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Effect of Exogenous Substances on Carthamin Red Coloration

Koshi Saito & Akiyoshi Fukushima

Department of Applied Chemistry, Faculty of Engineering, Tokai University, 1117 Kitakaname, Hiratsuka-shi, Kanagawa-ken 259-12, Japan

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ABSTRACT

Effect of exogenous substances on carthamin colour modification was examined in solutions containing the pigment and alcohols, ethers, ketones, esters, carboxylic acids, fatty acids, amino acids, amides or amines. Alcohols, amino acids, amines, carboxylic acids and ethers effectively promoted reddening of the aqueous acetone comprising carthamin. Ketones and amides had little or no effect on the bathochromic colour shift. Esters and fatty acids, except for formic and acetic acids, exerted hypsochromic effects on the carthamin red. Based on the experimental findings, a possible mechanism of the carthamin colour modification was briefly debated.

INTRODUCTION

Recently, interest in food colorants has increasingly centred on naturally occurring harmless pigments. However, one of the most difficult problems is that they are usually unstable and easily converted to undesired and/or distasteful substances. Carthamin is a water-soluble plant pigment distributed among reddened flower florets of safflower (*Carthamus tinctorius* L.). The chemical construction of the pigment has been extensively studied by many workers (Kuroda, 1930; Obara & Onodera, 1979; Takahashi *et al.*, 1982) and shown to be 6-β-D-glucopyranosyl-2-[[3-β-D-glucopyranosyl-2,3,4-trihydroxy-5-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-6-oxo-1,4-cyclohexadien-1-yl]-methylene]-5,6-dihydroxy-4-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-4-cyclohexene-1,3-dione (Scheme 1), though the stereochemistry of asymmetric carbon atoms at the 3- and 6-positions in the

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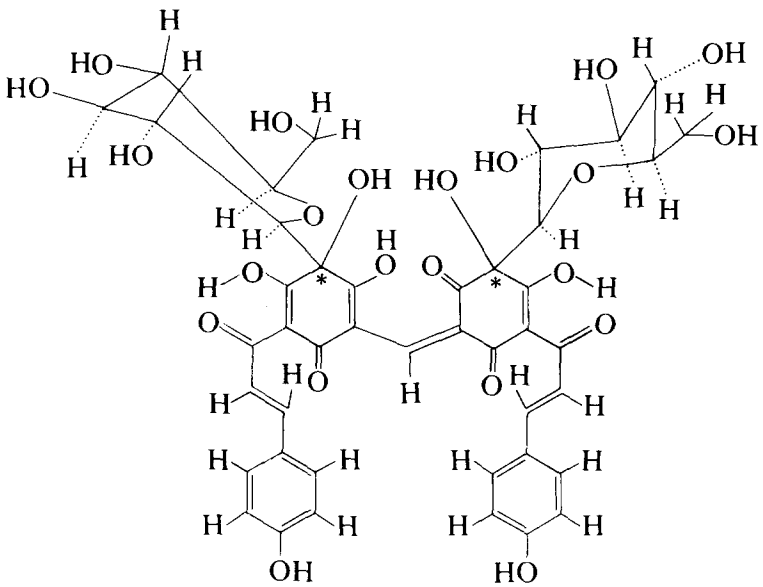
mother skeleton has not yet been fully determined. The novel bichalcone glycoside has long been used as a red dyestuff for cotton-cloths by the local populations of some countries; however, it can also be considered as a most useful tinctorial additive for colouring manufactured foods. Chemically, carthamin is quite unstable, both in solutions and cellular media. In solutions carthamin changes easily from its characteristic natural red to unknown reddish orange or orange yellow compounds and the discoloration proceeds more prominently in polar solvents than in less polar aprotic



Scheme 1. Chemical structure of carthamin.

ones, which represents a major obstacle to its application in food products or soft drinks. Many factors prevailing in the chromatism are considered to influence the coloration of the pigment. Saito & Fukushima (1986) have found that the unfavourable colour transition could be prevented to some extent by addition of certain amino acids and carboxylic acids to the carthamin solutions. These results indicate that exogenous substances take part in the colour modification through change in the physico-chemical environment in the aqueous solution of carthamin.

To ensure more widespread application of carthamin, this work was planned to study the kinetics of colour modification and to investigate some factors influencing the coloration of the pigment. We wish to report here new findings about the effects of many substances on carthamin red coloration in experimental model systems.

