Is carthamine a single component?

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A chromatographically pure sample of red pigment from dyer's saffron (*Carthamus tinctorius* L) was separated into two distinct reddish components on an insoluble polyvinyl polypyrrolidone column, developed with acetone/methanol/water. These components were isolated, purified and subjected to the analytical processes. Using standard analytical techniques, only slight differences could be detected between the two types of carthamine. However, a marked difference was displayed by optical rotary dispersion spectroscopy of the two samples. This provides evidence for the existence of two geometrical isomers of carthamine.

INTRODUCTION

Carthamine is a red vegetable dye which has long been used to dye textiles and to produce cosmetic rouges [1]. It was named originally in 1846 by Schlieper, who analysed the chemical structure [2]. Since then numerous reports on the structural and the tinctorial properties of the dye have appeared in the literature [3–15]. Kuroda proposed a monomeric chalcone structure [10], later superseded by a bichalcone formula (Figure 1) [16,17]. Figure 1 shows that carthamine has two asymmetric carbon atoms at positions 3 and 3′ on the molecular skeleton. Hence it could comprise stereochemically distinguishable forms, although the precise configuration has not yet been ascertained.

In this paper work undertaken to separate chromatographically the components of carthamine derived from

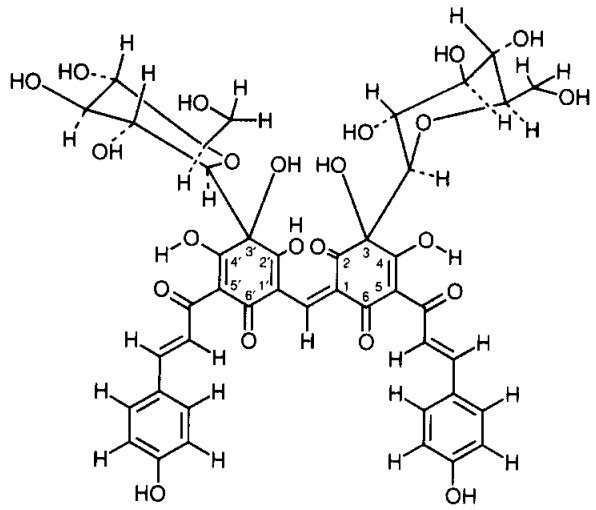

Figure 1 Structure of carthamine: 1-(2,6-diketo-3-β-D-glucopyranosyl-3,4-dihydroxy-5-*p*-hydroxycinnamoyl)-cyclohex-4-enylidene-1′-(2′,3′,4′–trihydroxy-3′-β-D-glucopyranosyl-5′-*p*-hydroxycinnamoyl-6′-keto)-cyclohexa-1′,4′-diethylmethane

dyer's saffron (*Carthamus tinctorius* L) using a synthetic vinyl polymer, poly-*N*-vinylpolypyrrolidone (PVPP) is described.

EXPERIMENTAL

Materials

Carthamine was prepared from the processed florets of dyer's saffron, purchased from a local market (Yamagata, Japan), according to the method of Saito *et al.* [18]. PVPP (Sigma), Toyo Pearl HW-40f (Toyo), Sephadex LH 20 (Pharmacia), and Chromatorex ODS (Fuji-Davison) cellulose and silica gel thin-layer plates (Merck) were used as supplied. Other chemicals and reagents used were all of analytical grade supplied by several commercial suppliers. Deionised/distilled water was used in all experiments.

Conditioning of PVPP

A weighed sample of PVPP (500 g) was thoroughly washed and suspended in an aqueous solution containing 0.75 g/l potassium ferricyanide, 40 g/l sodium carbonate and 9 g/l sodium chloride. The suspension was stirred for 120–150 h at room temperature and at intervals the solution was replenished. When freshly mixed yellow solution of ferricyanide/carbonate/chloride was no longer bleached, the PVPP was washed several times with distilled water, then stirred successively in sodium carbonate solution, hydrochloric acid, water and acetone. Finally the slurry thus obtained was washed with diethylether, dried in air and kept in a desiccator.

