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Journal of Chromatography A, 989 (2003) 249–255
JOURNAL OF CHROMATOGRAPHY A
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Purification of quinoline yellow components using high-speed counter-current chromatography by stepwise increasing the flow-rate of the mobile phase

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Received 8 October 2002; received in revised form 13 January 2003; accepted 14 January 2003

Abstract

Quinoline yellow (Color Index No. 47005) consists of multiple components that show a large difference in their partition coefficients (K), ranging from 0.03 to 3.3 in the solvent system *tert*.-butyl methyl ether (MTBE)–1-butanol–acetonitrile–aqueous 0.1 *M* trifluoroacetic acid (TFA). Consequently, it requires an excessively long elution time for the simultaneous separation of all components by the standard high-speed counter-current chromatography technique, which uses a constant flow-rate of the mobile phase. In order to overcome this problem, we increased the flow-rate of the mobile phase stepwise from 0.1 to 2.0 mL/min. Using this new procedure, six components (0.2–6.1 mg) were successfully isolated from 25 mg of a commercial quinoline yellow preparation in a single run using a two-phase solvent system composed of MTBE–1-butanol–acetonitrile–aqueous 0.1 *M* TFA (1:3:1:5, v/v). The purified components were analyzed by high-performance liquid chromatography, electrospray ionization mass spectrometry, and nuclear magnetic resonance spectroscopy.

Keywords: Counter-current chromatography; Flow-rate; Quinoline yellow; Dyes; Sulfonates

1. Introduction

Quinoline yellow (Fig. 1, Color Index No. 47005) is a quinophthalone synthetic yellow dye prepared by sulfonating 2-(2-quinolyl) indan-1,3-dione. It consists essentially of a mixture of sodium disulfonates, monosulfonates and trisulfonates [1,2], and the quantities of these components vary widely according to the synthesis conditions [2]. Since quinoline yellow is widely used for coloring foods, drugs and cosmetics, high-purity preparations of both main and subsidiary dyes are required for toxicological and structural evaluation for the purpose of sanitation and good manufacturing practice. Unfortunately, reference standards are not yet commercially available. Successful applications of high-speed counter-current chromatography (HSCCC) [3] have been reported for the purification of peptides [4–6], antibiotics

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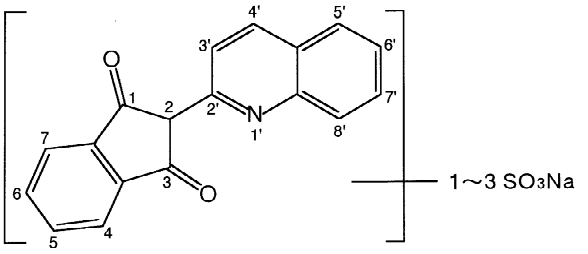
0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00118-3



Quinoline yellow	M. W.
Monosulfonate	375
Disulfonate	477
Trisulfonate	579

Fig. 1. Quinoline yellow. M.W., molecular mass.

[7–9], alkaloids [10,11], and dyes [12–15]. Although the purification of quinoline yellow components has been attempted using HSCCC with a pH-zone refining technique [14,15], only two monosulfonates were purified. Therefore, we applied HSCCC to the separation of quinoline yellow components to obtain multiple components. However, we failed in the simultaneous separation of all components in a single run, due to the large difference in their partition coefficients (K), ranging from 0.03 to 3.3.

In order to separate the components in a single-step operation, we increased the flow-rate of the mobile phase stepwise from 0.1 to 2.0 mL/min, resulting in the successful isolation of six components from a commercial quinoline yellow preparation. In this paper, we report in detail the separation of the quinoline yellow components using this new HSCCC technique.

2. Experimental

2.1. Reagents

Acetonitrile, 1-butanol, *tert*.-butyl methyl ether (MTBE), methanol, potassium dihydrogenphosphate, disodium hydrogenphosphate, and trifluoroacetic acid (TFA) were of analytical grade and purchased from Wako (Osaka, Japan). Quinoline yellow was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. HPLC analysis

A PU-970 chromatograph, equipped with a constant-flow pump (Jasco, Tokyo, Japan), was used with a variable-wavelength UV–Vis detector (UV-970, Jasco) operated at 254 nm. Separation was performed on Cosmosil $5C_{18}$ AR (5 μm, 150×4.6 mm I.D., Nacarai, Tokyo, Japan) under the following conditions: mobile phase, acetonitrile–0.05 *M* phosphate buffer (pH 6.0); gradient rate, acetonitrile 5–50%, linear 30 min; flow-rate, 1 mL/min.