MATERIALS

Carthamin used throughout this study was obtained from freshly collected *Carthamus* flowers. Bright yellow florets were gathered on our experimental field in 1985 and boiled in rectified methanol for a few minutes. After removing methanol, the florets were frozen with liquid nitrogen, followed by grinding with a pestle and mortar. The finely ground powder was dried in air and stocked in a vacuum desiccator over silica gel before use. Carthamin sample was prepared from the dried floret powder according to the enzymic method of Saito *et al.* (1983). All chemicals and reagents were of the highest purity obtainable from commercial sources. Water was carefully deionized, then distilled before using it to prepare solvent and to wash experimental implements.

METHODS

Carthamin and chemicals for testing were dissolved in 60% aqueous acetone to a concentration of 55·0 μM. An aliquot of the solution was mixed separately with the same volume of carthamin solution and the mixture (1·0–2·0 ml) was gently stirred in an incubator for 10 min at 24 $\pm$ 2°C. The optical density in the mixture was monitored by a Shimadzu spectrophotometer (type 150-02) connected with Rikadenki electrometric recorder (model R-21). The reaction velocities were determined from the data of spectral changes at ΔA 521 nm in test solutions observed during the course of the incubation period. Time-course of chemical shift was recorded automatically at 24°C by using a Shimadzu spectrophotometer (model MPS 2000) at four different intervals, 0–2, 20–22, 40–42 and 60–62 min, using 55·0 μM histidine or asparagine in 60% aqueous acetone containing 55·0 μM carthamin.

RESULTS

Ultraviolet- and visible-light absorption spectrum of carthamin in aqueous acetone

An ultraviolet- and visible-light absorption spectrum of carthamin was taken with a Shimadzu multi-purpose spectrophotometer as described in the 'Methods' section. Presetting of the absorption range was chosen between 0 and 3·0 and the spectrum recorded automatically from 310 to 600 nm in 60% aqueous acetone containing 55·0 μM carthamin. A typical result is shown in

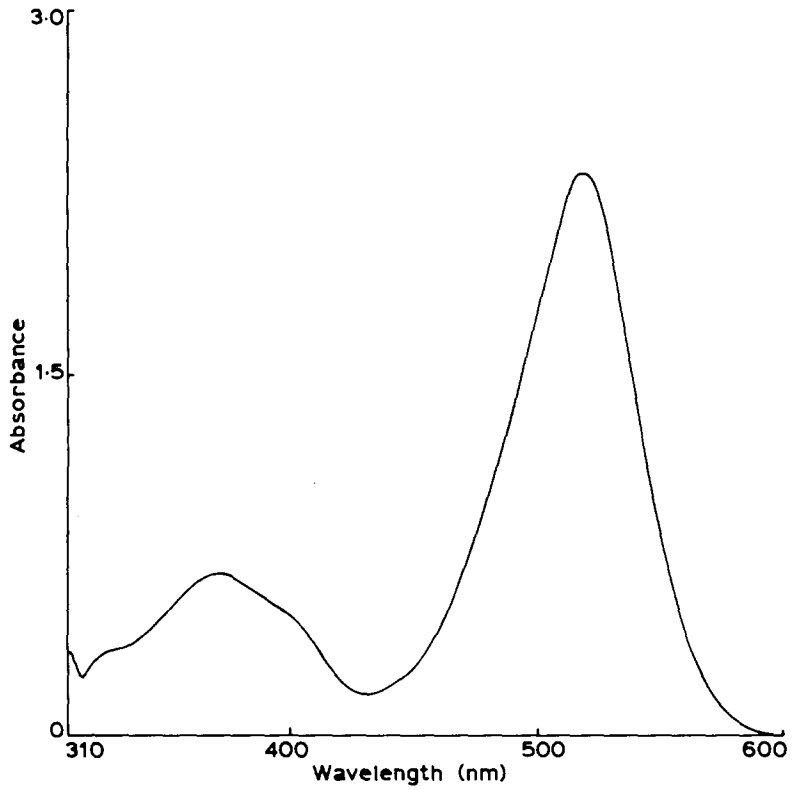


Fig. 1. UV spectrum of carthamin. The spectrum was recorded automatically with a Shimadzu spectrophotometer in aqueous acetone containing carthamin.

Fig. 1. $\lambda_{max}^{Me_2CO}$ observed in carthamin containing acetone solution was as follows; nm (ε): 314 (6100), 373 (5820) and 521 (18 900). The spectral pattern of carthamin is comparable with that of the pigment in methanol (Takahashi *et al.*, 1984; Saito & Takahashi, 1985; Saito *et al.*, 1985).

