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Separation of lac dye components by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography has been successfully applied to the separation of the lac dye components. A 25-mg quantity of the sample was separated using a two-phase solvent system composed of *tert*.-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5). The fractions were analyzed by high-performance liquid chromatography and electrospray tandem mass spectrometry. The separation yielded 2.6 mg of 97.2% pure laccaic acid C, 9.5 mg of 98.1% pure laccaic acid A, 3.6 mg of 98.2% pure laccaic acid B, and 0.5 mg of a 95.0% pure anthraquinonedicarboxylic acid with a molecular mass of 360.

Keywords: Counter-current chromatography; Food analysis; Laccaic acids; Lac dye; Dyes

1. Introduction

Lac dye is a natural food additive extracted from a stick lac which is a secretion of the insect *Coccus laccae* (*Laccifer lacca* Kerr) and is widely used for coloring food [1]. It is known that its red color is derived from a water-soluble pigment including laccaic acids A, B, C, and E (Fig. 1) [2–8]. The quantities of these components vary according to the locality and season. For food sanitation and safe manufacturing practice, therefore, the development of a simple and precise method for identification of each component is required. Although high-performance liquid chromatography (HPLC) can be used for the analysis of these components in commercial preparations and foods, pure compounds from lac dye, for use as the reference standards, are not commercially available. In addition, no effective method for isolation of these components has been reported.

Recently, high-speed counter-current chromatography (HSCCC), an advanced liquid–liquid partition

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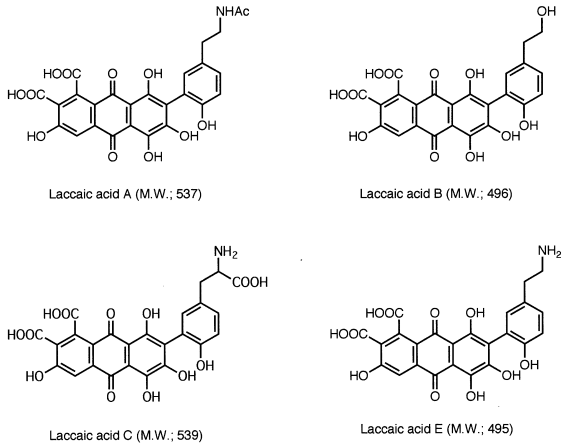

Fig. 1. Structures of laccaic acids. M.W.=molecular mass.

method that does not require a solid support [9], has been used for the separation of a natural food additive [10], synthetic dyes [11,12] and antibiotics [13–16]. In these studies, the method yielded useful amounts of pure standards. In the present study, four lac dye components were purified using HSCCC and each component was identified using HPLC and electrospray tandem mass spectrometry (ES-MS–MS).

2. Experimental

2.1. Reagents

Acetonitrile, *n*-butanol, *tert*.-butyl methyl ether, trifluoroacetic acid (TFA), and oxalic acid were of analytical grade and purchased from Wako (Osaka, Japan). Lac dye preparation for food additive was purchased from Wako.

2.2. HPLC analysis

A chromatograph equipped with constant flow pumps (LC-100P, Yokogawa, Tokyo, Japan) was used with variable-wavelength UV–VIS detectors (LC-100U, Yokogawa) operated at 280 nm. The separation was performed on LiChrosorb RP-18 (5 μm, 250×4.0 mm I.D., E. Merck, Darmstadt, Germany) with acetonitrile–0.01 *M* oxalic acid (1:4) as the mobile phase at a flow-rate of 0.8 ml/min.

2.3. ES-MS–MS conditions

The mass spectrometer and the data system used were a TSQ 7000 Triple-Stage Quadrupole (Finnigan MAT, San Jose, CA, USA) with an electrospray source and a DEC 3000 (Digital Equipment, Bedford, MA, USA), respectively. The instrument was operated at a scan rate of 400 u/s under a daughter-ion scan mode in the negative-ion mode. A gas sheath flow of 70 psi (483 kPa) nitrogen, electrospray voltage of 4.5 kV, and capillary temperature of 195°C were used. Collision induced dissociation was performed using argon as a collision gas at a pressure of 2.0 mTorr (0.27 Pa). The collision offset was +20 V.