2.3. Measurement of the partition coefficient

Approximately 1 mg of the test sample was weighed into a 10 mL test tube to which 2 mL of each phase of the equilibrated two-phase solvent system was added. The tube was stoppered and shaken vigorously for 1 min to equilibrate the sample thoroughly with the two phases. Equal volumes of each phase were then analyzed by HPLC to obtain the partition coefficients.

2.4. 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 synchronous planetary motion of the separation column at 800 rpm. The multi-layer coil was prepared by winding an approx. 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 MTBE–1-butanol–acetonitrile–0.1 *M* TFA (1:3:1:5) was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking three times each followed by disposing of the generated gas by inverting the vessel and manipulating its stopcock at room temperature.

In each separation, the column was first filled entirely with the upper non-aqueous stationary phase, then 25 mg of the sample dissolved in 1 mL of each phase 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 0.1 mL/min by a HPLC pump (LC-6A, Shimadzu). After 20 min, the flow-rate of the mobile phase was increased to 2 mL/min. The effluent from the outlet of the column was fractionated into test tubes at 1 min per tube using a fraction collector (DF-2000, Tokyo Rikakikai, Tokyo, Japan).

After the separation was completed, the 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.

2.5. Electrospray ionization (ESI) MS analysis

The ESI mass spectra were obtained with a Quattro II triple stage tandem mass spectrometer (Micromass UK, Altrincham, UK) equipped with a Z-spray atmospheric pressure ionization source. Sample solution was introduced into the mass spectrometer using a Model 100 syringe pump (KD Scientific, Boston MA, USA).

2.6. NMR analysis

NMR spectra were measured using a JEOL (Tokyo, Japan) JNM A400 NMR spectrometer operating at 400 MHz for ^{1}H in [$^{2}H_{6}$]dimethyl sulfoxide (DMSO-d_6). The ^{1}H chemical shifts were referenced to the solvent peaks (^{1}H: 2.49 ppm in DMSO-d_6).

3. Results and discussion

3.1. Selection of the two-phase solvent system

Successful separation by HSCCC depends upon the selection of a suitable solvent system which requires the following considerations [13]: (1) to obtain satisfactory retention of the stationary phase, the settling time of the solvent system should be considerably shorter than 30 s; (2) to avoid excessive waste of the solvent, the mixture should provide nearly equal volumes of each phase; and (3) for efficient separation, the partition coefficient (K) of the target compound should be close to 1 where the separation factor (α) between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) is larger than 1.5. In general, small K values result in a loss of peak resolution, while large K values tend to produce excessive sample band broadening. A minimum α value of 1.5 is required for baseline separation by semi-preparative HSCCC equipment which yields a moderate partition efficiency of around 800 theoretical plates. The K value of a pure compound can be obtained by computing the ratio of its UV absorbance between the two phases. When the compounds to be separated are not available in a pure form, as in the present case, the K values of individual components cannot be determined by the above method. In this case, the following HPLC method can be used. After partitioning the sample between the two solvent phases, aliquots of the upper and lower layers are analyzed by HPLC. From these two chromatograms, the K value of each component is determined by computing the ratio of the peak heights (or areas) between the corresponding peaks. Fig. 2 shows the HPLC

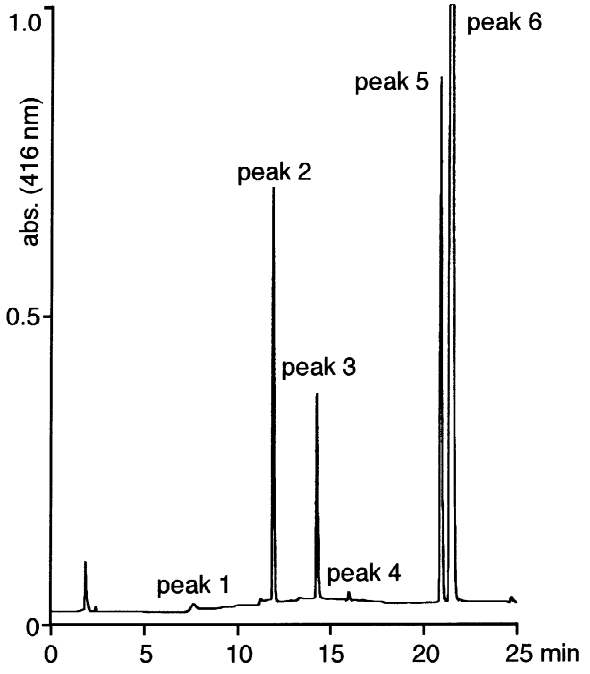


Fig. 2. HPLC separation of quinoline yellow components. For HPLC conditions, see Experimental. Injection volume 10 μL.

