Variation in Headspace Volatiles of Saffron Determined by GC×GC-ToF-MS

Busisiwe N. Zwane¹, Guy P. Kamatou¹, Alvaro M. Viljoen², Georges Betti³, and Mathias Schmidt⁴

Abstract
Saffron, obtained from the stigmas of Crocus sativus L. (Iridaceae), is the most expensive spice traded worldwide. In addition to its culinary uses, various medicinal properties have been confirmed for saffron, which has recently captured the interest of the phytotherapy industry. The quality of saffron is determined by the presence of 3 compounds, namely, crocin, picrocrocin, and safranal, with the latter being responsible for the distinct aroma characteristic of saffron. To determine the volatiles and assess possible geographical variation, headspace analysis using 1-dimensional and 2-dimensional gas chromatography (GC) on 26 samples collected from 9 countries was undertaken. The major constituents identified include safranal, 4-ketoisophorone, acetic acid, 2(5H)-furanone, and 1,4-cyclohexanedione-2,2,6-trimethyl. Quantitative rather than a qualitative variation was noted in the samples from different origins. The levels of safranal ranged from 22.1% to 62.4%. This study represents the first report on the headspace volatiles of saffron using GC×GC-time-of-flight-mass spectrometry and clearly demonstrates the superior chromatographic potential of 2-dimensional GC compared with conventional 1-dimensional GC.

Keywords
Crocus sativus, saffron, safranal, GC, GCxGC, spice

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Saffron is obtained from the stigmas of Crocus sativus L. (Iridaceae), a Mediterranean Crocus species with a mysterious origin.¹ Pharmacological studies on saffron have confirmed various biological properties such as antihypertensive,² anticonvulsant,³ and antitussive activities.⁴ Other biological activities of saffron include its effects on sexual behavior⁵ and its anxiolytic⁶ and relaxant properties.⁷ The antinociceptive and anti-inflammatory activities of saffron extracts have also been reported.⁸ In clinical studies, positive results have been demonstrated in the treatment of depression,⁹,¹⁰ Morbus Alzheimer,¹¹,¹² and sexual dysfunction in women and men.¹³,¹⁴ Furthermore, antigenotoxic and cytotoxic effects of saffron have been evaluated, and results indicated that saffron is not toxic.¹⁵ Regardless of these promising medicinal properties, saffron is still primarily used as a coloring and flavoring substance in food.¹⁶

Due to its popularity, tedious, and labor-intensive cultivation, it ranks as the most expensive spice in the marketplace. The majority of saffron on the world markets stems from the region of Khorasan (Eastern Iran/Western Afghanistan), with further noteworthy cultivations in northern Africa (Morocco and Tunisia), India, and, to a smaller scale, in France and China. Albeit Spain has lost most of its saffron-producing capacities, considerable quantities of saffron are traded on the world market as “Spanish saffron” to justify higher prices. Next to testing the applicability of a gas chromatography (GC)×GC–time-of-flight (ToF)-mass spectrometry (MS) method for quality control, we wanted to test whether regional and climatic differences are reflected in the composition of saffron constituents from sources with a traceable origin.

No single compound gives a food product its distinctive flavor and aroma, but it is the combination of volatile compounds that collectively impart the unique flavor and fragrance which defines a specific culinary herb or spice. The quality of saffron is determined by the ISO 3632 standard (1993)¹⁷ and is based on the levels and relative ratios of crocin (color), safranal

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(aroma), and picrocrocin (taste) determined by photometric methods. A high amount of these compounds is used as a parameter to benchmark for quality.\textsuperscript{18} The chemical composition of saffron may not only be geographically variable as saffron is cultivated in a range of climatic conditions which may impact the quality. This variation may also be a function of parameters such as the harvesting protocol, the drying method, and storage conditions.\textsuperscript{19-21}

Many chromatographic techniques have been used to isolate and identify the nonvolatile saffron compounds or to determine its volatile constituents which include high-performance liquid chromatography,\textsuperscript{22,23} GC coupled to MS (GC-MS),\textsuperscript{24,25} GC coupled to flame ionization detector.\textsuperscript{25,26} Headspace analysis has been widely used to generate flavor profiles for a variety of foods and spices. This technique is simple, non destructive, clean, and can be applied to both liquid and solid matrices. Usually, only the volatile fraction is sampled and transferred to conventional GC (1-dimensional GC) for analysis.\textsuperscript{7-20} However, this technique is less sensitive, and separation of isomers may not be achieved.

