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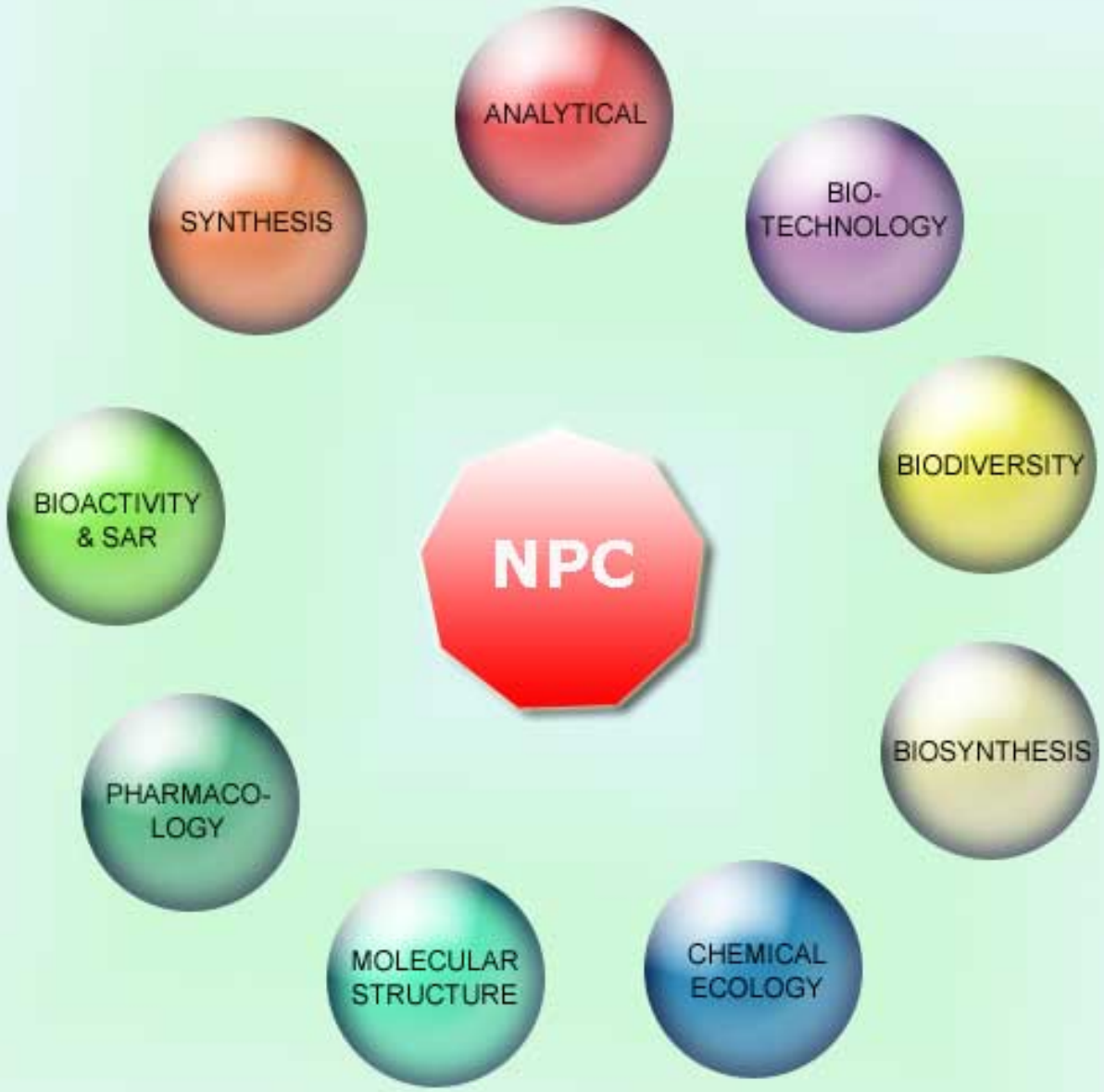

This Issue is Dedicated to
Professor Franco F. Vincieri
on the Occasion of his 70th Birthday

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Characterization of By-products of Saffron (*Crocus sativus* L.) Production

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The stigma, stamens and sepals of *Crocus sativus* L,. from two different geographical origins, were analyzed for their crocin and flavonol contents. Identification of crocins, safranal, picrocrocin, and flavonols was carried out by HPLC/DAD and HPLC/MS analysis. Both stigma samples, grown under natural conditions, exhibited high crocin contents (between 342 and 231 mg/g), while the stamens and sepals were rich in flavonols (between 6 and 10 mg/g). The stamens contain mainly kaempferol- 3-*O*-sophoroside, whereas the sepals contain mainly quercetin and methyl-quercetin glycosides. These data may be useful in order to find a possible exploitation of the by-products of saffron production, in which large quantities of *C. sativus* flowers are available.

