Note

Structural Determination of the Subsidiary Colors in Food Blue No. 1 (Brilliant Blue FCF) Aluminum Lake Detected by Paper Chromatography

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One of eight Food Blue No. 1 aluminum lakes (B-1Als) used in the official inspection of coal-tar colors in fiscal year 1999 had a violet sub-spot during paper chromatography and was rejected. To clarify the origin of the sub-spot, the violet subsidiary color (Sub-V) was isolated from the sample. On the basis of NMR and MS analyses and ion chromatography, the structure of the subsidiary color was elucidated to be 2-[[4-[*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl][4-hydroxyphenyl]methylio]benzenesulfonic acid. The relative content of Sub-V to that of *m,m*-B-1 in the rejected sample was determined to be 39.5% by HPLC. The relative contents in other submitted samples of B-1Al were in the range of 1.1–3.6%.

Key words: Food Blue No. 1 aluminum lake; brilliant blue FCF aluminum lake; paper chromatography; subsidiary color; MS; NMR; polyamide; HPLC

Introduction

In Japan, twelve synthetic food colors and eight aluminum lakes are permitted for use in foods. They can be sold after passing the official inspection tests.

Recently, from the viewpoint of international harmonization, several studies have been performed to identify and quantify the subsidiary colors in Food Red No. 40¹⁻⁴⁾, Food Red No. 2³⁻⁷⁾, Food Red No. 102³⁻⁹⁾, Food Red No. 104¹⁰⁾, Food Yellow No. 5^{3, 4), 6), 8), 11-13)}, Food Blue No. 1¹⁴⁻¹⁶⁾, and Food Green No. 3¹⁷⁾ in Japan. Japan's Specifications and Standards for Food Additives, 7th Edition (JSFA-VII) specified the subsidiary colors in Food Red No. 40 and Food Yellow No. 5¹⁸⁾.

Food Blue No. 1 (B-1; CI No. 42090, brilliant blue FCF, FD & C Blue No. 1) is classified as a triphenylmethane color prepared by the condensation of 1 mole of *o*-sulfobenzaldehyde (OSBA) and 2 moles of 3-*N*-ethyl-*N*-benzylaniline sulfonic acid (*m*-EBASA), and is called disodium salt of 2-[bis[4-[*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl]methylio]benzenesulfonic acid [OSBA-(*m*-EBASA)(*m*-EBASA); *m,m*-B-1]. Commercial B-1 product is a mixture of the isomers formed from *o*-, *m*-, and *p*-EBASA. Stein¹⁹⁾ and Kamikura¹⁴⁾ identified two subsidiary colors in commercial B-1 by comparison of the TLC or HPLC data with those of synthetic compounds; one was OSBA-(ethylbenzylaniline: EBA)(*m*-EBASA) [Sub-F] formed by the loss of one sulfonate group from B-1 and the other was OSBA-(*N*-ethyl-aniline: EA)(*m*-EBASA) [Sub-E] formed by the loss of

one sulfonatobenzyl group. Matsufuji *et al.* reported that recent commercial B-1 products contained five subsidiary colors, OSBA-(*m*-EBASA)(*p*-EBASA) [*m,p*-B-1; Sub-C], OSBA-(*o*-EBASA)(*m*-EBASA) [*o,m*-B-1; Sub-D], Sub-E and two unidentified subsidiary colors (Sub-A and B), other than *m,m*-B-1, which was the main compound¹⁵⁾. Furthermore, Kusaka *et al.* also identified a magenta subsidiary, OBSA-(4-oxo-2,5-cyclohexadienylidene: OCD)(*m*-EBASA) [Sub-M] in commercial B-1 products¹⁶⁾. The structures are shown in Fig. 1.

