Generation of Saffron Volatiles by Thermal Carotenoid Degradation

Manuel Carmona,* Amaya Zalacain, Mª Rosario Salinas, and
Gonzalo L. Alonso

Cátedra de Química Agrícola, ETSI Agrónomos, Universidad Castilla-La Mancha,
E-02071 Albacete, Spain

Generation of volatiles by thermal treatments has been studied in saffron spice for two reasons: (a) to determine volatile profile changes during simulated aging processes and (b) to study the volatile generation pathway. During the aging process, while the amounts of C10 compounds such as safranal and HTCC increase, the amounts of C9 compounds such as isophorone and 2,6,6-trimethylcyclohexane-1,4-dione decrease. A new compound tentatively identified as 4,5,6,7-tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone seems to play a very important role in the aging process. The importance of this compound, structurally similar to dihydroactindiolide, was also confirmed when the saffron volatile fraction was analyzed via the degradation of the linear chain of crocetin and crocetin esters and is reported for the first time in this paper. Thermal degradation studies of zeaxanthin, crocetin, and trans and cis crocetin esters isomers allowed us to propose different mechanisms which explain saffron volatile generation depending on the crocetin ester isomer structure.

KEYWORDS: Crocetin esters; picrocrocin; safranal; saffron volatiles; thermal degradation

INTRODUCTION

At present, there is a sole accepted hypothesis for generation of saffron volatiles. In this hypothesis, all compounds responsible for the organoleptic attributes of saffron, i.e., color, taste, and aroma, are formed from a unique precursor, with volatile generation being the last step in the sequence (**Figure 1**). Zeaxanthin, the original precursor proposed by Bucherer and Eugster (*1*), is broken down by an enzyme called *Cs*ZCD (*Crocus sativus* zeaxanthin cleavage dioxygenase) at both ends to generate crocetindialdehyde and picrocrocin (*2*). Subsequently, on the one hand, crocetindialdehyde would be oxidized and esterified by diverse glucosyltransferase (*3*, *4*) to generate the crocetin esters responsible for saffron color. On the other hand, picrocrocin, the compound mentioned in the literature as being responsible for saffron bitterness (*5*), is transformed by thermal treatment or alkaline–acid hydrolysis into safranal (*6*, *7*), the major compound in saffron's volatile fraction (*8–12*). Other relevant compounds contributing to this fraction would be produced from any of the glycoside compounds different from picrocrocin identified by Straubinger et al. (*13*, *14*).

Several research results support this hypothesis; for instance, in vivo studies demonstrated the disappearance of picrocrocin during anthesis and the formation of safranal and HTCC (4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) (*6*). Since both of these were undetectable until this time, they suggested that HTCC could be the intermediate compound for
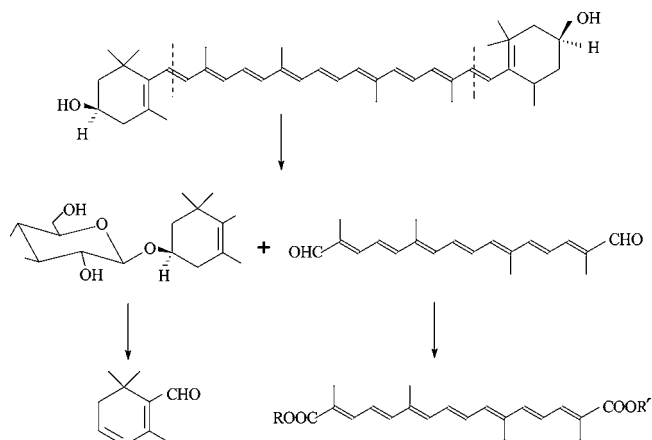

Figure 1. Accepted hypothesis for the generation of crocetin esters, picrocrocin, and safranal in *C. sativus* L. R and R' can be H, glucose, gentibiose, or neapolitanose (*31*).

the synthesis of safranal from picrocrocin. This fact is in accordance with the findings of Raina et al. (*15*), who submitted saffron samples to a soft dehydration process with a volatile profile rich with HTCC. During storage, HTCC was transformed, resulting in samples whose volatile profile was similar to the profiles of those samples dehydrated at high temperatures where greater safranal content was found.

On the other hand, there are other results that are not in accordance with the previous hypothesis. The enormous picrocrocin disappearance reported by Himeno and Sano (*6*) was not related to safranal production. When the fact that picrocrocin

* To whom correspondence should be addressed. E-mail: Manuel.Carmona@uclm.es. Fax: 34 96 75 99 238.

6826 *J. Agric. Food Chem.,* Vol. 54, No. 18, 2006

Carmona et al.

content according to Lozano et al. (*16*) is 60 times higher than that of safranal is taken into account, the proportion should be the opposite. On another hand, Loskutov et al. (*17*) reported safranal generation in saffron extract once picrocrocin had completely disappeared. Recently, different assays with Greek saffron showed how the HTCC content decreased during storage, as suggested by Raina et al. (*15*), even though there was no such increment in safranal content over the same period (*18*). Carmona et al. (*19*) showed that during a thermal aging process, safranal production only took place at high temperatures, even when a picrocrocin decrement was observed earlier. No relation between the picrocrocin decrement and safranal generation was found when the optimum dehydration conditions were studied (*20*).

In summary, while this is an accepted hypothesis in the international research community that works with isolated enzymes and compounds, there are serious doubts about the volatile generation mechanism that occurs within the spice matrix. As suggested by Wahlberg and Eklund (*21*), this could take place by means of oxidative degradation of crocetin esters, which is a possibility that has not been explored. The thermal degradation approach which was a very useful tool for elucidating volatile generation in other carotenoids [β-carotene, lycopene, lutein, and canthasanthin (*22*−*25*)] has not been applied to saffron crocetin esters.

The aim of this paper is to obtain new results that support or reject the accepted hypothesis for saffron volatile generation. To achieve this goal, the behavior of the most important volatiles present in saffron will be followed during an accelerated thermal aging process. Then, the compounds generated by thermal degradation of saffron crocetin esters, zeaxanthin and crocetin, will be assessed in an attempt to establish a relationship between carotenoid structure and the volatiles that have been identified.

MATERIALS AND METHODS

Samples. *Saffron (C. sativus L.).* Harvesting, removal, and dehydration of the stigmas from saffron flowers took place in Motilla del Palancar, Cuenca, Spain, by traditional procedures following the trade standard of the Protected Denomination of Origin "Azafrán de la Mancha" (*26, 27*).

Crocetin Esters Isolated from Saffron. Crocins were isolated according to the method of Patent P9700664 (*28*).

Crocetin Ester Fractions Enriched in Trans and Cis Isomers. Fractions were obtained by extracting 12 g of saffron in 120 mL of ultrapure water. The sample was shaken for 1 h in the dark and centrifuged after 5 min at 4500 rpm for 15 min (Selecta, Barcelona, Spain). The extract that was obtained was loaded in a preparative glass column filled with a preparative C_{18} reversed phase material (Waters, Milford, MA) of 125 Å and a particle size of 55−105 μm. The eluents were acetonitrile and water. Once the different fractions were obtained, they were brought to dryness for subsequent analysis.

Crocetin and Zeaxanthin. Commercial standards were supplied by Extrasynthese (Genay, France).

Standards. Toluene, acetic acid, 3,7-dimethyl-1,6-octadiene (linalool), 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal), and 3,5,5-trimethyl-2-cyclohexen-1-one (isophorone) were supplied by Sigma-Aldrich (Barcelona, Spain), and dihydroactinidiolide (DHA) was a gift from Givaudan.

Accelerated Saffron Aging Process. To simulate an accelerated aging process, a homogenized dehydrated saffron sample was placed in five Petri plates inside an oven (Selecta) at 50 °C. After 2 h, one sample was removed and the oven temperature was increased to 70 °C. Every 2 h, the process was repeated and the temperature was increased 20 °C above the previous step, finally reaching 130 °C. The reported data represent the average of three sample replicates.

Analytical Determinations. *Thermogravimetric Analysis.* Approximately 10 mg of powdered sample was uniformly placed in aluminum

pans and weighed automatically prior to its analysis with the TG/DTA 6200 equipment, SII model (Seiko Instruments Inc.). Samples were subjected to progressive heating at 10 °C/min in an interval from 10 to 400 °C. Tests were carried out in an atmosphere of synthetic air with a flow rate of 100 mL/min. Data were gathered every 2 s. Station ExStar 6000 (Seiko Instruments Inc.) was used for data interpretation.