separation of quinoline yellow components in which six peaks were resolved.

Quinoline yellow is a mixture of mono-, di-, and trisulfonates and is freely soluble in water, but hardly dissolves in organic solvents such as chloroform or ethyl acetate. When these components were partitioned in a mixture of 1-butanol and 0.1 *M* TFA, which has frequently been used for the separation of polar acidic compounds, they were mostly distributed into the lower aqueous phase. In order to improve the *K* values, we added MTBE and acetonitrile as modifiers to this solvent system, the results of which are shown in Table 1. In the solvent system composed of MTBE–1-butanol–acetonitrile–0.1 *M* TFA (2:2:1:5), *K* values of peaks 2, 3, 5 and 6 were 0.034, 0.051, 3.96, and 2.45, respectively, indicating that the separation of these components is difficult due to a large difference in *K* values between two groups, although the α values are appropriate. In general, it is difficult to resolve compounds having *K* values of less than 0.3, whereas it requires a long time to elute compounds having *K* values of >1.5. In an attempt to improve the *K* values of peaks 2 and 3, the concentration of TFA was increased to 0.5 *M*, but no significant change in the *K* values of these components was observed. Volume ratios of 1:3:1:5 and 0.5:3:1:5 were also tested without success. The above negative results prompted us to look for a new approach to solve this problem.

Recently, we reported that the use of slow flow-rates of the mobile phase (<0.5 mL/min) is effective for the separation of compounds having small *K* values [16]. On the other hand, the use of faster flow-rates of the mobile phase (>2 mL/min) is effective for a rapid separation of compounds with large *K* values. We combined these two favorable conditions to perform a successful separation of all the components in a single run. Using a solvent system composed of MTBE–1-butanol–acetonitrile–0.1 *M* TFA (1:3:1:5), the separation was started at a slow flow-rate of 0.1 mL/min to separate the first group (peaks 1 and 2). Then, the flow-rate was increased stepwise to 2.0 mL/min to elute the second group (peaks 5 and 6).

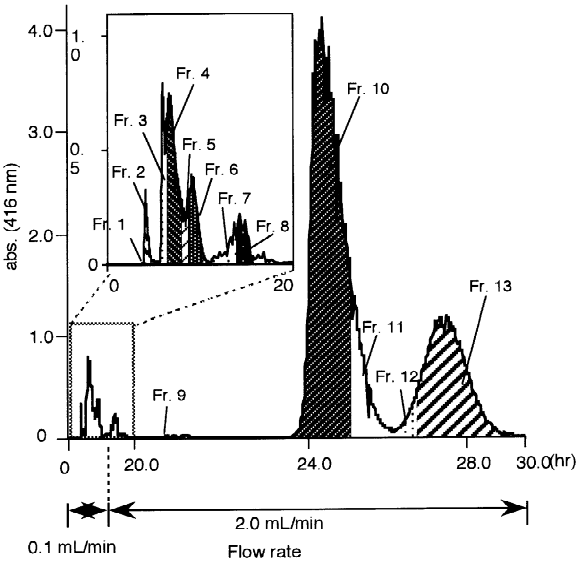


Fig. 3. Separation of quinoline yellow components by HSCCC. For HSCCC conditions, see Experimental.