2.4. Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10-ml test tube to which 2 ml of each phase of preequilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for 1 min to thoroughly equilibrate the sample with the two phases. Then, equal volumes of the upper and lower phases were analyzed by HPLC to determine the partition coefficient of each component.

2.5. HSCCC separation

The apparatus used was a HSCCC-1A prototype multi-layer coil planet centrifuge (Shimadzu, Kyoto, Japan) with a 10-cm orbital radius which produces a synchronous planetary motion at 800 rpm. The multilayer 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 composed of *tert*.-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5) was thoroughly equilibrated in a separation funnel by repeated vigorous shaking and degassing at room temperature. The column was first entirely filled with the upper nonaqueous 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 1 ml/min by HPLC pump (LC-6A, Shimadzu). The efluent from the outlet of the column was fractionated into test tubes at 1 ml per tube with a fraction collector (DF-2000, Tokyo Rikakikai, Tokyo, Japan). A 0.2-ml volume of the contents in each test tube was diluted with distilled water and the absorbance was determined with a UV–VIS spectrophotometer (Ubest-50, Japan Spectroscopic, Tokyo, Japan) at 495 nm. 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.

3. Results and discussion

3.1. Selection of two-phase solvent system

In HSCCC, successful separation necessitates the careful search for a suitable two-phase solvent system which provides an ideal range of the partition coefficients (K) for the applied sample [17–19]. K is defined as the solvent concentration in the stationary phase divided by that in the mobile phase. For efficient separation, K of the target compound(s) should be close to 1, where the separation factor (α) ($\alpha = K_1/K_2$ where K_1 and K_2 are partition coefficients of two solutes and $K_1 > K_2$) between the components should be greater than 1.5. If $K \ll 1$, the solutes would be eluted close together near the solvent front resulting in a loss of peak resolution and, if $K \gg 1$, the solutes would be eluted in excessively broad peaks with long elution time. A minimum α-value of 1.5 is required for the baseline separation in a semi-preparative CCC equipment providing a moderate partition efficiency of around 800 theoretical plates. The K value for a pure compound can be determined simply by measuring the UV absorbance of each phase after partitioning it between the two phases. When the compounds to be separated are not available in a pure form, as is the present case, their K values cannot be determined by the above method, and, instead, the following HPLC method should be used. As shown in Fig. 2A, HPLC can separate lac dye components into six peaks where the desired components corresponding to peaks 2, 3, 5, and 6 are laccaic acids C, E, B, and A, respectively [20]. After partitioning the sample between the two candidate phases, aliquots of the upper and lower zones were analyzed by HPLC. From these two chromatograms, the K value of each component was determined by comparing the peak heights between the corresponding peaks.

Laccaic acids have two or three carboxyl groups, five or six hydroxyl groups, and/or one amino group, and are freely soluble in water, but only slightly soluble in organic solvents such as chloroform and ethyl acetate. Based on these physical properties of laccaic acid, we selected a two-phase solvent system composed of *tert.*-butyl methyl ether–*n*-butanol–acetonitrile–water, which has been frequently used as the solvent system for the separation of polar compounds. In the *tert.*-butyl methyl ether–*n*-butanol–acetonitrile–water (4:2:3:8) system first examined, the K values of the components corresponding to peaks 1, 2, 3, 4, 5, and 6 were 0.08, 0.10, 0.19, 2.00, 0.84, and 0.60, respectively (Table 1). This indicates that peaks 1 and 2 would be eluted together near the solvent front because of their small K values. A more polar solvent composition of *tert.*-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5) yielded the best K values with desirable α values of over 1.5 for all peaks as indicated in Table 1. With this solvent system, all peaks will be completely resolved and eluted in the order of 1, 2, 3, 6, 5, and 4. The settling time of this solvent system is less than 30 s, which ensures a satisfactory retention level of the stationary phase in HSCCC [21]. Therefore, we selected this solvent system for the separation of the lac dye components.