Identification of Volatile Compounds by Thermal Desorption−Gas Chromatography−Mass Spectrometry (TD−GC−MS). A joined system made up of Perkin-Elmer (Norwalk, CT) ATD-400 thermal desorption equipment, a model HP-6890 gas chromatograph, and a model HP-5973 mass spectrometer provided with a NIST library (Hewlett-Packard, Palo Alto, CA) were used. A fused silica capillary column with stationary phase BP21 50 m in length, with an inside diameter of 0.22 mm, and 0.25 μm of film was employed (SGE). The carrier gas was helium of chromatographic purity (220 kPa). Twenty milligrams of sample was introduced to the desorption tube and desorbed at different temperatures (50, 70, 90, 110, 130, 150, 180, 210, and 240 °C) for 1 min. Other conditions for the thermal desorption equipment were as follows: oven temperature of 250 °C, cold trap temperature of −30 °C, and transfer line temperature of 200 °C. Conditions for gas chromatography were as follows: 100 °C (5 min) increased at a rate of 18 °C/min to 210 °C (15 min). In the mass spectrometer, the electron impact mode (EI) was set up at 70 eV. The mass range varied from 35 to 500 units, and the detector temperature was 150 °C. All compound identification was carried out using the NIST library and by comparison with those reported previously (*29*). The identification of 4,5,6,7-tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone and 2,6,6-trimethylcyclohepta-2,4-dien-1-one was achieved by following the method of Cadwallader (*30*). Quantification was carried out when the peak heights were 10 times greater than the baseline in the extracting single-ion mode. The reported data represent the average of three sample replicates.

Quantification of Safranal. Three series of safranal standard (Sigma-Aldrich, Madrid, Spain) solutions in ethanol (10, 20, 40, 80, and 120 mg/L) were prepared and analyzed by TD−GC−MS. A calibration curve was established for the series of safranal standards as a function of safranal's peak area ($A_{safranal}$) [concentration of safranal (milligrams per gram of dry matter) = $0.003A_{safranal}$; $R^2 = 0.993$].

Identification and Quantitation of Crocins and Picrocrocin by LC−DAD−MS. Twenty milligrams of crocetin esters was macerated for 1 h in 8 mL of ultrapure water previously bubbled with helium. The entire process was carried out in darkness and at room temperature. Twenty microliters of the extract filtered through a PVDF filter of 0.45 μm (Millipore) was injected into an Agilent (Palo Alto, CA) 1100 HPLC chromatograph equipped with a 5 μm Phenomenex Luna C18 column (150 mm × 4.6 mm) thermostated at 30 °C. The solvents were acidified water with formic acid (0.25%) (A) and acetonitrile (B), using the following gradient: 80% A for 5 min to 20% C in 15 min, at a flow rate of 0.8 mL/min. Double on-line detection was carried out with a diode array spectrophotometer and a quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 1100). The probe of the mass spectrometer was connected to the UV cell outlet. The DAD detector was set at 250, 330, and 440 nm. Both the auxiliary and sheath gases were nitrogen with a flow rate of 12 L/min. The gas drying temperature was set to 350 °C and the nebulizer pressure to 30 psi. The capillary voltage was ±2500 V and the capillary temperature 195 °C. Spectra were recorded in positive and negative ion mode between *m/z* 100 and 1500. Identification and quantification were carried out according to method described in ref *31*. The reported data represent the average of three sample replicates.

RESULTS AND DISCUSSION

The saffron volatile composition after an accelerated aging process was analyzed by TD−GC−MS. Interest was focused on five of the identified compounds: safranal, as it is known to be the most abundant compound in saffron aroma; 3,5,5-trimethyl-2-cyclohexenone (isophorone) and 2,6,6-trimethylcyclohexane-1,4-dione, which recently have been demonstrated to be useful in determining saffron geographical origin (*29*);
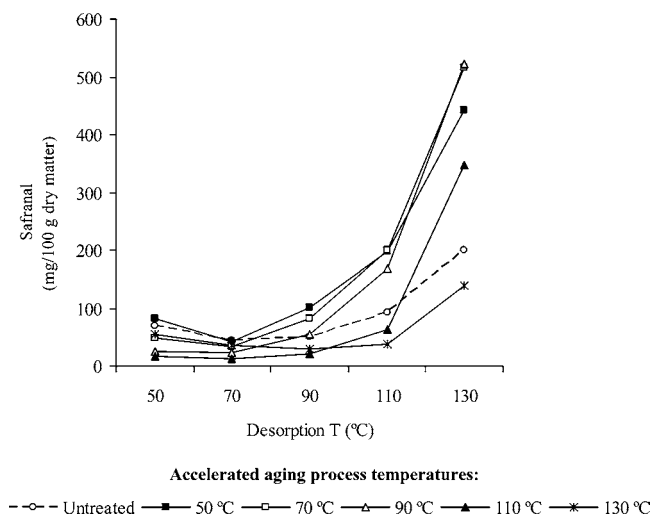
Generation of Saffron Volatiles

J. Agric. Food Chem., Vol. 54, No. 18, 2006 **6827**

Figure 2. Evolution of safranal content in saffron samples submitted to an accelerated aging process in the oven and analyzed by TD–GC–MS at various desorption temperatures.

4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC), as a precursor of safranal; and a compound tentatively identified as 4,5,6,7-tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone (TDOI) [retention time of 12.48 min and a mass fragmentation pattern of 109 (100), 123 (61), 137 (96), 152 (78), 165 (45), and 180 (84)]. These last two compounds were not detected in the initial saffron samples but were included in the study since they were found in abundance when samples were subjected to an accelerated aging process.

Some considerations concerning thermal treatment should be made, as the same temperatures were used for the aging process and the thermal desorption analysis. Whereas the sample remained in the oven for 2 h, time enough to generate aroma precursors, the desorption analysis time was 1 min, the time needed to desorb the volatiles generated in the oven.

Safranal. Successive analysis of all samples that were treated revealed that safranal content decreased when the desorption temperature increased from 50 to 70 °C and immediately increased from 90 °C (**Figure 2**), results that are in accordance with those of other authors. Alonso et al. (*32*) found that if a sample was successively analyzed at 70 °C, the safranal content decreased after each analysis, even though its content remained constant at 150 °C, suggesting that an intermediate temperature should exist to generate this compound. It has recently been shown that the best saffron dehydration temperature for safranal generation is between 80 and 90 °C (*20*).

In fact, **Figure 2** shows that the aged saffron samples subjected to temperatures of 70 and 90 °C for 2 h had the greatest potential for safranal production when higher desorption temperatures were applied (130 °C), indicating that safranal precursor generation took place during the accelerated aging process. These results point out the well-known importance of the dehydration process in saffron, with special regard for the temperatures reached during this process (*19, 33, 34*). However, from a technological and industrial point of view, the most relevant implication is that the generation of safranal precursors is not completed during dehydration. Thus, safranal can be regenerated by a soft thermal treatment as dehydrated samples were used in this study.

Isophorone and 2,6,6-Trimethylcyclohexane-1,4-dione. These are two of the most important C9 compounds in the saffron volatile fraction. The content for both compounds peaked in samples subjected to a temperature of 50 °C and then

decreased when higher temperatures were applied (**Figure 3**). From a desorption temperature of 90 °C, a small increment was detected, and as suggested for safranal, this may be produced by the precursors formed in the oven during the aging process. Yet while the content of isophorone in the sample aged at 90 °C was duplicated when the sample was thermally desorbed from 50 to 130 °C, the safranal content increased almost 20 times (**Figure 2**). The principles of aroma evolution would never allow these compounds to be found in a proportion greater than in the original conditions, regardless of storage conditions, at least not in saffron dried according to the Castilla-La Mancha tradition. Moreover, the similar evolution observed under all conditions (**Figure 3**) is remarkable, creating a possible relationship between both compounds, as already suggested in a previous study (*29*).

HTCC and 4,5,6,7-Tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone (TDOI). The new compound identified as TDOI coincided with the aglycone structure of a glycosidic compound described by Straubinger et al. (*10, 14*).

