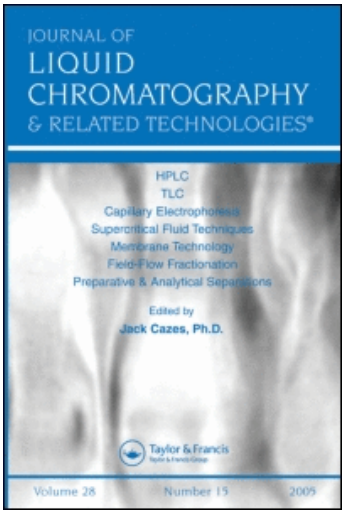

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Analytical and Preparative Separation of Kaoliang and Lac Colors by pH-Zone-Refining CCC

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Abstract: The natural coloring agents Kaoliang and Lac colors were separated using pH-zone-refining countercurrent chromatography (pH-z-CCC) on an analytical or preparative scale. The main components of Kaoliang color have been reported to be apigeninidin and luteolinidin, however, the structures of other compounds present in the color have not been elucidated as it is difficult to separate the compounds using conventional chromatographic techniques. Therefore, the separation of this color was done by pH-z-CCC that isolated three compounds. LC/MS and ^{1}H-NMR analyses were used to determine the structures established as a halogenated phenyl compound, *p*-coumaric acid and methylated apigenin. Lac color mainly consists of four anthraquinone derivatives, named laccaic acid A, B, C, and E. These four laccaic acids were separated on a large scale by pH-z-CCC. Purified Lac color (1 g) was submitted to pH-z-CCC with tridodecylamine as a ligand, and produced 790 mg, 41 mg, 109 mg, and 6 mg of laccaic acid A, B, C, and D, respectively, with a minimum purity of 96%.

Keywords: Kaoliang color, Lac color, pH-zone-refining countercurrent chromatography, Flavonoids, Anthraquinone

INTRODUCTION

Natural dyes have been widely used since ancient times, and they play an important role in the coloring of foods, clothes, and cosmetics. Although

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they have been used for such a long time, the exact molecular structures of many of these natural dyes are still unknown. HPLC is generally used to isolate compounds from natural materials; however, it is not the most suitable technique for the separation of complex or large amounts of materials. On the other hand, pH-zone-refining countercurrent chromatography (pH-z-CCC) has the potential to solve these problems, since it has a good resolution power associated to preparative capabilities.

Kaoliang color is a brown coloring agent used in East Asia, mainly China, Korea, and Japan. According to the "Japan Food Additives Association Standard", this color is prepared by extraction of kaoliang (*Sorghum nervosum* BESS.) seed coat, either in neutral or alkaline conditions and slightly warmed water or with acidic aqueous ethanol. The main components are two flavonoids; apigeninidin and luteolinidin (Figure 1).[1] Other flavonoids and their derivatives have been isolated from this color in previous studies,[2] but the structures of many components have not yet been determined. One reason for the lack of information to date is that the composition of this color is too complex to obtain only a single component under each separated peak on an HPLC chromatogram. The pH-z-CCC method promises greater resolution in comparison to HPLC, so we tried to separate the components of kaoliang color using the pH-z-CCC method.

Lac color is a red or orange color extracted from "Stick Lac", a secretion of lac scale insect (*Laccifer lacca* KERR.). Its main components are anthraquinone derivatives, named laccaic acid A, B, C, and E. These four laccaic acids have a phenol-conjugated anthraquinone structure in common, and the side chain attached to the phenol moiety defines each laccaic acid (Figure 1). In 2000, Oka and Ito separated these acids by high-speed countercurrent chromatography (HSCCC) from 25 mg of Lac color, and the laccaic acids were obtained at a purity of not less than 95%.[3] Since laccaic acids have a carboxylic acid group in their structure, the pH-CCC method is expected to be useful for the isolation of greater amounts at one time.

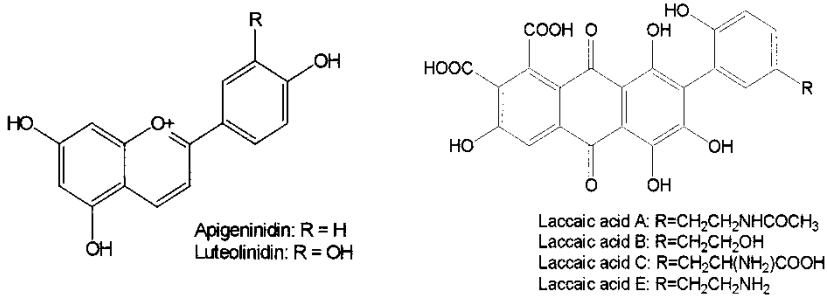

Figure 1. Chemical structures of the studied dyes: apigeninidin, luteolinidin, and laccaic acids.