3.2. Separation of quinoline yellow components by HSCCC with varying flow-rate of the mobile phase

A 25 mg quantity of quinoline yellow preparation was separated by the above technique using an upper organic phase as the stationary phase and a lower aqueous phase as the mobile phase. As described

Table 1
Partition coefficients of the components of quinoline yellow

Solvent system	s.t. (s)	Peak 2 *K*	Peaks 2–3 α	Peak 3 *K*	Peak 5 *K*	Peaks 5–6 α	Peak 6 *K*
MTBE–1-butanol–acetonitrile–0.1 *M* TFA (2:2:1:5)	23	0.03	(1.50)	0.05	3.96	(1.61)	2.45
MTBE–1-butanol–acetonitrile–0.2 *M* TFA (2:2:1:5)	23	0.03	(1.21)	0.04	3.36	(1.67)	2.01
MTBE–1-butanol–acetonitrile–0.3 *M* TFA (2:2:1:5)	21	0.04	(1.38)	0.05	2.48	(1.75)	1.41
MTBE–1-butanol–acetonitrile–0.5 *M* TFA (2:2:1:5)	22	0.05	(1.52)	0.08	3.69	(1.77)	2.08
MTBE–1-butanol–acetonitrile–0.1 *M* TFA (1:3:1:5)	17	0.15	(1.22)	0.21	4.78	(1.50)	3.28

s.t., settling time.

above, the flow-rate was initially adjusted to 0.1 mL/min to separate peaks 1, 2, 3, and 4, and then it was increased to 2 mL/min. The retention of the stationary phase was 72.2%, and the total separation time was 40 h. The HSCCC fractions were analyzed by HPLC, and their absorbances were measured at 416 nm to construct the elution curve (Fig. 3). All eluted fractions and both phases of the column contents were analyzed by HPLC, which indicated that the components were separated into six peaks.

Based on the HPLC analysis and the elution curve, all collected fractions were combined into 13 pooled fractions (fractions 1–13). The yields of pooled fractions were calculated by weighing the fractions, after evaporation of the solvents. Fig. 4 shows the HPLC analysis of these combined fractions: fraction 2 (1.4 mg), fraction 4 (2.6 mg), fraction 6 (1.3 mg), fraction 8 (0.2 mg), fraction 10 (6.1 mg), and fraction 13 (0.9 mg) contained almost pure components (>98%), corresponding to HPLC peaks 1, 2, 3, 4, 6, and 5, respectively. On the other hand, fractions 1 (0.1 mg), 3 (4.0 mg), 5 (2.4 mg), 7 (1.0 mg), 9 (1.0 mg), 11 (1.5 mg), and 12 (0.6 mg) contained multiple components.

3.3. Structural characterization of quinoline yellow components by ESI-MS and NMR spectrometry

Negative-ion ESI mass spectra of fractions 2, 4, 6, 8, 10, and 13 correspond respectively to peaks 1, 2,