Spectral change in visible-light absorption of carthamin red after addition of histidine or asparagine to the pigment solution

Two compounds, histidine and asparagine, were selected as typical inducers of chemical shift for carthamin solutions. Just after addition of the test compounds to the carthamin solution, the time course of the spectral change at 521 nm was followed repeatedly at given intervals of the examination period. On mixing histidine to the solution, the absorption peak continuously rises with duration of the incubation time (Fig. 2). If histidine is substituted with asparagine, a different picture arises, indicating that the selected absorption peak falls gradually with the lapse of time (Fig. 3). Under

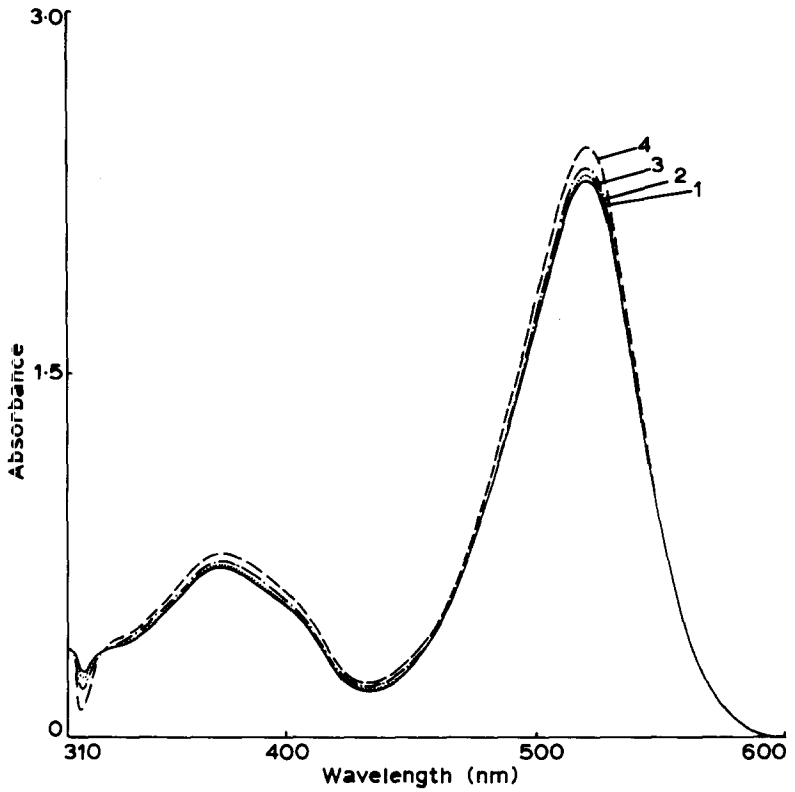


Fig. 2. Time dependence of spectral change in absorption maxima of carthamin after addition of histidine. The assay was carried out automatically at 24°C in a Shimadzu spectrophotometer equipped with a stoppered cuvette. Spectral change was taken at 0–2, 30–32, 60–62 and 90–92 min, respectively. The numbers 1, 2, 3 and 4 in the Figure, refer to the spectra recorded at 0–2, 30–32, 60–62 and 90–02 min, respectively.

these conditions, the rate of the chemo-shifts derived from histidine and asparagine is roughly computed to be 56·7 (bathochromic) and 23·3 nM (hypsochromic) carthamin/min, respectively.

Effect of alcohols on spectral change in carthamin solution

Five different alcohols were tested for spectral change in carthamin solutions. Figure 4 shows that all of the alcohols examined at the 55 nmol ml^{-1} level promote the batho-shift reaction in the incubation media. *n*-Propanol is the most efficient of the compounds tested, as shown by its greater bathochromic effect. Ethanol follows this, while *n*-butanol and methanol are both weak effectors. Mercaptoethanol shows a relatively high value, while it effects less than ethanol. This may be reflected by the fact that

