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COMPARATIVE ANALYSIS OF PIGMENTS IN PETALS AND STIGMATA OF *CROCUS ALMEHENSIS* C. BRICKELL AND B. MATHEW AND *CROCUS SATIVUS* L.

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Abstract

Crocus almehensis is one of the most beautiful and unique yellow flower varieties of the *Crocus* species in Iran. Spectrophotometry, thin-layer chromatography and densitometry methods were applied to analyze pigments in the petals and stigmata of this species. Absorption spectra of alcoholic and aqueous extracts of its petals and stigmata showed the existence of two groups of carotenoids (in particular water soluble carotenoids) and flavonoids, and other phenolic compounds that have high absorbance in UV. The absorption spectra of carotenoid pigments in petals and stigmata of *Crocus almehensis* were very similar to those of carotenoid pigments in *Crocus sativus*. Thin-layer chromatography of alcoholic extracts of petals and stigmata of *Crocus almehensis* showed seven distinguishable bands on silica gel plates. The absorption spectra of five bands were similar to the absorption spectrum of carotenoids. Chromatographic and spectrophotometric analysis determined that, like saffron stigmata, the main carotenoid pigments to be found in the petals and stigmata of *Crocus almehensis* is crocetin, an aglycon that exists as different glycosidic esters. Thin-layer chromatography of alcoholic extracts of petals and stigmata of *Crocus almehensis* also showed the existence of another glycosidic ester. This glycosidic ester either does not exist in saffron stigmata or its amount is too little to detect on TLC plates. It is the most frequently occurring glycosidic ester of crocetin in the stigmata and petals of *Crocus almehensis*.

Introduction

Crocus species are perennial, herbaceous and ornamental plants belonging to the Iridaceae family that are able to spend a dry, dormant period in the form of an underground corm. They are small plants with reduced inflorescences. Often they have a single flower and a very short stem which is subterranean.

Crocus species, in the botanical description, are very complex plants, with their taxonomy depending upon certain features such as cataphylls, prophylls, bracts and bracteoles. In fact they are plants of relatively simple

Keywords: *Crocus*; Phenolic compounds; Picrocrocin; Pigment

Figure 1. Flowers of *C. almehensis* (yellow-flowered *Crocus*) that often appear in the snow in late winter and early spring

make-up, as are many of the petaloid monocotyledones, and the apparent complexity of the *Crocus* arises mainly from much reduction of plants [5].

In Iran, eight wild and one cultivated species of *Crocus* have been determined. *Crocus almehensis* is one of the most beautiful *Crocus* species in Iran. It takes its name from Almeh which is situated in N.E. Iran and is the only yellow-flowered *Crocus* in this country. It was first discovered by Mrs. Ann Ala in 1970.

The flowers of *C. almehensis* usually appear in early spring, when snow starts to melt (Fig. 1). Perianth segments are orange-yellow in color and three exterior segments have bronze-purple stripes on their outside surface. The style is also yellow and is divided into three orange-red stigmata that each expand at the apex and become rugged. Upland areas with steppe vegetation and at a height of 1700-2000 meters above sea level form a suitable habitat for the germination of this plant, which grows only in Iran (2n = 20) [5].

Several studies have been carried out to analyze the carotenoidic composition of pigments in the stigmata of saffron and some wild species of *Crocus*. Consequently, relatively detailed and complete information is available about their structural and chemical characteristics, especially the water soluble type. Results show that besides crocin - the main yellow constituent of *Crocus* and digentiobioside ester of polyene dicarboxylic acid of crocetin - other compounds exist that could be mono - and di-glycosyl esters of crocetin (Fig. 2). Compared with saffron, new, more polar compounds have been found which seem to be tri-glycosyl esters of crocetin. Moreover, in addition to these compounds, saffron stigmata also contain very small amounts of crocetin aglycon in free form [1-4, 6-13, 15, 16].