Food Blue No. 1 aluminum lake (B-1Al) is prepared as a complex of B-1 and aluminum salt under alkaline conditions¹⁸⁾. In fiscal year 1999, eight samples of B-1Al were submitted for official inspection. Because one of them had a violet sub-spot in addition to the main spot during paper chromatography (PC), it was rejected²⁰⁾. Analytical HPLC of the rejected sample was performed under the conditions specified by Matsufuji *et al.*¹⁵⁾. The retention time (*Rt*) of the violet subsidiary color detected in it was similar to that of the Sub-M reported by Kusaka *et al.*¹⁶⁾. Since we could not obtain authentic Sub-M, we isolated the violet subsidiary color (Sub-V) by column chromatography to identify its chemical structure by MS and NMR. We also surveyed its content in the samples of B-1Als submitted for official inspection in fiscal year 1999.

Fig. 1. Chemical structures of the main component (*m,m*-B-1) and known subsidiary colors in commercial B-1 *m,m*-B-1 [OSBA-(*m*-EBASA)(*m*-EBASA)], the disodium salt of 2-[bis[4-*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl]methyl]obenzenesulfonic acid; Sub-C: *m,p*-B-1 [OSAB-(*m*-EBASA) (*p*-EBASA)], the disodium salt of 2-[[4-*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl][4-*N*-ethyl-*N*-(4-sulfophenylmethyl)amino]phenyl]methyl]obenzenesulfonic acid; Sub-D: *o,m*-B-1 [OSBA-(*o*-EBASA) (*m*-EBASA)], the disodium salt of 2-[[4-*N*-ethyl-*N*-(2-sulfophenylmethyl)amino]phenyl][4-*N*-ethyl-*N*-(4-sulfophenylmethyl)amino]phenyl]methyl]obenzenesulfonic acid; Sub-E [OSBA-(EA) (*m*-EBASA)], the monosodium salt of 2-[[4-*N*-ethylamino]phenyl][4-*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl]methyl]obenzenesulfonic acid; Sub-F [OSBA-(EBA)(*m*-EBASA)], the monosodium salt of 2-[[4-*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl][4-*N*-ethyl-*N*-(4-sulfophenylmethyl)amino]phenyl]methyl]obenzenesulfonic acid; Sub-M [OSBA-(OCD) (*m*-EBASA)], the disodium salt of 2-[[4-*N*-ethyl-*N*-(2-sulfophenylmethyl)amino]phenyl][4-oxo-2,5-cyclohexadienylidene]methyl]obenzenesulfonic acid.

Materials and Methods

Samples and reagents

Eight official Japanese B-1Al samples submitted for official inspection in fiscal year 1999 were used. The brilliant blue FCF reference standard was a standard product distributed by NIHS and was used as *m,m*-B-1. Polyamide C-200 for column chromatography was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water was obtained from a Milli-Q SP. TOC. Reagent Water System (Millipore Co., Massachusetts, USA), and all other chemicals were of reagent or HPLC grade.

Instruments

Analytical HPLC was performed with a JASCO 880 system equipped with a photodiode array detector MD 1515 (JASCO Co., Ltd., Tokyo, Japan). Ion chromatography (IC) was done with an ion chromatograph DX-320 (Dionex Co., CA, U.S.A.). Electrospray ionization (ESI)-MS was recorded on a Shimadzu QP-8000 mass spectrometer (Shimadzu Co., Kyoto, Japan) with 0.04% ammonium carbonate as the solvent. Fast-atom bombardment (FAB)-MS was recorded on a JEOL DX-300 mass

spectrometer (JEOL Co., Ltd., Tokyo, Japan) with glycerin as the matrix agent. ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker OMEGA600 (Bruker Co., Ltd., Germany) instrument (600 MHz for ¹H, and 150 MHz for ¹³C) in methanol-*d*₄. The UV-visible (UV-Vis) spectrum was determined with a Hitachi UV-Vis spectrometer U-3210 (Hitachi, Ltd., Tokyo, Japan).

PC

PC was carried out as described by JSFA-VII¹⁸⁾. The development solvent for the PC was a mixture of *n*-butanol, 1% ammonia solution and absolute ethanol (6 : 3 : 2), and Toyo filter paper No. 50 (Advantec Toyo Co., Tokyo, Japan) was used for the PC.

