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Purification of Food Color Red No. 106 (acid red) using pH-zone-refining counter-current chromatography

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Abstract

pH-Zone-refining counter-current chromatography was successfully applied to the separation of the main components of Food Color Red No. 106 (R-106, acid red, Color Index No. 45100). A 300-mg quantity of sample was separated using the following two-phase solvent system: *n*-butanol–water, 40 m*M* sulfuric acid in organic stationary phase and 30 m*M* ammonia in aqueous mobile phase. The obtained fractions were analyzed by high-performance liquid chromatography and fast atom bombardment mass spectrometry. The separation yielded 261.9 mg of main component of acid red with purity of 99.9%.

Keywords: Counter-current chromatography; Preparative chromatography; Food Color Red No. 106; Acid red; Dyes

1. Introduction

Many synthetic dyes widely used for coloring foods, drugs, and cosmetics contain impurities deriving from the reactants and side products during the manufacturing process [1–4]. For sanitation and good manufacturing practice, toxicological and structural studies are required for main and subsidiary dyes as well as colorless organic impurities. Although high-performance liquid chromatography (HPLC) has been used for the determination of main dyes and subsidiary dyes in commercial colors [5–8], foods [9,10], and cosmetics [11,12], high-purity standards of these components needed as reference standards are currently not commercially available. The recently developed preparative method for the separation of ionizable compounds, called pH-zone-refining counter-current chromatography (CCC) [13,14] is capable of yielding large quantities of highly pure components. It has been successfully applied to separation and/or purification of a variety of components including peptides [15,16], amino acids [17], alkaloids [18], and synthetic colors containing carboxylate [13,19–21] or sufonate [22,23] groups.

In this study, pH-zone-refining CCC was used for the purification of several hundred milligrams of Food Color Red No. 106 (R-106, acid red, Color

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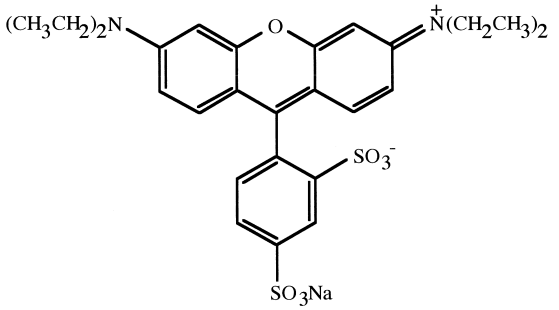

Fig. 1. Structure of the main component (acid red) in Food Color Red No. 106. Molecular mass: 580.

Index No. 45100), a xanthene-class color that contains two sulfonate groups (Fig. 1). In a previous study [24], using conventional high-speed CCC, separation of only a relatively small amount (25 mg) of Food Color Red No. 106 was possible. Further analysis using HPLC and fast atom bombardment mass spectrometry (FAB–MS) served for the identification of the major component.

2. Experimental

2.1. Reagents

Acetonitrile, *n*-butanol, trifluoroacetic acid (TFA), aqueous ammonia, sulfuric acid, and glycerol were of analytical grade and purchased from Wako (Osaka, Japan). Food Color Red No. 106 was purchased from San-ei Chemical Industry (Osaka, Japan).

2.2. HPLC analysis

A chromatograph, equipped with a constant flow pump (PU-970, Jasco, Tokyo, Japan), was used with a variable wavelength UV–Vis detector (UV-970, Jasco, Tokyo, Japan) operated at 254 nm. The separation was performed on Cosmosil $5C_{18}$ AR (5 μm, 150×4.6 mm, I.D., Nacarai, Tokyo, Japan) with acetonitrile–0.01 *M* TFA (27:73, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. A 2-μl of sample solution was injected into HPLC.

2.3. CCC apparatus

The apparatus used was an HSCCC-1A prototype multi-layer coil planet centrifuge (Shimadzu, Kyoto, Japan) with a 10-cm orbital radius which produces a type-J synchronous planetary motion at 800 rpm. The multi-layer coil was prepared by winding a ≈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.