In this study, headspace analysis by 2-dimensional GC coupled to MS (GC×GC-ToF-MS) was undertaken to achieve a comprehensive analysis of saffron volatiles. Results obtained from various samples sourced from different countries are compared with compositional data generated by conventional 1-dimensional GC.

**Materials and Methods**

**Sample Collection**

Twenty-six samples obtained from 9 countries, including Afghanistan, Algeria, China, France, Greece, India, Iran, Morocco, and Spain (Table 1), were selected for this study. The samples were supplied by Dr Mathias Schmidt and Georges Betti.

**Sample Preparation**

Saffron stigmas were ground into a fine powder. The powdered saffron samples (0.5 g) were weighed into the 20 mL headspace vials for analysis. The headspace analysis is a solvent-free technique aimed at sampling the gaseous or vapor phase in equilibrium (or not) with a solid phase in order to characterize its composition.\textsuperscript{27-29} However, this technique is less sensitive, and separation of isomers may not be achieved.

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**GC×GC-ToF-MS, GC-ToF-MS, and Headspace Analysis Procedure**

**GC×GC-ToF-MS and headspace analysis procedure.** The GC×GC system consisted of a multipurpose sampler (Gerstel) which was operated in the headspace mode. The system was equipped with a 1000 μL gas syringe and a tray for 20 mL vials (Gerstel). Each sample was heated and agitated at the same time for 5 minutes, at 50 °C in the preheating module. The 26 saffron samples (0.5 g) were placed in headspace vials and were analyzed using a Leco Pegasus 4 GC×GC-ToF-MS system.

Each sample (1000 μL) was injected into the GC×GC system with a 5:1 split ratio. The system consisted of an Agilent 7890 Gas Chromatograph with a cryogenic thermal modulator (Leco) and a secondary oven. A 30 m × 0.25 mm × 0.25 μm film thickness, Rxi-5Sil MS GC capillary column, was used as the first column analysis; the second column was 0.790 m × 0.25 mm × 0.25 μm film thickness, Rxi-5Sil MS GC capillary column. Helium was used as the carrier gas at a constant flow rate of 1.50 mL/min, front inlet septum purge flow at 3 mL/min, and purge valve time 60 seconds after the beginning of the run. The inlet temperature was 200 °C, from the beginning and throughout the run. The primary column was programmed with an initial oven temperature at 40 °C for 1 minute at the rate of 10 °C/min and was ramped to 220 °C for 2 minutes. The secondary column temperature program was set to an initial temperature of 60 °C for 0.50 minutes then ramped at 10 °C/min to 240 °C for 2 minutes. The thermal modulator’s initial temperature was set at 80 °C for 1 minute at the rate of 10 °C /min and was ramped to 260 °C for 2 minutes. Both the

**Table 1. Origin of Saffron Samples Studied.**

| Codes | Origin of the samples | Year |
|-------|-----------------------|------|
| S1    | Val de Voi Touraine (France, old cultivar) | 2011 |
| S2    | Limousin (France, old cultivar) | 2012 |
| S3    | France, cultivar introduced from unknown origins | 2008 |
| S4    | Algeria (Constantine, originally from France) | 2012 |
| S5    | Sold in Spain as “Spanish saffron,” non-traceable | 2012 |
| S6    | Sold in Spain as “Spanish saffron,” non-traceable | 2009 |
| S7    | Greece (Kozani, biocultivation, old cultivar) | 2011 |
| S8    | Greece (Kozani, biocultivation, old cultivar) | 2011 |
| S9    | Greece (Kozani, biocultivation, old cultivar) | 2009 |
| S10   | Greece (Kozani, old cultivar) | 2010 |
| S11   | Afghanistan (Herat, origin Khorasan) | 2011 |
| S12   | Afghanistan (origin Khorasan?) | 2010 |
| S13   | Afghanistan (origin Khorasan?) | 2011 |
| S14   | Morocco (Taliouine, old cultivar) | 2010 |
| S15   | Morocco (Taliouine, old cultivar) | 2008 |
| S16   | Morocco (Taliouine, old cultivar) | 2009 |
| S17   | Iran (North Khorasan, old cultivar) | 2010 |
| S18   | Iran (South Khorasan, old cultivar) | 2012 |
| S19   | Iran (Tarbat/Khorasan, old cultivar) | 2009 |
| S20   | Iran (Kurdistan, old cultivar originally from Khorasan) | 2010 |
| S21   | Iran (Torbate) | 2011 |
| S22   | Iran (Ghaenat) | 2012 |
| S23   | Iran (Gonabad) | 2010 |
| S24   | India (Mogra) | 2010 |
| S25   | India (Lacha) | 2010 |
| S26   | China | 2011 |
Table 2. Chemical Constituents (% Area) of Headspace Analysis of Saffron Using 1-Dimensional and 2-Dimensional GC.

