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Qualitative Determination of Volatile Compounds and Quantitative Evaluation of Safranal and 4-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek Saffron

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Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the main component of saffron's essential oil. It was obtained using microsimultaneous hydro distillation−extraction (MSDE) and by ultrasound-assisted extraction (USE), which is a mild method. 4-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) is a precursor of safranal and was obtained in considerable amounts only by USE. Five C_{13}-norisoprenoids were found in saffron for the first time. Using a gas chromatography technique, safranal and HTCC were quantified from Greek saffron samples. The quantity of safranal isolated by MSDE ranged between 288.1 and 687.9 mg/100 g of saffron, whereas in the case of USE safranal and HTCC ranged between 40.7 and 647.7 mg/100 g of saffron and between 41.7 and 397.7 mg/100 g of saffron, respectively. Freeze-drying was also tested as an alternative drying method. Over years of storage at 4 °C the quantity of safranal remained mostly constant while the quantity of HTCC decreased over the same periods.

KEYWORDS: Ultrasound extraction; GC-MS; saffron; freeze-drying; quantitative analysis; safranal; HTCC

INTRODUCTION

Saffron is the commercial name of the dried stigmas of *Crocus sativus* L. flowers. It is used both as a spice in foods and as a drug in traditional medicine. In food, saffron performs the functions of a spice, adding its faint, delicate aroma, pleasing flavor, and magnificent yellow color. In Greek traditional medicine, a small quantity of saffron is thought to regulate menstruation and help with conception. Saffron is the main constituent of saitocrocin, a tincture medicine mixture used in Japanese traditional medicine. It is assumed that saffron enhances brain activity. In addition, the biological activity of saffron as a natural preventing substance in anticancer research is in development (*1*−*8*).

C. sativus L. is cultivated in many countries such as Greece, Spain, Iran, India, and Jordan. Saffron's quality is determined by its color, taste, and aroma, which depend on many factors such as soil, climate, rainfall, harvest time, and postharvest treatments.

The substances responsible for its characteristic quality are crocins, picrocrocin, and safranal. Crocins, glucosyl esters of crocetin, are uncommon water-soluble carotenoids and represent the yellow pigments of saffron. Picrocrocin [$C_{16}H_{26}O_7$, 4-(β-D-gluco-pyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde], the glucoside precursor of safranal, is responsible for saffron's bitter taste (*9*). Safranal ($C_{10}H_{14}O$, 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), the main component of the distilled essential oil of saffron, is a monoterpene aldehyde, responsible for its characteristic aroma (*9*). Besides safranal, other major constituents of saffron's aroma are 3,5,5-trimethyl-2-cyclohexene-1-one (isophorone), 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC), 3,5,5-trimethyl-3-cyclohexen-1-one (an isomer of isophorone), 2,6,6-trimethyl-2-cyclohexene-1,4-dione, and 2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde (an isomer of safranal) (*10*, *11*).

The drying methods, applied to reduce the moisture of *C. sativus* L. stigmas to 10−12% and thus produce commercial saffron, include toasting (Spain and Iran), sun-drying (India) (*12*), and putting the stigmas into specific dark rooms for 9−10 h at 30−35 °C, which is the traditional method practiced in Greece. During the drying process changes occur in terms of color, taste, and especially aroma. Concerning color, a relatively low temperature (<30 °C) leads to a longer drying period and a poorer quality material because of prolonged enzymatic activity resulting the biodegradation of crocins. On the other hand, drying at a higher temperature (>60 °C) takes less time, but thermal degradation of pigments results in a poor-quality product (*12*). Besides temperature during the processing period, increasing relative humidity and an a_w value within the range

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of 0.11−0.64 during the storage period accelerate the rate of pigment decomposition (*13*).

Safranal occurs either from picrocrocin by acid hydrolysis or by the action of β-glucosidase producing HTCC, which converts to safranal after dehydration by heating (*14*, *15*). Alonso et al. (*16*) mentioned that the difference in the safranal content of samples of different origin might be due to the dissimilar drying processing methods. Also, many other volatiles are generated from the degradation of saffron carotenoids. Tarantilis and Polissiou (*10*) mentioned that several volatile constituents of saffron's essential oil were presumably degradation components of saffron's lipophilic carotenoids such as zeaxanthin, resulting from the action of heat and oxygen on these ingredients.

Very recently, freeze-drying has been used to dry a large number of foodstuffs such as spices, coffee, fruits, and other high-quality foods. The advantage of this technique is due to the absence of liquid water from the final product, and thus microbial reactions and deterioration are completely stopped without the loss of basic constituents (*17*). Also during the drying process the temperature is below zero. Apparently, the freeze-drying method can be also applied to *C. sativus* L. stigmas.