EXPERIMENTAL

Instruments and Reagents

The separations were preformed using a commercial HSCCC centrifuge (Model CCC-1000, Pharma-Tech Research Co., MD, USA). The pH-CCC system consisted of a horizontal flow-through planar centrifuge with an Ito Multilayer coil (volume 120 mL), a pump (PU614, GL-Science Inc., Tokyo, Japan), UV detector (UV620, GL-Science Inc.), a Microflow pH sensor (Model 14, Broadley-James, CA, USA), a manual injection valve with a 12 mL loop, and a fraction collector (SF-212N, JASCO).

The LC/MS analyzing system consisted of a Waters Alliance 2695 HPLC separation module, Waters 2996 photodiode array (PDA) detector, and Waters Micromass ZQ 2000 (ESI-MS). Instrument control and data acquisition was performed using Micromass MassLynx software (version 3.5) (Waters Co., Milford, MA, USA). The ^{1}H-NMR analysis was performed at 600 MHz by using JNM-ECA600 (JEOL, Tokyo, Japan).

tert-Butyl methyl ether (MTBE) and ammonium hydroxide were purchased from Kishida Chemicals Co., Ltd. (Osaka, Japan). Tridodecylamine (TDA) and deuterated dimethyl sulfoxide, DMSO-d_6, were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Sigma Aldrich Japan K.K. (Tokyo, Japan), respectively. Lac color, POWDERED SAN RED NO. 3 was provided by San-Ei Gen F.F.I., Inc. (Osaka, Japan). All other reagents were purchased from Wako Pure Chemical Industry (Osaka, Japan). Water purified with the Easy Pure RO System (Barnstead, IA, USA) and Ultra Pure Water System CPW-101 (ADVANTEC, Tokyo, Japan) was used.

Kaoliang Color Preparation

The sample of kaoliang used for separation was extracted five times from 100 g of the kaoliang seed coat with 1.0 L of 0.5% aqueous ammonium carbonate at 60°C for 1 hour. The extractions were combined, concentrated *in vacuo*, and the aqueous residue was lyophilized. This dried residue was used for separation.

pH-z-CCC Separation

When separating Kaoliang color, a two-phase solvent system consisting of diethyl ether–acetonitrile–water (4:2:5) was used. The lower aqueous phase was the basic retaining phase with ammonium hydroxide (20 mM) used as the mobile phase. The upper organic phase was the refraining acid stationary phase with 20 mM trifluoroacetic acid (TFA). In the separation of Lac color, the solvent system consisted of MTBE–*n*-butanol–acetonitrile–water

(2:2:1:5); the lower aqueous phase with 20 mM of ammonium hydroxide was used as the retaining stationary phase and the upper organic phase contained TFA (20 mM) as a displacer with 17.5 mM TDA as a ligand.

The sample solution was prepared by dissolving the color into the mixture of upper and lower phases of the individual separating solvent system, and then passing the sample through a PTFE membrane filter (pore size is 0.5 μm). When Kaoliang color was used, the sample solution was passed through a filter paper before PTFE MF filtration.

In all experiments, the separation protocol was as follows: after filling the coil tube with the stationary phase, rotation was started at 1000 rpm. Then, the sample solution was injected into the coil tube, and recording of the absorbance and pH of eluted phase was started. The upper mobile phase was pumped at a rate of 1.5–3 mL/min. in a "Head to Tail" direction (for Kaoliang color) or "Tail to Head" direction (for Lac color).

LC/MS Analysis

Analytical samples were directly injected into the LC/MS apparatus. When Lac color was analyzed, TDA was removed by extraction with aqueous ammonia from the organic mobile phase before analysis.

The reverse phase column, Develosil C30-UG-5 (2.0 i.d. x 150 mm, Nomura Chemical Co., Ltd., Aichi, Japan), was used with a mobile phase of 0.05% (v/v) aqueous TFA and acetonitrile. The gradient programs are as follows: acetonitrile was added at a concentration of 0 to 100% in 30 min., 100% for 10 min. (for Kaoliang), or 10 to 100% in 40 min. (for Lac). The column was re-equilibrated by elution at the initial solvent condition for 15 min. The flow rate was 0.15 mL/min., and the temperature was 30°C (for Kaoliang), or 40°C (for Lac).