| Name                                                   | 1st Dimension (RT, s) | 2nd Dimension (RT, s) | % Area | GC×GC-ToF-MS | GC-ToF-MS |
|--------------------------------------------------------|-----------------------|-----------------------|--------|--------------|-----------|
| Pentanal                                               | 210 0.57              | 0.8                   | nd     |
| 5-(1,1-dimethylpropyl)-1,3-cyclopentadiene             | 210 0.75              | 1.3                   | 1.4    |
| 2-methyl-3-Pentanone                                   | 212 0.61              | 1.1                   | nd     |
| Hexanal                                                | 256 0.62              | 2.8                   | 0.9    |
| 2,3-Dihydro-3-methyl-Furan                            | 296 0.56              | 0.4                   | nd     |
| 3-Methyl-2-butanal                                     | 300 0.56              | 0.7                   | nd     |
| 1-Butanol                                              | 306 0.52              | 1.2                   | nd     |
| 1-Penten-3-ol                                          | 318 0.52              | 1.8                   | nd     |
| 2,3,4,5-tetramethyl-2-cyclopenten-1-one                | 324 0.76              | 0.3                   | nd     |
| Heptanal                                               | 352 0.65              | 0.2                   | 0.1    |
| 3-Methyl-2-butanol                                     | 354 0.55              | 0.1                   | nd     |
| 1-Pentanol                                             | 390 0.53              | 0.4                   | 0.2    |
| 2-Pentyl-furan                                         | 390 0.73              | 0.4                   | nd     |
| 6-Methyl-2-heptanone                                   | 392 0.67              | 0.2                   | 0.1    |
| 4,4-Dimethyl-2-cyclopenten-1-one                       | 414 0.59              | 0.6                   | 0.3    |
| Acetoin                                                | 418 0.5               | 0.4                   | nd     |
| Ethanone, 1-((2-methyl-1-cyclopent-1-yl)-              | 418 0.64              | 0.5                   | 0.1    |
| 1-Hydroxy-2-propanone                                  | 428 0.49              | 0.08                  | nd     |
| Octanal                                                | 436 0.68              | 0.1                   | 0.1    |
| 1-((1-Cyclohexen-1-yl)-ethanone                       | 450 0.63              | 0.2                   | 0.3    |
| Unidentified                                           | 456 0.68              | 0.8                   | 0.5    |
| Propanoic acid, 2-hydroxy-, ethyl ester, (S)-          | 462 0.52              | tr                    | nd     |
| Unidentified                                           | 468 0.64              | 0.4                   | 0.2    |
| Hemellitol                                             | 474 0.68              | 0.6                   | 0.2    |
| α-Phellandrene                                         | 510 0.64              | 0.3                   | 0.2    |
| 1,4-Cyclohexadiene, 3,3,6,6-tetramethyl-               | 516 0.68              | 0.3                   | 0.2    |
| Nonanal                                                | 520 0.71              | 0.3                   | 0.1    |
| β-Isophorone                                           | 522 0.67              | 1.8                   | nd     |
| 3 Furfural                                             | 530 0.5               | 0.1                   | 0.05   |
| Unidentified                                           | 538 0.64              | 0.1                   | nd     |
| Unidentified                                           | 540 0.65              | 0.5                   | nd     |
| *Arba* Cyclocitratal                                   | 546 0.69              | 0.9                   | 0.1    |
| 1-Octen-3-ol                                           | 550 0.57              | 0.1                   | nd     |
| 1-Heptanol                                             | 554 0.56              | 0.1                   | nd     |
| Acetic acid                                            | 556 0.46              | 11.0                  | 3.9    |
| 3,4,4-Trimethyl-2-Cyclohexen-1-one                     | 556 0.65              | 0.1                   | 0.1    |
| Unidentified                                           | 558 0.5               | 0.1                   | nd     |
| Unidentified                                           | 564 0.53              | 0.1                   | tr     |
| 3,4 Dimethylacetophenone                               | 568 0.66              | 0.1                   | 0.1    |
| 1H-Pyrazole, 4,5-dihydro-5,5-dimethyl-4-               | 576 0.64              | 0.1                   | 0.3    |
| 2-Acetyl-5-methylfuran                                 | 580 0.56              | 0.1                   | tr     |
| 1-Hexanoyl, 2-ethyl-                                   | 582 0.58              | tr                    | nd     |
| 5-Methyl furfural                                      | 584 0.53              | tr                    | nd     |
| 2-Caren-10-al                                          | 594 0.65              | 1.8                   | 1.8    |
| t-Decalin                                              | 598 0.57              | 0.1                   | 0.1    |
| Benzaldehyde                                           | 608 0.55              | 0.1                   | 0.1    |
| Unidentified                                           | 622 0.63              | 2.4                   | 0.9    |
| Propanoic acid                                         | 626 0.46              | 0.1                   | 0.1    |
| 3,7-Dimethyl-1,6-Octadien-3-ol                         | 626 0.6               | tr                    | nd     |
| Unidentified                                           | 630 0.49              | 0.2                   | nd     |