TDOI was found in greater amounts than HTCC, reaching its highest values at 50 °C (desorption temperature) for all aged samples (**Figure 4a**). Under those conditions, a linear relationship between the content of TDOI and the temperature used for the treated samples existed. By representing the 50 °C desorption temperature analysis in terms of the oven temperature, we obtained a good adjustment to a straight line ($y = -133257T^a_{oven} + 2 \times 10^6$; $R^2 = 0.995$): the greater the temperature, the lower the content of TDOI. At higher desorption temperatures, its content evolution showed practically no slope, suggesting that it was simultaneously transformed into another compound as it was generated.

Certain analogies existed between the behavior of TDOI and HTCC which made the latter compound a candidate for the transformation of TDOI. At the lowest desorption temperature (50 °C), the HTCC content also decreased linearly, depending on temperature ($y = -28169T^a_{oven} + 194173$; $R^2 = 0.98$) (**Figure 4b**). Curiously, the order of abundance of the aged samples at a desorption temperature of 130 °C was the inverse of that at 50 °C, which also occurs in the case of TDOI, although less markedly. It is difficult to offer a reasonable explanation for this fact. The HTCC contents in all samples decrease to the same point as the desorption temperature increases from 50 to 70 °C.

Regardless of the sample treatment, a relationship between HTCC and the TDOI compound was found. In all cases, a linear relationship with a negative slope was maintained, while the regression coefficient (R^2) in all cases was higher than 0.97. This behavior suggested that when a determined concentration of TDOI was reached it was transformed into HTCC in proportion to temperature. A higher oven temperature will indicate a lower ratio of HTCC and TDOI at all desorption temperatures. This relationship also could explain the parallel behavior of HTCC and TDOI at a desorption temperature of 50 °C (**Figures 4a,b**); the content of HTCC was high since the content of TDOI was also high.

These results do not support the accepted volatile generation pathway, as it seems to be the opposite. The classic hypothesis affirmed that the compound responsible for safranal is picrocrocin, which forms during stigma maturation and disappears from the anthesis to generate safranal. Now, it has been shown that the level of safranal precursors increases with thermal treatment, whereas picrocrocin disappears rather than increases in content with similar treatments (*19*). In addition, the appearance of TDOI in large amounts, which is structurally
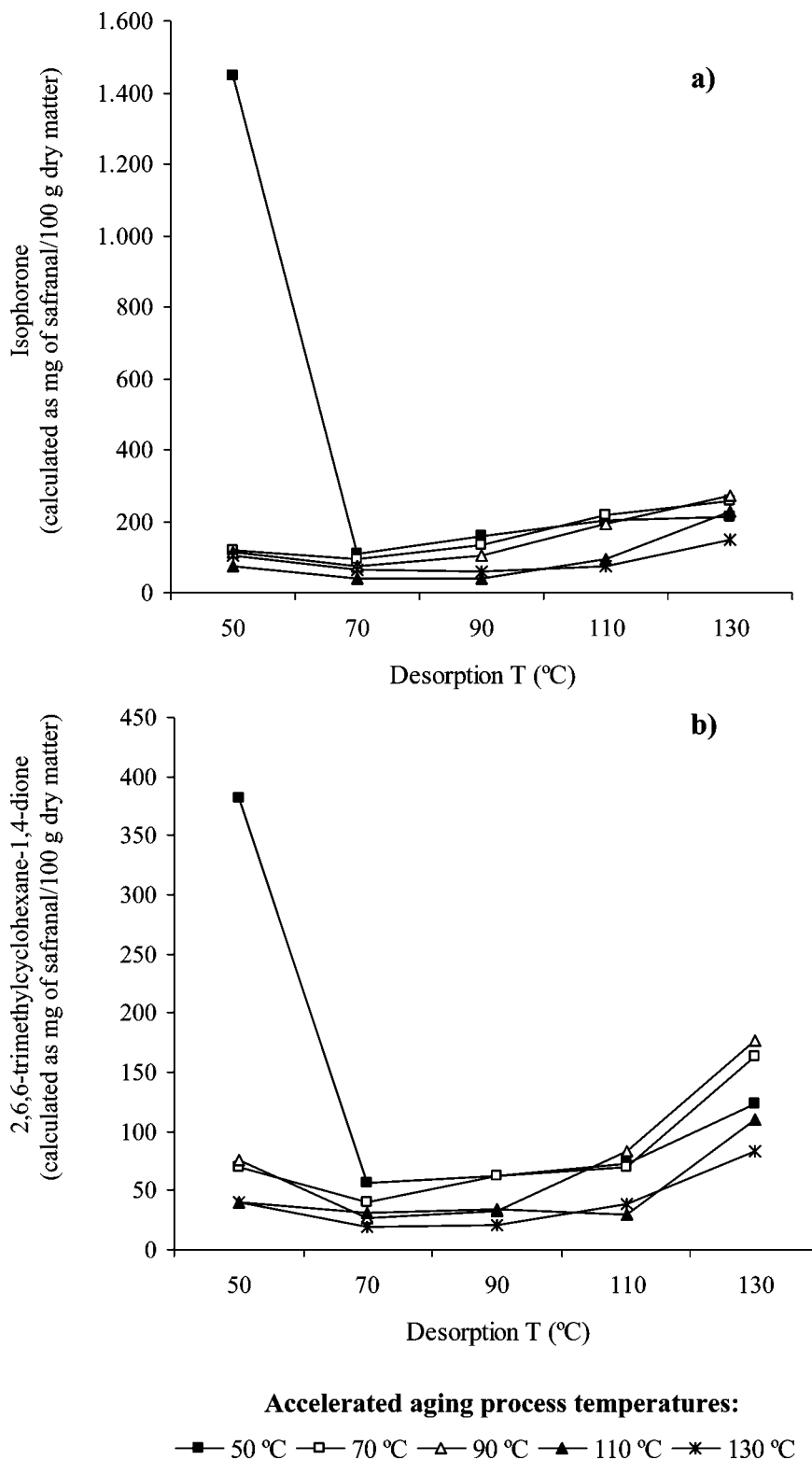
6828 *J. Agric. Food Chem.,* Vol. 54, No. 18, 2006

Carmona et al.

Figure 3. Content evolution of (**a**) 3,5,5-trimethyl-2-cyclohexenone (isophorone) and (**b**) 2,6,6-trimethylcyclohexane-1,4-dione in saffron samples subjected to an accelerated aging process and analyzed at various desorption temperatures.

related to DHA, created great expectations since DHA, found in several plants, is especially relevant as a thermal and enzymatic β-carotene degradation product (*35*, *36*). These reasons were an important stimulus for focusing research efforts on the thermal degradation of main saffron carotenoids.

Crocetin Ester Thermal Degradation. Isolation of crocetin ester from *C. sativus* L. stigmas was carried out following the method of Patent P9700664 (*28*), which employs an aqueous

extraction that does not allow co-extraction of water-insoluble carotenoids such as zeaxanthin, phytofluene, tetrahydrolycopene, phytoene, or others found in saffron (*37*). First, the freeze-dried isolated sample containing a mixture of different crocetin esters was subjected to thermogravimetric analysis to determine the temperature at which their structure breaks or changes (**Figure 5a**). At low temperatures, between 30 and 120 °C, a dip in the DTA curve was observed, and moisture and volatile compound
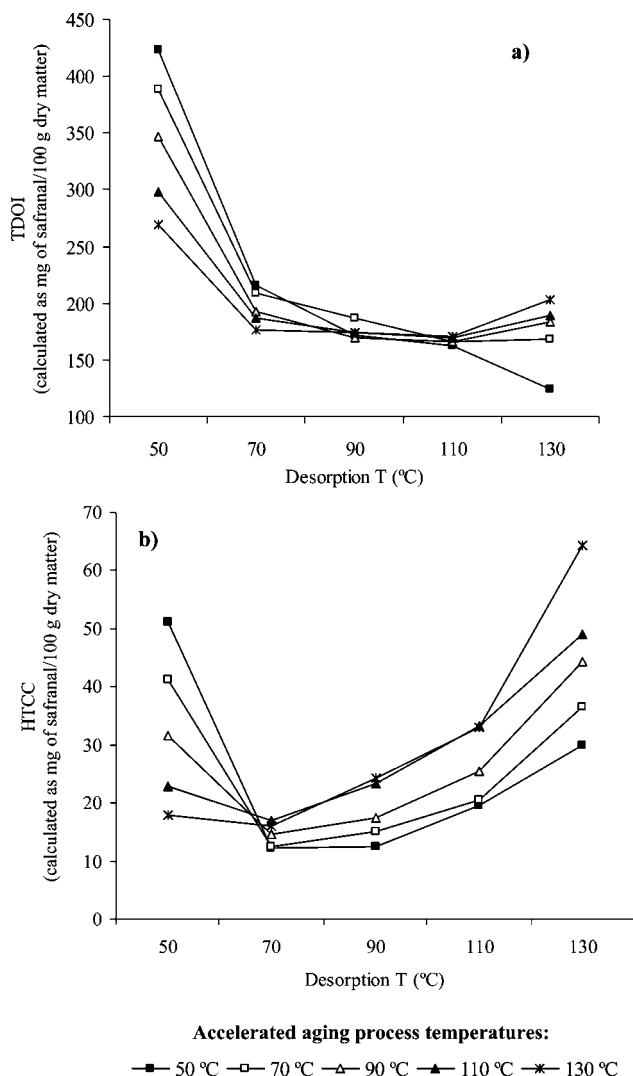
Generation of Saffron Volatiles