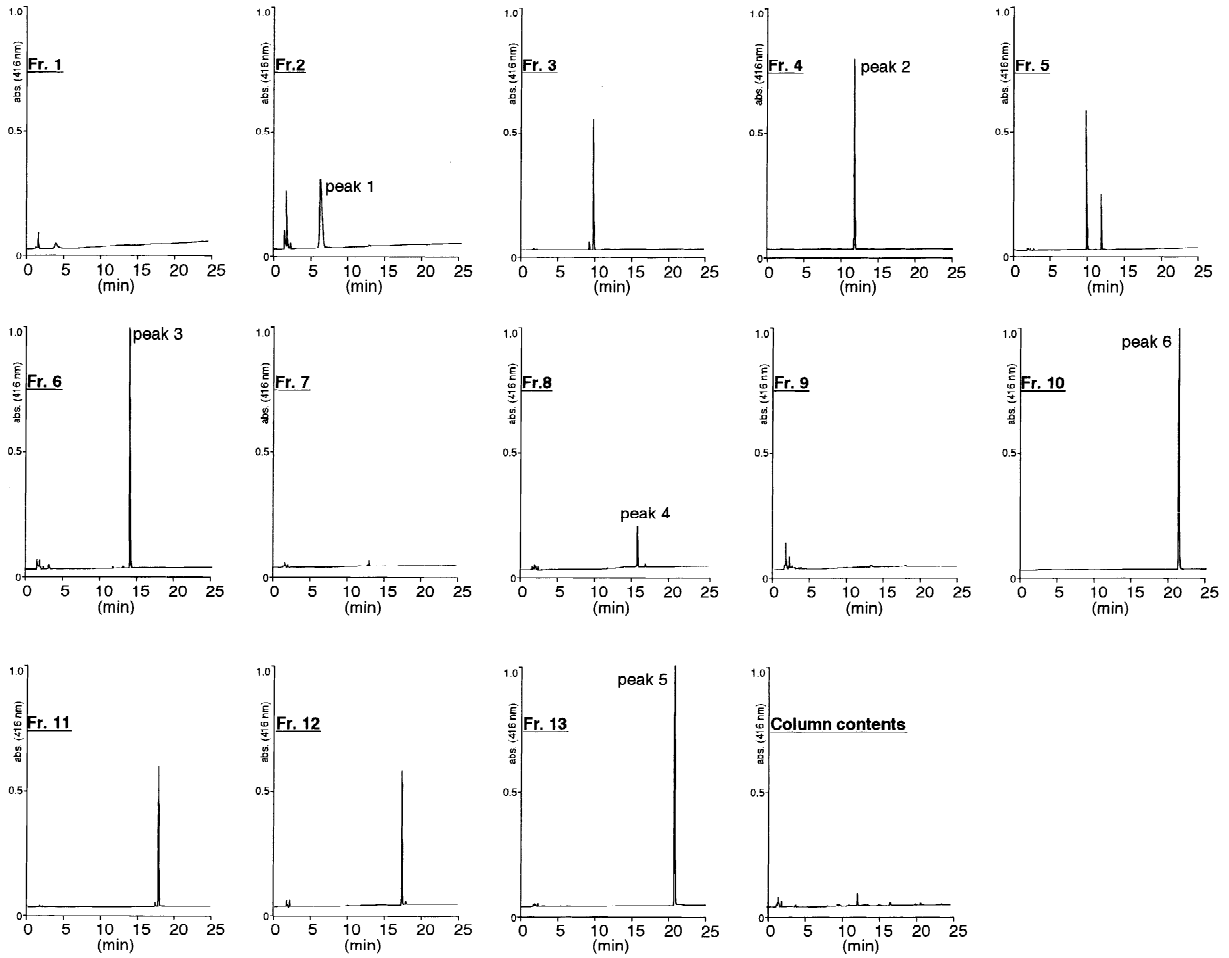


Fig. 4. HPLC separation of fractionated components of quinoline yellow. For HPLC conditions, see Experimental. Injection volume 10 μL.

3, 4, 6, and 5 in the chromatogram of the original sample in Fig. 2. The spectrum of fraction 2 (peak 2) showed an ion at m/z 556 with moderate intensity and an ion at m/z 578 with weak intensity. When it is taken into consideration that quinoline yellow is a mixture of sulfonates of quinophthalone with a molecular mass of 273 (Fig. 1), this result suggests that the ions at m/z 556 and 578 can be assigned to the $[M-Na]^-$ and $[M-H]^-$ of the sodium salts of the trisulfonic acids of quinophthalone. Therefore, we conclude that peak 1 corresponds to the sodium salts of the trisulfonic acids of quinophthalone. In the spectra of fractions 4 (peak 2), 6 (peak 3) and 8 (peak 4), the $[M-Na]^-$ was clearly observed at m/z 454, suggesting that peaks 2, 3, and 4 correspond to the sodium salts of the disulfonic acids of quinophthalone. Furthermore, according to the $[M-Na]^-$ at m/z 352 in the mass spectra of fractions 10 (peak 6) and 13 (peak 5), peaks 5 and 6 should correspond to the sodium salts of the monosulfonic acids of quinophthalone.

The aromatic protons observed in the NMR spectra of the isolated components are summarized in Table 2. In the NMR spectrum of the major component (fraction 10) there are nine protons, although these signals are somewhat broadened. While the four protons of the indandione moiety appeared as a multiplet, the remaining signals for the quinoline moiety were assignable as shown in Table 2. This spectral pattern strongly suggests that a sulfonate group has been introduced at the 6′ position of the quinoline moiety. Fraction 13 corresponds to another monosulfonate at the 8′ position, because the signal at the 6′ position is observed as a triplet ($J = 7.6$ Hz). As shown above, fractions 4 and 6 were found to be disulfonates from the mass spectral data, and the NMR data suggest that the second sulfonate groups were introduced into the indandione moiety in both compounds. Although the signals were broadened as for the case of fraction 10, each signal could be assigned as shown in Table 2, indicating that the two sulfonate groups are positioned at C-5 and C-6′. Similarly, the assignment of the aromatic protons allowed us to determine the structure of fraction 6 as shown in Fig. 5, and it was confirmed that those of the sulfonate groups were located at C-5 and C-8′.