(Continued)
| Name                                                                 | 1<sup>st</sup> Dimension (RT, s) | 2<sup>nd</sup> Dimension (RT, s) | % Area     |
|----------------------------------------------------------------------|----------------------------------|----------------------------------|------------|
| 2,2,4,4-Tetramethyl-1,3-cyclobutanedione,                           | 630                              | 0.56                             | 0.2        |
| Isophorone epoxide                                                  | 630                              | 0.61                             | tr         |
| 1-Octanol                                                           | 632                              | 0.57                             | 0.3        |
| Unidentified                                                        | 636                              | 0.56                             | nd         |
| Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-                          | 640                              | 0.6                              | 0.1        |
| Propanoic acid, 2-methyl                                           | 644                              | 0.47                             | 0.1        |
| Unidentified                                                        | 660                              | 0.57                             | 0.3        |
| 4,4-Dimethyl-cyclohex-2-en-1-ol                                     | 660                              | 0.58                             | 0.3        |
| α-Isophorone                                                        | 664                              | 0.61                             | 2.5        |
| Ethanol, 2-(2-ethoxyethoxy)-                                        | 670                              | 0.53                             | tr         |
| Unidentified                                                        | 670                              | 0.54                             | tr         |
| Oxalic acid                                                         | 676                              | 0.44                             | 8.4        |
| Butanoic acid, 4-hydroxy-                                           | 680                              | 0.5                              | 0.3        |
| Benzaldehyde, 2-methyl                                             | 682                              | 0.56                             | tr         |
| 2-Isopropylidene-3-methylhexa-3,5-dienal                            | 688                              | 0.61                             | tr         |
| Acetophenone                                                        | 698                              | 0.56                             | tr         |
| 2,5,5-Trimethyl-1-hexen-3-ynes                                      | 700                              | 0.66                             | 0.4        |
| Safranal                                                            | 702                              | 0.64                             | 11.2       |
| 2-Decenal, (Z)                                                     | 704                              | 0.67                             | 0.2        |
| 2-Hydroxy-3,5,5-trimethyl-cyclohex-2-ene                             | 708                              | 0.59                             | 0.6        |
| Unidentified                                                        | 712                              | 0.58                             | 0.1        |
| Unidentified                                                        | 716                              | 0.59                             | 0.1        |
| 5-Hexen-2-one                                                       | 720                              | 0.5                              | tr         |
| 4-Ketoisophorone                                                    | 728                              | 0.58                             | 18.2       |
| Unidentified                                                        | 738                              | 0.61                             | 0.1        |
| 2(5H)-Furanone, 3-methyl                                            | 740                              | 0.5                              | tr         |
| Unidentified                                                        | 750                              | 0.58                             | 0.1        |
| 1,4-Cyclohexanediene-2,2,6-trimethyl                                | 752                              | 0.57                             | 0.4        |
| Unidentified                                                        | 758                              | 0.45                             | tr         |
| Benzaldehyde, 2,5-dimethyl                                          | 758                              | 0.58                             | 0.2        |
| 2,4-Cycloheptadien-1-one, 2,6,6-trimethyl-                          | 758                              | 0.62                             | 0.7        |
| 2(5H)-Furanone                                                      | 762                              | 0.48                             | 2.5        |
| Benzaldehyde, 2,5-dimethyl                                          | 762                              | 0.59                             | tr         |
| Benzenemethanol, α,α-dimethyl-                                     | 766                              | 0.53                             | tr         |
| Unidentified                                                        | 768                              | 0.61                             | tr         |
| Unidentified                                                        | 772                              | 0.58                             | 0.1        |
| 2-Undecenal                                                         | 778                              | 0.69                             | 0.1        |
| Unidentified                                                        | 784                              | 0.56                             | 2.8        |
| Unidentified                                                        | 786                              | 0.57                             | tr         |
| Unidentified                                                        | 790                              | 0.46                             | tr         |
| Unidentified                                                        | 832                              | 0.6                              | tr         |
| Hexanoic acid                                                       | 834                              | 0.48                             | 0.1        |
| Dimethyl sulfoxide                                                  | 852                              | 0.47                             | 0.2        |
| Propanoic acid, 2-methyl−1-(1,1-dimethyl-ethyl)−2-methyl-1,3-propanediyl ester | 882                              | 0.83                             | 0.3        |
| Phenylethyl alcohol                                                 | 864                              | 0.51                             | 0.2        |
| 3,5-Octadiene, 4,5-diythyl−(E,Z)                                    | 870                              | 0.59                             | 0.09       |
| Unidentified                                                        | 892                              | 0.52                             | tr         |
| Ethanone, 1-(1H-pyrrol-2-yl)-                                       | 898                              | 0.48                             | tr         |
| Unidentified                                                        | 900                              | 0.57                             | 0.3        |
| Phenol                                                              | 918                              | 0.46                             | 0.3        |

