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An Improved Method for Large-Scale Isolation of a Water-Soluble Safflor Pigment from Dyer's Saffron Flowers

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ABSTRACT

An improved method for large-scale isolation of a water-soluble safflor pigment was assessed. The pigment was separated from the alcoholic extract of the bright-yellow florets of dyer's saffron by fixing it with BD-cellulose in an acidic solution and purified through successive column chromatographies. Thus, a chromatographically pure sample obtained was analyzed for confirming the identity with an authentic safflor yellow B, then it was mixed separately with BN-, BND-, QAE- and ECTEOLA-cellulose in an acidic buffer solution, and the trapping capacity by the anion-exchange celluloses was compared. The arrested pigment was released by diluted methanol, ethanol and acetone, and its solubility rate could be elevated further by mixing formic acid or acetic acid into the liberation media. Data were evaluated in reference to practical utilization of the synonymous colouring matters as colour additives for processed foods or soft drinks.

INTRODUCTION

Application of ionic binding forces to the isolation of metabolic products is familiar to chemists and biochemists or manufacturing engineers of naturally occurring substances, and it has long been used in practice for obtaining amino acids, organic acids, nucleic acids and alkaloids from various biological materials (Walton, 1975). However, no evidence is accessible in the literature as to useful techniques for separating C-glucosidic quinoid chalcones and related pigments from any vascular plant extracts.

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Recently, red or orange–yellow safflor pigments have been attracting increasing attention as harmless colourants for foods or soft drinks. Although classical methods are now routinely used for obtaining the saffron dyes, they are all laborious and often hazardous in practice. Particularly, orange–yellow pigments including safflor yellow A, safflor yellow B and precarthamin are easily soluble in water and less affinitive for cross-linked macromolecular substances (Saito & Fukushima, 1988*a*). This further complicates the process of the isolation and purification of these phenolic metabolites.

In our preceding studies we found that several anion-exchange celluloses were usable for arresting the water-soluble colouring matters (Saito & Fukushima, 1988*b*). Some organic acids and bases were effective for recovering the ionically trapped vegetative dyes and could be released almost quantitatively without appreciable change in their chromatographic, spectrophotometric and tinctorial characteristics. The reported method, therefore, was surely useful and safely applicable for practical isolation of the quinoidal chalcones and related components. However, the use of acids and bases as the dissociating initiators seemed to be somewhat disadvantageous for removing the solvents smoothly and it often compelled us to waste long hours to ensure successful evaporation.

During studies on large scale isolation of the quinoid chalcone pigments we found that bound substances could be dissociated by relatively higher concentrations of methanol and ethanol. In this report an improved method for obtaining *Carthamus* pigments from the floral extract is documented.

ABBREVIATIONS

BD = benzoyl diethyl aminoethyl, BND = benzoyl naphthoyl diethyl aminoethyl, QAE = diethyl-(2-hydroxypropyl)-aminoethyl, ECTEOLA = epichlorhydrin triethanolamine, PVP = cross-linked insoluble polyvinyl-pyrrolidone.

MATERIALS AND METHODS

Materials

A standard sample of safflor yellow B used throughout the present studies was from the stocked collection which was obtained in our laboratory from younger bright-yellow flower florets of dyer's saffron (*Carthamus tinctorius* L.). Ionically charged BD-, BND-, QAE- and ECTEOLA-cellulose were

purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Sephadex HL-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Avicel cellulose was supplied by Asahi Kasei Kogyo Co., Ltd (Tokyo, Japan). Toyo Pearl HW-40f was provided from Toyo Soda Kogyo Co., Ltd (Tokyo, Japan). Silica gel (Wako gel C-20) was obtained from Wako Pure Chemical Ind., Ltd (Osaka, Japan). Cellulose thin-layer plate, No. 5716 (20 cm × 20 cm, 0·10 mm thick, without fluorescent indicator), silica gel thin-layer plates; 60, No. 5721 (20 cm × 20 cm, 0·25 mm thick, without fluorescent indicator) and $60F_{254}$, No. 5715 (20 cm × 20 cm, 0·25 mm thick, with fluorescent indicator) were purchased from E. Merck (Darmstadt, FRG). All organic solvents were obtained from several commercial sources and some of them were glass distilled just before using for dissociation and/or chromatographic operation on the bound-released substances.