Finally, the NMR spectra of these components displayed the following characteristic features. (1) While the protons of compounds where a sulfonate group is substituted at C-6′ appear as broadened signals, they are sharply observed in the C-8′ derivatives. (2) In the case of disulfonated compounds, H-4 and H-7 in fraction 6 are observed as two singlet peaks and two couples of a doublet, respectively, whereas this phenomenon does not occur in fraction 4. These results indicate that the first sulfonation occurs at C-6′ or C-8′ of the

Table 2
^{1}H-NMR spectral data[a] for the isolated components of quinoline yellow

Fraction	Indandione				Quinoline					
	H-4	H-5	H-6	H-7	H-3′	H-4′	H-5′	H-6′	H-7′	H-8′
4	7.80 s	–	7.87 d $J = 7.6$	7.59 d $J = 7.6$	8.52 s	8.52 s	8.15 s	–	7.92 d $J = 8.2$	7.92 d $J = 8.2$
6	7.77[b] s 7.83[b] s	–	7.85 d $J = 7.6$	7.60[c] d 7.58[c] d $J = 7.6$	8.41 d $J = 9.6$	8.70 d $J = 9.6$	8.08 d $J = 7.6$	7.48 t $J = 7.6$	7.90 d $J = 7.6$	–
10	7.55–7.67 m				8.46 d $J = 10.0$	8.49 d $J = 10.0$	8.14 s	–	7.97 d $J = 8.6$	7.86 d $J = 8.6$
13	7.60–7.70 m				8.41 d $J = 9.6$	8.69 dd $J_1 = 9.6$ $J_2 = 1.2$	8.08 dd $J_1 = 7.6$ $J_2 = 1.2$	7.48 t $J = 7.6$	7.90 d $J = 7.6$	–

[a] Chemical shift (ppm), multiplicity (s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet), coupling constant (Hz).
[b] This singlet signal was observed as two peaks, probably due to the restricted rotation.
[c] This signal was observed as two couples of a doublet, probably due to the restricted rotation.

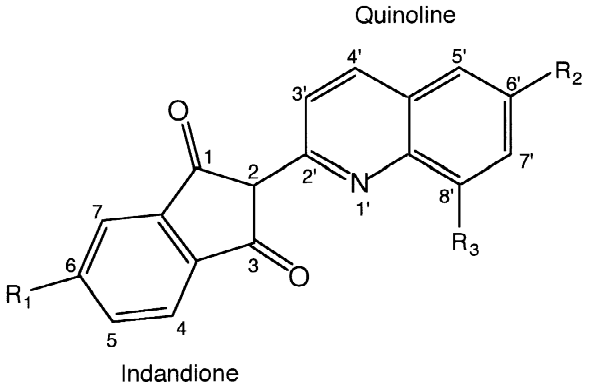


Fig. 5. Structures of the isolated quinoline yellow components. Fraction 4, $R_1 = SO_3Na$, $R_2 = SO_3Na$, $R_3 = H$; fraction 6, $R_1 = SO_3Na$, $R_2 = H$, $R_3 = SO_3Na$; fraction 10, $R_1 = H$, $R_2 = SO_3Na$, $R_3 = H$; fraction 13, $R_1 = H$, $R_2 = H$, $R_3 = SO_3Na$.

quinoline moiety followed by a second substitution at position C-5.

4. Conclusions

Since the components of quinoline yellow show large differences in partition coefficients ($K = 0.03$–3.3), it is difficult to separate all components simultaneously using the standard HSCCC technique with a constant flow-rate of the mobile phase. This problem was solved by applying a stepwise increase of the flow-rate starting at 0.1 mL/min and ending at 2.0 mL/min. Using this new HSCCC technique we were able to purify six components of quinoline yellow in a single run with a two-phase solvent system consisting of MTBE–1-butanol–acetonitrile–0.1 *M* TFA (1:3:1:5). The purified components were shown to be one sodium trisulfonate, three sodium disulfonates, and one sodium sulfonate of quinophthalone by analysis with HPLC, ESI-MS, and NMR. The overall results of our studies indicate that this new HSCCC technique performed by stepwise increasing the flow-rate of the mobile phase is a powerful technique for the separation of quinoline yellow, the components of which exhibit large differences in their partition coefficients.

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