(Continued)
front inlet and transfer line temperature were constant at 200 °C and 225 °C, respectively. The total analysis time for the GC method was 21 minutes. The MS mass range was 45-400 m/z with an acquisition rate of 100 spectra/s. The ion source chamber was set at 200 °C. To confirm the identity of the major volatile constituent, safranal was purchased from Sigma Aldrich and also injected under similar conditions.

**GC-ToF-MS analysis.** For the GC-ToF-MS analysis, the procedure was the same as described above with the exception that only the 30 m × 0.25 mm × 0.25 μm film thickness capillary column (Restek) was used. The GC conditions are those described for the GC×GC analysis above with the secondary oven turned off.

**Data Processing and Analysis**

Data were processed automatically using LECO ChromaTOF software version 4.50. The minimum signal to noise ratio (S/N) cut off >500 based on “unique mass,” the most specific mass usually extracted for analyte after deconvolution of the mass spectra signal. Identification of peaks was based on NIST Mass Spectral Library (NIST 11). Library similarity factors were reported on a scale ≥800 in the MS fragmentation in the database (high match is associated with better match) for both forward and reverse search and only compounds with the similarity higher than 80% were tentatively assigned a name. The percentage area on the chromatogram and the retention time were obtained using the ChromaTOF software version. Relative abundance (% area) calculations were based on the ratio between the peak area of each compound and the sum of areas of all selected compounds.