Preparation of safflor yellow B for the trapping test

Younger bright-yellow flower florets of dyer's saffron (4·7 kg fresh weight) were harvested in a local field on 7–17 July in 1987. They were immersed in 7440 ml of 99·5% methanol for several days at 3–4°C and, at intervals, they were stirred, then extracted three times; each time fresh methanol was used. The combined extracts (19 900 ml) were reduced to about one-twentieth volume with a rotary evaporator (below 30°C). The dark yellowish brown concentrate was filtered through Celite, washed with ether and the mother solution was further condensed to one-fourth part of the original volume. An equivalent portion of the concentrate was loaded onto a column (4·4 cm × 40 cm) of BD-cellulose (50 g), which had been pre-washed and activated previously. It was passed through the column with gravity flow and the pigment-adsorbed cellulose ion-exchanger was washed thoroughly with deionized–distilled water to wash out water-soluble impurities. The cellulose was then washed with 3·3M acetic acid in acetone. The eluate (2980 ml) was evaporated (at less than 35°C) and the concentrate was subjected to the process of chromatographic purification of the pigment.

Chromatography of the released safflor pigments

An aliquot of the yellow brown concentrate was loaded onto four glass columns and chromatographed successively by using the following packings and solvents: Avicel cellulose—*n*-butanol/acetic acid/water (4:1:2, by vol.), Sephadex LH-20—distilled water, Toyo Pearl HW-40f—methanol/water (6·5:3·5, by vol.), silica gel C-20—*iso*-propanol/water (7:3, by vol.) or *n*-butanol/acetic acid/water (3:1:1, by vol.). During the chromatographic operation, pigments were often spotted on thin-layer plates and developed

in *n*-butanol/acetic acid/water (4:1:2, by vol.) and acetic acid/water (3:7, by vol.), then R_f-values and the colours were checked by reference to authentic specimens under an ultra-violet light lamp (354/366 nm, Compact 4-W UV lamp, model UVGL-15; San Gabriel, Ca., USA) and daylight before or after exposing to ammonia vapour. The chromatographically pure sample was used for the analytical processes described below.

Identification of the dissociation compound

IR-spectra were recorded in KBr disks using a Shimadzu spectrophotometer, model IR-345. R_f-values were examined on air-dried thin-layer plates developed mainly with *n*-butanol/acetic acid/water (3:1:1, by vol.), acetic acid/water (1·5:8·5, by vol.) and phenol saturated with water. Detection reagents such as liquid ammonia and alcoholic ferric chloride solution were sprayed on the chromatograms, and the resulting tinctorial characteristics between the released sample and an authentic specimen were compared under visible light.

Trapping of the recovered safflor yellow B by anion-exchangers

A 25 nmol/ml solution of the recovered safflor yellow B in 50 mM citrate-phosphate buffer was made up at pH 3·0 and each 4 ml solution was treated with 50 mg wet weight of ion-exchangers. Several equivalent portions of cellulose–pigment complexes were prepared in this manner. The pigment-adsorbed celluloses in test tubes were poured on funnels and washed with 5 ml of deionized–distilled water, which was then removed by suction.

Liberation of the fixed pigment

Five millilitres of the desired concentration of methanol, ethanol or acetone was severally added dropwise on the funnel (with the damp complex) and resulting eluate was kept for the process of spectrophotometric estimation. Sometimes, a given composition of formic acid or acetic acid was mixed with 67% (v/v) aqueous acetone and an aliquot of the formic acid–acetone or acetic acid–acetone mixture was used as the liberation medium for the bound dye. The amount of the pigment arrested by the ion-exchangers was estimated by comparing optical density of the eluate with that of the original solution before treating with the anion-exchange celluloses. The recovery rate of the pigment was determined from the spectral change at 411 nm with a Shimadzu, type UV 150-02 spectrophotometer.