Because *Crocus almehensis* is an endemic plant of

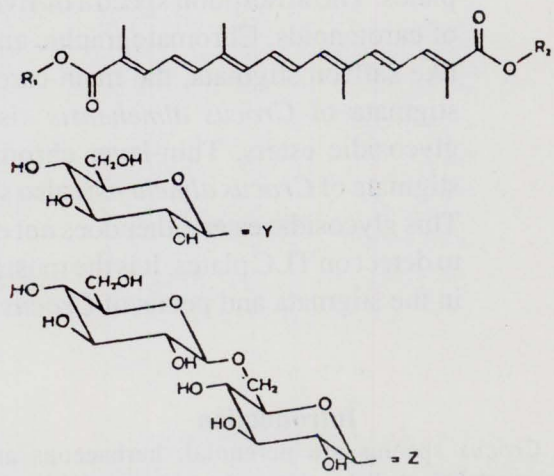

Figure 2. Structures of crocetin glycosyl esters from saffron (*Crocus sativus* L.). 1) R_1=R_2=z(crocin); 2) R_1=z, R_2=y; 3) R_1=z, R_2=H, 4) R_1=R_2=y; 5) R_1=y, R_2=H; 6) R_1=R_2=H (crocetin) (11)

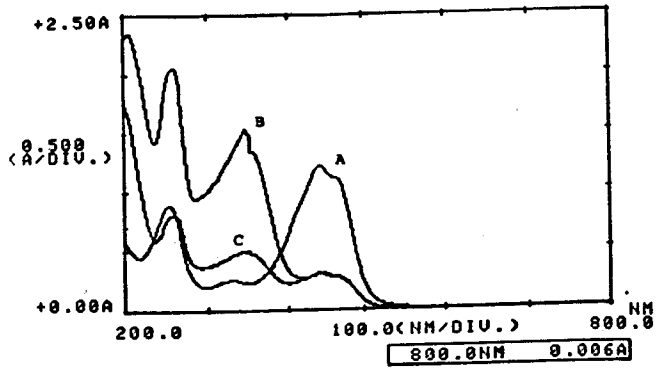

Figure 3. Comparison of absorption spectra of aqueous extracts of *Crocus sativus* and *Crocus almehensis*
A) Absorption spectrum of aqueous extract of *Crocus sativus* stigma; absorbance maxima are at 440, 325 and 255 nm (5 mg/5 ml W×15)
B) Absorption spectrum of aqueous extract of *Crocus almehensis* stigma; absorbance maxima are at 442 and 370 nm (5 mg/5 ml W×2)
C) Absorption spectrum of aqueous extract of *Crocus almehensis* petal; absorbance maxima are at 442, 346 and 258 nm (5 mg/5ml W×2)

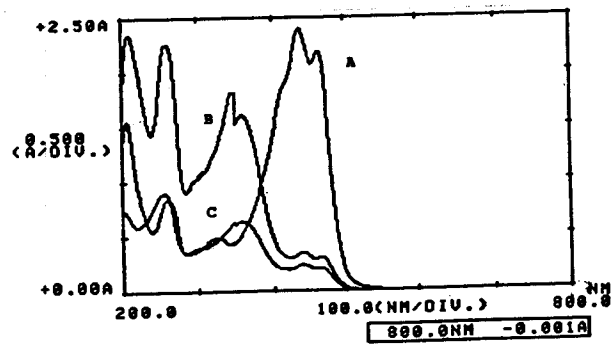

Figure 4. Comparison of absorption spectra of alcoholic extracts of *Crocus sativus* and *Crocus almehensis*
A) Absorption spectrum of alcoholic extract of *Crocus sativus* stigma; absorbance maxima are at 462, 436 and 252 nm (5 mg/5 ml 80% EtOH×15)
B) Absorption spectrum of alcoholic extract of *Crocus almehensis* petal; absorbance maxima are at 462 and 437, 251 and 217 nm (10 mg/2 ml 80% EtOH×15)
C) Absorption spectrum of alcoholic extract of *Crocus almehensis* stigma; absorbance maxima are at 464, 437, 361 and 259 nm (10 mg/10 ml 80% EtOH×15)

Iran, and so far similar analyses have not been done on the pigments of its flowers, we have focused our studies on the analysis of pigments in the petals and stigmata of this *Crocus* and on comparing those with the pigments found in compounds in saffron stigmata.

Materials and Methods

Stigmata and petals of *Crocus sativus* L. brought from a field in Gonabad, and also stigmata and petals of *Crocus almehensis* C. Brickell and B. Mathew supplied from the Almeh region were used in the analysis of carotenoid pigments. Stigmata and petal samples were dried on air and then stored at 4°C.

Two alcoholic and aqueous extraction procedures were used for the spectrophotometric study of the pigments. For obtaining alcoholic extracts, certain amounts of plant powder were first well ground in a certain volume of cold 80% ethanol (V/V) and stored in the dark for 24 h. Then the mixtures were homogenized and finally the homogenates were centrifuged at 600 xg for 15 minutes using an IEC-Centra-8R instrument. The supernatants were retained at 4°C [1-3, 15-17].