Coloring strength (crocins), bitterness (picrocrocin), and safranal are determined commercially according to ISO 3632-2 (*18*) by measuring their absorbances in aqueous solution at 440, 257, and 330 nm, respectively. However, in the case of safranal this method has a big disadvantage because safranal is not very soluble in water. In addition, at 330 nm, the *cis*-crocin isomers also absorb (*9*). Thus, the absorption value for safranal must be erroneous with overestimated values. Several alternative methods have been used for the determination of safranal such as gas chromatography (GC), gas chromatography−mass spectrometry (GC-MS), liquid chromatography (LC), and liquid chromatography−mass spectrometry (LC-MS) (*9*−*11*, *19*−*24*). Due to the volatile nature of the sample the best technique is gas chromatography.

The classic method for the isolation of volatile constituents from plant materials is hydrodistillation. Several other methods have also been applied, for example, microsimultaneous hydrodistillation−extraction (MSDE), vacuum headspace (VHS) (*10*), supercritical fluid extraction (*19*), thermal desorption (*20*), and extraction with organic solvents (*9*, *21*, *25*). In recent years, ultrasound-assisted extraction (USE) has been used for the isolation of bioactive principles from plant materials using organic solvents (*26*). Ultrasound has been used for the isolation of aroma compounds from aromatic plants and foods at room temperature (*27*, *28*).

The aim of this work was to determine the volatile constituents and the content of safranal and HTCC in Greek saffron, with respect to the drying processing and storage period at 4 °C by GC-MS and GC-FID analysis. Saffron was dried by the traditional method, whereas stigmas were dried by freeze-drying. The isolation of the volatile constituents was performed using USE with organic solvent and MSDE.

MATERIALS AND METHODS

Samples. Stigmas of *C. sativus* L. harvested in the years 1998, 1999, 2001, and 2002 were dried by the traditional method. The samples of stigmas were kept at 4 °C in the absence of light until their analysis in 2003.

Freshly harvested stigmas in 2002 were freeze-dried using a Christ Alpha 1-2 freeze-dryer. After the freeze-drying process, the stigmas were kept at −18 °C in the absence of light until their analysis in 2003.

Dried and fresh stigmas were provided directly from the "Cooperative of saffron, Krokos Kozanis" in order to avoid the case of adulteration of commercial samples. The stigmas were randomly taken from different producers and fields.

Standards. Safranal with a purity of 98% was supplied by IES. Three series of safranal standard solutions in diethyl ether (seven samples each at 0.5, 5.5, 10.4, 15.7, 20.6, 25.5, and 30.7 mg/5 mL, respectively) were prepared.

Isolation of the Essential Oil. *MSDE.* MSDE was carried out in a Likens−Nickerson apparatus (in low-density solvent configuration) (*29*, *30*). The extraction solvent was diethyl ether (5 mL). The sample flask was charged with 5 g of stigmas of red Greek saffron in 50 mL of deionized water. The MSDE procedure was carried out for 2 h. For the condensation of the steam the condenser was cooled with a solution of water/glycol (−10 °C). The volume of the final collected samples in diethyl ether was 5 mL.

USE. USE was performed in a Sonorex, Super RK 255H type (300 × 150 × 150 mm internal dimensions) ultrasound water bath (indirect sonication), at the fixed-frequency of 35 kHz. The temperature of the sonicated water bath was 25 °C. The sample flask was charged with 5 g of saffron as in the MSDE procedure. The solvent system extractant was 100 mL of water/diethyl ether (1:1, v/v). Each saffron sample was sonicated five times for 10 min (five fractions per saffron sample). For each sonication a new volume of the solvent system extractant was added in the sample flask. After the end of each sonication, the solvent system extractant was introduced in a separation funnel, and 20 mL of a saturated solution of NaCl was added. The funnel was well shaken and then left to rest. When the two layers were separated, the organic layer was collected. The water layer was washed with another 20 mL of diethyl ether. The whole organic extract was introduced again in the separation funnel and washed with 20 mL of a saturated solution of NaCl. The organic extract was concentrated by a gentle flow of nitrogen up to 5 mL volume, and a minor quantity of anhydrous magnesium sulfate was added. The volume of the final aromatic extract in diethyl ether was 5 mL.