The mass spectrometer conditions were as follows: capillary voltage, 3.5 kV; cone voltage, 20–60 eV; source temperature, 100°C; desolvation temperature, 300°C; desolvation gas flow, 600 L/h.

RESULTS AND DISCUSSION

Separation of Kaoliang Color

Optimization of Separating Conditions

The kaoliang extract is soluble in neutral to alkaline water or in an acidic organic solvent, so the separation should be performed with a neutral/alkaline inorganic and an acidic organic phase. Several types of solvent system were examined; however, the kaoliang extract easily formed emulsions in a variety of solvent systems, especially when a hydrophilic

solvent system such as *n*-butanol–water or MTBE–acetonitrile–water system was used. When such a solvent system was used, the stationary phase was eluted from the coil tube. Therefore, a more hydrophobic solvent system was selected such as the diethyl ether–acetonitrile–water system. Emulsification due to the sample was responsible for the poor stationary phase retention. It could be reduced or even suppressed by filtering the sample solution. Insoluble particles seem the cause of the organic reduced stationary phase retention.

Separation of Components

Under the optimized conditions, pH leaped at about 230 min. and immediately stopped at around pH 11. A stair-like area was seen in the elution zone of 228–231 min. It contained a variety of compounds, and the following high pH zone contained apigenin, which was detected by LC/MS. The amount of each compound in the stair-like area was minute and they could not be fractionated directly. So, the separation of 1 g of kaoliang extract was performed several times and the corresponding fractions were pooled. The pooled fractions were concentrated *in vacuo*, and submitted again to pH-z-CCC. An expanded stair area was observed and six fractions were collected (Figure 2).

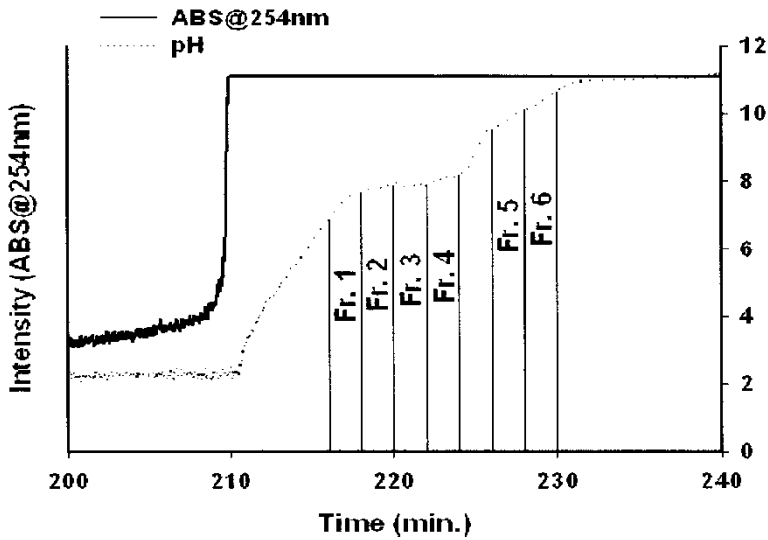

Figure 2. Magnification of the stair-like region in the CCC chromatogram of pooled sample. The solid line indicates the absorbance at 254 nm, and the dotted line shows pH. The solvent system consisted of diethyl ether–acetonitrile–water (4:2:5). The aqueous phase with 20 mM ammonium hydroxide was used as the mobile phase at 2.0 mL/min. The organic stationary phase contained 20 mM TFA. The revolution speed was 1000 rpm.

Analysis

Each fraction was analyzed directly by LC/MS. Three fractions contained several peaks, which were hardly detectable before pH-z-CCC separation. Fractions 2, 4, and 6 contained mainly a single product. These fractions were further purified by HPLC again, and analyzed by NMR.

Compound A, located in Fraction 2, has a local maximum absorption at around 250 nm (Figure 3A), which indicates that the compound will have a non-conjugated phenyl group. An isotopic pattern was observed on the mass spectrum in negative ion mode, which indicated that the compound had chlorines and/or bromines in the structure. Furthermore, only one pair of doublet appears on the ^{1}H-NMR spectrum, although the mass spectrum indicates the molecular weight is more than 400m/z. One possible structure, that is consistent with these results, is a symmetric structure. In summary, this compound is estimated to be non-conjugated, have a chlorine and/or bromine-bonded phenyl group, and a symmetric structure.