2.4. Preparation of solvent system and sample solution

The solvent pairs were prepared as follows: *n*-butanol and distilled water at an arbitrary volume ratio were thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated. The upper organic phase was acidified with sulfuric acid (retainer) at 40 m*M* and used as the stationary phase. Aqueous ammonia (eluter) was added to the lower aqueous phase at 30 m*M* which was used as the mobile phase. A sample solution was prepared by dissolving 300 mg of Food Color Red No. 106 in 4 ml of each phase and adjusting at pH 0.2 with sulfuric acid.

2.5. Separation procedure

The column was first entirely filled with the upper non-aqueous stationary phase, then the sample solution was loaded. The column 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 with different densities, either lighter or heavier than the surrounding medium, are driven toward the head of the coil) at a flow-rate of 1 ml/min by a HPLC pump (LC-6A, Shimadzu). The effluent from the outlet of the column was fractionated into test tubes at 1 ml per tube using a fraction collector (DF-2000, Tokyo Rikakikai, Tokyo, Japan).

After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas

under slow coil rotation in the tail-to-head elution mode. The absorbance at 554 nm and the pH of each eluted fraction were measured to draw the elution curve and pH profile.

2.6. FAB–MS analysis

The FAB mass spectrum was obtained with a double-focusing mass spectrometer (JMS-HX110, Jeol, Tokyo, Japan). A xenon ion gun was operated at 10 kV. The matrix used was glycerol.

3. Results and discussion

3.1. Selection of two-phase solvent system

Successful separation by pH-zone-refining CCC largely depends upon the selection of a suitable solvent pair. The step-by-step systematic search of the suitable solvent system for acidic analyte is described below [14]:

(1) Select an appropriate two-phase solvent system; *n*-butanol–water system, methyl *tert*.-butyl ether–water system, hexane–ethyl acetate–methanol–water system, etc., according to the polarity of the target compounds.

(2) Add ammonia (eluter) to the lower phase at an appropriate concentration (usually 10–40 m*M*).

(3) A 2-ml volume each of the upper phase and the basified lower phase is delivered into a test tube.

(4) Add a small amount of the sample, apply a stopper and vortex several times to equilibrate the contents.

(5) Measure the analyte concentration in the upper and the lower phases using a spectrophotometer, and obtain the partition coefficient (K_{base}) by dividing the analyte concentration in the upper phase by that in the lower phase.

(6) If $K_{base}<0.3$, add appropriate amount of retainer acid (TFA for carboxylic acid sample and sulfuric acid for sulfuric acid sample) to the test tube to bring the pH down to around 2 and 0.5, respectively.

(7) Measure the analyte concentration and K using procedure 5.

(8) If $K_{acid}>3$, the solvent composition is suitable for separation.

(9) If K_{base} is not small enough, repeat the whole procedure using a more hydrophobic solvent system.

(10) If K_{acid} is not large enough, repeat the whole procedure using a more polar solvent system.

(11) For a basic analyte, substitute hydrochloric acid for ammonia in procedure 2 to test $K_{acid}<0.3$, and substitute triethylamine for TFA in procedure 6 to test $K_{base}>3$.

Acid red has a dipolar ion structure and is freely soluble in water, but it is not well soluble in an organic solvent such as chloroform and ethyl acetate. We tried to use a mixture of *n*-butanol and water as a two-phase solvent system, which is commonly used for the separation of polar compounds. According to the procedure described above, we measured K values. When TFA (10–40 m*M*) was used as a retainer, K_{acid} values are no larger than 3.0. Therefore, we repeated the whole procedure using sulfuric acid as a retainer. As summarized the K values in Table 1, when the difference in the concentrations between sulfuric acid and ammonia is 10 m*M*, K_{acid} values became greater than 3.0, i.e. 20 m*M* sulfuric acid and 10 m*M* ammonia, 30 and 20 m*M*, or 40 and

Table 1
Partition coefficients of the components of Food Color Red No. 106

Solvent system [*n*-Butanol–water]	Retainer [H_2SO_4 (m*M*)]	Eluter [NH_4OH (m*M*)]	Partition coefficient (K)[a]	
			Acid	Base
	10	10	1.97	0.23
	20	10	4.3	0.21
	20	20	1.72	0.23
	30	20	3.32	0.23
	30	30	2.53	0.23
	40	30	4.88	0.19

[a] $K_{(U/L)}$.