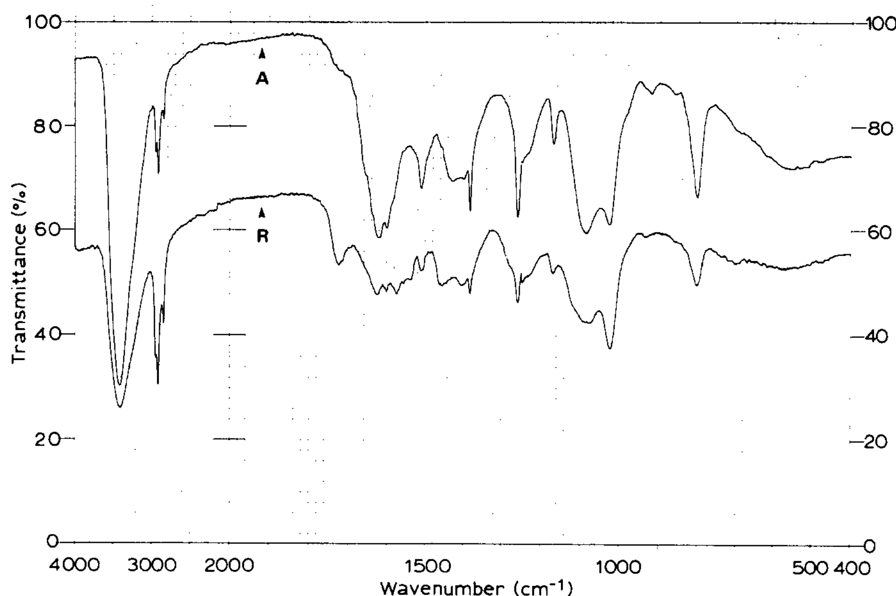

Fig. 1. IR-spectra in KBr disks of a BD-cellulose trapped and recovered safflor yellow pigment, and an authentic safflor yellow B. R, BD-cellulose trapped and recovered safflor yellow pigment; A, authentic safflor yellow B.

RESULTS

Safflor pigments from bright-yellow dyer's saffron flowers were fixed with BD-cellulose in an acidic solution and recovered by using diluted acetic acid in aqueous acetone. The released pigment was then subjected to the chromatographic processes with different column packings in various developing solvents. Thus, a chromatographically pure sample was obtained through the several purification steps. It migrated with the same R_f-values as those of an authentic safflor yellow B on thin-layer plates developed in different solvent systems. No difference could be found between the recovered compound and a standard specimen in their tinctorial properties and/or chromatographic and spectrophotometric behaviours (Fig. 1). From the analytical data we concluded that the newly isolated pigment was safflor yellow B.

Test solutions were prepared by dissolving an equivalent weight of the pigment into 50 mM citrate–phosphate buffer at various pH ranges. The effect of pH values on the formation of pigment–cellulose complex was observed in the mixtures (4 ml) of activated BD-cellulose (100 mg) and

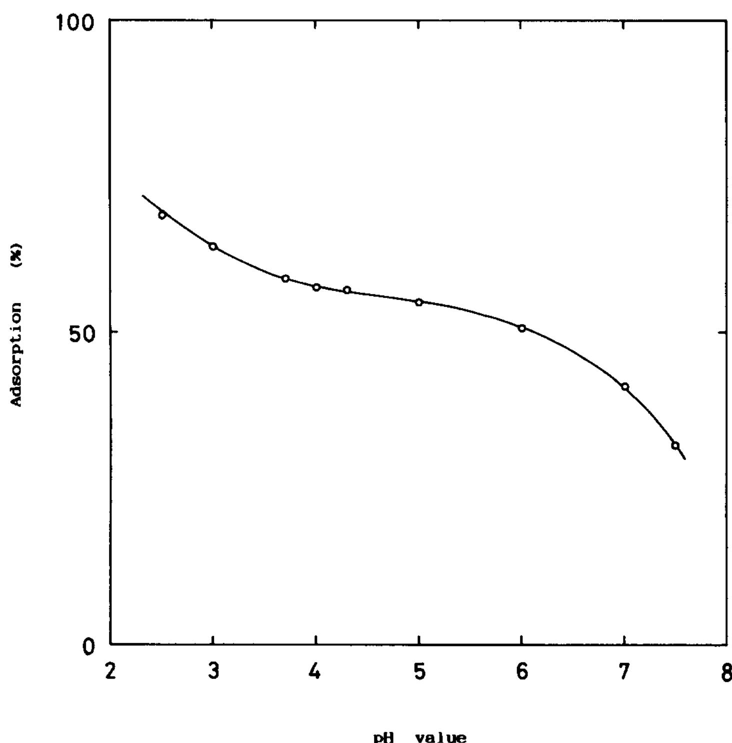

Fig. 2. Effect of pH values of a buffer on the trapping of a safflor yellow pigment by activated BD-cellulose.