Using the same method, water extraction was obtained with cold distilled water. Spectra of diluted alcoholic and aqueous extracts were obtained by a Shimadzu UV 160 spectrophotometer and were compared with each other.

Suitable amounts of alcoholic extracts were chromatographed on TLC plates. The stationary phase was an activated silica gel G60 and the mobile phase a solvent system of *n*-butanol, acetic acid and water (4:1:1 by vol.). The thickness of the silica gel on 20×20 cm standard-sized plates was 0.3 mm [13, 15, 17]. Finally, after the developed plates were dried, the separation mode, number, R_f and color of the bands formed were

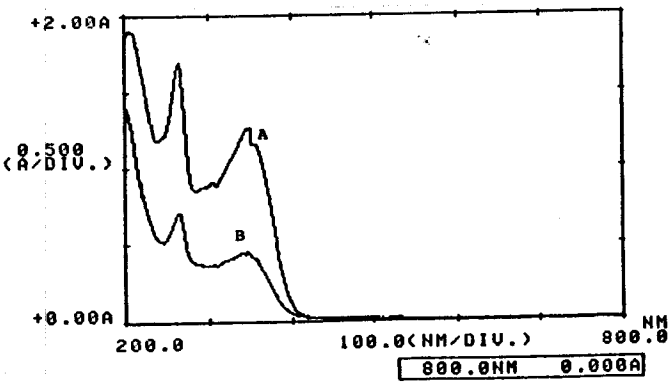

Figure 5. Comparison of absorption spectra of alcoholic and aqueous extracts of *Crocus sativus* petal
A) Absorption spectrum of aqueous extract of *Crocus sativus*. petal; absorbance maxima are at 533, 345, 263 and 208 nm (1 g/5 ml W×60)
B) Absorption spectrum of alcoholic extract of *Crocus sativus* petal; absorbance maxima are at 564, 349, 265 and 211 nm (1 g/5 ml 80% EtOH×10)

studied under visible and UV light. The densitometric spectra of separated bands on TLC plates for each sample were obtained at 440 nm by Shimadzu CS-9000 densitometer and then the spectra of different samples were compared with each other. The percentage of constituent substances of each band in the ratio of total were also determined based on the area of each peak by densitometer.

Results

Spectrophotometry

Spectra of alcoholic and aqueous extracts of petals and stigmata of *Crocus sativus* L. and *Crocus almehensis* and also their absorbance maxima in the range of 200-800 nm are shown in Figures 3,4 and 5. Absorption maxima in the range of 400-500 nm along with the shape of spectra suggest the existence of carotenoid pigments in the stigmata and petals of *Crocus almehensis*. Absorbance in the UV region (below 400 nm) is due to the existence of non-carotenoid compounds such as bitter glycoside, picrocrocin (absorbance at 250-260 nm) and colorless flavonoids (flavonols, flavones, etc.). The shape of absorption spectra of alcoholic and aqueous extracts of petals in *Crocus sativus* L. shows an absence of carotenoid pigments and absorbance at wavelengths higher than 500 nm which is due to the existence of anthocyanin violet pigments (Fig. 5).

When extraction is done with water (Fig. 3), only water soluble carotenoids are solubilized and liposoluble carotenoids are not extracted. Therefore, the shift that is observed in the absorbance maxima in the visible region and also the shape of spectra of aqueous extracts as compared with the spectra of alcoholic extracts (Fig. 4) could be due to this. An interesting aspect of the above results is the possibility of the aqueous extraction of the sample suggesting the water solubility of carotenoid pigments in these samples.

Thin Layer Chromatography

Figures 6 and 7 show the results of thin-layer chromatography of alcoholic extracts of *Crocus sativus* and *Crocus almehensis* stigmata and petals, and the separation mode, number and color (yellow or violet) in visible light in TLC chromatograms of formed bands. Figure 7 shows a schematic illustration of thin-layer chromatographic separation of samples. Features of separated bands such as R_f, color intensity, color in UV (366 nm) and visible light, as well as the type of pigment in *Crocus sativus* stigmata and the stigmata and petals of *C. almehensis* are shown in Table 1. Figures 8-12 also show the absorption spectra of pigments that exist in separated bands on TLC plates obtained from extracts of *Crocus sativus* and *Crocus almehensis* stigmata and petals. Six distinguishable bands appear on the plates when alcoholic extracts of *Crocus sativus* stigmata are chromatographed so that their R_f values are 0.29, 0.43, 0.56, 0.63, 0.80 and 0.96 respectively. All of the bands are yellow-orange in visible light and dark brown in UV (366

