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Purification of Food Color Red No. 106 (Acid Red) using high-speed counter-current chromatography

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ABSTRACT

High-speed counter-current chromatography (HSCCC) has been successfully applied to the separation of the components of Food Color Red No. 106 (R-106). The separation was performed using 25 mg of the sample with a two-phase solvent system composed of *n*-butanol and 0.01 *M* trifluoroacetic acid (1:1, v/v). Analyses by thin-layer chromatography, high-performance liquid chromatography and fast atom bombardment mass spectrometry confirmed that HSCCC was effective in the purification of the components of R-106. The separation gave 21 mg of a 99.9% pure main component (Acid Red) and 0.9 mg of 98.0% pure subsidiary dye which is probably a des-ethyl derivative.

INTRODUCTION

Many synthetic dyes are widely used for coloring foods, drugs and cosmetics. However, these synthetic colors have impurities deriving from the reactants and side products during the manufacturing process [1–4]. In the investigation of colorless organic impurities, main and subsidiary dyes are required for toxicological and structural evaluation for the purposes of sanitation and good manufacturing practice (GMP). High-performance liquid chromatography (HPLC) has been shown to be a powerful technique for the determination of main dyes and also subsidiary dyes in commercial colors [5–8], foods [9,10] and cosmetics [11,12]. Unfortunately, highly pure components of colors as reference standards are not commercially available for

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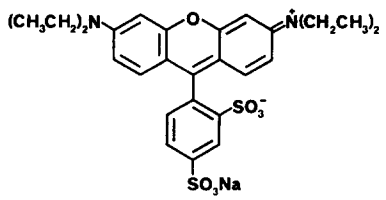

Fig. 1. Structure of the main component (Acid Red) in Food Color Red No. 106. Molecular weight: 580.

chemical analysis. Recently, however, the successful application of counter-current chromatography, a liquid–liquid partition method developed by Ito *et al.* [13], has been reported for the purification of synthetic colors [14–16].

In this study, high-speed counter-current chromatography (HSCCC) [13] was used for the purification of Food Color Red No. 106 (Acid Red, Color Index No. 45 100), which contains a synthetic xanthene-class color (Fig. 1) and at least one major subsidiary dye. Further analysis using HPLC and fast atom bombardment mass spectrometry (FAB-MS) served for the identification of the main and subsidiary dyes.

EXPERIMENTAL

Reagents

Acetonitrile, *n*-butanol, ammonium acetate, sodium chloride, potassium chloride, glycerol and trifluoroacetic acid (TFA) were of analytical-reagent grade and were purchased from Wako (Osaka, Japan). Food Color Red No. (R-106) was obtained from San-ei Chemical Industries (Osaka, Japan).

High-performance liquid chromatography

A chromatograph equipped with a constant-flow pump (LC-6A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (SPD-6A; Shimadzu) operated at 254 nm and a chromatographic data system (C-R3A; Shimadzu). The separation was performed on Wakosil $5C_{18}$ (5 μm) (250 × 4.6 mm, I.D.) (Wako) with 0.01 *M* TFA–acetonitrile (70:30, v/v) as the mobile phase at a flow-rate of 1.0 ml/min.

Thin-layer chromatography (TLC)

After applying a sample to a C_{18}-modified silica gel TLC plate (E. Merck, Darmstadt, F.R.G.; 15389), the plate was developed with 1 *M* sodium chloride–acetonitrile (67:33, v/v).

Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10-ml test-tube and 2 ml of each phase of pre-equilibrated two-phase solvent system was pipetted in. The test-tube was stoppered and shaken vigorously for 1 min to equilibrate the sample thoroughly with the two phases. The resulting upper and lower phases were analysed by HPLC. Each partition coefficient was determined by dividing the corresponding peak area of the upper phase by that of the lower phase.