Gas Chromatography (GC). A Hewlett-Packard 5890 series II chromatograph equipped with a flame ionization detector (FID) and an HP-5ms capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with helium as carrier gas at 1 mL/min was used for the analysis of saffron's essential oil. Column temperature was initially kept for 3 min at 50 °C, then gradually increased to 180 °C at a rate of 3 °C/min, and finally increased to 250 °C at a rate of 15 °C/min and kept for 5 min. The injector and detector temperatures were set at 220 and 290 °C, respectively. One microliter of the sample was injected manually in the splitless mode.

Gas Chromatography−Mass Spectrometry (GC-MS). The analysis was performed under the same conditions as GC, using a Hewlett-Packard 5890 series II chromatograph equipped with a 5972 series mass selective detector (MSD) in the electron impact mode (70 eV).

RESULTS AND DISCUSSION

Qualitative Analysis. The chemical composition of the volatiles of saffron obtained by the two isolation methods was determined by GC-MS. Volatile constituents were tentatively identified by comparing their elution order and mass spectra with data from the NBS75K mass spectra library and published data (*10*, *11*, *25*, *31*−*35*). **Table 1** demonstrates the volatile constituents in a characteristic sample of saffron's essential oil (saffron harvested in 2002 and dried by the traditional method) and in stigmas (harvested in 2002) previously dried by freeze-drying, obtained by MSDE and by USE, and their corresponding relative percentage.

In the case of saffron dried by the traditional method, the main volatile constituents were present in the extracts isolated by both methods, but quantitative differences in their relative amounts were observed. The percentage of safranal was reduced from 64.7% using MSDE to 12.1% using USE. HTCC was determined in the percentage of 0.1% by MSDE, whereas by

Table 1. Tentatively Identified Volatile Constituents of Saffron's Essential Oil Obtained by MSDE and by USE and Their Corresponding Relative Percentages

			isolation method				
			MSDE %[b]		USE %[c]		
compd	KI[a]	volatile compound	TS[d]	FS[e]	TS	FS	identified by
1	873	(2-furanyl)-1-ethanone	–[f]	3.0	–	2.7	NBS75K[h]
2	939	β-myrcene	tr[g]	–	–	–	NBS75K, (*31*)
3	971	1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene (limonene)	2.9	–	–	–	NBS75K, (*31*)
4	984	3,5,5-trimethyl-3-cyclohexen-1-one (isomer of isophorone)	2.4	23.5	0.1	0.7	NBS75K, (*31*)
5	1004	2-methylene-6,6-dimethylcyclohex-3-ene-1-carboxaldehyde	–	–	tr	–	(*11*)
6	1005	2,2-dimethylcyclohexane-1-carboxaldehyde	0.3	–	0.1	–	(*11*)
7	1035	3,7-dimethyl-1,6-octadien-3-ol (linalool)	–	–	0.1	–	NBS75K
8	1042	2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde (isomer of safranal)	2.3	1.7	0.3	–	(*10*)
9	1058	phenylethyl alcohol	–	–	3.9	3.9	NBS75K
10	1058	3,5,5-trimethyl-2-cyclohexen-1-one (isophorone)	14.3	8.5	3.9	3.9	NBS75K
12	1080	2,6,6-trimethyl-2-cyclohexene-1,4-dione (4-ketoisophorone)	4.0	3.1	2.1	2.2	NBS75K, (*31*)
13	1085	3,3,4,5-tetramethylcyclohexan-1-one	–	0.6	–	–	(*10*)
14	1098	2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one	0.9	1.5	0.2	0.4	(*35*)
15	1104	2,2,6-trimethyl-1,4-cyclohexanedione	1.0	0.2	4.0	0.7	NBS75K, (*10*), (*11*)
16	1131	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal)	64.7	52.4	12.1	0.3	NBS75K
17	1151	4-methylene-3,5,5-trimethylcyclohex-2-enone (isophorone-4-methylene)	1.8	0.9	0.8	–	(*31*)
18	1163	2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	0.1	–	–	–	(*25*)
19	1225	unknown	–	–	–	3.7	
20	1226	2,6,6-trimethyl-3-oxo-1-cyclohexene-1-carboxaldehyde	–	–	0.5	–	(*10*)
21	1231	unknown	0.1	0.1	-	5.0	
22	1237	isomer of 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one	–	–	8.4	3.8	(*10*)
23	1241	2,3-dihydroxy-1,4-naphthochinon	0.1	–	–	–	(*11*)
24	1246	2-hydroxy-3,5,5-trimethyl-4-methylenecyclohex-2-en-1-one	tr	–	–	–	(*11*)
25	1258	4-hydroxy-2,6,6-trimethyl-3-oxocyclohex-1-ene-1-carboxaldehyde	0.2	0.1	2.2	5.4	(*11*)
26	1273	1-(but-2-enylidene)-2,6,6-trimethylcyclohex-2-ene [megastigma-4,6(*E*),8(*E*)-triene]	0.1	0.1	–	–	NBS75K
27	1294	8-hydroxylinalool	–	–	0.2	–	(*28*)
28	1298	4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde	0.6	0.1	5.9	16.8	(*11*)
29	1307	unknown	–	–	4.6	10.3	
30	1320	4-(2,2,6-trimethylcyclohexan-1-yl)-3-buten-2-one	0.7	0.5	–	–	(*10*)
31	1327	4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC)	0.1	-	35.2	36.8	(*11*), (*25*)
32	1337	3-(but-1-enyl)-2,4,4-trimethylcyclohex-2-en-1-ol	tr	0.2	–	–	(*11*)
33	1342	3-(but-1-enyl)–2,4,4-trimethylcyclohexan-1-ol	tr	-	–	–	(*11*)
34	1375	4-(2,6,6-trimethyl-1-cyclohexene-1-yl)-3-buten-2-one (β-ionone)	0.1	2.66	–	–	NBS75K
35	1379	1-(4-hydroxy-2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-one (3-hydroxy-β-damascone)	–	–	tr	–	(*32*)
36	1382	5-(buta-1,3-dienyl)-4,4,6-trimethylcyclohexa-1,5-dien-1-ol	tr	–	–	–	(*11*)
37	1382	(*E*)-4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]-heptan-1-yl)but-3-en-2-one	–	–	4.2	1.6	(*32*)
38	1490	4-(3-hydroxybut-1-enyl)-3,5,5-trimethylcyclohex-3-enol (3-hydroxy-β-ionol)	–	–	0.1	–	(*33*)
39	1503	4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-ol (3,4-didehydro-β-ionol)	–	–	0.1	–	(*33*)
40	1518	4-(3-hydroxy-but-1-enyl)-3,5,5-trimethylcyclohex-3-ene-1,2-diol (megastigma-5,7-diene-3,4,9-triol)	–	–	tr	–	(*34*)
41	1534	unknown	–	–	0.2	–	(*34*)
		total %	96.7	99.2	89.5	98.2	

