr.*, **347**, 163—172 (1985).
4) Uematsu Y., Hirokado M., Nakajima K., Takahashi I., Matsui K., Kazama M., *Ann. Rep. Tokyo Metr. Res. Lab. P. H.*, **38**, 245—252 (1987).
5) Uematsu Y., Hirokado M., Nakajima K., Takahashi I., Matsui K., Kazama M., *Ann. Rep. Tokyo Metr. Res. Lab. P. H.*, **39**, 151—155 (1988).
6) Bailey J. E., Jr., Calvey R. J., *Assoc. Off. Anal. Chem.*, **58**, 1087—1128 (1975).