High-speed counter-current chromatography

The apparatus used was a Shimadzu HSCCC-1A prototype multi-layer coil planet centrifuge with a 10-cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multi-layer coil was prepared by winding a *ca.* 160 m length of PTFE tubing onto the column holder with a 10-cm hub diameter and a 15-cm hub length, making six coiled layers with a total capacity of about 300 ml. The two-phase solvent system used was *n*-butanol–0.01 *M* TFA solution (1:1), which was thoroughly equilibrated in a separating funnel by repeated vigorous shaking and degassing at room temperature. The column was first entirely filled with the upper non-aqueous stationary phase, then 25 mg of the sample dissolved in 2 ml of both phases was loaded. The centrifuge was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the head of the column (the head–tail relationship of the rotating coil is conventionally defined by the Archimedean screw force, where all objects of different densities are driven toward the head of the coil) at a flow-rate of 2 ml/min by HPLC pump (LC-6A). The effluent from the outlet of the column was continuously monitored with an SPD-6A UV detector at 254 nm and then fractionated into test-tubes at 2 ml per tube with a fraction collector (DF-2000; Tokyo Rikakikai, Tokyo, Japan). When separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas combined with slow rotation of the coil in the tail-to-head elution mode. A 0.2-ml volume of the contents of each test-tube was diluted with distilled water and the absorbance was determined with a Ubest-50 UV–visible spectrophotometer (Japan Spectroscopic, Tokyo, Japan) at 254 nm.

Fast atom bombardment (FAB) mass spectrometry

The FAB mass spectra were obtained on a JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan). A xenon ion gun was operated at 6 kV. The matrix used was glycerol and the samples were dissolved in distilled water.

RESULTS AND DISCUSSION

Selection of two-phase solvent system

The partition coefficient (K) of the solute is the most important factor in selecting a suitable solvent system for HSCCC, because it determines the retention time and the resolution of the solute peak [13]. In general, the K value is determined simply by measuring the UV absorbance of the solute in both phases after partitioning in the two-phase solvent system [15–17]. However, when the sample is a mixture of various components such as Food Color Red No. 106 (R-106), a precise K value of each component cannot be determined by the above method. Although TLC could separate the orginal R-106 into only three spots, as shown in Fig. 2A, HPLC was able to separate it into nine components under the conditions described under Experimental. The components corresponding to peaks 7 and 9 are assumed to be a major subsidiary dye and Acid Red, respectively. After partitioning with the two phase solvent system, the resulting upper and lower phases were analysed by HPLC and the K values of peaks 7 and 9 could be determined.

Acid Red has a dipolar ion structure and is freely soluble in water, but it is difficult to dissolve it in organic solvents such as chloroform and ethyl acetate. First,