Chromatographic purification of sample carthamine

Carthamine was purified by column chromatography. A known weight of crude carthamine was added to a column (3 × 48.5 cm, bed volume 322.2 ml) of Toyo Pearl HW-40f and developed in 65% methanol. The red band eluate was collected manually and carthamine obtained after evaporation *in vacuo*. The dark red mass was then chromatographed three times on a Sephadex LH 20 column (3 × 48.5 cm), with gradually increasing concentrations of aqueous methanol used as the developing solvent (30, 60, 80% respectively). The chromatographically purified carthamine was kept at –20°C in the dark.

Separation of carthamine on PVPP column

The dried PVPP was soaked in water for 24 h and the paste filtered. The damp residue was suspended in acetone/methanol/water (24:3:16) and the suspension deaerated by treating on an ultrasonic oscillator. It was then packed carefully in a glass column (6.5 × 50 cm, bed vol. 1460 ml). A weighed sample (0.5–2.0 g) of carthamine was dissolved into 10–30 ml acetone/methanol/water. Column eluates were collected manually.

Purification of carthamines

Two main fractions from the PVPP column chromatography were evaporated separately at below 35°C. The condensate was loaded onto a Chromatorex octadecyl silane (ODS) column (3.8 × 35 cm, bed vol. 340.1 ml) and developed in 70% acetone. Each resulting fraction was collected, evaporated to dryness and kept in a freezer at –20°C in the dark.

Characterisation of separated carthamines

Chromatography was carried out using cellulose or silica gel TLC plates in chromatography chambers at 26±0.5°C. Developing solvents applied were:

A *n*-Butanol/acetic acid/water (4:1:2)
B Acetic acid/water (15:85)
C Acetone/methanol/water (24:3:16)
D Phenol saturated with water.

Carthamine spots were detected on the chromatograms with a UV lamp (365 nm) or under a 40 W fluorescent light lamp.

HPLC was conducted on an ODS column (Wakosil $5C_{18}$, 5 μm, 4 × 250 mm i.d.) connected to a Jasco 880 system. Carthamine samples (20 μg/ml, 3–4 μl) were injected with a Hamilton microsyringe after filtration through a membrane filter (Millipore SLFH 013 NS, 0.5 μm) and developed in isopropanol/acetone/water (73:7:20) with a flow rate of 0.3 ml/min at a pressure of 8.2–8.3 MPa at 40°C. Column eluates were monitored at 520 nm.

UV/VIS spectra were recorded on a Shimadzu MPS 2000 spectrophotometer in 65% methanol. IR spectra were registered on potassium bromide disks using a Shimadzu IR-400 spectrometer. Proton NMR spectra were run on a Jeol JNM FX-400 machine in pyridine-d_5/methanol-d_4 (95:5) mixtures. Trimethyl silane was used as internal standard.

Optical rotary dispersion (ORD) spectra were measured in 55% methanol solution at 25°C.

RESULTS

Separation of carthamine on a calibrated PVPP column

A chromatographically purified sample of carthamine was found to be separable into several resolved bands on a PVPP column when developed in acetone/methanol/water. Among the red bands separated, two were major constituents and the rest minor. Each carthamine fraction migrated on the polyamide gel column with its own R_f value; one ran faster towards the front of the gel column, referred to as carthamine 1, with the other called carthamine 2.

Characterisation of resolved carthamines

The reddish bands corresponding to carthamine 1 and carthamine 2 were recovered separately from the PVPP column and purified by chromatographic techniques using Toyo Pearl HW-40f, Sephadex LH 20 and Chromatorex ODS in several developing solvent systems. The carthamine samples thus purified were examined using other techniques in an attempt to identify differences between them.

R_f values

Table 1 lists the TLC results conducted on cellulose and silica gel plates. It is clear that the carthamines ran with similar R_f values on both TLC plates, even though they were developed in different solvents, indicating that both samples had the same solubility rate in the developing solvents tested. They were also detectable under UV (365 nm) as an orange-yellow fluorescence.

Table 1 Comparison of the chromatographic behaviour of carthamine 1 and carthamine 2

	Carthamine 1		Carthamine 2	
Solvent[a]	Cellulose	Silica gel	Cellulose	Silica gel
A	0	0.41	0	0.40
B	0	0.68	0	0.67
C	0.43	0.93	0.43	0.94
D	0	0.32	0	0.33

a For details see Experimental section

HPLC profiles

Both carthamine samples had similar retention times on an HPLC ODS column (Figure 2). Complete separation of carthamines on the silica gel column could not be achieved when isopropanol/acetone/water was replaced with other developing solvent systems.