**Results and Discussions**

In this study, headspace GC×GC-ToF-MS was applied for the first time to determine the volatile constituents of saffron from

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**Table 3.** Maximum and Minimum Relative Percentage Area of the 5 Major Volatile Constituents of Saffron From Various Origins.

| Compounds                                           | Minimum | Maximum | Average |
|-----------------------------------------------------|---------|---------|---------|
| Safranal, 2,6,6-Trimethyl-1,3-cyclohexadiene-1-carboxaldehyde | 22.1    | 62.4    | 41.2    |
| 4-Ketoisophorone, 2,6,6 trimethyl-2-cyclohexene-1,4 dione     | 5.0     | 13.0    | 8.9     |
| Acetic acid                                                  | 1.0     | 6.2     | 2.7     |
| 2(3H)-Furanone, dihydro-4-hydroxy-                          | 0.7     | 6.8     | 2.7     |
| 1,4-Cyclohexanedione-2,2,6-trimethyl                        | 1.6     | 6.8     | 4.4     |
different origins. Out of the 26 saffron samples investigated from 9 countries, quantitative rather than a qualitative variation of chemical constituents was recorded. Since no significant qualitative variation was obtained, 1 sample (from France, S2) was arbitrarily selected as a representative sample for the headspace of saffron using 1-dimensional and 2-dimensional GC presented in Figure 1. The chemical constituents on 1-dimensional and 2-dimensional GC of saffron are presented in Table 2. The major compounds detected in all the samples were safranal, 4-ketoisophorone, acetic acid, 2,2,6-trimethyl-1,4-cyclohexanedione, and 2(5H)-furanone. The amount of these compounds ranged as follows: safranal (22.1%-62.4%), 4-ketoisophorone (5.0%-13.0%), acetic acid (1.0%-6.2%), 2(5H)-furanone (0.7%-6.8%), and cyclohexanedione-2,2,6-trimethyl-1,4 (1.6%-6.8%) (Table 3, Figure 2). Safranal, one of the compounds used to determine the quality of the spice, was found in significant amounts in the samples investigated.

Saffron volatile constituents are divided into 2 groups depending on their structures and/or precursors. The first is made up of groups of compounds having structures that bear a distinct similarity as safranal, such as isophorone and 4-ketoisoprophorone. The second group, known as C_{13}-norisoprenoids, proceeds from the degradation of lipophilic carotenoids and includes the constituents with a partially unsaturated 4-hydroxy-2, 6, 6-trimethyl-3-oxycyclohexa-1, 4-diene-1-carboxyaldehyde and an isomer of safranal.
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(2,6,6-trimethyl-1, 4-cyclohexadiene-1-carboxaldehyde).\textsuperscript{31-34} Isophorone also present in saffron plays a noticeable role in the generation of new compounds during the aging process.\textsuperscript{27}

Safranal is responsible for the aroma of this spice, and the quality of saffron is based on the amount of this compound. Safranal is an important key odorant in saffron as determined by GC-MS-Olfactometry in previous studies.\textsuperscript{35} The ISO norm 3632 classifies the quality of saffron by safranal content. No valid conclusions can, however, be drawn from the safranal determination as the samples were not standardized by harvesting technique and storage time. The latter has a major impact on safranal contents.\textsuperscript{22,36} Fresh stigmas do not contain important quantities of safranal; this compound is formed during drying and storage through liberation from picrocrocin.\textsuperscript{20,37,38} Said transformation is possibly the reason why in the sequence of safranal contents the Spanish samples range higher than the Iranian and Moroccan samples, although there is a high probability that the Spanish samples were in fact just relabeled saffron from Iran or Morocco and reached the higher safranal levels only by longer storage and transit times.

A comparison between the analysis using 1-dimension and 2-dimension showed a high sensitivity of 2-dimensional chromatography with more compounds identified. Table 2 shows some compounds on 1-dimension eluted at the same retention time, while on 2-dimension, they eluted at different retention times. Furthermore, some isomers such as safranal isomer, 3-furfural (Table 2), could not be separated using 1-dimensional GC.

Based on the results obtained in this study, the different chemical constituents in saffron are highly variable as there are major quantitative differences of the same compounds from saffron obtained even from the same country.

Conclusions

Safranal was identified as the major constituent in all the samples investigated. The 26 samples analyzed showed a general similarity in chemical constituents but differed quantitatively. Such differences in a complex matrix such as saffron can be detected and quantified using the superior separation abilities of 2-dimensional GC. Unfortunately, the comparison of samples of different origins did not yield parameters and specifications typical for a given geographic origin, which might have been used to detect fraudulent labeling in saffron samples.

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