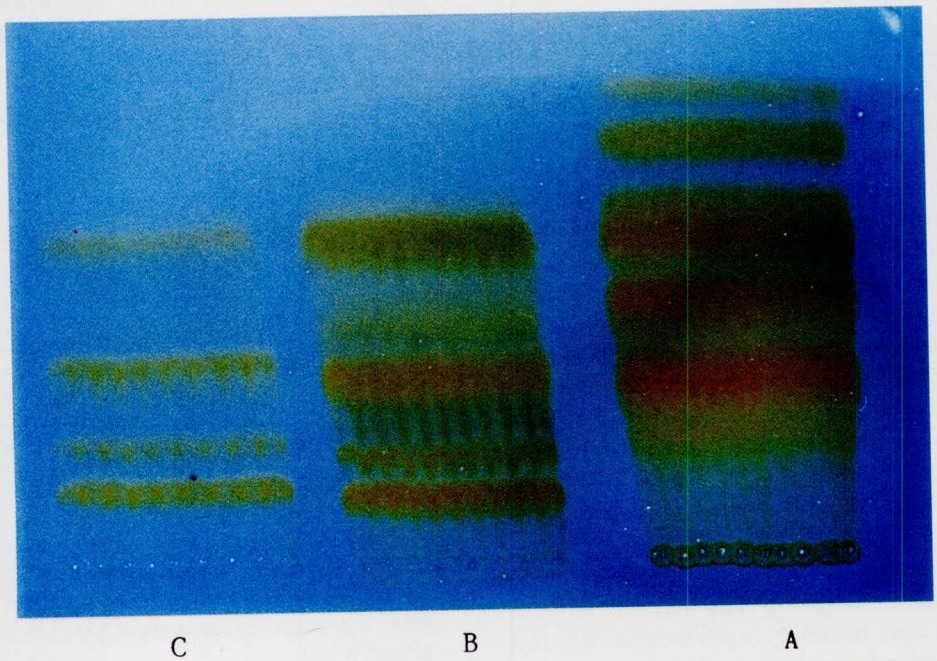

Figure 6. Comparison of separated bands on TLC plates of alcoholic extracts of *Crocus* stigma (A), *Crocus almehensis* petal (B) and *Crocus almehensis* stigma (C)

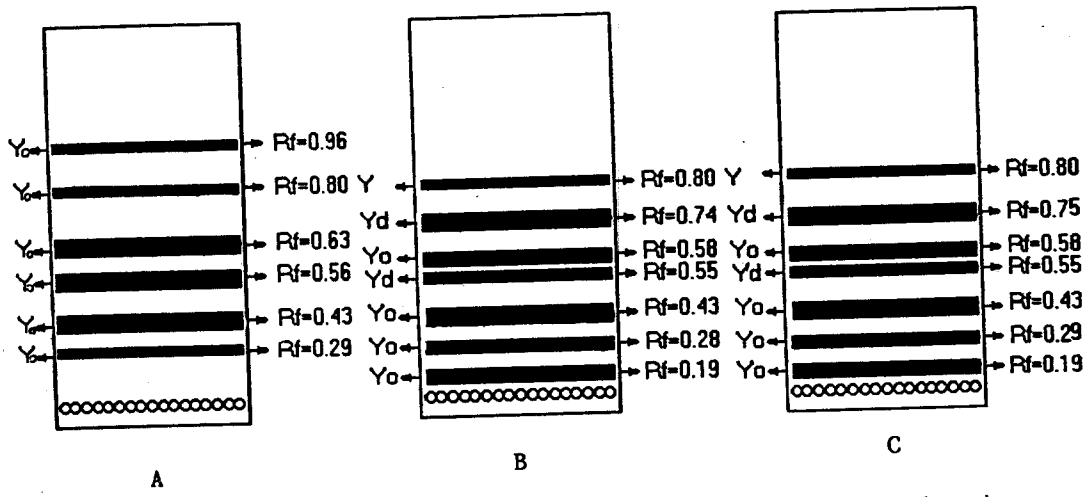

Figure 7. Schemata of separated bands on TLC plates of alcoholic extracts of *Crocus sativus* stigma A) *Crocus almehensis* stigma (B) and *Crocus almehensis* petal (C); bands with 0.19 and 0.74 R_f values are absent in *Crocus sativus* stigmata and *Crocus almehensis* stigmata and petals do not have a band with 0.96 R_f value

nm). Each of these bands shows the existence of one of the glycosidic esters of crocetin.