J. Agric. Food Chem., Vol. 54, No. 18, 2006   **6829**

Figure 4. Content evolution of (**a**) 4,5,6,7-tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone (TDOI) and (**b**) HTCC in saffron samples subjected to an accelerated aging process in the oven and analyzed at various desorption temperatures.

Figure 5. Diagrams of thermogravimetric analysis (TGA), differential thermal analysis (DTA), and differential thermogravimetric analysis (DTG) for (**a**) crocetin esters isolated from saffron [also including thermogravimetric analysis (TG)] and (**b**) crocetin standard.

elimination was observed (approximately 2.5%, TG curve) with maximum evaporation at 70 °C (local maximum in the DTG curve). Molecules begin to break down around 180 °C, which is similar to 186 °C, as reported previously (*38*), even though the maximum in decomposition speed was observed at 205 and 265 °C (**Figure 5a**, DTG curve). The analysis of a standard of commercial crocetin, using the same technique and conditions, revealed a single peak of rupture in the chain at 265 °C (**Figure 5b**). Therefore, it was deduced that sugars which esterify crocetin played an important role in crocin degradation at 205 °C. Then, the crocetin and crocetin ester pool were thermally desorbed at a higher temperature, 210 °C, to analyze the compounds that were generated (**Figure 6**). Contrary to what was expected, safranal was generated in both cases, but the occurrence of sugar moieties at crocetin ends favored its generation. In this case, the noticeable compounds in order of abundance were as follows: HTCC and TDOI and two megastigma isomer compounds (megastigma-7,11,13-triene and megastigma-2,6,8-triene) (**Figure 6a**) (*39*). Their presence supports the idea that several of the extraction techniques used for the isolation of the volatile fraction in the past were much too exhaustive, changing the real saffron volatile profile. For
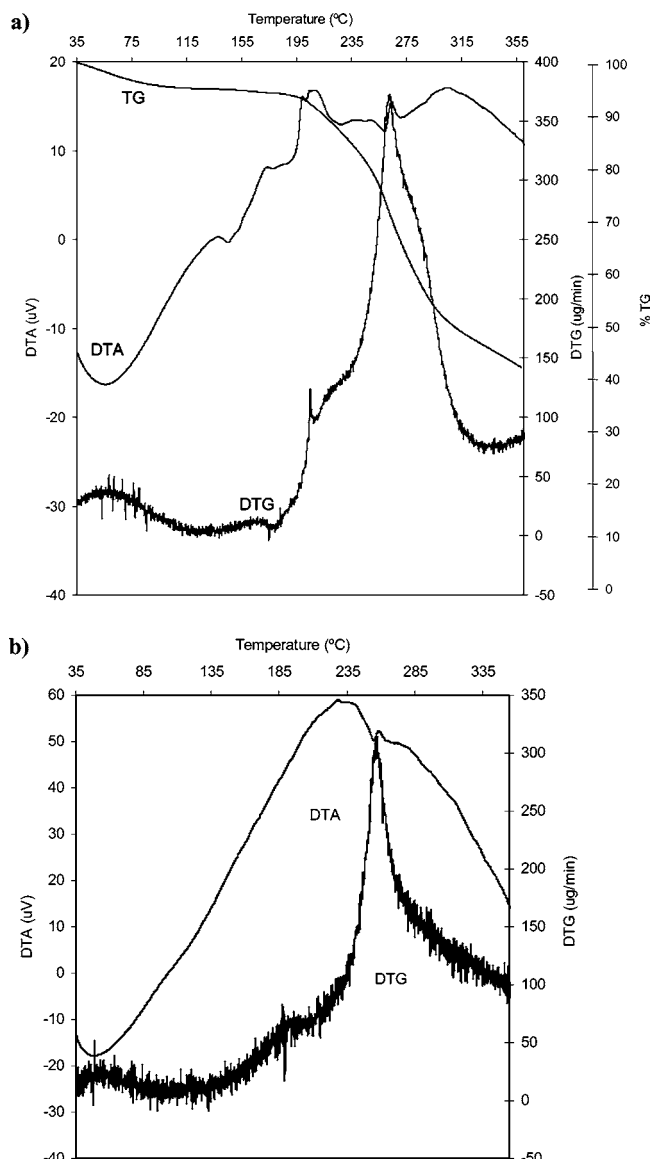
crocetin degradation, a C7 cyclic compound [fragmentation pattern of 91 (81), 197 (100), 121 (73), and 150 (57)] also previously identified by Cadwallader and co-workers (*39*) as 2,6,6-trimethylcyclohepta-2,4-dien-1-one was identified as the most abundant compound.

In both cases, toluene detection is remarkable. Its generation could be explained by mechanisms already described for β-carotene (*40*) and annatto (*41*). According to Scooter (*41*), toluene and *m*-xylene production is furthered by the multi-cis isomer structure and hindered by the all-trans structure. It is logical to assume that this could take place easily at 210 °C due to the quantity of energy transmitted to the samples.

To explore the possibility of different behavior of cis or trans crocetin esters when they are thermally degraded, the pool of crocetin esters was passed through the C18 chromatography column to obtain one fraction enriched with trans isomers and another one enriched with the cis isomers (**Figure 7**). The enriched cis fraction had some traces of the trans isomer crocetin (β-D-gentibiosyl) ester between the two more important cis
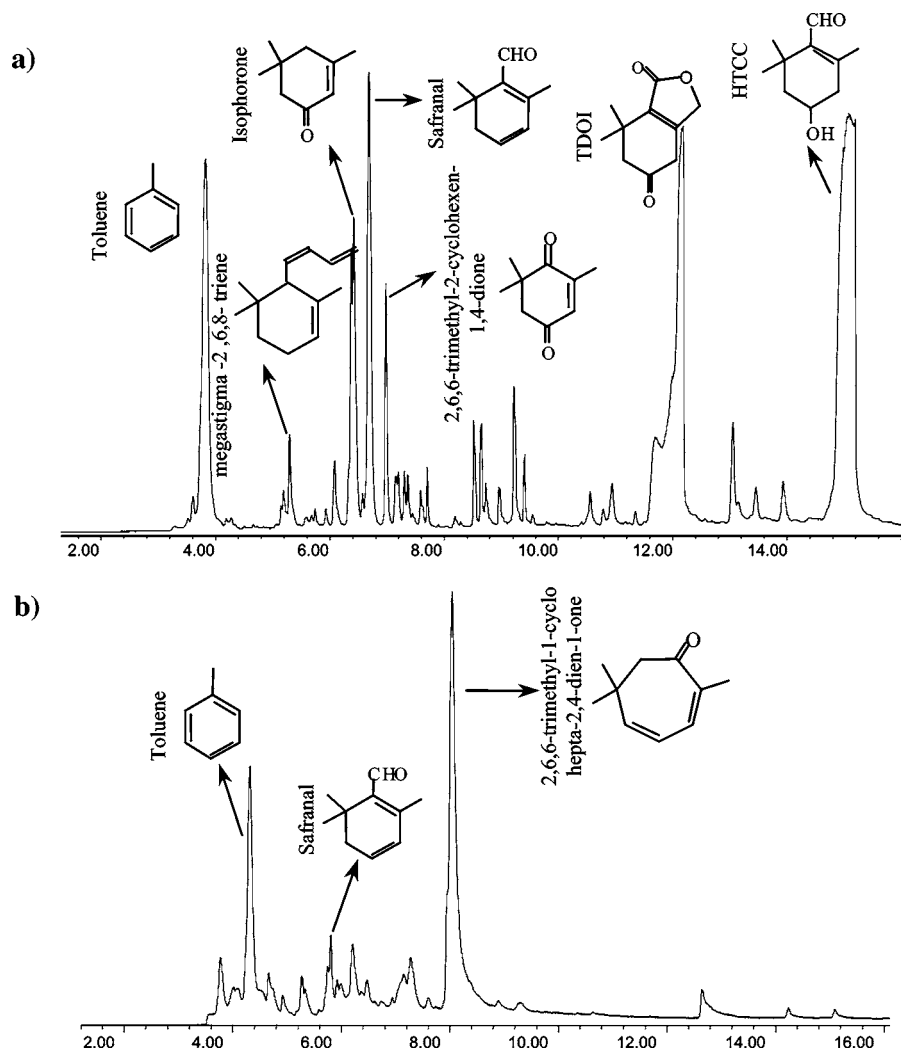
Figure 6. TD–GC–MS chromatograms of (**a**) saffron crocetin esters and (**b**) crocetin standard, when a desorption temperature of 210 °C was applied. Structures of the most relevant compounds identified by mass spectrometry are shown.