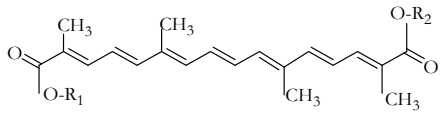
Keywords: Crocins, flavonols, HPLC/DAD/MS, sepals, stamens, stigma.

The dried, red stigmas of *Crocus sativus* L. are a very expensive spice known as saffron, which is used as a food flavoring and coloring agent and as a traditional herbal medicine [1a]. Crocus is cultivated in India, Iran, Spain, Greece and Italy. The production process involves a large amount of manual work and cannot be completely mechanized. In Italy, from a 1000 m^2 area, about 120,000-150,000 flowers can be obtained (4000-5000 kg), which give rise to 5-7 kg of fresh stigma, i.e. 1.0-1.3 kg of dried product.

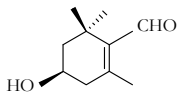
Many papers deal with methods for the separation and determination of the biologically active [1b-1f] and aroma components [2a-2c]. The quality control of commercial saffron is checked using spectrophotometric [3a,3b], TLC [3c], GC [3d], HPLC [3e], and CE [3f] methods.

The purpose of this paper is the analysis of stigmas from *C. sativus* cultivated in Italy (Perugia and Fiesole) in order to characterize this commercial saffron from a quality point of view. In these areas, cultivation is effected under natural conditions and without the use of any chemical product in the drying and conservation phases.

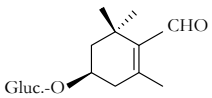
However, the most important part deals with the characterization of the biologically active components of the stamens and sepals in order to find a possible use for this material, which forms the major part of *C. sativus* flowers. The exploitation of stamens and sepals, notwithstanding their availability as by-products in the production of saffron, has not been taken into account, with the exception of one paper dealing with the isolation of flavonoids from crocus petals to study their tyrosinase inhibition action [4a]. Notwithstanding the lack of information on the polyphenol content of these tissues, petal extracts were used to control rat blood pressure [4b] and to test their antitussive effect in guinea pigs [1b]. The major biologically active components of saffron are crocin analogues, which are all glycosides of



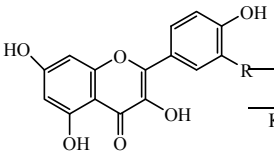
COMPOUND	R1	R2	MW
Crocin-5 ; $C_{50}H_{74}O_{29}$	Three β-glucosyl	β-D-gentiobiosyl	1138
Crocin-4 ; $C_{44}H_{64}O_{24}$	β-D-gentiobiosyl	β-D-gentiobiosyl	976
Crocin-3 ; $C_{38}H_{54}O_{19}$	β-D-gentiobiosyl	β-D-glucosyl	814
Crocin-2 ; $C_{33}H_{44}O_{14}$	β-D-gentiobiosyl	H	652
Crocin-2' ; $C_{33}H_{44}O_{14}$	β-D-glucosyl	β-D-glucosyl	652
Crocin-1 ; $C_{26}H_{34}O_9$	β-D-glucosyl	β-D-glucosyl	490
Crocetin ; $C_{20}H_{24}O_4$	H	H	328



Safranal $C_{10}H_{14}O$
MW= 150



Picrocrocin $C_{16}H_{26}O_7$
MW= 330



COMPOUND	R	MW
Quercetin ; $C_{15}H_{10}O_7$	OH	302
Kaempferol ; $C_{15}H_{10}O_6$	H	286

Figure 1: Chemical structures of saffron components

trans-crocetin, a carotenoid derivative, and which are responsible for the color. Safranal (2,6,6-trimethyl-1,3-cyclohexadien-1-carboxaldehyde), which is responsible for the characteristic aroma of saffron, is formed during storage by dehydration of picocrocin, which is responsible for its bitter taste. Flavonoids are found in stigma, sepals, and stamens (Figure 1).

As regards stigma, the composition of the extract was similar to that found by other authors regarding crocins, picocrocins, and safranal. Three kaempferol derivatives (two triglycosides and one diglycoside) were identified, according to previous findings [1f,5a]. In the case of stamens, a lesser number of crocins was found and quercetin, as well as kaempferol derivatives were detected. Also, methyl-quercetin derivatives in quite large amounts were recorded. There were no differences, from a qualitative point of view, between the two sampling zones; in fact only a quantitative variation was found in the samples from the different geographic regions [5a].

Table 1 reports the quantitative data for the dried stigma. It should be noted that the two samples differ are present in largest amount in the two samples. These compounds, together with *cis*-crocin 4, were

Table 1: Quantitative data for dried stigma. Average value ± SD of three samples. Data are expressed as mg/g fresh sample.