UV/VIS absorption patterns

UV/VIS absorption spectra of the two carthamine samples were measured over the range 200–600 nm in 65% methanol (Figure 3). Both showed three major peaks (nm): band I (520.0, 519.2), band II (382.6, 383.0), band III (243.8, 244.0). Band I was attributed to the cinnamoyl systems at the 5 and 5′ positions, band II was derived from the benzoyl system, while band III was identifiable with the double bonds of the quinochalcone structures.

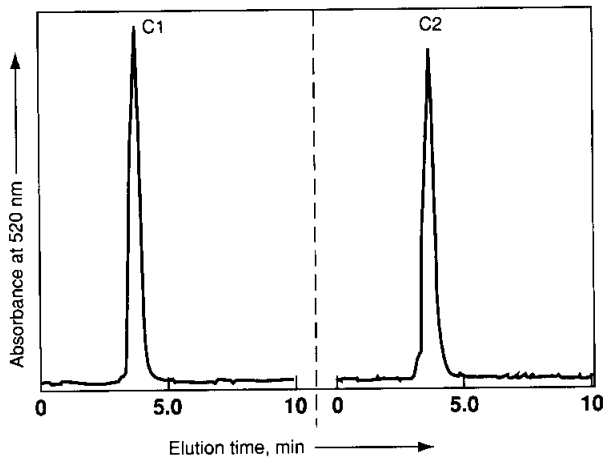

Figure 2 HPLC profiles of carthamine 1 (C1) and carthamine 2 (C2); for details see Experimental section

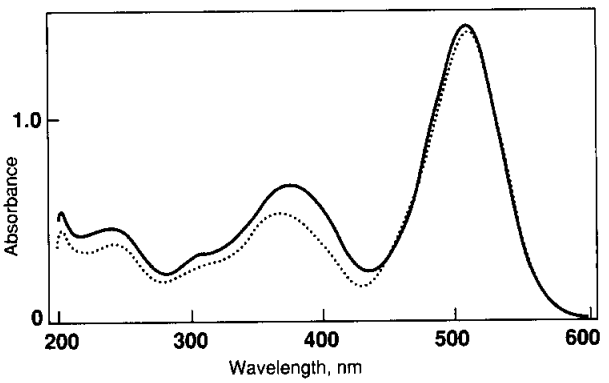

Figure 3 UV/VIS spectra of carthamine 1 (solid line) and carthamine 2 (dotted line)

IR and NMR spectral patterns

Only small differences were noted in the IR and NMR spectra of the two types of carthamine.

ORD patterns

Table 2 summarises the date from ORD measurements of two carthamine samples. The values differ quite markedly

Table 2 Difference between carthamine 1 and carthamine 2 observed by ORD analysis

Carthamine	Specific rotation
1	+527
2	+615

with each other, providing evidence that the two carthamines are optical isomers.

DISCUSSION

Chromatograms clearly indicate that a purified sample of carthamine is not homogeneous, since it is separable on a PVPP column when developed in an aqueous solvent system. However, this heterogeneity is difficult to detect when using other analytical techniques. Slight differences in the proton NMR results indicates that the signal disparity could arise from the attachment of the glucopyranosyl unit at the 3 or the 3′ position. It is this structural difference that distinguishes the two stereoisomers of carthamine, which have different optical activities. The ORD spectra obtained support this conclusion (Table 2).

The results suggest that carthamine 1 has an anti conformation while carthamine 2 has a skew conformation, the former running faster through the PVPP column, the skew type being more strongly attracted to the polyamide gel. Carthamine 1 is more stable than carthamine 2.

CONCLUSION

Carthamine exists in the floral tissues of dyer's saffron as geometric isomers. Poly-*N*-vinylpolypyrrolidone is a promising column packing for separating stereoisomers of carthamine. The carthamine sample thus obtained are amenable to crystallographic analysis or other close examination. Work is continuing to determine unequivocally the absolute configurations of carthamine 1 and carthamine 2.

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