crocetin esters, crocetin di(*β*-D-gentibiosyl) ester and crocetin (*β*-D-gentibiosyl) (*β*-D-glucosyl) ester. The two enriched fractions, together with zeaxanthin and crocetin standards, were subjected to increasing desorption temperatures every 30 °C starting with 150 °C, and the volatile fraction that was generated was studied (**Figure 8**). Crocetin results confirmed the ability to generate safranal from the linear chain. The first compounds liberated were acetic acid and 2,6,6-trimethylcyclohepta-2,4-dien-1-one. At higher temperatures, 2,6,6-trimethylcyclohepta-2,4-dien-1-one and generation of other volatiles were not found, with the degradation of the complete chain being responsible for toluene formation.

When zeaxanthin, the bibliographic precursor compound of crocetin esters, was thermally degraded, safranal was obtained, although it was a less advantageous process than chain degradation for producing toluene (**Figure 8b**). A priori, the ends of the chain would be a point of tension and therefore of breakdown. It has been shown that the 9−10 and 9′−10′ bonds are a thermodynamically favored point for oxidative degradation of a very close carotenoid such as *β*,*β*-carotene (*42−44*). The lack of safranal isomers, neither C13 nor C9 compounds related to isophorone, suggested that the generation of safranal took place from the ends of the molecule and not from the linear chain as in the case of crocetin.

While the trans isomer enriched fraction degradation (**Figure 8c**), the first compounds to be generated were HTCC and TDOI,

confirming their important role in saffron volatile generation. When the aged samples were studied, a higher oven temperature indicates a higher HTCC content in relation to that of TDOI. The trans fraction showed, in the thermogravimetric analysis (**Figure 9**), a constant disappearance beginning at 180 °C (DTA curve). The DTG curve was quite blunt without a defined temperature when transformation into volatile compounds was more accentuated, suggesting a progressive formation of volatile compounds. The formation of safranal and other volatiles began at 210 °C and could take place through TDOI and HTCC, as proposed in **Figure 10**.

However, this mechanism cannot explain the safranal generation from crocetin degradation. The appearance of safranal at an earlier stage of heating and the presence of HTCC and 4,5,6,7-tetrahydro-7,7-dimethyl-5-oxo-3*H*-isobenzofuranone (TDOI) at very low levels (**Figure 8a** vs **Figure 8c**) suggest different pathways, maybe through the major C7 compound according to the hypothesis shown in **Figure 11**.

When the formation of volatiles in the fraction enriched with saffron cis crocetin esters was studied, several isomers from the megastigma family were found in very significant quantities (**Figure 8d**). The meager presence of HTCC and TDOI, probably coming from trans crocetin esters remaining, did not seem to be responsible for the noticeable content of safranal at 240 °C. A third mechanism is proposed under these harsh circumstances (**Figure 12**). Volatile generation would take place
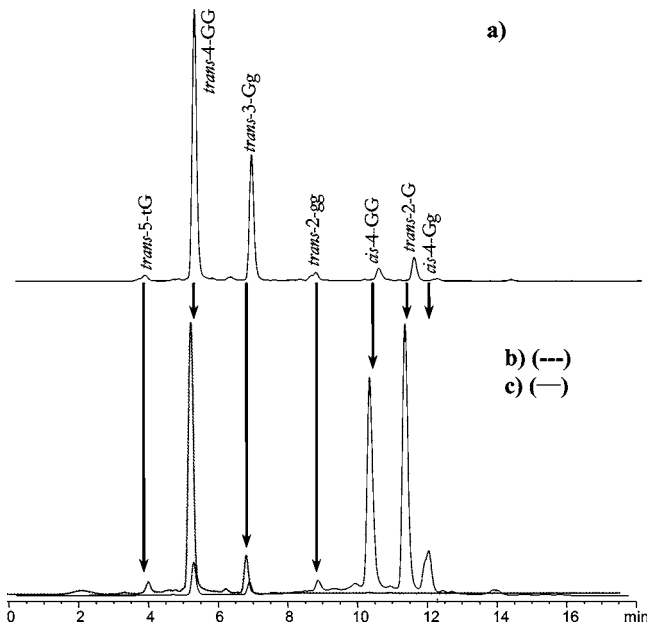
Generation of Saffron Volatiles

J. Agric. Food Chem., Vol. 54, No. 18, 2006 **6831**

Figure 7. LC–DAD–MS chromatograms of (**a**) a saffron crocetin ester pool, (**b**) its enriched trans fractions, and (**c**) its enriched cis fractions. Identification of the most relevant compounds by mass spectrometry is shown. Abbreviations: trans-5-tG, trans crocetin (β-D-triglucoside) (β-D-gentibiosyl) ester; trans-4-GG, trans crocetin di(β-D-gentibiosyl) ester; trans-3-Gg, trans crocetin (β-D-glucosyl) (β-D-gentibiosyl) ester; trans-2-gg, trans crocetin di(β-D-glucosyl) ester; cis-4-GG, cis crocetin di(β-D-gentibiosyl) ester; trans-2-G, trans crocetin di(β-D-gentibiosyl) ester; cis-5-Gg, cis crocetin (β-D-glucosyl) (β-D-gentibiosyl) ester.

from megastigmas which at the same time would occur from other intermediaries, as could be deduced from the thermogravimetric analysis. The cis fraction presented an evolution similar to that of the trans fraction up to 180 °C, changing completely beyond 205 °C (**Figure 9**, DTA curve). Crocetin esters from the cis fraction exhibited a tendency to increase that could be explained by the formation of new compound intermediaries in the generation of volatiles.

In summary, if the results obtained at high temperatures are transferred to the degradation process of crocetin esters which takes place during storage, the volatile generation in saffron would begin from trans isomers and not from cis isomers, as previously thought. This is just the opposite of the proposal by other authors for relevant members of the carotenoid family such as β-carotene (*45*). Perhaps the special properties of saffron crocetin esters in relation to their polarity behavior with other carotenoids could be responsible for this behavior.