30 m*M*. Among them, a pair of 40 m*M* sulfuric acid and 30 m*M* ammonia shows the largest K_{acid} value and the smallest K_{base} value, therefore, we selected the following solvent system: *n*-butanol–water, 40 m*M* sulfuric acid in organic stationary phase and 30 m*M* ammonia in aqueous mobile phase.

3.2. Separation of acid red components by pH-zone-refining CCC

A 300-mg quantity of acid red was dissolved in 4 ml of each phase and its pH was adjusted at 0.2 with sulfuric acid. Then, according to the procedures described in Section 2, the sample solution was separated. The retention of the stationary phase was 28.8%, and the total separation time was 3.8 h. The absorbance at 554 nm and pH of the CCC fractions were measured to draw the elution and pH curves (Fig. 2).

At first, we attempted to separate the components by injecting the sample solution without adjusting its pH, however, we could not obtain the satisfactory separation. Since the sample is a Na salt, an addition of sulfuric acid is required to neutralize Na^+ to lower the pH so that target compounds are distributed largely in the upper stationary phase. Varying the pH between −0.2 and 0.8, the separation was repeated. As the results, the successful separation and the clear pH curve were obtained when adjusting pH at 0.2.

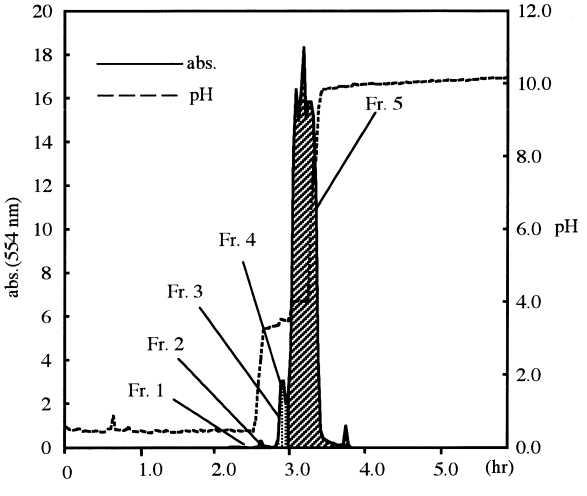

Fig. 2. Separation of the components of Food Color Red No. 106 by pH-zone-refining CCC. Conditions: see Section 2.

Based on the HPLC analysis and the elution curve, all collected fractions were combined into five pooled fractions (fractions 1–5). Fig. 3 shows the HPLC analysis of these combined fractions and the original sample: fraction 5 (261.9 mg) contained almost pure component corresponding to acid red. On the other hand, fraction 1 (1.4 mg), 2 (8.6 mg), 3 (1.3 mg), and 4 (4.6 mg) contained multiple components.

In the HPLC analysis of the original sample (Fig. 3), acid red constituted about 95.0% of the total peak area at 254 nm. After only one step operation by pH-zone-refining CCC, the purity of acid red was increased to 99.9%. These results demonstrate a high resolving power of pH-zone-refining CCC achieved by the careful selection of the proper solvent system.

3.3. Identification of acid red components by FAB–MS

Fig. 4 shows the FAB mass spectrum of fraction 5 corresponding to acid red. Acid red has a molecular mass of 580 and the two molecular ion species, $[M+H]^+$ and $[M+Na]^+$, are clearly observed at *m*/*z* 581 and 603, respectively, and $[M+2H-Na]^+$, $[M+H-SO_3]^+$ and $[M+2H-Na-SO_3]^+$ also appear at *m*/*z* 559, 501, and 479, respectively. Therefore, these results indicate that the compound in fraction 5 is acid red.