[a] Kovats indices on nonpolar HP-5ms column in reference to *n*-alkanes. [b] Microsimultaneous steam distillation–extraction. [c] Ultrasound-assisted extraction. [d] Saffron dried according to the traditional Greek method. [e] Freeze-dried saffron. [f] Not detected. [g] Relative percentage < 0.05%. [h] Mass spectral library of the GC-MS system.

USE this component was determined in the percentage of 35.2% (**Table 1**). Moreover, the relative percentage of safranal ranged between 50 and 70.8% and between 8.7 and 41.2% in all samples obtained by MSDE and USE, respectively. HTCC was detected in small amounts in all samples obtained by MSDE. HTCC ranged between 7.3 and 50.3% in all samples in the case of USE. In the case of the freeze-dried stigmas, the percentage of safranal was reduced from 52.4% using MSDE to 0.3% using USE. HTCC was not detected in freeze-dried stigmas when MSDE was the isolation method. Interestingly, HTCC content in the USE-extracted freeze-dried stigmas was determined at 36.8%.

In addition to the above quantitative differences, **Table 1** shows qualitative differences among the two isolation methods and the two drying methods. In the case of stigmas dried by the traditional method, 24 volatile compounds were identified in the essential oil obtained by MSDE and 25 in the essential oil obtained by USE. In stigmas previously dried by freeze-drying, 17 volatile compounds were identified in the essential oil obtained by MSDE and 16 in the essential oil obtained by USE.

Concerning the stigmas dried by the traditional method and obtained by MSDE as the isolation method, safranal (compound **16**) was the main volatile component followed by isophorone (compound **10**), an isomer of isophorone (compound **4**), and an isomer of safranal (compound **8**). When USE was performed, HTCC (compound **31**) was the main volatile component, followed by safranal, 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde (compound **28**), and isophorone. Isomers of safranal and isophorone were isolated in low percentages.