pigment (25 nmol/ml) at different pH values (2·5–7·5). The results are illustrated in Fig. 2. It can be seen from the figure that, at pH 2·5, more safflor yellow B (68·4%) is trapped by the exchanger than that (31·6%) fixed at pH 7·5. The effect of incubation time on the amount of the pigment adsorbed was investigated in acidic mixtures. The results indicate that an equilibrium between bound and unbound safflor yellow B occurs within a 10-min incubation period. Longer periods of incubation, up to 30 min, had little or no effect on the amount of the compound arrested (data not shown). Safflor yellow B was also adsorbed by other anion-exchange celluloses, though the rate was varied. Both BD- and BND-cellulose fixed the pigment more efficiently than QAE- and ECTEOLA-cellulose. Their ratios ranged as follows: 2·5:2·5:1·3:1·0, respectively (0·24 μM safflor yellow B/mg cellulose ion-exchanger/min, in an average of four different determinations). The fixed dye was separable from the trappers with aqueous organic solvents. The relationship between pigment solubility and solvent concentration was examined using three different solvents—methanol, ethanol and acetone—at 10, 20, 30, 50, 70 and 90% (v/v), respectively. Figure 3 shows that the

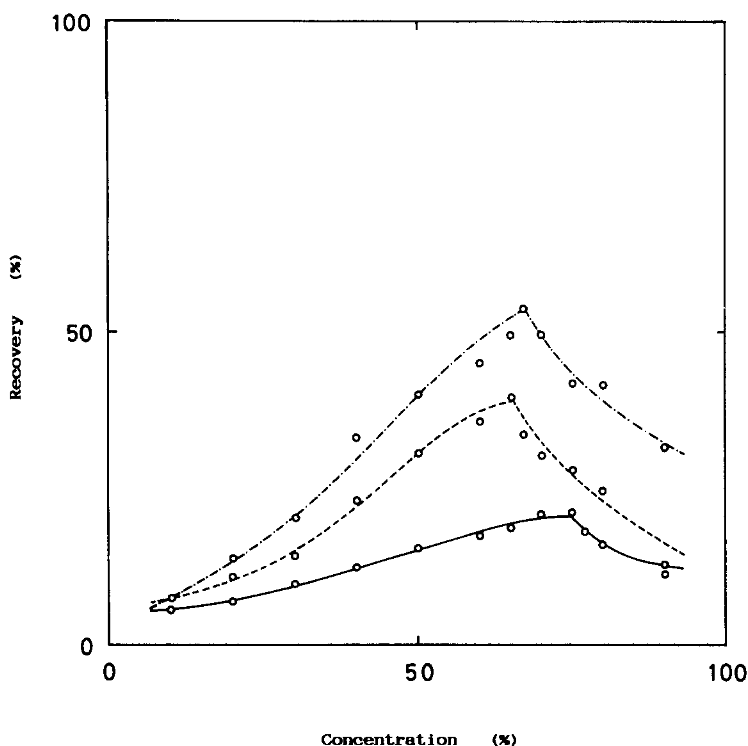

Fig. 3. Relationship between solvent concentration and pigment solubility: methanol (○——○), ethanol (○– – – –○) and acetone (○–·–·–○).

amount of the released matter correlates well with the dilution rate of the solvent used. It increases linearly, reaches a maximal level at 65–75%, and decreases sharply over the limited range of the solvent concentration. For aqueous methanol there is a sharp elution optimum at 75%, while both ethanol and acetone show somewhat lower values. For the optimal separation of the bound safflor yellow B, 67% acetone was the most effective among the three. With acetone at this concentration, 54% of the pigment could be recovered.