Seven distinguishable bands are observed on TLC plates of *Crocus almehensis* stigmata, whose R_f values are 0.19, 0.28, 0.43, 0.55, 0.58, 0.74 and 0.80 respectively. Two of these bands, with R_f values of 0.55 and 0.74, contain flavonoid pigments and are a dirty yellow color in visible light and dark brown in UV light. Considering their absorption spectrum, the other bands must contain glycoside esters of crocetin. Eight distinguishable bands are also separated on TLC plates of *Crocus almehensis* petals, whose R_f values are completely similar to those of separated bands on TLC plates of *Crocus almehensis* stigmata (except one band with R_f= 0.34). Two bands of those seen to be dirty yellow in visible light show the presence of flavonoid pigments. The alcoholic spectrum of one of these bands shows the existence of both flavonoid and carotenoid pigments which, because of the similarity in their R_f value, accumulate in the same region on TLC plates.

As a result of TLC analysis of petal alcoholic extracts of *Crocus sativus* L., several bands were separated most of which were violet in color. These bands probably contain glycosidic esters of anthocyanin pigments. One dirty yellow band is also placed near the solvent line and because its spectrum has no similarity with that of

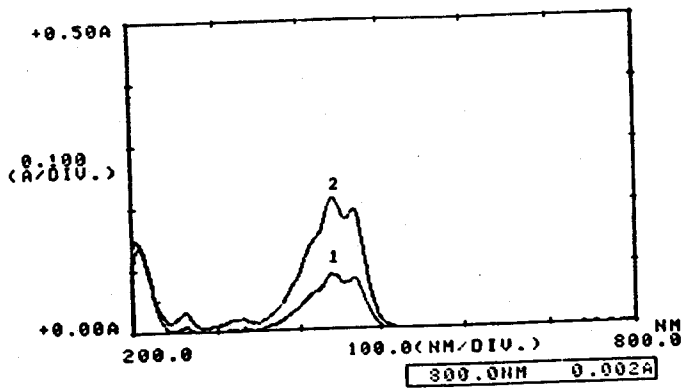

Figure 8. Absorption spectra of pigments in first separated bands on TLC plates of *Crocus almehensis* stigma (1) and petal (2); absorbance maxima are at 465, 438, 263 and 207 nm. Spectra show the existence of carotenoid pigments in these bands.

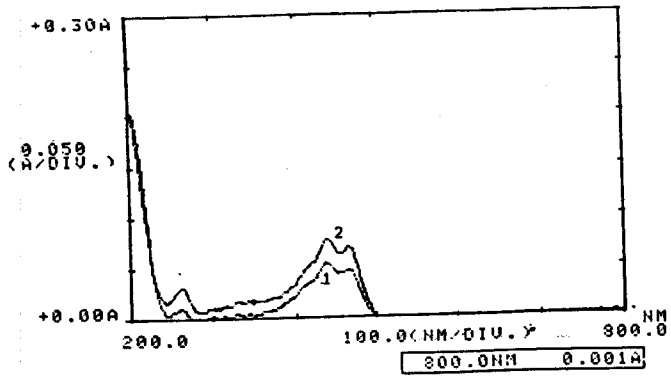

Figure 9. Absorption spectra of pigments in second separated bands on TLC plates of *Crocus almehensis* stigma (1) and petal (2); absorbance maxima are at 465, 437, 262 and 207 nm. Spectra show the existence of carotenoid pigments in these bands.