3.2. Separation of the components of lac dye by HSCCC

A 25-mg quantity of lac dye was separated using the above solvent system. The retention of the stationary phase was 83.6%. The total separation time was 8.3 h with the total elution volume of 500 ml. The fractionated effluents from the HSCCC were applied to HPLC to analyze the contents, and the

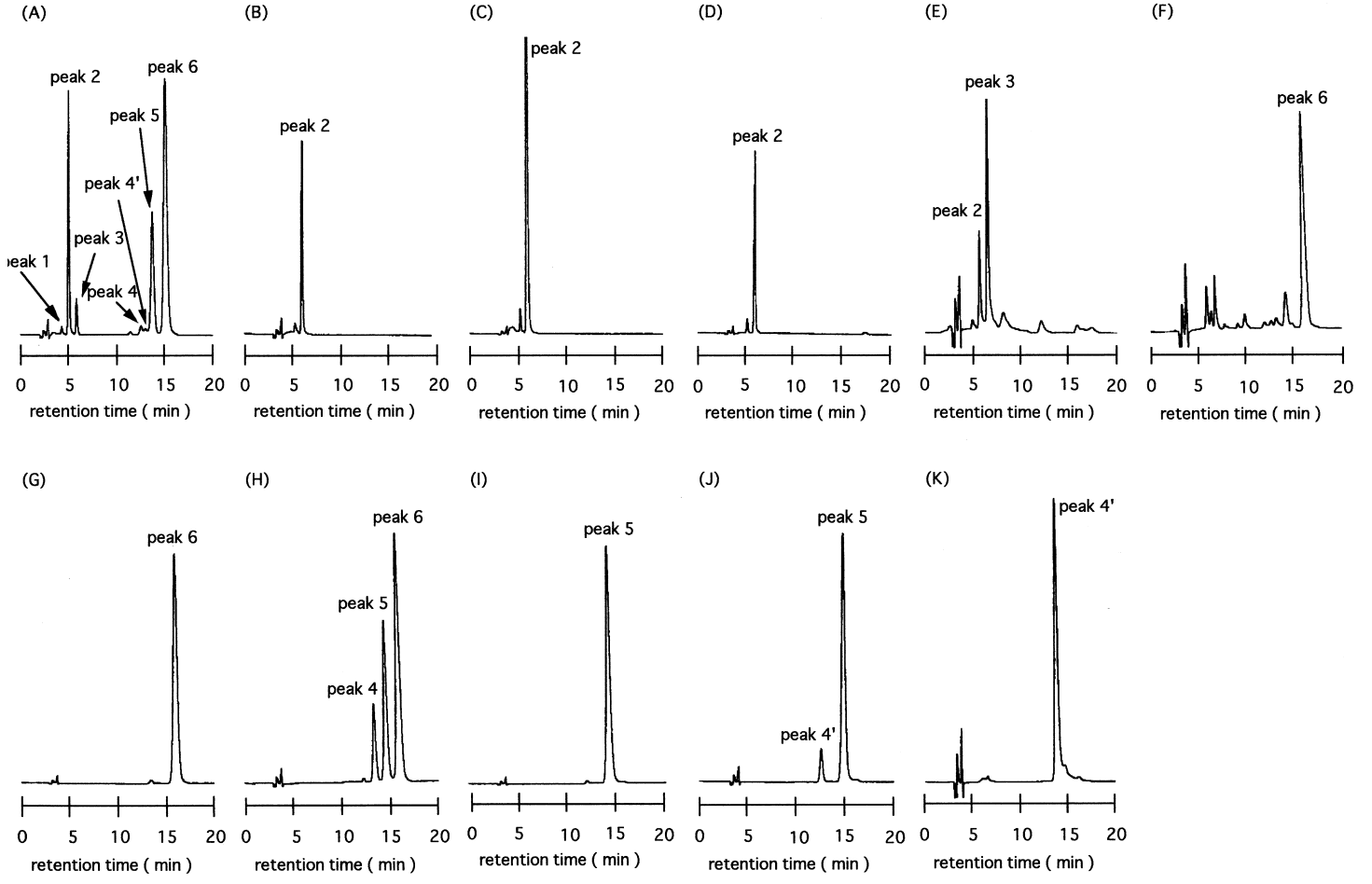

Fig. 2. HPLC separation of the lac dye components. (A) Original lac dye, (B) fraction I, (C) fraction II, (D) fraction III, (E) fraction IV, (F) fraction V, (G) fraction VI, (H) fraction VII, (I) fraction VIII, (J) fraction IX, (K) fraction X. HPLC conditions: see Section 2.