Secondary Metabolite Generation Mechanism. The results obtained change the view of volatile generation in saffron and consequently suggest that the accepted pathway for crocetin ester formation shared with saffron volatile generation has to be reconsidered (**Figure 1**). If the generation of six-membered ring cyclic compounds takes place from the lineal chain, a new hypothesis can be proposed, leaving aside the discussion of whether zeaxanthin is the original precursor. This new pathway should attempt to explain the paradox of *C. sativus* and *Gardenia jasminoide*. While both plants contain the same crocetin esters, although in a different proportion (*31*), *C. sativus* stigmas contain picrocrocin and safranal whereas *G. jasminoide* fruit does not. It is reasonable to suppose that to produce structurally identical compounds both plants should use enzymes belonging to a single metabolic route. Then, if the accepted
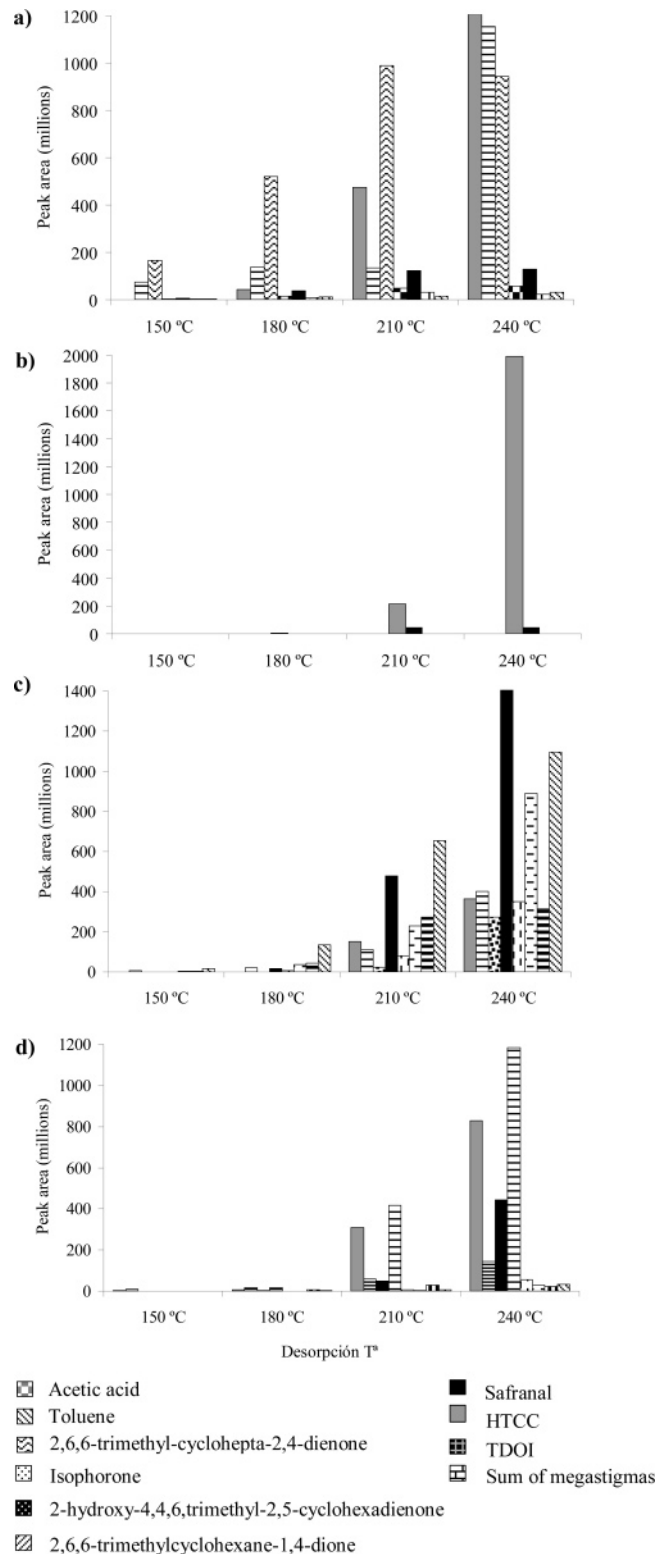

Figure 8. Compounds generated from (**a**) a crocetin standard, (**b**) a zeaxanthin standard, (**c**) a saffron fraction enriched with trans crocetin esters, and (**d**) a saffron fraction enriched with cis crocetin esters, when subjected to degradation by thermal treatment at increasing desorption temperatures.

hypothesis for *C. sativus* L. metabolite generation is correct (**Figure 1**), *G. jasminoide* should possess an extremely efficient enzymatic complex that would quantitatively transform the picrocrocin formed to the point of making it undetectable.

Another possibility is that both plants may share the crocetin ester formation route, but the volatile generation is different
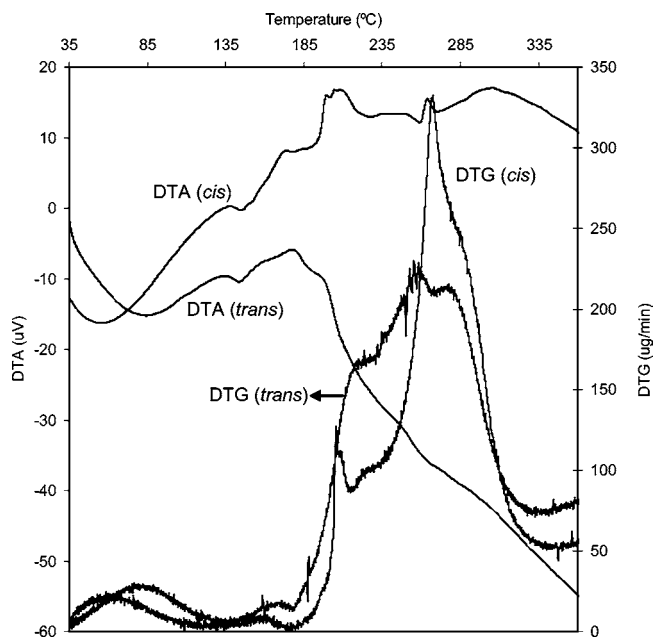
6832 *J. Agric. Food Chem.,* Vol. 54, No. 18, 2006

Carmona et al.

Figure 9. Diagrams of thermogravimetric analysis (TGA) of saffron fractions enriched with trans and cis crocetin esters, where differential thermal analysis (DTA) and differential thermogravimetric analysis (DTG) are included.
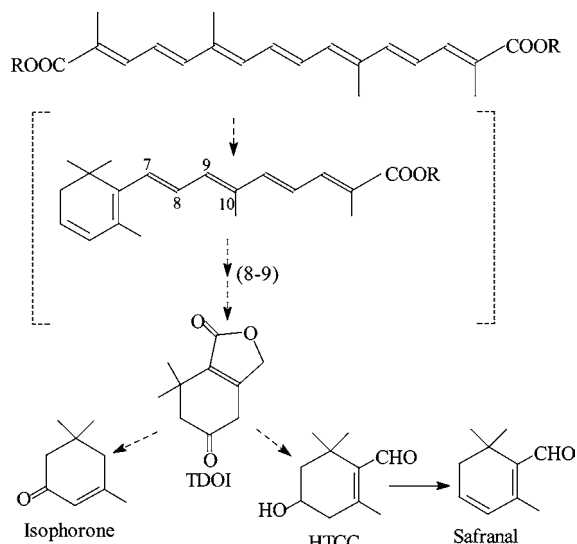

Figure 11. Proposed mechanism for the generation of safranal, isophorone, and related compounds from crocetin when subjected to thermal degradation.

Figure 10. Pathway proposed for the generation of safranal and isophorone from a saffron trans crocetin-enriched fraction when subjected to thermal degradation. R and R′ can be H, glucose, gentibiose, or neapolitanose (*31*).

Figure 12. Proposed mechanism for the generation of safranal, isophorone, and related compounds from megastigmas, by submitting an enriched cis crocetin saffron fraction to to thermal degradation. R and R′ can be H, glucose, gentibiose, or neapolitanose (*31*).