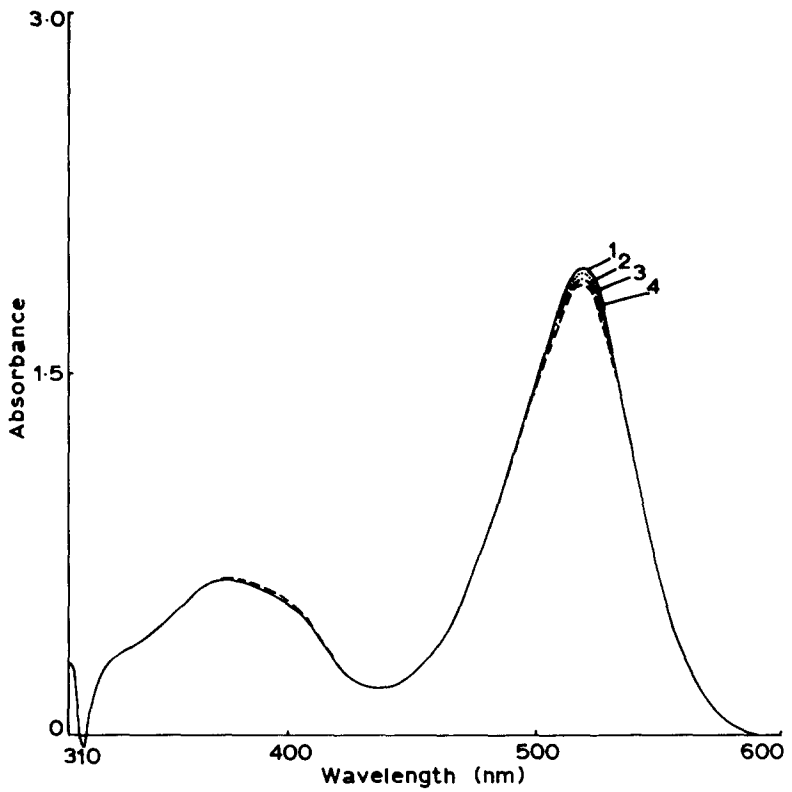


Fig. 3. Time dependence of spectral change in absorption maxima of carthamin after addition of asparagine. The assay was carried out automatically at 24°C in a Shimadzu spectrophotometer equipped with a stoppered cuvette. Spectral change was taken at 0–2, 30–32, 60–62 and 90–92 min, respectively. The numbers 1, 2, 3 and 4 in the Figure refer to the spectra recorded at 0–2, 30–32, 60–62 and 90–92 min, respectively.

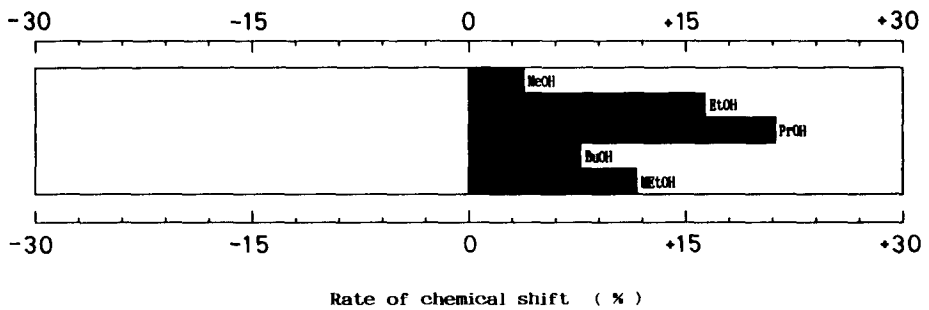


Fig. 4. Effect of alcohols on spectral change in carthamin solution. MeOH: methanol, EtOH: ethanol, PrOH: *n*-propanol, BuOH: *n*-butanol, MEtOH: mercaptoethanol. (+): bathochromic shift, (−): hypsochromic shift.

the sulphydryl residue in the alcohol acts competitively in the batho-shift reaction. The ratio of the bathochromic effect from each alcohol is estimated to be 1·0 (methanol):4·3 (ethanol):5·6 (*n*-propanol):2·0 (*n*-butanol):3·0 (mercaptoethanol).

Effect of ethers on spectral change in carthamin solution

In this study five ethers were chosen for testing the effect of the change in the carthamin solution (Fig. 5). Ethyleneglycol monomethyl ether, diethyl ether, cyclohexanone oxime methyl ether and isopropyl ether are all bathochromic effectors, while chloromethyl methyl ether shows the opposite effect on carthamin solution. Possibly, the chloride residue in this ether works as a negative effector. On the whole, the bathochromic effect of ethers is much less than that of alcohols.

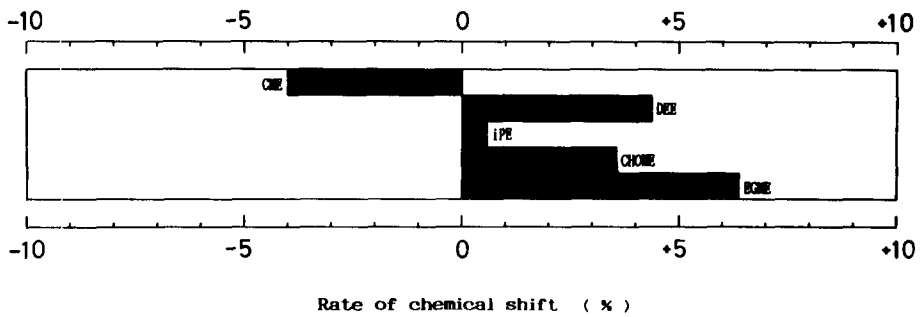


Fig. 5. Effect of ethers on spectral change in carthamin solution. CME: chloromethyl-methyl ether, DEE: diethyl ether, iPE: isopropyl ether, CHOME: cyclohexanone oxime methyl ether, EGME: ethyleneglycol monomethyl ether.