COMPOUNDS (Rt)	Stigma (FI)	Stigma (PG)
trans crocin-5 (10.30)	2.4±0.09	2.0±0.07
crocin derivative (11.14)	2.1±0.08	0.8±0.04
crocin derivative (11.46)	0.3±0.01	0.3±0.01
crocin derivative (11.87)	0.3±0.009	0.1±0.007
trans crocin-4 (12.84)	238.9±2.86	148.5±2.66
crocin derivative (13.88)	1.3±0.06	0.5±0.02
trans crocin-3 (14.39)	65.6±1.84	46.2±1.38
crocin derivative (14.99)	0.2±0.01	0.2±0.009
crocin derivative (15.90)	0.6±0.03	0.5±0.02
t*rans* crocin-2' (16.17)	2.1±0.07	1.5±0.06
crocin derivative (17.37)	0.3±0.01	0.3±0.009
cis crocin-4 (17.79)	9.5±0.33	14.1±0.49
trans crocin-2 (19.33)	16.9±0.51	14.8±0.50
crocin derivative (20.40)	0.2±0.009	traces
crocin derivative (21.11)	0.3±0.01	traces
cis crocin-1 (22.02)	1.0±0.05	0.8±0.04
crocin derivative (22.81)	0.2±0.01	traces
crocin derivative (23.17)	traces	0.5±0.02
TOTAL	**342.02**	**231.1**
Picrocrocin (6.34)	111.1±2.33	68.9±1.79
Safranal (24.87)	2.2±0.09	2.6±0.09
K-3-sophoriside -7- glucoside (3.78)	4.7±0.2	3.3±0.14
K -3,7,4'-triglucoside (5.90)	1.2±0.05	0.9±0.04
K-3-sophoroside (8.49)	6.2±0.22	5.4±0.17
TOTAL	**12.1**	**9.64**

mainly in *trans*-crocin 4, *trans*-crocin 3 and picrocrocin contents, i.e. the three compounds which also the main compounds found by Caballero-Ortega et al. [5b] in a study of 11 saffron samples from different origins. The crocins content of the two samples is quite high giving evidence for the very good quality of the two samples. Among flavonols, kaempferol-3-*O*-sophoroside was the main compound reported for a Spanish sample analyzed by Carmona *et al*. [5a].

Table 2 reports the crocin contents of sepals and stamens. The amount of crocins is low, while that of flavonols (Table 3) ranged from 10.1 to 6.1 mg/g. Stamens and sepals differ mainly in their kaempferol-3-*O*-sophoroside content, which is the most abundant flavonol in the sepals.

The flavonols composition of the two tissues is different: in sepals, kaempferol derivatives ranged between 91 -93 %, whereas in stamens, quercetin and methyl-quercetin derivatives ranged between 52-71%. From all these data the possible exploitation of alternative tissues like stamens and sepals as phytochemical resources can be pointed out. For each kg of stigma, about 1000 kg of flowers are processed; therefore, sepals and stamens are important by-products of saffron production and their use could increase the economic value of *C. sativus* flowers.

Table 2: Crocins content of sepals and stamens. Average value ± SD of three samples.

COMPOUNDS (Rt)	Sepals (FI)	Sepals (PG)	Stamens (FI)	Stamens (PG)
trans crocin-4 (12.84)	3.1±0.17	traces	112.2±5.65	4.0±0.19
crocin der. (13.88)			1.7±0.09	traces
trans crocin-3 (14.39)	0.8±0.04	traces	33.4±1.74	traces
crocin der. (14.99)			traces	traces
crocin der. (15.99)			1.3±0.07	
trans crocin-2' (16.17)			3.3±0.18	traces
cis crocin-4 (17.79)	traces		22.0±1.14	0.1±0.006
trans crocin-2 (19.33)	traces	traces	20.7±1.07	1.3±0.08
cis crocin-1 (22.02)			7.0±0.38	traces
crocin der. (22.81)			0.3±0.02	traces
crocin der. (23.17)			0.1±0.008	traces
cis crocin-2 (24.82)	0.3±0.02		0.6±0.03	
TOTAL	**4.2**	**traces**	**196.3**	**5.4**

Experimental

Sample preparation: Sepals, stamens and dried stigma samples were obtained from plants harvested in 2005 from Fiesole (FI, Italy) and Perugia (PG, Italy). Sepals and stamens (500 mg) were suspended in 50 mL of 70% ethanol, adjusted to pH 2.0 with formic acid, and left overnight. After extraction, the samples were filtered to eliminate plant residues, and the filtrate evaporated to dryness under vacuum at room temperature. The residue was redissolved in EtOH/H_2O (70:30) and adjusted to pH 2.0 with formic acid to a final volume of 3 mL.