The ^{1}H-NMR spectrum of compound B (in Fraction 4) has two double-doublets at 6.75 ppm and 6.25 ppm, and two doublets at 7.48 ppm and 7.45 ppm (Figure 3B). Additionally, the mass spectrum indicates that its molecular weight is 164; this compound is determined to be *p*-coumaric acid.

Compound C, found in Fraction 6, has a large peak at 285 m/z on the mass spectrum in positive ion mode, and has a local maximum absorption at around 330 nm on the UV/Vis spectrum (Figure 3C). On the ^{1}H-NMR spectrum, two pairs of doublet and two singlets were observed. The chemical shifts, integrals, estimated positions, and coupling constants are as follows: δ(ppm) 7.79 (*d*, 2*H*, H-3′ and H-5′, $J_{(2',3')} = J_{(5',6')} = 8.2$ Hz), 6.86 (*d*, 2*H*, H-2′ and H-6′), 6.50 (*d*, 1*H*, H-8, $J_{(3,8)} = 2.1$ Hz), 6.48 (*s*, 1*H*, H-3), 6.34 (*d*, 1*H*, H-6), 3.75 (*s*, 3*H*, CH_3O). Since the amount of this compound obtained was too small for determination, Heteronuclear Multiple Bond Correlation (HMBC) and Heteronuclear Multiple Quantum Coherence (HMQC) spectra could not be obtained, therefore, the structure could not be completely elucidated. However, these results clearly indicated this compound was 5 or 7 or 4′-methylated apigenin.

Preparative Separation of Lac Color

Optimization of Separation Conditions

To determine the separation conditions for the Lac sample, we referred to Oka's report.[3] In Oka's study, the solvent system consisted of MTBE–*n*-butanol–acetonitrile–water (2:2:1:5) and was used in "Head to Tail" direction (aqueous basic mobile phase). So, we examined the same solvent system and direction with a retainer and an eluter at a concentration of 10–50 mM. When the sample was 200 mg, the operation was completed

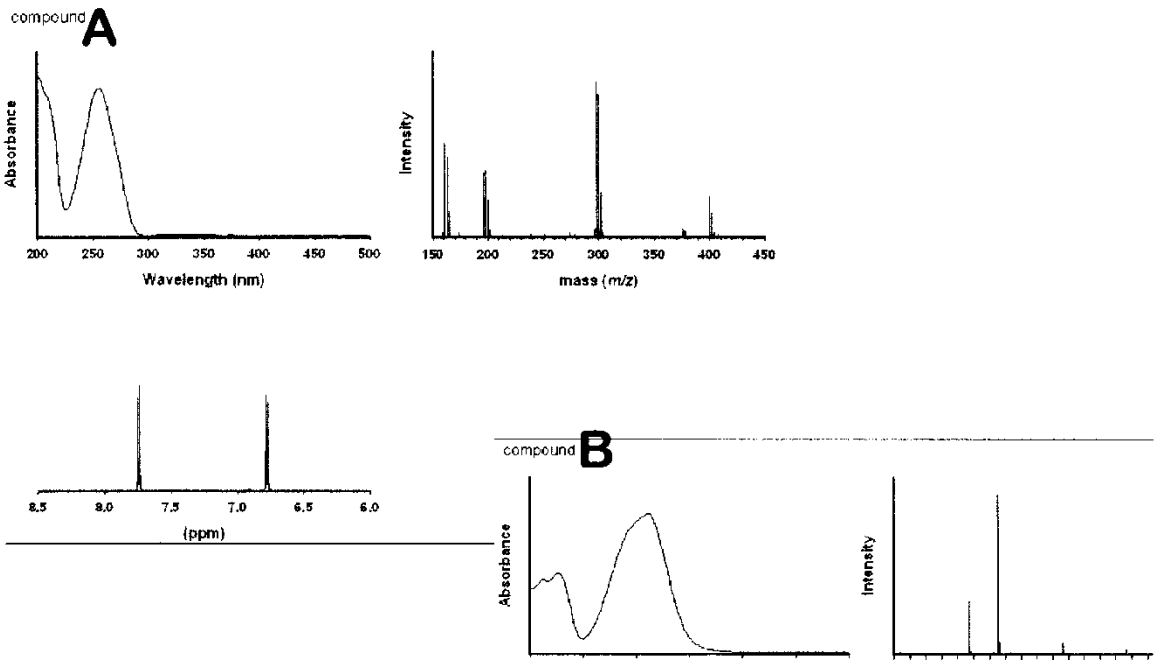

Figure 3. The UV/Vis, mass, ^{1}H-NMR spectra, and determined structures of the three compounds obtained from kaoliang extract.