from what is traditionally accepted. In **Figure 13**, a new model is proposed for this second hypothesis. Volatile formation would take place from these esters due to the action of a unknown enzyme, explaining why odorous C9 and C10 compounds are found in one plant and not in the other. This hypothesis, where the key compound could be TDOI, is compatible with existing knowledge of the formation of six-carbon rings in carotenoid linear ends (*46*). At the same time, it is consistent with the work of Himeno and Sano (*6*), who found that at the moment of anthesis, in addition to the decrease in picrocrocin content, a very significant decrease in crocin content was produced. However, they could not find an adequate explanation for this. In fact, while in vitro the transformation of picrocrocin into
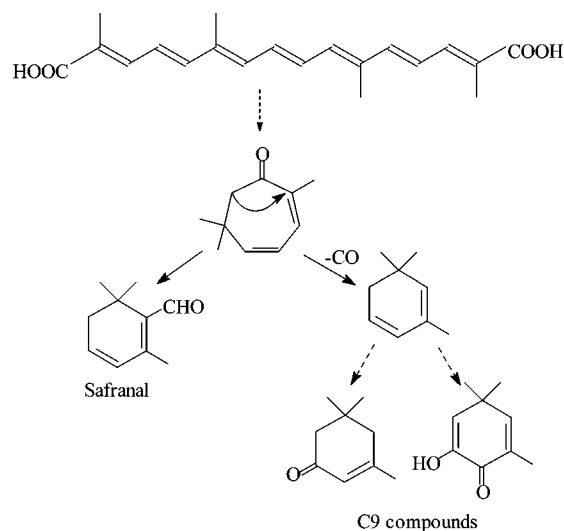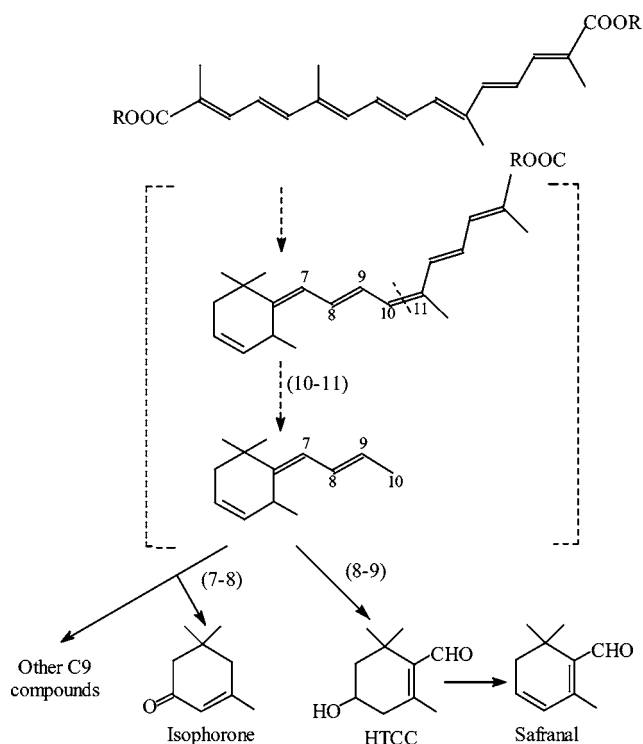
safranal has been demonstrated (*7*), no one has been able to confirm proportional safranal generation consistent with the disappearance of picrocrocin in the spice. Also, during stigma development, crocetin esters and picrocrocin would maintain a more or less constant proportion since the HTCC formed by an enzymatic action, type of carotenase, would be glycosylated, being transformed into picrocrocin and other minor glycosides (*6*). Then, it is possible that at the time of anthesis one or several, specific or unspecific, glycosidases would act on picrocrocin, changing it again into HTCC, but also because the mentioned enzyme would actively work on crocetin esters.
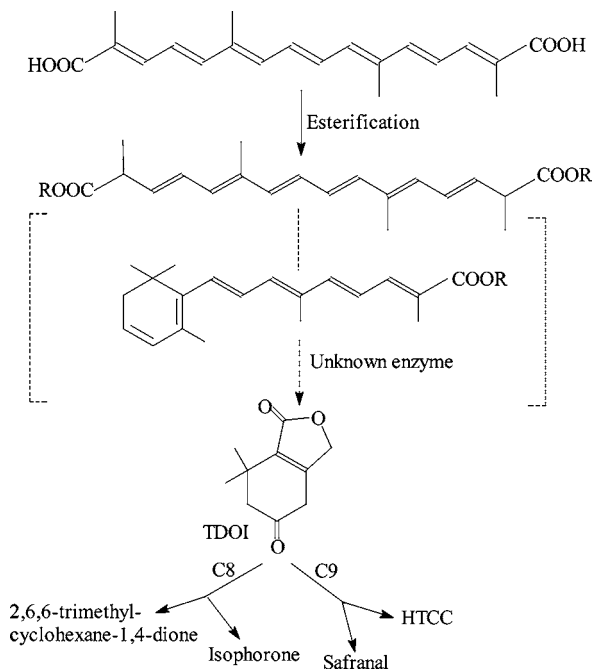
Generation of Saffron Volatiles

J. Agric. Food Chem., Vol. 54, No. 18, 2006 **6833**

Figure 13. New hypothesis for generation of volatiles from *C. sativus* L. crocetin esters. R and R′ can be H, glucose, gentibiose, or neapolitanose (*31*).

In the case of saffron dehydrated at high temperatures, the activity of these enzymes would be short, but in saffron dehydrated at room temperature, they could remain active for many hours over the several days that the drying processes last. Under these conditions, it could certainly be said that picrocrocin is the precursor for safranal, provided that enzymatic activity is viable. Once saffron is considered a spice, dehydrated stigmas, safranal formation would no longer depend on picrocrocin. Safranal and other C9 and C10 volatiles would be generated, contrary to what could be expected, from trans crocetin esters.

Work to isolate the various crocetin esters to determine which of them generate aroma and which do not is still pending. At this moment, the best candidate would be the trans crocetin di-(β-D-glucosyl) ester that recently has been detected in saffron but not in gardenia (*31*). Also, structure confirmation must be awarded to compound TDOI, which seems to play such an important role in the generation of aroma. Evaluation of the possible contribution of megastigma compounds and the generation of these volatiles at very high temperatures remains to be done.

The hypotheses formulated throughout this paper will be endorsed or rejected in the near future with the definitive characterization of one or more active enzymes on carotenoids, the correct identification of the substrates upon which they act, the discovery of an ultimate answer for the role played by glycosides in saffron, and their transformation into aroma or not during anthesis or dehydration.

LITERATURE CITED

(1) Buchecker, R.; Eugster, C. H. Absolute configuration of picro-crocin. *Helv. Chim. Acta* **1973**, *56* (3), 1121−1125.

(2) Bouvier, F.; Suire, C.; Muttener, J.; Camara, B. Oxidative remodeling of chromoplast carotenoids: Identification of the carotenoid dioxygenase CsCCD and CSZCD genes involved in crocus secondary metabolite biogenesis. *Plant Cell* **2003**, *15*, 47−62.

(3) Rubio, A.; Fernández, P.; Fernández, J. A.; Gómez-Gómez, L. Glucosilation of the saffron apocarotenoid crocetin by a glu-cotrasferase isolated from *Crocus sativus* stigmas. *Planta* **2004**, *219*, 955−966.

(4) Castillo, R.; Fernández, J. A.; Gómez-Gómez, L. Implications of carotenoid biosynthetic genes in apocarotenoid formation during stigma development of *Crocus sativus* and its closer relatives. *Plant Physiol.* **2005**, *139*, 674−689.

(5) Giaccio, M. Crocetin from saffron: An active component of an ancient spice. *Crit. Rev. Sci. Nutr.* **2004**, *44*, 155−172.

(6) Himeno, H.; Sano, K. Synthesis of crocin, picrocrocina and safranal by saffron stigma-like structures proliferated in vitro. *Agric. Biol. Chem.* **1987**, *9* (51), 2395−2400.

(7) Iborra, J. L.; Castellar, M. R.; Canovas, M.; Manjón, A. Picrocrocin hydrolysis by inmobilized β-glucoidase. *Biotechnol. Lett.* **1992**, *14* (6), 574−580.

(8) Carmona, M.; Zalacain, A.; Salinas, M. R.; Alonso, G. L. A new approach to saffron aroma. *Crit. Rev. Food Sci. Nutr.* **2006**, in press.

(9) Rödel, W.; Petrzika, M. Analysis of volatile components of saffron. *J. High Resolut. Chromatogr.* **1991**, *14*, 771−774.

(10) Straubinger, M.; Bau, B.; Eckestein, S.; Jezussek, M.; Winter-halter, P. Isolation of new saffron constituents using counter-current chromatography. In *Natural Product Analysis*; Schreier, P., Herderich, M., Humpf, H. U., Schwab, W., Eds.; Vieweg: Braunschweig/Wiesbaden, Germany, 1998; pp 27−34.

(11) Zarghami, N. S.; Heinz, D. E. Monoterpene aldehydes and isophorone-related compounds of saffron. *Phytochemistry* **1971**, *10*, 2755−2761.

(12) Zarghami, N. S.; Heinz, D. E. The volatile constituents of saffron (*Crocus sativus* L.). *Lebensm.-Wiss. -Technol.* **1971**, *4*, 43−45.