Table 1
Partition coefficients (K) of the lac dye components

Solvent systems	Peak no.					
	1	2	3	4	5	6
tert.-Butyl methyl ether–*n*-butanol–acetonitrile–water (4:2:3:8)	0.08	0.10	0.19	2.00	0.84	0.60
tert.-Butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5)	0.19	0.36	0.54	5.00	2.38	1.67

absorbance of effluents in every tube was measured at 495 nm to draw the elution curve (Fig. 3). HPLC analysis of each fraction revealed that, as expected, the components elute in the order of peaks 1, 2, 3, 6, 5, and 4.

Based on the HPLC analysis and the elution curve, all collected fractions were combined into ten pooled fractions (I to X). Fig. 2B–K shows the HPLC analysis of these combined fractions: Fraction II (tube No. 70–100, 2.6 mg), fraction VI (tube No. 210–334, 9.5 mg), fraction VIII (tube No. 355–390, 3.6 mg), and fraction X (tube No. 409–420, 0.5 mg) contained almost pure components corresponding to HPLC peaks 2 (laccaic acid C), 6 (laccaic acid A), 5 (laccaic acid B), and 4′, respectively. On the other hand, fractions I (tube No. 44–69. 2.5 mg), III (tube No. 101–104, 1.2 mg), IV (tube No. 105–137, 0.4 mg), V (tube No. 138–209, 1.3 mg), VII (tube No. 335–354, 1.8 mg), and IX (tube No. 391–408, 0.5 mg) contained multiple components.

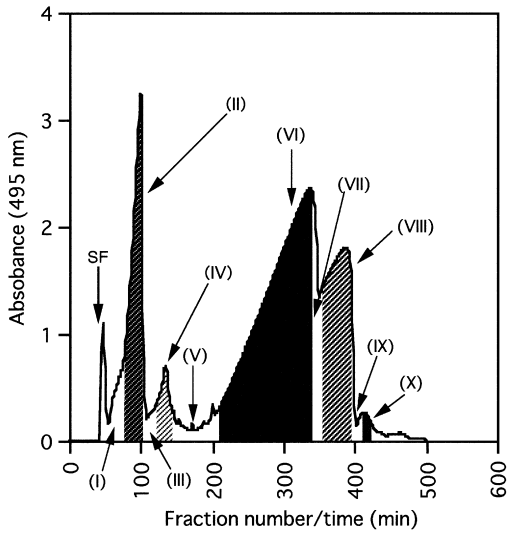

Fig. 3. Separation of lac dye by HSCCC. SF=solvent front. HSCCC conditions: see Section 2.

In the HPLC analysis of the original sample (Fig. 2A), peaks 2, 6, 5, and 4′ constituted about 35.5, 37.1, 18.0, and 0.8% of the total peak area at 280 nm, respectively. After only one step operation by HSCCC, the purity of the above four components were increased to 97.2% (Fig. 2C), 98.1% (Fig. 2G), 98.2% (Fig. 2I), and 95.0% (Fig. 2K), respectively. These results demonstrate the high resolving power of HSCCC achieved by the careful selection of the proper solvent system.

3.3. Identification of the lac dye components by ES-MS–MS

First, we measured the ES mass spectra of the isolated components in fractions II, VI, VIII, and X to identify the contents. Deprotonated molecules, $[M-H]^-$, clearly appeared at m/z 538, m/z 536, m/z 495, and m/z 359 in the mass spectra of fractions II, VI, VIII, and X, respectively, indicating that their molecular masses are estimated to be 539, 537, 496, and 360. However, no structural information was available from these mass spectra, because they form no fragment ions. In order to identify the components, therefore, we measured the ES tandem mass spectra under the conditions described in Section 2 using $[M-H]^-$ as the precursor ions. The tandem mass spectra of the components are shown in Fig. 4. The components in fractions II (Fig. 4A), VI (Fig. 4B), and VIII (Fig. 4C) clearly gave $[M-H]^-$, $[M-H-CO_2]^-$, $[M-H-2CO_2]^-$, and/or $[M-H-2CO_2-H_2O]^-$. These results precisely coincided with the ES tandem mass spectra of the laccaic acids reported earlier [20]. Hence, the components in fractions II, VI, and VIII are laccaic acids C, A, and B, respectively.