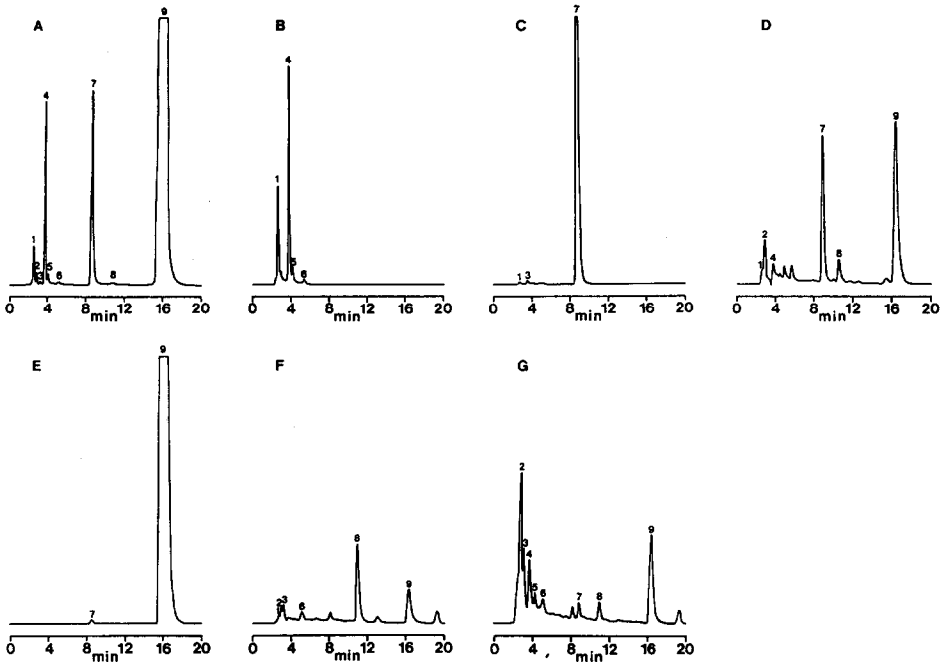

Fig. 2. HPLC separation of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV; (F) fraction V; (G) fraction VI.

we tried to use a mixture of *n*-butanol and water, which has been frequently applied as the solvent system in the separation of hydrophilic compounds [15,16]. However, the *K* value of the component corresponding to the peak 9 was 0.23, as shown in Table I. This indicates that the component is dissolved mostly in the lower aqueous phase and suggests that it is impossible to obtain a satisfactory separation using this solvent system, because the best separation is attained when the solute is partitioned evenly into both phases, namely $K = 1$ [13]. Next, *n*-butanol–0.01 *M* ammonium acetate solution (1:1), which is often used for the separation of synthetic peptides, was tested

TABLE I

PARTITION COEFFICIENTS (*K*) OF THE COMPONENTS OF FOOD COLOR RED NO. 106

K = Peak area of upper phase divided by peak area of lower phase.

Solvent system	Peak No.								
	1	2	3	4	5	6	7	8	9
n-Butanol–water (1:1)	0.89	0.19	0.84	0.00	0.00	0.00	0.13	∞	0.23
n-Butanol–0.01 *M* ammonium acetate solution (1:1)	1.45	0.19	1.46	0.00	0.02	0.00	0.34	∞	0.53
n-Butanol–0.01 *M* TFA solution (1:1)	0.05	0.15	11.70	0.00	0.00	0.00	0.65	∞	1.10

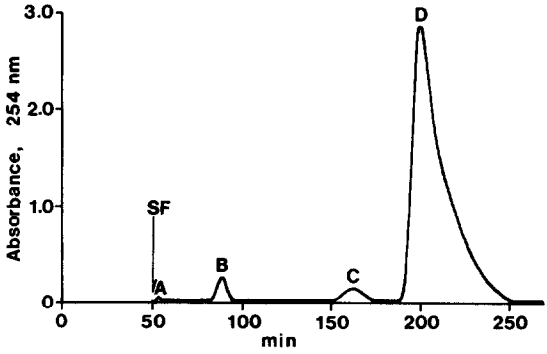

Fig. 3. Separation of Food Color Red No. 106 by HSCCC. SF = solvent front.

[18,19] but it still showed insufficient K values. In order to partition the components more readily into the upper organic phase, aqueous TFA solution was used [19]. The resulting k values were much improved using n-butanol–0.01 M TFA (1:1). These K values indicate that peaks 1, 2, 4, 5 and 6 are first eluted at the same time, peaks 3 and 8 remain in the column, but peaks 7 and 9 can be completely separated and eluted. Therefore, this system was selected as the solvent system for the purification of the components of R-106.

Purification of the components of Food Color Red No. 106 using HSCCC

A 25-mg amount of R-106 was separated using the selected solvent system. The retention of the stationary phase was 54.7%. The separation took 4.5 h and the elution volume of the entire run was 540 ml. The elution curve of the R-106 components at 254 nm is shown in Fig. 3. The components were separated into four peaks and each fraction and each phase of the column contents were analysed by TLC and

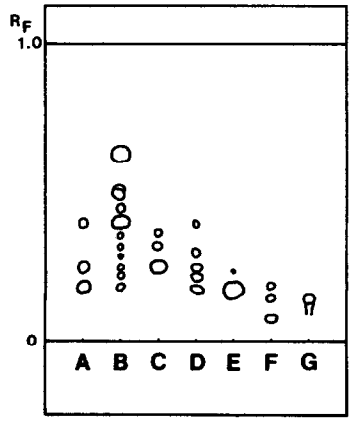

Fig. 4. TLC separation of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction I; (C) fraction II; (D) fraction III; (E) fraction IV; (F) fraction V; (G) fraction VI.