Effect of ketones and esters on spectral change in carthamin solution

Two ketones and eight esters were used in this study. Both methyl ethyl ketone and methyl isobutyl ketone are weak bathochromic effectors. All esters tested exhibit hypsochromic effects on carthamin red (Fig. 6). The rate observed with esters ranges from 1·4% to 4·3% and the ratio is as follows: 1·0 (methyl acetate):2·0 (ethyl acetate):2·3 (stearic acid ethyl ester):2·7 (methyl oleate):2·7 (myristic acid methyl ester):2·7 (ammonium acetate):3·0 (ethyl formate):3·2 (ethyl palmitate).

Effect of carboxylic acids on spectral change in carthamin solution

Of the carboxylic acids tested, it is mainly the members of the tricarboxylic acid cycle, in the metabolic pathway of living cells, which accelerate

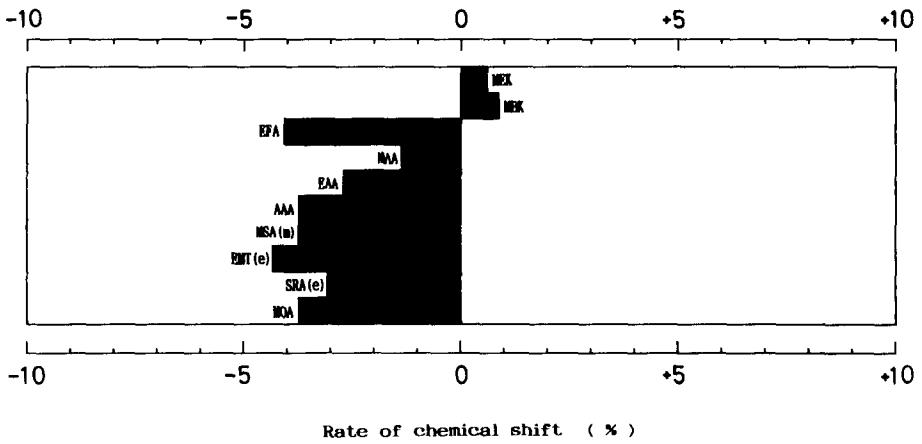


Fig. 6. Effect of ketones and esters on spectral change in carthamin solution. MEK: methyl ethyl ketone, MBK: methyl isobutyl ketone, EFA: ethyl formate, MAA: methyl acetate, EAA: ethyl acetate, AAA: ammonium acetate, MSA (m): myristic acid methyl ester, EMT (e): ethyl palmitate, SRA (e): stearic acid ethyl ester, MOA: methyl oleate.

bathochromic shifts of carthamin solution. Among these acids, malic acid promotes by 18·0% compared with the control. Isocitric and oxalic acids follow this with 8·2% and 6·2%, respectively. α-Ketoglutaric, fumaric and citric acids also shift the carthamin peak to some extent. Terephthalic and maleic acids affect the shift only slightly. Other carboxylic acids, including succinic acid, reduce the absorption peak of carthamin; however, the rates are relatively low (Fig. 7).

Effect of fatty acids on spectral change in carthamin solution

Conspicuous characteristics are seen among fatty acids tested in the present observation (Fig. 8). Formic acid and acetic acid elevate the absorption maximum at 521 nm by 8·1% and 5·1%, respectively. On the other hand, nine other acids exert hypsochromic effects on the carthamin peak with different rates. The ratios of the hypsochromic effects put the fatty acids in the following order: isobutylic, oleic, palmitic, caproic, myristic, stearic and butyric acids.

Effect of amino acids on spectral change in carthamin solution

In total, twenty amino acids were examined at a given concentration in aqueous acetone containing carthamin. The data are illustrated in Fig. 9. On the whole, amino acids are safely said to be bathochromic promoters for