(13) Straubinger, M.; Jezussek, M.; Waibel, R.; Winterhalter, P. Novel glycosidic constituents from saffron. *J. Agric. Food Chem.* **1997**, *45*, 1678−1681.

(14) Straubinger, M.; Bau, B.; Eckestein, S.; Fink, M.; Winterhalter, P. Identification of novel glycosidic aroma precursors in saffron (*Crocus sativus* L.). *J. Agric. Food Chem.* **1998**, *46*, 3238−3242.

(15) Raina, B. L.; Agarwal, S. G.; Bhatia, A. K.; Gaur, G. S. Changes in pigments and volatiles of saffron (*Crocus sativus* L.) during processing and storage. *J. Sci. Food Agric.* **1996**, *71*, 27−32.

(16) Lozano, P.; Delgado, D.; Gomez, D.; Rubio, M.; Iborra, J. L. A non-destructive method to determine the safranal content of saffron (*Crocus sativus* L.) by supercritical carbon dioxide extraction combined with high performance liquid chromatog-raphy and gas chromatography. *J. Biochem. Biophys. Methods* **2000**, *5* (43), 367−378.

(17) Loskutov, A. V.; Beninger, C. W.; Hosfield, G. L.; Sink, K. C. Development of an improved procedure for extraction and quantification of safranal in stigmas of *Crocus sativus* L. using high performance liquid chromatography. *Food Chem.* **2000**, *69*, 87−95.

(18) Kanakis, C. D.; Daferera, D.; Tarantilis, P. A.; Polissiou, M. G. Qualitative determination of volatile compounds and quantitative evaluation of safranal and 4-hidroxy-2,6,6-trimethyl-1-carbox-aldehyde (HTCC) in Greek saffron. *J. Agric. Food Chem.* **2004**, *52*, 4515−4521.

(19) Carmona, M.; Zalacain, A.; Pardo, J. E.; López, E.; Alvarruiz, A.; Alonso, G. L. Influence of different drying and aging conditions on saffron constituents. *J. Agric. Food Chem.* **2005**, *53*, 3974−3979.

6834 *J. Agric. Food Chem.,* Vol. 54, No. 18, 2006

Carmona et al.

(20) Gregory, M. J.; Menary, R. C.; Davies, N. W. Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. *J. Agric. Food Chem.* **2005**, *53*, 5969−5975.

(21) Wahlberg, I.; Eklund, A. In *Carotenoids. Volume 3. Biosynthesis and metabolism*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, 1998; pp 195−216.

(22) Kanasawud, P.; Crouzet, J. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β-Carotene degradation. *J. Agric. Food Chem.* **1990**, *38*, 237−243.

(23) Kanasawud, P.; Crouzet, J. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 2. Lycopene degradation. *J. Agric. Food Chem.* **1990**, *38*, 1238−1242.

(24) Roshdy, T. H.; Daun, H. Use of GC-MS technique for identification of oxygenated volatile thermal degradation products of canthaxanthin. *J. Agric. Food Chem.* **1990**, *38*, 1391−1396.

(25) Maldonado-Robledo, G.; Rodríguez-Bustamante, E.; Sánchez-Contreras, A.; Rodríguez-Sanoja, R.; Sánchez, S. Production of tobacco aroma from lutein. Specific role of the microorganisms involved in the process. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 484−488.

(26) Diario Oficial de Castilla-La Mancha; 1999,10 (Feb 11), pp 1098−1112.

(27) Official Journal of the European Communities; 2000, June 22, pp 4−8.

(28) Escribano, J.; Alonso, G. L.; Salinas, M. R.; Fernández, J. A. (Universidad de Castilla-la Mancha). Método de aislamiento de sustancias colorantes y saborizantes del azafrán especia: crocinas y picrocrocina. Patent P9700664, 1997.

(29) Carmona, M.; Martínez, J.; Zalacain, A.; Rodríguez-Méndez, M. L.; de Saja, J. A.; Alonso, G. L. Analysis of saffron volatile fraction by TD-GC-MS and e-nose. *Eur. Food Res. Technol.* **2006**, *223*, 96−101.

(30) Cadwallader, K. R. Flavor Chemistry of Saffron. In *Carotenoid-Derived Aroma Compounds*; Winterhalter, P., Rouseff, R., Eds.; American Chemical Society Symposium Series 802; American Chemical Society: Washington, DC, 2002; pp 220−239.

(31) Carmona, M.; Zalacain, A.; Sánchez, A. M.; Novella, J. L.; Alonso, G. L. Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoide* fruits. Tentative identification of seven new compounds by LC-ESI-MS. *J. Agric. Food Chem.* **2006**, *54*, 973−979.

(32) Alonso, G. L.; Salinas, M. R.; Sánchez, M. A.; Garijo, J. Safranal content in Spanish saffron. *Food Sci. Technol. Int.* **2001**, *7* (3), 225−229.

(33) Ordoudi, S.; Tsimidou, M. Saffron quality: Effect of agricultural practices, processing and storage. In *Production practices and quality assessment of food crops*; Dris, R., Jain, S. M., Eds.; Kluver Academic Publishers: Dordrecht, The Netherlands, 2004; pp 209−260.

(34) Winterhalter, P.; Straubinger, R. M. Saffron. Renewed interest in an ancient spice. *Food Rev. Int.* **2000**, *16* (1), 39−59.

(35) Crouzet, J.; Kanasawud, P.; Sakho, M. Thermal generation of carotenoids-derived compounds. In *Carotenoid-Derived Aroma Compounds*; Winterhalter, P., Rouseff, R., Eds.; American Chemical Society Symposium Series 802; American Chemical Society: Washington, DC, 1998; pp 115−129.

(36) Bosser, A.; Paplorey, E.; Beling, J. M. A simple way to (±)-dihydroactinidiolide from β-ionone related to the enzymatic co-oxidation of β-carotene in aqueous solution. *Biotechnol. Prog.* **1995**, *11*, 689−692.

(37) Pfander, H.; Schurtenberger, H. Biosynthesis of C20-carotenoids in *Crocus sativus* L. *Phytochemistry* **1982**, *21* (5), 1039−1042.

(38) Kamikura, M.; Nakazato, K. Natural yellow colors extracted from gardenia fruits (*Gardenia jasminoides* ELLIS) and colors found in commercial gardenia fruit extract color. Analysis of natural yellow colors by high performance liquid chromatography. *J. Food Hyg. Soc. Jpn.* **1985**, *26* (2), 150−159.

(39) Cadwallader, K. R.; Baek, H. H.; Cai, M. Characterization of saffron flavor by aroma extract dilution analysis. In *Spices: Flavor Chemistry and Antioxidant Properties*; Riach, S. J., Ho, C. T., Eds.; American Chemical Society Symposium Series 660; American Chemical Society: Washington, DC, 1997; pp 66−79.

(40) Bonnie, T. Y. P.; Choo, Y. M. Oxidation and thermal degradation of carotenoids. *J. Oil Palm Re*s. **1999**, *2* (1), 62−78.

(41) Scotter, M. J. The colour content and degradation products of annatto. The 2nd International Symposium on Natural Colorants, Puerto de Acapulco, Mexico, 1996.

(42) Mohamed, N.; Hashim, R.; Rahman, N. A.; Zain, S. M. An insight to the cleavage of β-carotene to vitamin A: A molecular mechanics study. *J. Mol. Struct.* **2001**, *538*, 245−252.

(43) Zorn, H.; Langhoff, S.; Scheibner, M.; Berger, R. G. Cleavage of β,β-carotene to flavour compounds by fungi. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 331−336.

(44) Zorn, H.; Langhoff, S.; Scheibner, M.; Nimtz, M.; Berger, R. G. A peroxidase from *Lepista irina* cleaves β,β-carotene to flavour compounds. *Biol. Chem.* **2003**, *384*, 1049−1056.

(45) Wache, Y. Effect of cis/trans isomerism of β-carotene on the ratios of volatile compounds produced during oxidative degradation. *J. Agric. Food Chem.* **2003**, *51*, 1984−1987.

(46) Britton, G. Overview of carotenoids biosynthesis. In *Carotenoids. Volume 3. Biosynthesis and metabolism*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser Verlag: Basel, Switzerland, 1998; pp 13−147.