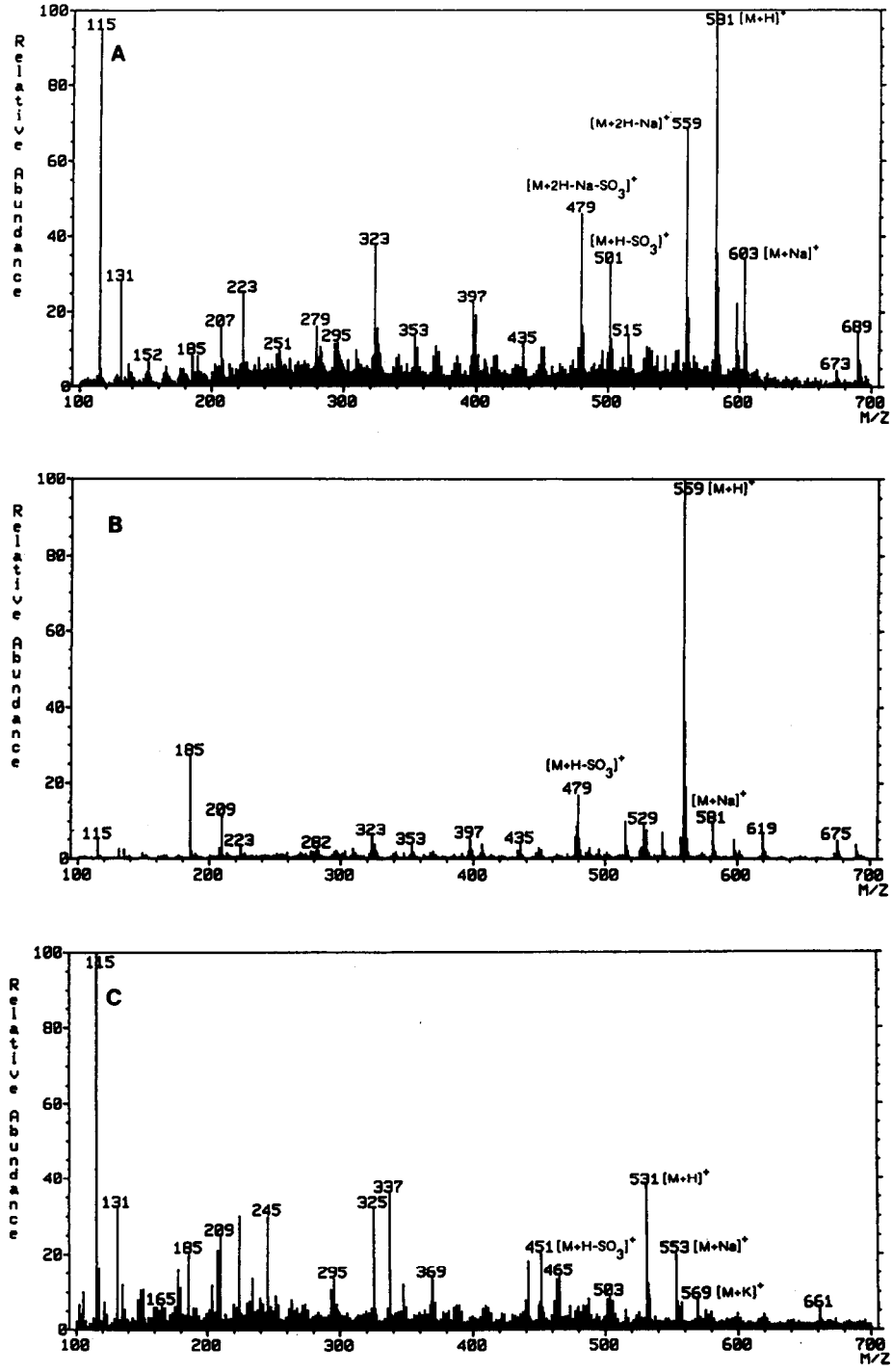

Fig. 5. FAB mass spectra of the components of Food Color Red No. 106. (A) Original Food Color Red No. 106; (B) fraction IV; (C) fraction II.