HPLC

B-1Al (0.1 g) was dissolve in 5 mL of dilute sulfuric acid (5→20) and then made up to 100 mL with 0.1 mol/L ammonium acetate solution. After removal of any insoluble material by centrifugation, the supernatant was subjected to analytical HPLC. A 20-μL aliquot of the sample solution (1 mg/mL as B-1Al) was directly chromatographed on a Develosil ODS-UG-5 column (4 mm i.d.×250 mm, Nomura Chemical Co., Aichi, Japan)

at 40°C with photodiode array detection from 200 to 700 nm, and the flow rate was set at 1.0 mL/min. Solvents A, A' and B were methanol, acetonitrile and 0.02 mol/L ammonium acetate solution, respectively. The mobile phases used were two isocratic conditions, a methanol-ammoniumacetate solutionsystem (A/B (38 : 62)) and an acetonitrile-ammonium acetate solution system (A'/B (21 : 79)).

Isolation of subsidiary colors

B-1Al (sample: I-i, 0.5 g) was dissolved in 5 mL of dilute sulfuric acid (5→20) and then made up to 50 mL with water. The solution was then loaded onto a polyamide C-200 column (30 g in nylon foil, 25 mm i.d.×40 cm). This column was washed with 10% acetic acid and

then developed with 800 mL of a mixture of ethanol and 10% acetic acid (7 : 3). After elution, the violet band in the column was cut out with scissors, and eluted with a mixture of ethanol and 10% acetic acid (7 : 3). The eluate was co-evaporated with ethanol and acetone to give crude Sub-V (20 mg).

Preparative HPLC was performed several times under the above HPLC conditions using a 500-μL of injection volume, with a methanol-ammonium acetate solution system as the mobile phase to afford Sub-V (5 mg). To obtain other subsidiary colors, preparative HPLC of the sample I-i was also performed under the conditions described by Matsufuji *et al.*¹⁵⁾. The fractions at the *Rt* of each color were collected and concentrated to give Sub-A~E and Sub-M.

Fig. 2. Chemical structure of Sub-V [free acid of OSBA-HP (*m*-EBASA)]

Sub-V, 2-[[4-[*N*-ethyl-*N*-(3-sulfophenylmethyl)amino]phenyl][4-hydroxyphenyl]methyl]obenzenesulfonic acid.

Ion chromatography

Ion chromatography (IC) was used to identify the counter ions of Sub-V. Sodium chloride and ammonium chloride used as standards were separately dissolved in water. The cationic ions in each 5-μL aliquot of the solutions were chromatographed on an IonPac CG12A guard column (4 mm i.d.×50 mm, Dionex) and an IonPac CS12A analytical column (4 mm i.d.×250 mm, Dionex), with 20 mmol/L methanesulfonic acid at flow rate of 1.0 mL/min at 35°C, and were detected by conductivity.

Quantification of Sub-V and *m,m*-B-1 by HPLC

The analytical HPLC was performed with the methanol-ammonium acetate solution system under the conditions described above. Five-point calibration curves were linear from 0.1 to 15 mg/mL for Sub-V (at 580 nm), and from 0.1 to 100 mg/mL for *m,m*-B-1 (at 630 nm).