Saffron stigmas (50 mg) were extracted with 10 mL of 70% ethanol, adjusted to pH 2.0 with formic acid, left overnight and then filtered to eliminate plant residues. The extracts were analysed by HPLC/DAD/MS for the determination of saffron components.

Authentic standards of crocin were purchased from Fluka (St. Louis, USA), safranal from Sigma-Aldrich (St. Louis, USA), and *p*-hydroxybenzoic acid, kaempferol 3-*O*-glucoside, rutin and curcumin from Extrasynthèse S.A. (Lyon, France). All solvents were of HPLC grade purity (BDH Laboratory Supplies, United Kingdom).

HPLC/DAD analysis:. Analysis for flavonols and crocins was carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). Flavonols and crocins were separated by using a 150 × 3.9 mm i.d. 4 μm Nova-Pak C18 column (Waters) operating at 27°C. UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 250, 308, 350 and 440 nm. The mobile phase was a

Table 3: Flavonols content of sepals and stamens. Average value ± SD of three samples. Data are expressed as μg/g fresh sample.

COMPOUNDS (Rt)	Sepals (FI)	Sepals (PG)	Stamen(FI)	Stamen(PG)
Kaempferol derivative (3.71)	76±4.10			
Kaempferol-3-sophoroside-7-glucoside (3.78)			511±22.84	923±41.53
Kaempferol derivative (5.81)			24±1.18	77±4.15
Kaempferol diglucoside (5.89)	97±4.85	113±5.6		
Kaempferol derivative (6.49)	15±1.03	34±1.83		
Kaempferol diglucoside (7.30)			416±21.16	755±33.75
Quercetin diglucoside (7.30)	480±22.08	738±32.16	1037±37.32	1227±47.81
Methyl quercetin diglucoside (7.82)	82±4.16	84±4.21	628±28.88	2091±61.74
Quercetin derivative (8.15)			27±1.15	39±2.14
Methyl quercetin di glucoside (8.42)			209±10.03	249±11.73
Kaempferol-3-sophoroside (8.49)	6415±192.45	8304±215.9	1702±64.7	377±17.72
Kaempferol glucosyl rhamnoside (9.29)	41±2.13	66±3.20		
Methyl quercetin derivative (9.34)			691±31.09	1188±46.32
Quercetin derivative (9.44)			239±11.47	303±14.54
Quercetin diglucoside (9.58)	24±1.27	60±3.18		
Kaempferol sinapoyl glucoside (10.59)	306±14.38	309±14.25	140±5.81	
Kaempferol derivative (10.86)				39±2.25
Kaempferol glucoside (10.98)	421±19.78	399±18.75	93±4.65	
Methyl quercetin glucoside (11.13)			52±2.75	176±8.62
Quercetin derivatives (11.55-12.21)			26±1.19	66±3.43
Kaempferol derivative (12.99)	21±1.15	17±0.078		
Quercetin *p*-cumaroyl glucoside (13.76)			199±9.75	237±110.61
Quercetin derivative (14.09)			4±0.22	26±1.20
Kaempferol *p*-cumaroyl glucoside (15.42)			35±2.05	52±2.65
Methyl quercetin *p*-cumaroyl glucoside (15.61)			26±1.21	40±2.12
Kaempferol (18.43)	20±1.16	14±0.74		8±0.44
TOTAL	**7998**	**10138**	**6059**	**7873**

one-step linear solvent gradient system, starting from 90% H_2O (adjusted to pH 3.2 with HCOOH) up to 100% CH_3CN during a 60-min period; flow rate 0.8 mL min^{-1}.

HPLC/MS analysis: HPLC/MS analysis was performed using a HP 1100L liquid chromatograph linked to a HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer operating conditions were: gas temperature, 350°C; nitrogen flow rate, 10.5 L/min, nebulizer pressure, 40 psi; quadrupole temperature, 30°C; and capillary voltage, 3500 V. The mass spectrometer was operated in positive mode at 120 eV.

Identification and quantification of individual polyphenols: Quantification of individual compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.998$) in the range 0-30 μg on the basis of authentic standards. In particular, crocin derivatives were determined at 440 nm using curcumin as reference compound; safranal was determined at 308 nm using safranal as reference compound and picrocrocin was determined at 250 nm using *p*-hydroxybenzoic acid as reference compound. Flavonols, like kaempferol and quercetin derivatives, were determined at 350 nm using kaempferol-3-*O*-glucoside and rutin, respectively, as reference compounds. In all cases, actual concentrations of the derivatives were calculated after applying corrections for differences in molecular weight.

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