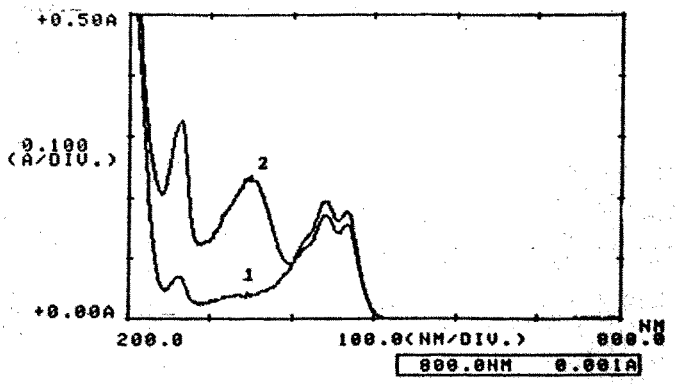

Figure 10. Absorption spectra of pigments in fourth separated bands on TLC plates of *Crocus almehensis* stigma (1) and petal (2); absorbance maxima in spectrum of No. 1 are at 466, 438 and 261 nm and in spectrum of No. 2 at 464, 438, 366 and 261 nm. Absorption spectra of bands in petals show the existence of both carotenoid and flavonoid pigments.

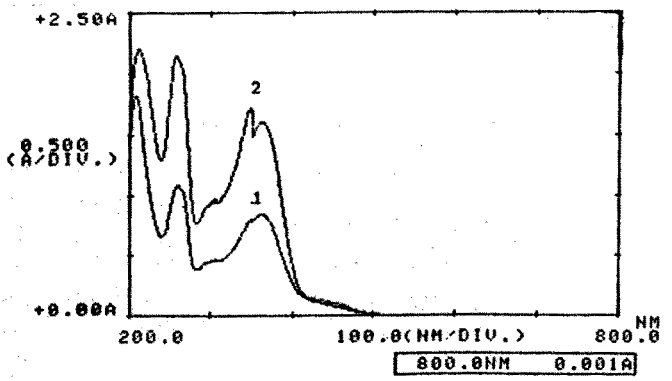

Figure 12. Absorption spectra of pigments in seventh separated bands on TLC plates of *Crocus almehensis* stigma (1) and petal (2); absorbance maxima are at 364, 258.5 and 217.5 nm. Spectra show the existence of flavonoid pigments in both samples.

carotenoid, it may be related to a yellow flavonoid pigment.

Densitometry

Results of densitometric analysis are complete and support the chromatographic and spectrophotometric analysis. The density of separated bands on TLC plates which correspond to pigments of *Crocus sativus* and *Crocus almehensis* stigmata and petals has been obtained at 440 nm by densitometer. Densitograms are shown in Figure 13. Densitometric spectra show six, seven and eight distinguishable peaks in *Crocus sativus* stigmata and *Crocus almehensis* stigma and petal extracts respectively. R_f values that were obtained by densitometry correspond to those obtained by chromatography. The amount of pigment in each band is represented as a percentage and is presented in Table 1.

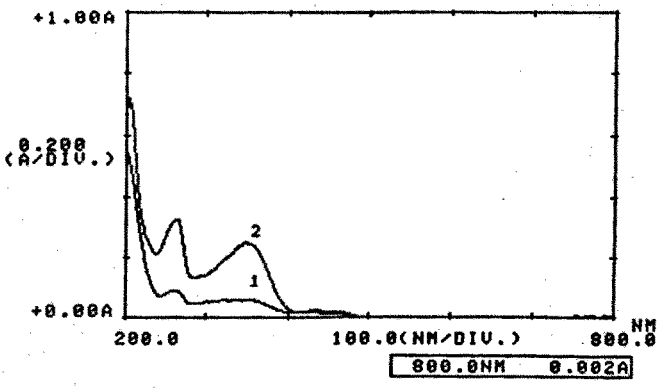

Figure 11. Absorption spectra of pigments in fifth and sixth separated bands on TLC plates of *Crocus almehensis* stigma (1) and petal (2); absorbance at 200-400 nm is due to the existence of flavonoid pigments in the fifth band of both samples and absorbance at 400-500 nm is due to the existence of carotenoid pigments in the sixth bands of both samples.

Discussion

The main pigment of *Crocus sativus* stigma is a yellow coloring pigment and C20 carotenoid of crocetin that exist as different glycosidic esters. α-Crocin is the most important of those and is a digentiobioside ester of crocetin. Glycosidic esters of crocetin are water soluble.

Absorption spectra of alcoholic and aqueous extracts of *Crocus sativus* stigmata are very similar to the absorption spectrum of β-Carotenoid and are different only at absorbance maxima.

Absorption spectra of alcoholic and aqueous extracts of stigmata and petals of the yellow flower *Crocus almehensis* show the presence of two groups of carotenoid and flavonoid pigments. The main carotenoid pigment in the stigmata and petals of *Crocus almehensis* is also crocetin which exists as different glycoside esters.

