A simple protocol for metabolite extraction from saffron and its application to authentication of market samples by HPLC fingerprinting

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Summary
Being an expensive spice, saffron is highly prone to adulteration. Crocin, picrocrocin and safranal, which are commonly used as chemical markers for authentication, suffer from a lack of stability and uniqueness to saffron. Inexpensive synthetic safranal poses an additional concern in adulteration. Therefore, identification of other metabolites becomes essential for saffron authentication. Available metabolite extraction methods are laborious, time-consuming and require a large quantity of samples. We report a simple protocol for extracting the metabolites from as little as 5 mg of saffron using 100 μl of solvent in 2 h. High-performance liquid chromatography (HPLC) of the metabolites at 1 ml·min^{-1} flow rate with 70% acetonitrile for 10 min and detection at 308 nm yielded an HPLC fingerprint specific to saffron's stigma. The fingerprint was stable after at least 6 years of storage at room temperature and successfully differentiated the authentic from inauthentic market samples.

Keywords
adulteration; extraction; high performance liquid chromatography; fingerprint; safranal; saffron

Crocus sativus L., commonly known as saffron, has several culinary and medicinal uses but is the world's most expensive spice [1, 2]. Crocin, picrocrocin and safranal are the major secondary metabolites in saffron. They are responsible for its colour, taste and aroma. Although saffron is in high demand worldwide, its production is limited to very few places around the world. Iran is the primary producer of saffron, accounting for more than 90 % of the world's production [3]. Extensive labour requirements for processing, limited production and saffron's high market value make it an attractive target for adulteration [4]. It is reported to be one of the most adulterated food commodities worldwide [5]. Therefore, proper quality control and authentication of the market samples are essential to ensure the availability of genuine saffron for human consumption.

HPLC is a popular method for authentication of saffron. However, currently available protocols to extract the metabolites from saffron are labour- and time-consuming, as they involve sonication, drying in vacuum, incubation in a dark room and extraction lasting up to 24 h. These protocols also require as much as 2 g of saffron, which is prohibitively expensive, considering its market price [6, 7]. Therefore, we optimized the metabolite extraction protocol to become simple and to require less sample.

Saffron's major secondary metabolites, crocin, picrocrocin and safranal, are often used as chemical markers for authentication of saffron [8]. According to ISO 3632-1:2011 [9] and ISO 3632-2:2010 [10], saffron is authenticated by estimating the quantity of crocin, picrocrocin and safranal using the UV-Vis spectrometric absorption of light at 440 nm, 257 nm and 330 nm, respectively. However, crocin was reported to absorb light also at 257 nm and 330 nm [11]. Picrocrocin was reported to degrade and get converted to safranal [12–14]. The UV-Vis spectrometric method often over-estimates safranal content due to the presence of *cis*-crocetin esters and other compounds, which also absorb light at 330 nm [15]. Further, safranal, which is the most widely used chemical marker for saffron's authentication,

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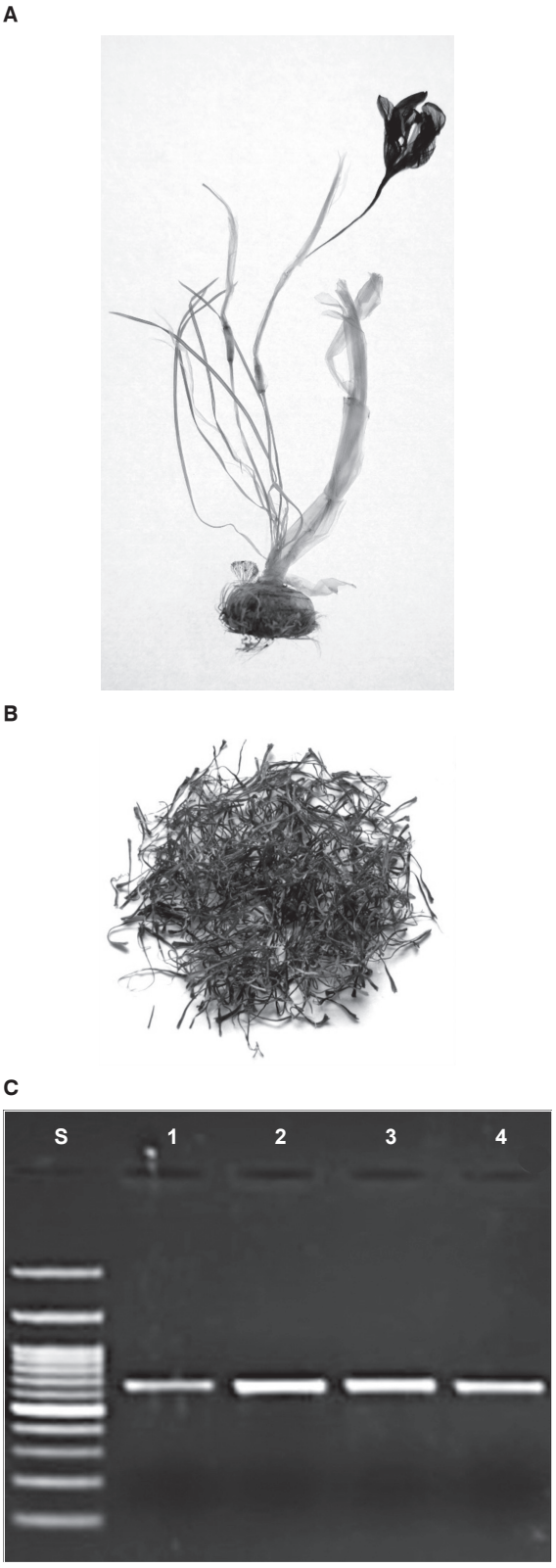

Fig. 1. Collection and authentication of reference saffron plants and stigma.