Table 1. ¹H-NMR Data (δ Value, 600 MHz/TMS) for Sub-V in MeOH-*d*₄^{a)}

Position	Sub-V	Reported data	
		Sub-M ^{b)}	Sub-E ^{c)}
3	7.09 (dd, <i>J</i> =1.1, 8.0)	7.09 (dd, <i>J</i> =1.2, 8.1)	7.09 (dd, <i>J</i> =1.1, 7.7)
4	7.55 (td, <i>J</i> =1.1, 8.0)	7.52 (td, <i>J</i> =1.2, 8.1)	7.55 (td, <i>J</i> =1.1, 7.7)
5	7.63 (td, <i>J</i> =1.1, 8.0)	7.58 (td, <i>J</i> =1.2, 8.1)	7.63 (td, <i>J</i> =1.1, 7.7)
6	8.12 (dd, <i>J</i> =1.1, 8.0)	8.10 (dd, <i>J</i> =1.2, 8.1)	8.12 (dd, <i>J</i> =1.1, 7.7)
2', 6'	7.38 (d, <i>J</i> =9.0)	7.32 (d, <i>J</i> =8.1)	7.40 (d, <i>J</i> =7.8)
3', 5'	6.79 (d, <i>J</i> =9.0)	6.89 (d, <i>J</i> =8.1)	6.78 (d, <i>J</i> =9.0)
2''	7.74 (s)	7.75 (s)	7.74 (s)
4''	7.77 (d, <i>J</i> =7.7)	7.74 (d, <i>J</i> =7.6)	7.75 (d, <i>J</i> =7.7)
5''	7.44 (t, <i>J</i> =7.7)	7.40 (t, <i>J</i> =7.6)	7.42 (t, <i>J</i> =7.7)
6''	7.34 (d, <i>J</i> =7.7)	7.32 (d, <i>J</i> =7.6)	7.32 (d, <i>J</i> =7.7)
2''', 6'''	7.50 (d, <i>J</i> =8.8)	7.39 (d, <i>J</i> =8.1)	7.37 (d, <i>J</i> =7.8)
3''', 5'''	7.09 (d, <i>J</i> =8.8)	6.53 (d, <i>J</i> =8.1)	6.94 (d, <i>J</i> =9.0)
N-CH ₂ -CH ₃	1.34 (t, <i>J</i> =7.3)	1.32 (t, <i>J</i> =6.6)	1.31 (t, <i>J</i> =7.2)
N-CH ₂ -CH ₃	3.85 (q, <i>J</i> =7.3)	3.65 (q, <i>J</i> =6.6)	3.74 (q, <i>J</i> =7.2)
N-CH ₂ -Ar	5.03 (s)	4.87 (s)	4.90 (s)
N'-CH ₂ -CH ₃			1.29 (t, <i>J</i> =7.2)
N-CH ₂ -CH ₃			3.44 (q, <i>J</i> =7.2)

^{a)} Multiplicities and coupling constants (δ in ppm from TMS) are given in parentheses.

^{b)} Kusaka *et al.*¹⁶⁾

^{c)} Matsufuji *et al.*¹⁵⁾

Results and Discussion

HPLC and structural determination of Sub-V

The violet sub-spot in one sample (I-i: the product (i) produced by manufacturer "I") of B-1Als, which was rejected in fiscal year 1999, was detected at a slightly higher *R_f* value than the main spot by PC²⁰⁾. The HPLC with a diode array detector was performed under the HPLC conditions of Matsufuji *et al.*¹⁵⁾. The chromatogram of sample I-i at 625 nm had five subsidiary colors corresponding to Sub-A~E²⁰⁾ and an additional subsidiary color named Sub-V. The Sub-V peak at *R_t* 37.6 min was seen at 580 nm. Kusaka *et al.* obtained a small amount of a magenta color, Sub-M, with an absorption maximum at 585 nm from a commercial B-1 product¹⁶⁾. The *R_t* was similar to that of Sub-V²⁰⁾. The absorption maximum of Sub-V was at 584.0 nm; this was compatible with that of Sub-M. These findings suggested that the structure of Sub-V might be analogous to that of Sub-M.

In the FAB-MS spectra, one quasi-molecular ion peak of Sub-V was obtained at *m/z* 550 [M-H]⁻, though Kusaka *et al.*¹⁶⁾ reported two quasi-molecular ion peaks of Sub-M at *m/z* 550 [M-2Na+H]⁻ and *m/z* 572 [M-Na]⁻ that have the formulas C₂₈H₂₄N₁O₇S₂ and C₂₈H₂₃N₁-NaO₇S₂, respectively. The ESI-MS spectrum of Sub-V had three quasi-molecular ion peaks at *m/z* 274 [M-2H]²⁻/2, *m/z* 550 [M-H]⁻ and *m/z* 572 [M-2H+Na]⁻. Of them, the peak at *m/z* 550 which corresponds to C₂₈H₂₄N₁O₇S₂ characterizes the spectrum of Sub-V, compared with that of Sub-M. Namely, Kusaka *et al.*¹⁶⁾ reported only two quasi-molecular ion peaks at *m/z* 274 and *m/z* 572 in the Sub-M spectrum. These data suggested that a univalent anionic form of Sub-V did not have the OCD moiety with a sodiumsulfonato group, but had the center cation with a sulfonato group. Ion chromatographic analysis revealed that Sub-V did not contain enough sodium as counter cation. These data indicated that Sub-V took acidic form. Therefore, its molecular formula is considered to be as C₂₈H₂₅N₁O₇S₂.