Thin-layer chromatography of alcoholic extracts of *Crocus sativus* stigma shows the existence of mono - and di-glycosidic esters of crocetin which, based on their polarities, are placed on TLC plates. Digentiobioside ester of crocetin, which is the most polar of them all, is also the most frequent pigment found in saffron stigma C [4, 5, 7-10, 12, 13, 15-17].

When alcoholic extracts of *Crocus almehensis* stigmata and petals are chromatographed, seven and eight bands are distinguished on TLC plates respectively. With the exception of one or two bands, the others all contain carotenoid pigments. The spectral similarity of many of

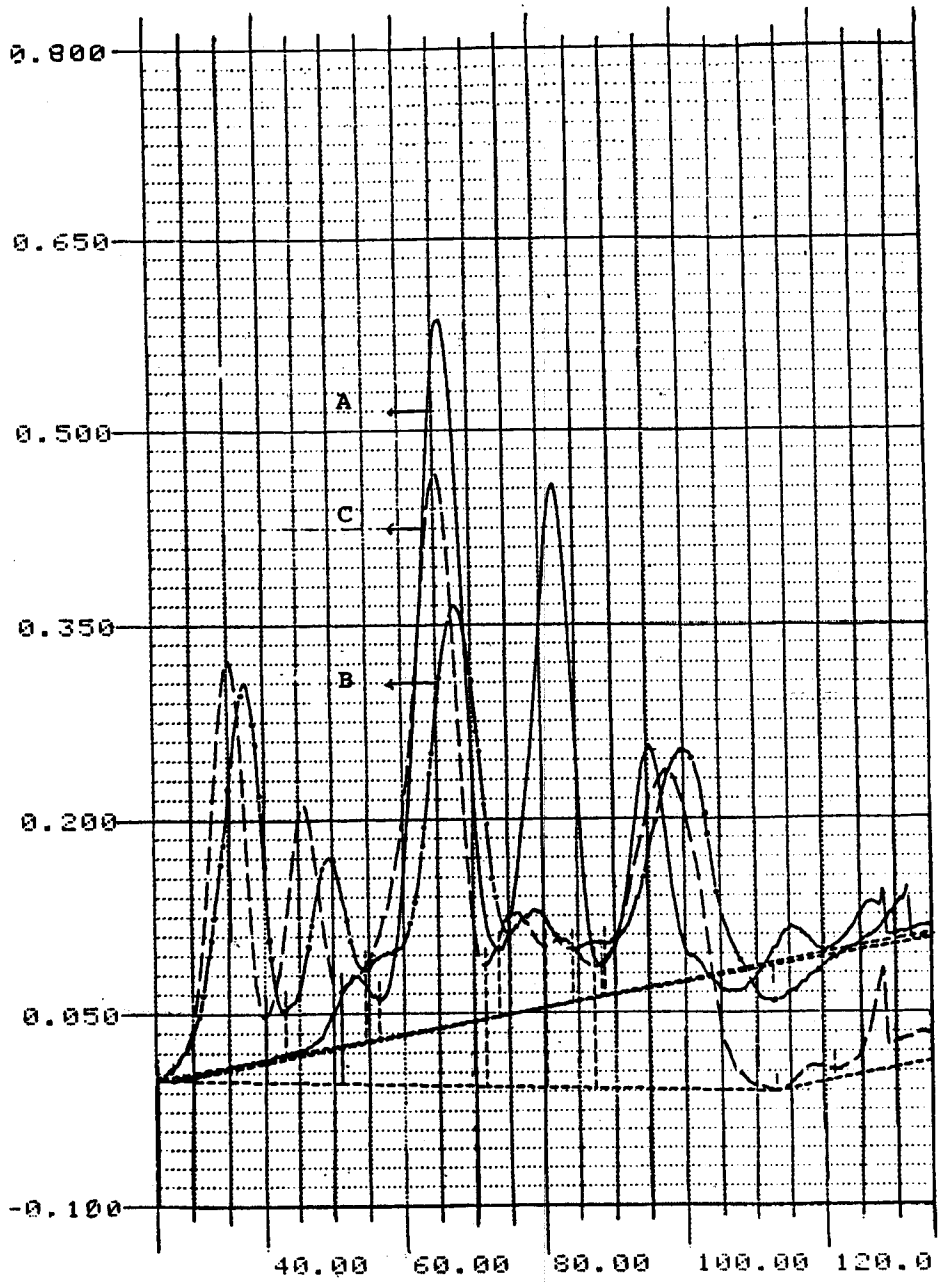

Figure 13. Comparison of densitograms; A, *Crocus sativus* stigma; B, *Crocus almehensis* stigma and C, *Crocus almehensis* petal.