Because facilitated liberation of the tied matters from the ionically charged trappers has often been noticed after addition of polar solvents (Saito & Fukushima. 1988*a*, b), work has been done to examine the effect of organic acids upon the amount of safflor yellow B recovered. Formic and acetic acids were mixed independently with 67% aqueous acetone at various proportions and added dropwise onto the BD–cellulose–pigment complex. The results listed in Table 1 show that the solubility of the bound dye is increased as the acid concentration goes higher. The stripping force of the bound matter by formic acid is stronger than that by acetic acid, which is

TABLE 1
Effect of Increasing Concentration of Formic Acid and Acetic Acid on the Solubilization of BD-Cellulose-Trapped Safflor Yellow B by Aqueous Acetone

Solvent	*Concentration (%)*[a]	*Pigment recovered (% of fixed amount)*
Formic acid	10	57·2
	30	67·8
	50	72·3
Acetic acid	10	54·3
	30	58·0
	50	65·1

[a] v/v. Formic acid and acetic acid were separately mixed with 67% (v/v) aqueous acetone at the concentration indicated in the table.

well comparable with the data reported in our preceding work (Saito & Fukushima, 1988*b*). The recovery rate of the bound pigment by formic acid–acetone or acetic acid–acetone preponderates clearly over that of each solvent tested alone (47·5%) and, moreover, this synergistic effect is further intensified by rising acid concentrations.

DISCUSSION

Safflor pigments from dyer's saffron flowers have long been used in some districts of our country for their attractive and fascinating tinctorial properties. They are usually obtained as amorphous pastes or yellowish-brown powders from alcoholic extracts of the yellow flowers and purified following the methods of lead salt precipitation and/or column chromatographies through combinations of certain polymer packings with various solvent systems. However, their yields are eventually low, though the pigments are accumulated in the floral tissues at relatively higher levels (Saito & Fukushima, 1988*b*). The low recovery rate of the colouring matters could perhaps be caused mainly by degradation and transformation through the complicated, subdivided and prolonged purification processes.

In this study we have established an improved protocol for large scale isolation of a safflor pigment by using new solvent systems. The pigment was trapped swiftly by anion-exchange cellulose(s) at lower pH ranges and the fixed matter was smoothly released with aqueous methanol, ethanol or acetone. Addition of some polar solvents to the aqueous dissociators

facilitated the recovery of the arrested compound more prominently. In general, phenolic compounds are known to be adsorbed on polymeric substances through hydrogen bonding. Several hydrogen bonding solvents, on the other hand, in mixture with water, have been shown to be more or less effective in stripping phenolics from the insoluble complexes. For example, in the case of bound tannins the stripping capacity by the aqueous solvents increased in the order: methanol, *iso*-propanol, ethanol, *tert*-butanol, methyl cellosolve, methyl cellosolve acetate, acetone and dioxane (Merrill *et al.*, 1947). Polyphenols have also been shown to be readily taken up by ionically-charged polymers (Lam & Shaw, 1970; Croteau *et al.*, 1973; Loomis, 1974; Gray, 1978; Fukushima *et al.*, 1987). No report on the recovery of bound phenols is obtainable in the literature. In the case of the ionically-tied safflor yellow B, it was separable from the trapper with non-polar solvent alone or with mixtures of non-polar and polar solvents. These are indicative of the possibility that the hydrogen bonding force is cooperative in the binding mechanism, though ionically affinitive strength may play the leading role in the process of pigment association. The data summarized in Table 1 support this assumption, indicating that the release of the arrested matter was synergistically promoted through addition of certain non-polar solvents to the polar dissociation reagent used. No difference could be detected between the trapped–recovered safflor pigment and an authentic safflor yellow B through the data from chromatographic and spectrophotometric analyses.

The currently established method is superior to that previously reported (Saito & Fukushima, 1988*b*) in the following respects: the bound pigment can be recovered under mild conditions, removal of solvents from the liberated matter is easier and the operation processes are greatly simplified and shortened. The technique described in this paper will undoubtedly help the large-scale isolation of safflor yellows and related pigments which are safely applicable to processed foods and soft drinks as natural harmless colour additives.

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