In stigmas dried by freeze-drying when MSDE was the isolation method, safranal was the main volatile component followed by the isomer of isophorone, isophorone, and the isomer of safranal. HTCC was not detected. When USE was performed, HTCC was the main volatile component followed

Table 2. Mass Spectral Data of Compounds **19**, **21**, and **29**

compd	prominent MS peaks
19	83 (100), 112 (90), 43 (90), 41 (81), 98 (77), 55 (72), 69 (43), 97 (36), 56 (36), 111 (25), 70 (20), 67 (19), 125 (16), 140 (3)
21	82 (100), 111 (50), 170 (38), 41 (32), 67 (21), 123 (18), 138 (17), 53 (17)
29	83 (100), 43 (55), 98 (40), 55 (36), 71 (16), 169 (10), 111 (10), 151 (7), 125 (7), 137 (2), 180 (1)

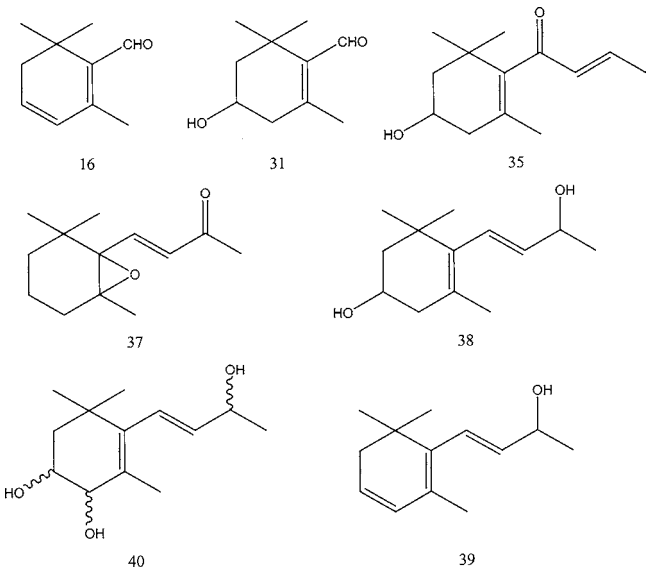

Figure 1. Structure data of safranal, HTCC, and the C_{13}-norisoprenoids found for the first time in saffron's essential oil.

by 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-diene-1-carboxaldehyde, isophorone, an isomer of isophorone, and safranal in decreasing order. In addition, an isomer of safranal was not detected. Also, in this case three constituents (compounds **19**, **21**, and **29**) were detected in considerable amounts and their spectral data are presented in **Table 2**.

Independently of the drying or isolation technique, 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (compound **14**), a known volatile compound of saffron, has been detected in all extracts, in percentages ranging between 0.2 and 1.5%. This compound is a character-impact component of saffron, as reported by Cadwallader et al. (*35*), which was detected at the highest $\log_3$FD factor, followed closely by safranal.

In the literature (*10*, *11*, *36*) it has been reported that several volatile constituents of saffron come from the thermal degradation of lipophilic carotenoids. Such constituents as 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (compound **14**), 2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione (compound **18**), and an isomer of 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one (compound **22**) belong to the C_9 group of compounds (*36*). In the C_{13} group of compounds, which are called C_{13}-norisoprenoids, several constituents have been identified in the essential oil of saffron studied in this work (**Table 1**). Megastigma-4,6(*E*),8(*E*)-triene (compound **26**), 4-(2,2,6-trimethylcyclohexan-1-yl)-3-buten-2-one (compound **30**), 3-(but-1-enyl)-2,4,4-trimethylcyclohex-2-en-1-ol (compound **32**), 3-(but-1-enyl)-2,4,4-trimethylcyclohexan-1-ol (compound **33**), β-ionone (compound **34**), and 5-(buta-1,3-dienyl)-4,4,6-trimethylcyclohexa-1,5-dien-1-ol (compound **36**) were previously identified to be saffron's constituents (*10*, *11*, *35*). Except for the above, additional C_{13}-norisoprenoids were also detected in saffron's essential oil (**Figure 1**), which has been reported in the literature to participate in essential oil composition of other origins. Compound **35** belongs to the 3-hydroxy-5-megastigmenes and has been tentatively identified as 3-hydroxy-β-damascone due to the presence of *m*/*z* 43, 69, 123, and 177 (*32*). Compound **37** belongs to the 5,6-epoxy-9-oxo compounds. Its mass spectrum presented the characteristic basic ion at *m*/*z* 123 and the molecular ion $[M]^+$ 208, and it has been tentatively identified as (*E*)-4-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl)but-3-en-2-one (*32*). Both of the aforementioned compounds were found also in tobacco. Compounds **38** and **39**, which were tentatively identified as 3-hydroxy-β-ionol and 3,4-didehydro-β-ionol, respectively, were found in quince fruit (*Cydonia oblonga* Mill.) (*33*). Also, compounds **40** and **41** are reported to be C_{13}-norisoprenoids of *Vitis vinifera* grapes (*34*). The first one was named megastigma-5,7-diene-3,4,9-triol, whereas to the second one no name was given. The mass spectral data of compound **41** were *m*/*z* 41 (23), 43 (82), 55 (14), 65 (9), 77 (14), 79 (15), 91 (20), 105 (19), 107 (11), 109 (21), 121 (15), 131 (16), 147 (12), 157 (6), 175 (22), *193* (100), 194 (15), *208* (7).