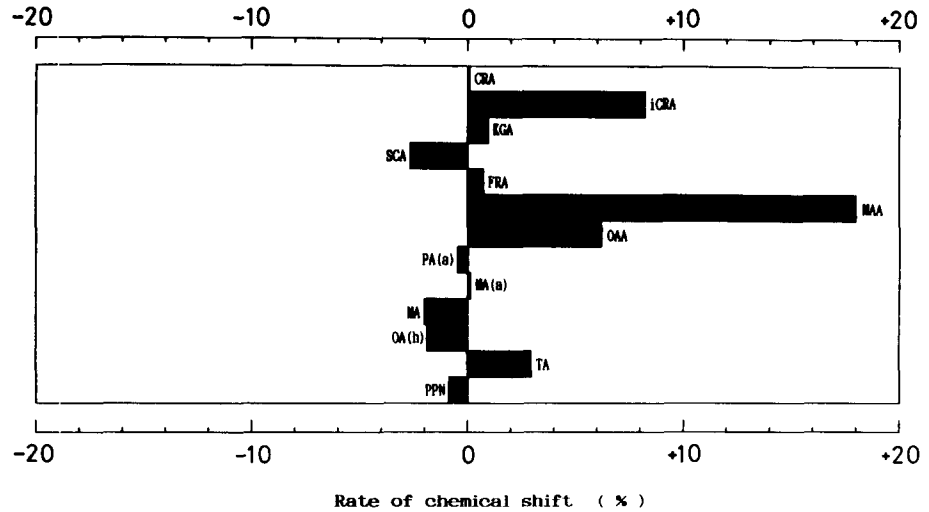


Fig. 7. Effect of carboxylic acids on spectral change in carthamin solution. CRA: citric acid, iCRA: isocitric acid, KGA: α-ketoglutaric acid, SCA: succinic acid, FRA: fumaric acid, MAA: malic acid, OAA: oxalacetic acid, PA (a): phthalic acid anhydride, MA (a): maleic acid anhydride, MA: maleic acid, OA (h): oxalic acid dihydrate, TA: terephthalic acid, PPN: propionic acid.

carthamin. These acids are usually classified according to the chemical nature of their R groups with appropriate sub-classes; namely (1) non-polar or hydrophobic, (2) polar but uncharged, (3) positively charged and (4) negatively charged R groups (Conn & Stumpf, 1963). The effect of the amino acids on colour modification of carthamin red is shown to be correlated with

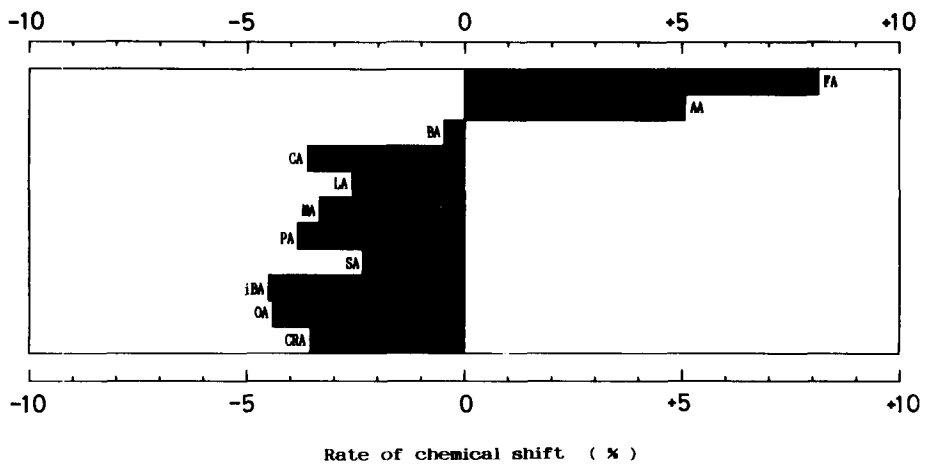


Fig. 8. Effect of fatty acids on spectral change in carthamin solution. FA: formic acid, AA: acetic acid, BA: butyric acid, CA: caproic acid, LA: lauric acid, MA: myristic acid, PA: palmitic acid, SA: stearic acid, iBA: isobutyric acid, OA: oleic acid, CRA: caprylic acid.