HPLC. At peak A on the elution curve, more hydrophilic compounds than the component corresponding to peak 1 were eluted and these compounds were hardly visible in HPLC and TLC of the original sample owing to the low concentrations. The compounds corresponding to the peaks 1, 2, 4, 5 and 6 were eluted at the same time at peak B. Peaks 7 and 9 were eluted at peaks C and D, respectively, on the elution curve, and peaks 3 and 8 remained in the column contents.

All contents in the fractionated test-tubes and column were combined to make six fractions on the basis of the results of TLC and HPLC analyses. Figs. 2B–G and 4 show the HPLC and TLC results, respectively. Although fractions I (test tube No. 50-15), III (test tube No. 183-188), V (upper phase of column contents) and VI (lower phase of column contents) contained various small amounts of the components, the components corresponding to peak 7 in fraction II (test tube No. 156-182) and peak 9 in fraction IV (test tube No. 189-290) were almost isolated from other components. In HPLC analyses, peaks 7 and 9 constituted about 2 and 95% of the total peak area at 254 nm, respectively, in the original sample, but after only one purification run by HSCCC the purity of peak 7 in fraction II (Fig. 2C) and peak 9 in fraction IV (Fig. 2E) increased to over 98.0 and 99.9%, respectively. Peak 9 was 21 mg of a pure compound and peak 7 was 0.9 mg of a pure compound. The results from TLC and HPLC analyses clearly indicate that the components of R-106 are easily purified by HSCCC.

Identification of acid red and an impurity by FAB-MS

The FAB mass spectra of the original sample, the compounds in fractions II and IV corresponding to peaks 7 and 9, respectively, are shown in Fig. 4 A–C. Acid Red has a molecular weight of 580 and the two molecular ion species, $[M + H]^+$ and $[M + Na]^+$, are clearly observed at *m/z* 581 and 603, respectively, and $[M + H - SO_3]^+$ also appears at *m/z* 501 in the spectrum of the original sample (Fig. 5A). The prominent ion at *m/z* 559 is assigned to $[M + 2H - Na]^+$. In the spectrum of the compound in fraction IV, the base peak is observed at *m/z* 559 together with the characteristic ions at *m/z* 479 and 581 (Fig. 5B). Further, when potassium chloride was added to the sample solution, the $[M + K]^+$ appeared at *m/z* 597. Hence the molecular weight was determined to be 558 and this compound in fraction IV is Acid Red, which possesses a free sulphonic acid instead of the sodium salt.

Fig. 5C shows the spectrum of the compound in fraction II. The three related molecular ions, $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$, are observed at *m/z* 531, 553 and 569, respectively. The last ion was much enhanced when potassium chloride was added, so the molecular weight was determined to be 530, which is 28 less than that of the parent food dye. The $[M + H - SO_3]^+$ is also observed at *m/z* 451. These results indicate that the structure of the subsidiary dye in fraction II is a des-ethylated derivative of R-106.

CONCLUSION

We were able to purify the components of R-106 using HSCCC. The HSCCC separation of the components was performed using 25 mg of sample with the two-phase solvent system *n*-butanol–0.01 *M* TFA (1:1). The confirmation studies by TLC, HPLC and FAB-MS showed that HSCCC is successful in the purification of the

major components of R-106. We obtained 21 mg of 99.9% pure main component (Acid Red) and 0.9 mg of a 98.0% pure subsidiary dye whose structure was shown to be a des-ethylated derivative. Hence the present HSCCC methodology can provide a useful separation technique for the study of the chemistry, composition and manufacture of synthetic colors.

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