Generally, the GC-MS results verified that the volatile constituents of saffron's essential oil belong to two groups. As Cadwallader (*36*) mentioned, these compounds are the liberated aglycons of glycosidic aroma precursors of saffron. These glycosides are generated from the degradation of lipophilic carotenoids such as zeaxanthin. The first group involves constituents having structures that bear a distinct similarity to that of safranal and are reported also as isophorone-related compounds (C_9 and C_{10} group of compounds) (*11*, *25*). It was suggested by Zarghami and Heinz (*25*) that isophorone-related compounds might be formed through the oxidation and decarboxylation of safranal. Furthermore, they suggested that those compounds could be formed enzymatically. The second one (C_{13}-norisoprenoids) involves the constituents with a partially unsaturated C_4 chain in the 1-position, which are generated from lipophilic carotenoids as well (*10*, *11*). As Rödel and Petrzika (*11*) mentioned, the existence of these compounds may be explained by cleavage of the double bonds of the polyene chain in positions removed from the ionone ring. It is possible that stepwise terminal degradation of carotenoids takes place. Tarantilis and Polissiou (*10*) mentioned that these constituents result from heat and oxygen during the distillation process. The detection of a number of constituents from freeze-dried stigmas after the application of MSDE verified this hypothesis (compounds **25**, **29**, **31**, and **33**, **Table 1**). During the freeze-drying process the temperature was kept very low (−45 °C), so these constituents must be the products of heat in the MSDE procedure. Also, several of these constituents (compounds **34** and **36**−**40**, **Table 1**) were isolated from traditional saffron by the use of USE at room temperature. Thus, it can be assumed that these resulted from the degradation of carotenoids during the drying process of the stigmas, at temperatures of 30−35 °C. These constituents contribute to the aroma of saffron, but they were detected in small amounts. On the contrary, the main bulk of saffron's essential oil is composed by the C_9 and C_{10} group of compounds.

Quantitative Analysis. Safranal was isolated in different contents by both MSDE and USE, whereas HTCC was isolated in considerable amounts only by USE, so a quantitative determination of these compounds was performed by GC-FID.

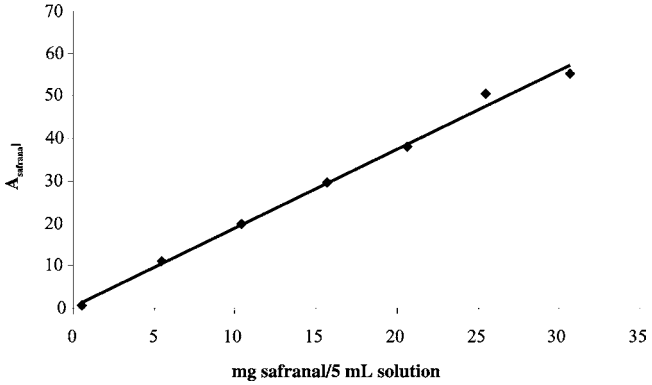

Figure 2. Calibration curve of safranal.

Table 3. Safranal and HTCC Content in 100 g of Saffron (Dried by Traditional Method) in Each Saffron Sample

		safranal (mg/100 g)		HTCC (mg/100 g)
year	sample	MSDE[a]	USE[b]	USE
1998	A1	575.4	197.2	118.8
	A2	554.1	188.9	117.0
	A3	494.4	208.2	102.0
	av	541.5 ± 42.0	198.1 ± 9.7	112.6 ± 9.2
1999	B1	397.9	132.8	46.4
	B2	343.0	109.0	108.9
	B3	302.5	111.3	71.9
	B4	288.1	70.9	41.7
	av	332.9 ± 49.2	106.0 ± 25.7	67.2 ± 30.8
2001	C1	657.6	647.7	170.2
	C2	313.6	40.7	146.5
	C3	441.6	105.8	53.1
	C4	687.9	491.8	125.9
	av	525.2 ± 178.7	321.5 ± 294.8	123.9 ± 50.6
2002	D1	430.4	68.4	143.3
	D2	294.8	73.9	376.2
	D3	359.9	97.9	397.7
	D4	234.4	58.2	336.9
	D5	512.7	158.0	154.7
	av	366.4 ± 109.7	114.1 ± 40.0	301.8 ± 123.2
	D6[c]	254.2	1.6	296.7

[a] Microsimultaneous steam distillation–extraction. [b] Ultrasound-assisted extraction. [c] Stigmas dried by freeze-drying.