Based on ¹H-¹H-correlation spectroscopy (COSY),

heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) studies, the ¹H- and ¹³C-NMR assignments for Sub-V with the quoted data for Sub-M and Sub-E are summarized in Tables 1 and 2. The ¹H- and ¹³C-NMR spectra of Sub-V were comparable with those of Sub-M and Sub-E, as shown in Tables 1 and 2, suggesting that it has a structure composed of a 4-[*N*-ethyl-*N*-(3-sulfophenyl-methyl)amino]phenyl group and a 2-substituted benze-

Table 2. ¹³C-NMR Chemical Shift Values (δ Value, 150 MHz/TMS) of Sub-V in MeOH-*d*₄

Position	Sub-V	Reported data	
		Sub-M ^{a)}	Sub-E ^{b)}
1	146.1	145.7	146.5
2	137.5	138.9	138.5
3	132.6	132.6	132.8
4	130.5	130.7	130.7
5	130.8	130.5	131.2
6	129.4	129.3	129.4
1'	130.4	129.0	129.5
2', 6'	140.6	139.6	141.3
3', 5'	119.3	114.0	114.3
4'	158.0	157.7	156.8
1''	138.4	139.2	138.6
2''	125.4	125.3	125.3
3''	147.4	147.0	147.2
4''	126.6	126.2	126.3
5''	130.2	129.9	130.0
6''	129.7	129.6	129.5
1'''	127.8	129.8	128.9
2''', 6'''	143.4	142.2	141.3
3''', 5'''	116.3	123.7	114.3
4'''	162.0 ^{c)}	Not observed	160.0
N-CH ₂ -CH ₃	13.0	13.5	12.7
N-CH ₂ -CH ₃	47.8	47.8	47.5
N-CH ₂ -Ar	55.3	54.9	54.7
N'-CH' ₂ -CH' ₃			14.3
N'-CH' ₂ -CH ₃			39.3
C ⁺	176.0	172.0 ^{c)}	178.2

a) Kusaka *et al.*¹⁶⁾

b) Matsufuji *et al.*¹⁵⁾

c) Observed in the HMBC spectrum

Table 3. Contents of Sub-V in Food Blue No. 1 Aluminum Lakes (B-1Als)

Sample	Contents of Sub-V and <i>m,m</i> -B-1 in test solutions ^{a)}		Contents of B-1 in B-1 Als ^{b)} (%)	Relative contents of Sub-V to <i>m,m</i> -B-1 (%)	Contents of Sub-V in B-1 (%)
	Sub-V (μg/mL)	<i>m,m</i> -B-1 (μg/mL)			
I-i	30.0	76.1	15.4	39.5	19.5
ii	0.8	69.9	12.0	1.1	0.7
II-i	0.8	75.8	12.5	1.1	0.6
ii	2.3	78.8	11.3	2.9	2.0
iii	2.0	78.0	13.4	2.5	1.5
iv	1.8	77.8	13.3	2.3	1.3
v	2.1	82.1	14.0	2.6	1.5
III-i	2.8	77.0	11.2	3.6	2.5

a) Data were obtained by HPLC using a mixture of methanol-ammonium acetate solution as the mobile phase. See footnotes of Fig. 3-① for HPLC conditions.

b) Contents of B-1 in B-1Als were determined by the titanium trichloride method.

Fig. 3. High performance liquid chromatograms of Food Blue No. 1 aluminum lake (sample I-i) with different mobile phases

m,m-B-1 and its subsidiary colors: 1, Sub-A; 2, Sub-B; 3, Sub-C; 4, *m,m*-B-1; 5, Sub-V; 6, Sub-D; 7, Sub-E

Sub-A~D were isolated from sample I-i using Matsufuji's HPLC conditions¹⁵). The presence of Sub-F was not recognized. The structure of Sub-A and B are not identified.