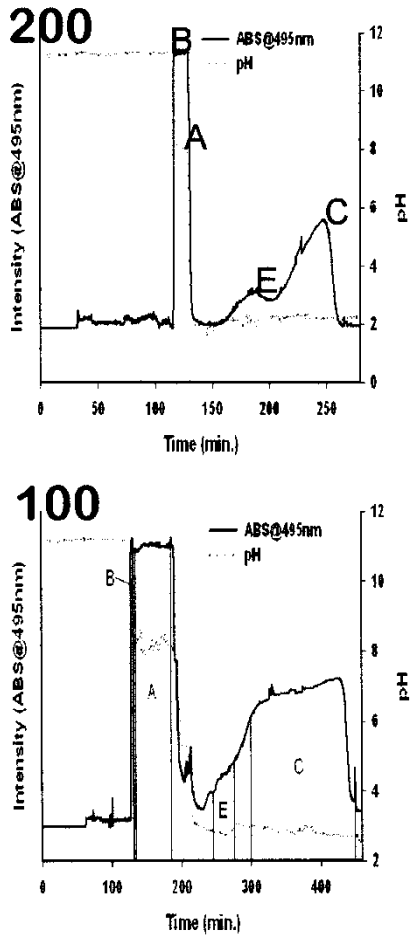

Figure 4. pH-zone refining CCC separation of a laccaic acid extract. Solvent system: MTBE–*n*-butanol–acetonitrile–water (2:2:1:5). Stationary phase: aqueous lower phase with 20 mM ammonium hydroxide; mobile phase: organic upper phase with 20 mM TFA and 17.5 mM TDA ligand, 3 mL/min T → H direction. Dotted line: pH and right scale. Full line: 495 blue light and left scale. Top: 200 mg of extract injected in 10 mL aqueous phase; bottom: 1 g injected in the same conditions.

with little elution of the stationary phase; increasing the sample to 1 or 2 g resulted in a significant loss of the organic stationary phase. However, using the organic upper phase as mobile phase and reversing the direction to "Tail to Head" improved this elution.

Separation of Lac color was performed under these conditions. The laccaic acids were eluted in the order B, A, E, C. Three laccaic acids were isolated, but E remained in the mixture with C. We assumed that the solubility

of the laccaic acids C and E was very similar in the mobile phase so that the selectivity was poor.

The only structural difference between laccaic acid C and E is a carboxylic acid moiety on the ethanol amine of C that E does not have (Figure 1). Some basic and hydrophobic agents, such as a polyalkylamine, may interact with the laccaic acids and change their solubility. Tridodecylamine (TDA), which is described as a ligand in Ito's review,[4] was added into the organic phase of the solvent system described above. The concentration of TDA was varied between 10 and 45 mM in the organic mobile phase. The best chromatogram was obtained with 17.5 mM TDA. The four laccaic acids were isolated under these conditions on a 200 mg scale (Figure 4 top).

Preparative Separation and Analysis of Laccaic Acids

Separation of 1 g Lac color was conducted using the same conditions as previously described (Figure 4 bottom). The eluted solvent was fractionated, and analyzed by LC/MS to determine the purity of the laccaic acids. The fractions containing pure laccaic acids were collected and weighed after removal of TDA and solvent. Analysis of each collected laccaic acid was performed with LC/MS to measure their purity. The results were as follows; laccaic acid A, 790 mg, purity 99%; B, 41 mg, 96%; C, 109 mg, 96%; E, 6.4 mg, 98%. The purity of each laccaic acid obtained was at least 96%.

CONCLUSIONS

pH-zone refining CCC is capable of separating the components of the Kaoliang color, and to isolate on a preparative scale, the laccaic acids of the Lac color. Three compounds were isolated from Kaoliang color, and analyzed by LC/MS and ^{1}H-NMR. Although further investigations are required to elucidate their precise structure, they were determined to be a chlorine or bromine-bonded, non-conjugated phenyl compound, *p*-coumaric acid and monomethylated apigenin. As it has been impossible to detect them using other methods, this is evidence of the high resolution potency of pH-z-CCC. The technique may be equally applicable to other natural colors made of ionizable compounds, providing a way to detect their components.

The four laccaic acids of the Laquer color were separated on a preparative scale using pH-z-CCC. Each laccaic acid was successfully isolated using TDA as a ligand, and the purity was at least 96%. This method can be used to obtain pure laccaic acids with ease.

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