A calibration curve (**Figure 2**) was established for the series of safranal standards as the function of safranal's peak area ($A_{safranal}$ = safranal's peak area/10^6 in GC-FID chromatograms) and safranal's content. The empirical equation of calibration curve for safranal was $A_{safranal} = (1.85 \pm 0.06) \times$ (mg of safranal) + (0.6 ± 1.1), and the R^2 multiple correlation was 0.995.

On the basis of the above calibration curve, equation, and GC-FID analysis, the content of safranal was estimated for each isolated aromatic extract (5 mL of etheric solution) from 5 g of saffron using the two isolation methods (MSDE and USE). To calculate the amount of HTCC of each aromatic extract, safranal's calibration curve was used as the two compounds have similar chemical structures. The above collected data were used for the calculation of safranal and HTCC content in 100 g of saffron (**Table 3**).

According to the literature, using MSDE the major amount of saffron's essential oil is obtained after 2 h (*10*, *29*, *30*). The kinetic study of USE was performed by the GC-FID analysis and the above empirical equation. After five extractions (total time = 50 min), the major amount of essential oil is obtained (98% expressed as safranal) (**Figure 3**). At the same time, after five extractions 98% of HTCC was obtained (**Figure 3**). Comparing the results of three kinetic studies tested the repeatability of the USE extraction procedure. The relative standard deviation (RSD) for safranal was 12.8% and for HTCC was 7.9%.

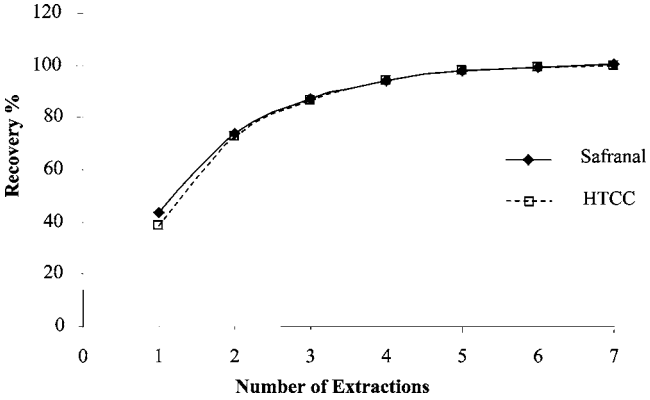

Figure 3. Kinetic study of the isolation of safranal and HTCC, using ultrasound-assisted extraction.

The amounts of safranal obtained by MSDE and USE were 288.1−687.9 and 40.7−647.7 mg/100 g of saffron, respectively. The amount of HTCC obtained by USE was 41.7−397.7 mg/100 g of saffron (**Table 3**). Also, **Table 3** shows the average safranal and HTCC contents per year. The amount of safranal remained more or less constant, increasing slightly over years of storage at 4 °C. Alonso et al. (*20*) mentioned that the amount of safranal in Spanish saffron was 206.47 mg/kg when a thermal desorption method was applied. In addition, they mentioned (*16*) that the safranal content (parts per thousand of dry matter) varied between 1.07 and 5.67 in samples analyzed by the thermal desorption−gas chromatography (TD-GC) technique. In all of our samples the quantity of safranal in the oil isolated by the USE is lower than that oil isolated by MSDE, but it follows the same trend as in the MSDE. On the other hand, in the USE extracts HTCC was detected in considerable amounts. The amount of HTCC was lower than the amount of safranal overall, slightly decreasing over years of storage (2002, 2001, 1999, and 1998 harvests). In freshly harvested saffron (2002) the amount of HTCC was higher than safranal (**Table 3**). It is reported that HTCC was converted to safranal enzymaticaly during the storage period even at 4 °C (*12*). So safranal, except in the year 2002, was found in larger amounts in Greek saffron's essential oil and not equal to or less than HTCC as found in the literature for Spanish samples (safranal, 1.49 ± 0.02 mg/g; HTCC, 3.06 ± 0.05 mg/g) (*19*, *23*). From the above results, it can be assumed that the greater the original amount of HTCC, the greater the amount of safranal that can be produced, and it remains in the stigmas over years of storage at 4 °C.