HPLC conditions

Column, Develosil ODS UG5 (4.6 mm i.d.×250 mm); column temp., 40°C; detected wavelengths, 580 nm and 625 nm; flow rate, 1.0 mL/min; sample size, 20 μ L

Solvents A, A' and B are methanol, acetonitrile and 0.02 mol/L ammonium acetate solution, respectively.

Two mobile phases: methanol-ammonium acetate solution system is a mixture of solvents A and B (① A/B (38: 62)), and the other is an acetonitrile-ammonium acetate solution system (② A'/B (21 : 79)).

nesulfonic acid group. The ^1H - and ^{13}C -NMR spectra of Sub-V and Sub-E were analogous except for the lack of an *N*-ethyl group [δ 1.29 (3H, t, $J=7.2$ Hz), δ 3.44 (2H, q, $J=7.2$ Hz)]. The main difference in the ^1H -NMR spectra between Sub-V and Sub-M was a significant downfield shift for H-3''' and H-5''' (δ 7.09 $\leftarrow$ δ 6.53). Furthermore, the ^{13}C -NMR spectrum of Sub-V also showed distinctive upfield shifts for C-3''' and C-5''' [δ 116.3 $\leftarrow$ δ 123.7], analogous to those for Sub-E. Considering these chemical shifts and coupling patterns, it was suggested that Sub-V has a hydroxybenzene moiety rather than the OCD moiety of Sub-M. This conclusion is consistent with the estimated formula. Therefore, the structure of Sub-V was concluded to be 2-[4-[*N*-ethyl-*N*-(3-sulfo-phenylmethyl)amino]phenyl][4-hydroxyphenyl]methyl]benzenesulfonic acid (Fig. 2). Considering the structures of Sub-M and Sub-V, their origin should be the same, and Sub-M might be converted to Sub-V during the preparation of B-1Al.

Quantification of Sub-V and *m,m*-B-1 in tested B-1Al by HPLC

Matsufuji *et al.* used a methanol-ammonium carbonate solution system as the mobile phase¹⁵). On the other hand, the HPLC conditions for azo food colors as described in JSFA-VII¹⁸) have used a methanol-ammonium acetate solution system or an acetonitrile-ammonium acetate solution system as the mobile phase. If HPLC of food azo colors and B-1 can be simultaneously performed, it would save time in routine analyses. Therefore, we examined the HPLC using the same mobile phases as described in JSFA-VII.

With the methanol-ammonium acetate solution system (see Fig. 3-①), *m,m*-B-1 and subsidiary colors peaks were eluted with *R*_ts similar to those cited by Matsufuji *et al.*¹⁵). The diode array spectrum of Sub-V had an absorption maximum at 574 nm which was slightly shorter than that of Sub-M at 580 nm. Sub-B, Sub-C and Sub-D showed the same absorption maximum at 634 nm as that of *m,m*-B-1. The maxima of Sub-A and E were at 622 nm and 616 nm, respectively, and these wavelengths are longer than the correspond-

ing ones reported by Matsufuji *et al.*¹⁵⁾.

In an acetonitrile–ammonium acetate solution system (see Fig. 3-②), the Sub-V peak was eluted before the *m*, *m*-B-1 peak. Moreover, Sub-B and C, and the *m*, *m*-B-1 peaks were eluted at the same *Rt*. Sub-C and Sub-D are the *m*, *p*- and *o*, *m*-isomers of *m*, *m*-B-1, respectively.

The contents of Sub-V in 8 samples of B-1Als in an official inspection were determined using HPLC with the methanol–ammonium acetate solution system; the results are shown in Table 3. The content of Sub-V in the rejected sample was remarkably high. However, those in other samples were in the range of 1.1–3.6% and were similar to those of Sub-M (0.1–0.8%) in commercial B-1 products¹⁶⁾.

The manufacturer “I” was instructed to use B-1 certified according to the requirement of JSFA-VII for the preparation of B-1Al. Subsequently, the content of Sub-V in sample I-ii prepared by the same manufacturer was reduced to a very low level.

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