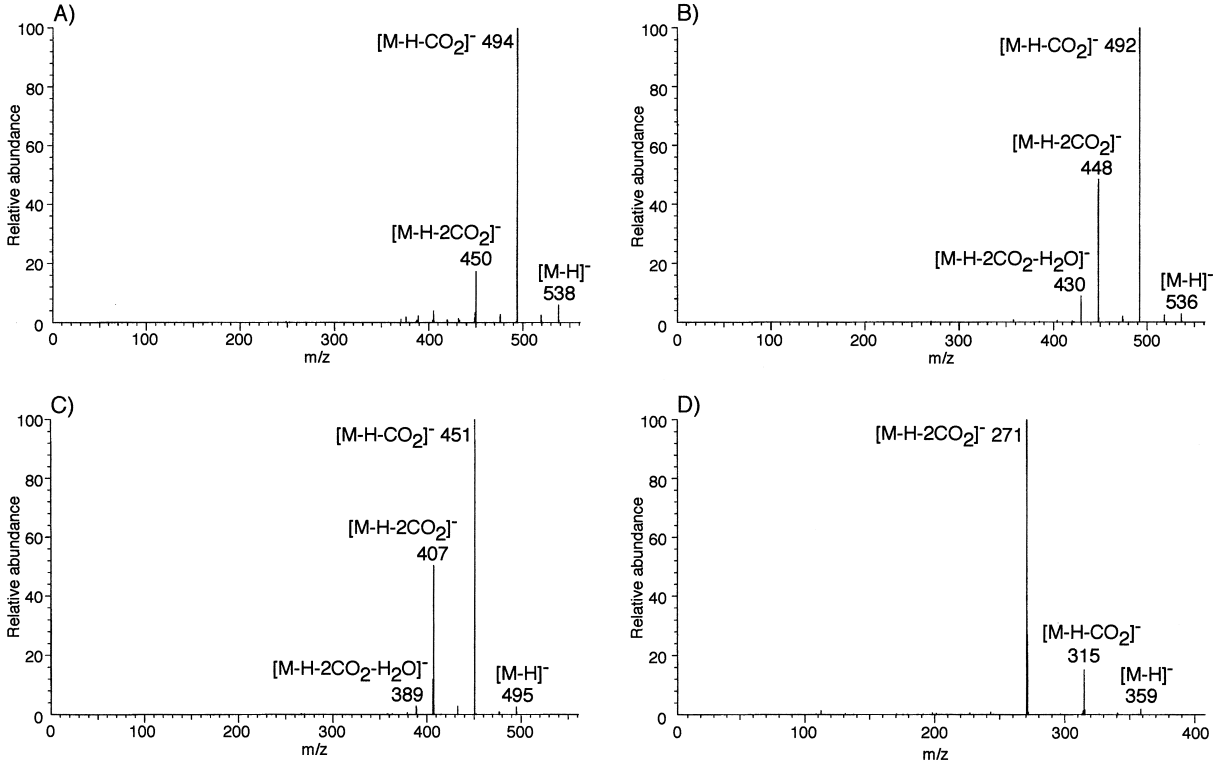

Fig. 4. ES mass spectra of the lac dye components. (A) Fraction II, (B) fraction VI, (C) fraction IIX, (D) fraction X. ES-MS–MS conditions: see Section 2.

Fig. 4D shows the tandem mass spectrum of the component in fraction X. As observed in the spectra of the laccaic acids, three product ions $[M-H]^-$, $[M-H-CO_2]^-$, and $[M-H-2CO_2]^-$, clearly appeared at m/z 359, m/z 315, and m/z 271, respectively. This result suggests that the component in fraction X is an anthraquinonedicarboxylic acid with a molecular mass of 360.

4. Conclusions

Using HSCCC, we were able to purify four components of lac dye with a two-phase solvent system composed of *tert*.-butyl methyl ether–*n*-butanol–acetonitrile–water (2:2:1:5). From 25 mg of the crude sample, we obtained 2.6 mg of 97.2% pure laccaic acid C, 9.5 mg of 98.1% pure laccaic acid A, 3.6 mg of 98.2% pure laccaic acid B, and 0.5 mg of a 95.0% pure anthraquinonedicarboxylic acid with a molecular mass of 360. The overall results of our studies indicate that HSCCC is a powerful technique for the purification of lac dye components.

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