4. Conclusion

Using pH-zone-refining CCC we were able to purify the main component of Food Color Red No. 106 with the following solvent system: *n*-butanol–water, 40 m*M* sulfuric acid in organic stationary phase and 30 m*M* ammonia in aqueous mobile phase. From 300 mg of the sample, we obtained 261.9 mg of 99.9% pure acid red. The overall results of our studies indicated that pH-zone-refining CCC is

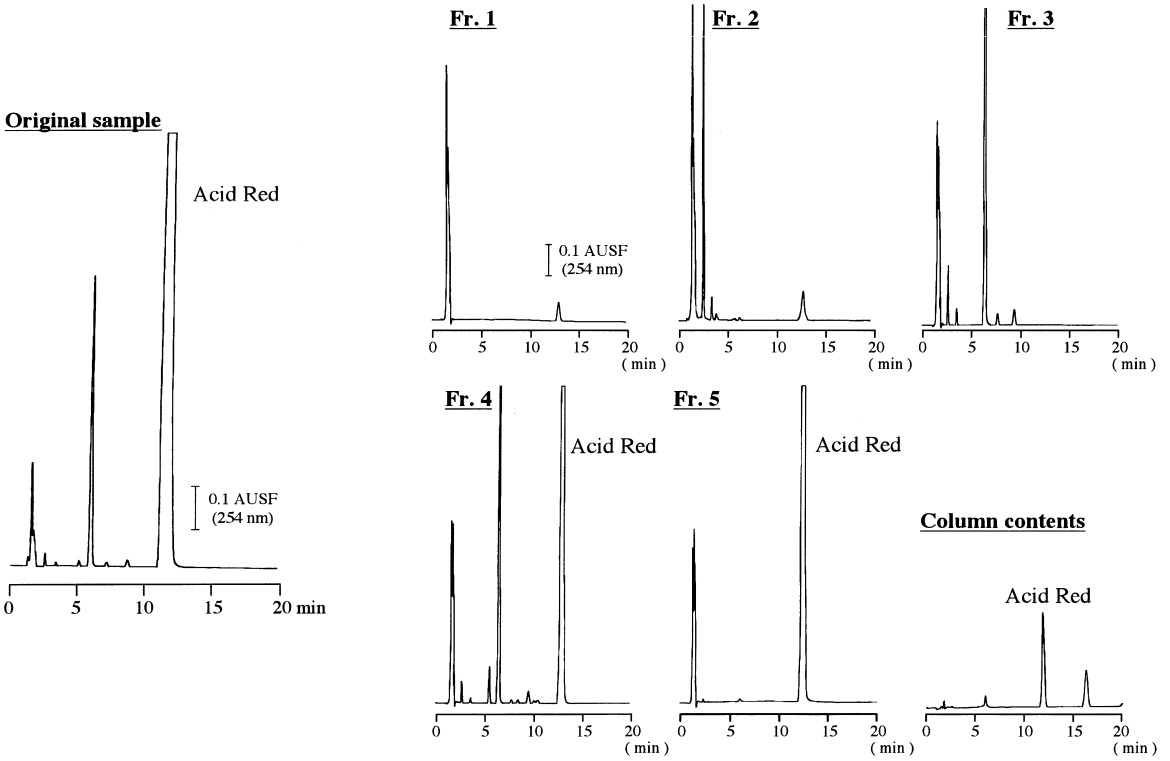

Fig. 3. HPLC separation of the components of Food Color Red No. 106. HPLC conditions: see Section 2.

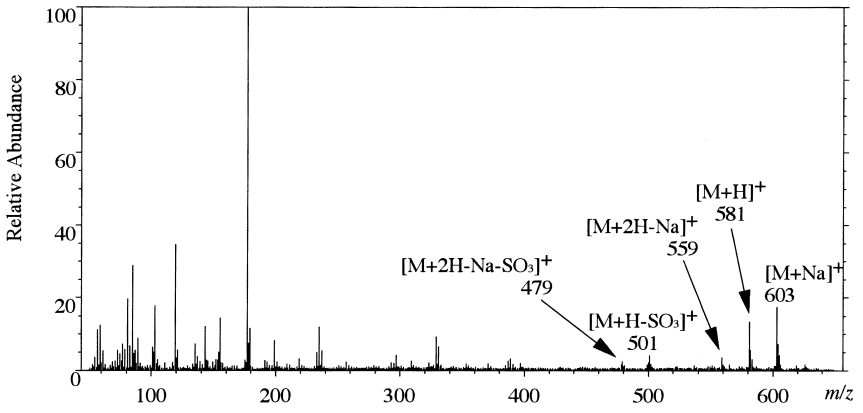

Fig. 4. FAB mass spectrum of the isolated component of Food Color Red No. 106. Conditions: see Section 2.

a powerful technique for the purification of acid red components.

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