On the other hand, it seems that HTCC was not converted to safranal during USE because of the mild temperature conditions. To verify that, stigmas of *C. sativus* L. flower were freeze-dried instead of dried by the traditional method. These stigmas had a bright red color, and their shape did not change. Those stigmas were subjected to both methods for the isolation of the volatile fraction. From GC-FID analysis it emerged that in the saffron volatile fraction isolated by USE the relative percentage of safranal was 0.3%, (1.6 mg/100 g of stigmas), whereas the relative percentage of HTCC was 36.8% (296.7 mg/100 g of stigmas). In saffron essential oil isolated by MSDE, HTCC was not detected, whereas the relative percentage of safranal was 52.4% (254.2 mg/100 g of stigmas), obviously produced by HTCC conversion due to the high temperature of the distillation

(**Table 3**). The above observation was verified by the extraction of saffron's distillation residue with diethyl ether (saffron dried by the traditional method). Safranal and HTCC were detected in very low amounts.

Obviously, during the freeze-drying process safranal is not generated from HTCC as happens during the traditional drying method. The amount of HTCC obtained by USE corresponds to the amount already present in the stigmas (*14*). In the case of MSDE, safranal was produced from HTCC due to the high temperature (*19*, *20*). Saffron's aroma was enriched with safranal during distillation because of HTCC dehydration at high temperature. USE extracts contained both safranal and HTCC, which had not been converted to safranal because of the mild temperature conditions of USE extraction. Consequently, after the USE extraction, safranal estimates reflect the real amount of safranal content in saffron samples, as no additional safranal was produced. The application of USE does not affect safranal. Safranal standard solutions were sonicated under the same conditions (35 kHz, 25 °C) as saffron samples, and the GC results did not show any change.

According to the literature (*37*) HTCC converts not only to safranal but also to 4-methylene-3,5,5-trimethylcyclohex-2-enone (compound **17**), with safranal being an intermediate product. Compound **17** (**Table 1)** was detected in all extracts obtained by the MSDE method, in percentages of 0.9−1.8%. It was also detected in the USE extract from saffron dried by the traditional method (0.8%), whereas it was absent from the corresponding extract of the freeze-dried stigmas. It seems that HTCC was converted to 4-methylene-3,5,5-trimethylcyclohex-2-enone due to the temperature of the distillation procedure or to the traditional method applied for the drying of the material.

Table 3 suggests it is difficult to compare the amounts of safranal and HTCC of saffron samples from different harvest years as the amount depends not only on storage temperature but also on the climatic conditions, soil, and overall drying temperature. However, it can be easily observed that the quantity of safranal isolated from all samples remained mostly constant, increasing slightly over years of storage when the samples were kept at 4 °C while the quantity of HTCC decreased during the same period.

With respect to the drying process, the freeze-drying method can be applied in saffron without the loss of its basic constituents. In that case HTCC is the major compound of the isolated essential oil and not safranal, which is present in minor amounts. However, when a freeze-dried saffron sample is heated or distilled, during that process, HTCC is easily converted to safranal.

In conclusion, the aroma of Greek saffron has been characterized by two major categories of volatile constituents. The first one involves constituents having structures bearing a distinct similarity to that of safranal, and the second one involves the volatiles, which are generated by the degradation of lipophilic carotenoids and are called C_{13}-norisoprenoids. From the second one, five volatile constituents were reported for the first time in saffron's aroma. With respect to the isolation method, by the MSDE technique safranal was the major volatile constituent, whereas in the USE technique HTCC was also a major volatile constituent in samples dried by the traditional method. Concerning the drying processes, freeze-drying can be applied to *C. sativus* L. stigmas as an alternative drying method, without the loss of the basic aroma constituents. Greek saffron is rich in safranal. The quantity of component isolated by MSDE ranged between 288.1 and 687.9 mg/100 g of saffron, whereas in the case of USE safranal and HTCC ranged between 40.7 and 647.7 mg/100 g of saffron and between 41.7 and 397.7 mg/100 g of saffron, respectively, in samples dried by the traditional method. From the freeze-dried stigmas the quantity of safranal isolated by MSDE was 254.2 mg/100 g of stigmas, whereas in the case of USE the quantities of safranal and HTCC were 1.6 and 296.7 mg/100 g of stigmas, respectively. The quantity of safranal isolated from all samples remained mostly constant, increasing only slightly over years of storage at 4 °C (1−5 years) while the quantity of HTCC decreased over the same storage periods. Finally, for compounds **19**, **21**, and **29** further work has to be performed for their identification.

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