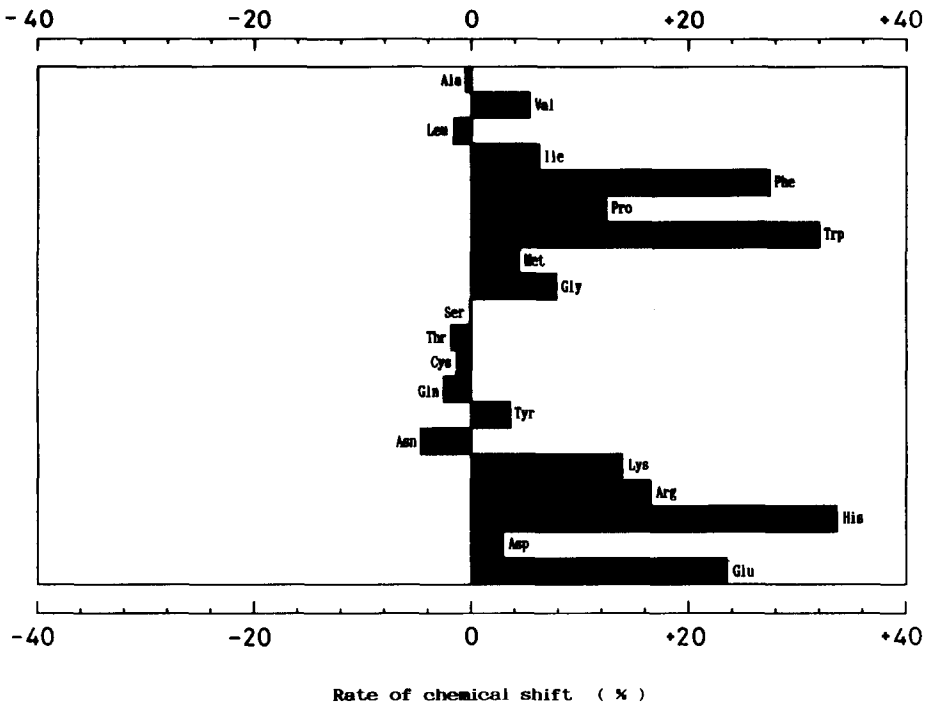


Fig. 9. Effect of amino acids on spectral change in carthamin solution.

their chemical structures. Groups (1), (3) and (4) contain effective bathochrome stimulators in their sub-groups, while almost all members of group (2) reduce the absorption peak of carthamin, though only slightly.

Effect of amines on spectral change in carthamin solution

Figure 10 presents the effects of amines on the carthamin colour modification. These basic substances act as bathochromic effectors, though some discrepancies are seen among the compounds examined. Dimethylamine, diethylamine and Tris(hydroxymethyl)amino methane are greater than methylamine, trimethylamine and monoethanolamine in their bathochromic effects. On the contrary, ethylenediamine, ethylamine and triethylamine work on as weak hypsochromic effectors.

Effect of amides on spectral change in carthamin solution

Only thioacetamide displays bathochromic activity on carthamin solution. It promotes by 8·4% compared with the blank test. Acetamide, laurylamide and *n*-butylamide suppress the maximal peak of carthamin; however, the rate is slight (Fig. 11).

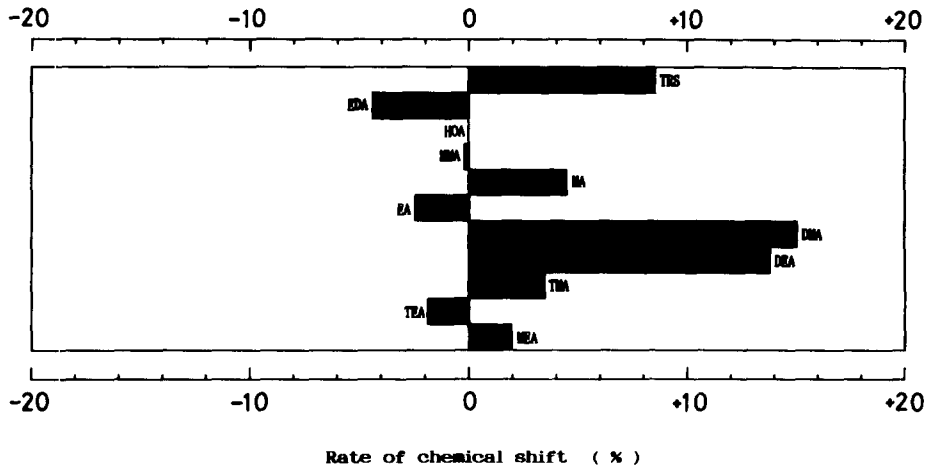


Fig. 10. Effect of amines on spectral change in carthamin solution. TRS: Tris-(hydroxymethyl)amino methane, EDA: ethylene diamine, HOA: hydroxylamine hydrochloride, MMA: monomethylamine hydrochloride, MA: methylamine, EA: ethylamine, DMA: dimethylamine, DEA: diethylamine, TMA: trimethylamine, TEA: triethylamine, MEA: monoethanolamine.

Relative rate of the chemical shift induced in the solution of carthamin in the presence of exogenous substances

A rate average of the chemical shift caused by exogenous substances in carthamin solutions was calculated from the data illustrated in Figs 4 to 11. The results are summarized in Table 1. As seen in the Table, alcohols are the most effective promoter for carthamin red coloration. Amino acids come next. Amines, carboxylic acids and ethers follow this, while both ketones and amides promote the bathochromic effect only slightly. The ratio of the shift induced by these effectors was roughly estimated to be 27·5:20·1:8·8:5·2:4·9:1·7:1·0, respectively. Esters and fatty acids, except for formic acid and acetic