the yellow bands (Figs. 8-12) is due to the type of their pigments which are different glycosyl esters of crocetin. The most prominent glycosidic ester of crocetin in these samples is probably its tri-glycosyl ester (R_f= 0.19). The existence of this pigment has been reported in some wild species of *Crocus* [11]. Because of its high polarity, this pigment is placed in the lowest region of the plate. The amount of this pigment in *Crocus sativus* stigma is too little to detect on the plate.

The amounts of carotenoid and flavonoid pigments and other colorless phenolic compounds in the petals of *Crocus almehensis* are more than in its stigma and the amounts of carotenoid pigments in those are, of course, less than these pigments in saffron stigmata. It must be noted that to verify the accuracy of proposed views, complementary studies must be done in this area using the most precise and progressive procedures.

In view of the results that were obtained from the

Table 1. Features of separated bands on TLC plates and amounts of pigments in each band obtained by densitometry

Band No.	R_f	Band intensity	Color in visible light (366 nm)	Color in UV	Type of pigment	Amount as compared with total (%)
1) *Crocus sativus* stigma	0.19	--	--	--	--	--
2) *Crocus sativus* stigma	0.29	distinguishable	yellow-orange	brown	carotenoid	5%
3) *Crocus sativus* stigma	0.34	--	--	--	--	--
4) *Crocus sativus* stigma	0.43	high	yellow-orange	brown	carotenoid	45%
5) *Crocus sativus* stigma	0.56	high	yellow-orange	brown	carotenoid	32%
6) *Crocus sativus* stigma	0.63	high	yellow-orange	brown	carotenoid	14%
7) *Crocus sativus* stigma	0.74	--	--	--	--	--
8) *Crocus sativus* stigma	0.80	pale	yellow-orange	brown	carotenoid	2%
9) *Crocus sativus* stigma	0.96	very pale	yellow-orange	brown	carotenoid	0.5%
1) *Crocus almehensis* stigma	0.19	high	yellow-orange	brown	carotenoid	21%
2) *Crocus almehensis* stigma	0.28	pale	yellow-orange	brown	carotenoid	21%
3) *Crocus almehensis* stigma	0.34	--	--	--	--	--
4) *Crocus almehensis* stigma	0.43	high	yellow-orange	brown	carotenoid	38%
5) *Crocus almehensis* stigma	0.55	pale	dirty yellow	opaque brown	flavonoid	45%
6) *Crocus almehensis* stigma	0.58	very pale	yellow-orange	pale brown	carotenoid	3%
7) *Crocus almehensis* stigma	0.74	very high	dirty yellow	opaque brown	flavonoid	17%
8) *Crocus almehensis* stigma	0.80	very pale	yellow	very pale brown	carotenoid	≅
9) *Crocus almehensis* stigma	0.96	--	--	--	--	--
1) *Crocus almehensis* petal	0.19	high	yellow-orange	brown	carotenoid	30%
2) *Crocus almehensis* petal	0.29	pale	yellow-orange	brown	carotenoid	11%
3) *Crocus almehensis* petal	0.34	pale	purple	blue-purple	anthocyanin	4%
4) *Crocus almehensis* petal	0.43	high	yellow-orange	brown	carotenoid-flavonoid	32%
5) *Crocus almehensis* petal	0.55	pale	dirty yellow	opaque brown	flavonoid	3%
6) *Crocus almehensis* petal	0.58	very pale	yellow	pale brown	carotenoid	5%
7) *Crocus almehensis* petal	0.75	high	dirty yellow	opaque brown	flavonoid	12.5%
8) *Crocus almehensis* petal	0.80	very pale	yellow	pale brown	carotenoid	≅
9) *Crocus almehensis* petal	0.96	--	--	--	--	-

pigment analysis of *Crocus almehensis*, and considering that the petals of this species like its stigmata, indeed, even more so, have water soluble carotenoid pigments, it is deemed possible to use this *Crocus* as a saffron source once more accurate and complete analyses of the quality and quantity of these pigments have been carried out and once their economic importance has been evaluated. Tests for the extensive plantation of this *Crocus* and other similar species, as well as efforts towards the domestication and cultivation of these plants, will be a positive step in this field.

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