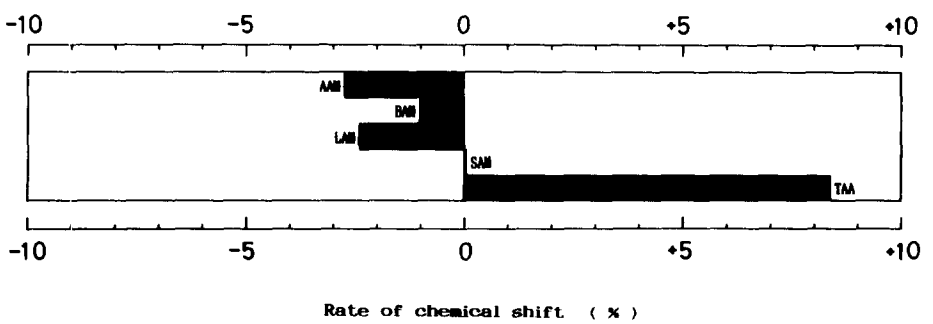


Fig. 11. Effect of amides on spectral change in carthamin solution. AAM: acetamide, BAM: *n*-butylamide, LAM: laurylamide, SAM: stearamide, TAA: thioacetamide.

TABLE 1
Relative Rate of the Chemical Shifts Induced in Carthamin Solutions by Presence of Exogenous Substances

Compound	*Rate of bathochromic shift (% of control)*	*Rate of hypsochromic shift (% of control)*
Alcohols	12·10 ± 6·88	—
Ethers	2·17 ± 3·61	—
Ketones	0·73 ± 0·12	—
Esters	—	3·35 ± 0·90
Carboxylic acids	2·28 ± 5·47	—
Fatty acids	—	1·71 ± 3·97
Amino acids	8·86 ± 11·66	—
Amines	3·89 ± 6·32	—
Amides	0·44 ± 4·08	—

acid, act as hypsochromic effectors in carthamin solutions, though the rate is relatively low.

DISCUSSION

It has been shown in the present study that the red coloration of carthamin in aqueous acetone is greatly affected by the presence of many substances. Among these compounds most alcohols, ethers, ketones, carboxylic acids, amino acids and amines have been found to be bathochromic promoters for carthamin red coloration, while esters, amides and fatty acids, except for formic and acetic acids, exert hypsochromic effects.

Solvent-induced chemical shifts, caused by solvatochromic dyes with electron-donating groups, have already been reported in the literature. Among them, hypsochromic shifts, derived from benzamides and thiobenzamides, have been suggested to be correlated with thionyl bonds (Edward, 1973). Ultraviolet spectra of *o*- and *p*-phenylenediamine have revealed that a blue shift is related to pH value in aqueous solutions (Garcia Manrique *et al.*, 1973). The merocyanine dyes exhibit sizeable bathochromic red shifts in their electronic spectra in less polar solvents (Brooker *et al.*, 1965) and in a limited number of hydrogen bonding systems (Figueras, 1971; Kolling & Goodnight, 1973). Furan derivatives have been measured in water, ethanol, dioxane and *n*-heptane solutions. Except for the acids, a bathochromic shift of λ_{max} with increased dielectronic constant of the solvents has been observed (Borisova & Kul'nevich, 1973). Kamlet & Taft (1976) have identified a relationship between solvatochromic

shifts of 4-nitroanilines and 4-nitrophenols interacting with a wide range of hydrogen bond donor–acceptor pairs. They have found that their β-scale for solvent basicities is linearly related to pK_f values of the same equilibrium. Kolling (1976), using three typical solvatochromic indicators, which share a common $\pi \rightarrow \pi^*$ mechanism for photon absorption, has compared them to determine thermodynamic data for hydrogen bond donor–acceptor interactions involving *N*- and *O*-atom acceptor sites. Simple parameter correlations for the blue-shift indicators are poorest in non-polar and polar aprotic solvents, indicating little sensitivity toward changes in Lewis basicity. By contrast, the red shift solvatochromic indicators are highly sensitive to change in Lewis basicity for polar and non-polar aprotic media and for the lower alcohols.

From the data presented above it is clear that many substances affect the red coloration of carthamin. For carthamin colour modification, the following effects might be considered to operate. In esters and fatty acids, except formic and acetic acids, carthamin will partially lose its biphenyl-oxostyryl ketone character, and the resulting suppression of the electron acceptor ability should cause a sizeable hypsochromic shift. On the other hand, batho-shift promoters (alcohols, amino acids etc.) might cause bathochromic displacement through a $\pi \rightarrow \pi^*$ mechanism for photon absorption, and thus a positive charge-transfer transition to longer wavelengths.

Recently it has been reported that, in visible spectra of anthocyanins, hypsochromism or bathochromism occurs during molecular association of the pigments in aprotic solvents (Hoshino *et al.*, 1981). A wide variety of factors must be involved in the colour modification process, by which a characteristic tinctorial expression is induced in carthamin solutions and/or in cellular media containing carthamin.

The data from the present studies will surely contribute to the successful commercial application of carthamin in food products or soft drinks.

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