A – saffron plant, B – saffron stigma, C – agarose gel electrophoresis of DNA barcode markers.
S – DNA size marker, lanes 1–4 – rbcL DNA barcode markers from four saffron samples.

also has limitations as a marker. It is not unique to saffron, it was detected in other plants such as *Sambucus nigra*, *Citrus limon*, *Camellia chinensis, Calycopteris floribunda* and *Achillea distans* [6, 16]. It is also possible to fake saffron's authentication by adding synthetic safranal, which is more than a million times cheaper than saffron. Therefore, it becomes essential to explore other metabolites for authentication of saffron.

Sujata et al. [17] previously proposed an extraction method in which 100 mg of saffron was extracted with 5 ml of 80% ethanol. Hadizadeh et al. [18] extracted 100 mg of saffron with 20 ml of ethanol by sonicating for 20 min. Ethanol, which was used as a solvent in these methods, can convert picrocrocin to safranal. Therefore, ethanol was replaced by methanol as a solvent in other methods. Corti et al. [6] extracted 2 g of powdered saffron with 200 ml of methanol, which was refluxed in a dark room under nitrogen, dried under vacuum, fractionated in silica columns and eluted in chloroform-methanol-acetic acid (1 : 0.1 : 0.013). Ortega et al. [7] added 50 mg of saffron to 20 ml of 50% methanol, and extraction was done by stirring in the dark for 24 h. Pathan et al. [19] extracted 200 mg of saffron with 25 ml of methanol by sonication and added another 25 ml of methanol before completing the extraction process. The above-described methods are complex and require a large quantity of samples and solvents, which are expensive. The use of large amounts of solvents increases the cost of the analytical method and raises environmental concerns, too. Moreover, these methods are cumbersome when handling a large number of samples for routine authentication of the market samples. Therefore, it was essential to develop a simple method that requires the least amount of sample and solvent, and does not require additional procedures like drying under vacuum, sonication and refluxing in the dark under nitrogen.

Here, we report a simple method for metabolite extraction from saffron, and an HPLC fingerprint comprising six compounds, which can be reliably used for saffron's authentication and quality control.

MATERIALS AND METHODS

Chemicals and reagents

Safranal standard (88% purity) was obtained from Sigma Aldrich (St. Louis, Missouri, USA). All the solvents for HPLC and chemicals for DNA isolation were obtained from HiMedia (Mumbai, India).

Reference material and market samples

The saffron plants and stigma used as reference in this study were collected from Pampore Valley in Kashmir, India (Fig. 1). Saffron samples (n = 25), which were 1 to 6 years old, were obtained from local markets.

Authentication of samples

Authentication of saffron's reference and market samples using pharmacognostic methods was done by Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute (Chennai, India) and Centre for Medicinal Plants Research (Kottakkal, Kerala, India). For authentication by DNA barcoding, genomic DNA was isolated from reference materials and market samples as described before [20]. Using the genomic DNA as a template, *rbcL* DNA barcode markers were amplified with rbcLa-F and rbcLajf634-R primers [21, 22] (Fig. 1). The PCR-amplified DNA barcodes were purified and subjected to Sanger sequencing. The raw DNA sequences were edited, assembled and searched for in GenBank database by Basic Local Alignment Search Tool (BLAST; National Institute for Biotechnology Information, Bethesda, Maryland, USA) analysis for species identification and authentication.

Standardization of extraction

For the extraction of saffron, 5–25 mg of the sample was directly added to 100–500 µl of acetonitrile without grinding. Then it was incubated at 40, 50, 60 or 70 °C for 5, 15, 30, 60, 90, 120 or 150 min. The extract was filtered through a 0.2 µm pore size nylon filter (Sartorius, Göttingen, Germany) and 20 µl of the filtered sample was injected into HPLC device. The samples were analysed by the system consisting of a 515 HPLC pump with a reverse-phase Sunfire C18 column (100 Å, 5 µm, 250 mm × 4.5 mm) and a photodiode-array detector (all from Waters, Milford, Massachusetts, USA). Chromatographic separation was carried out in an isocratic flow of 60, 70, 80, 90 or 100% acetonitrile as the mobile phase at a flow rate of 0.5, 0.8 or 1.0 ml·min^{-1} for 30 min. The separated compounds were monitored spectrophotometrically at a wavelength of 308 nm, which had been reported to detect the major phytochemicals in saffron [13, 17, 23]. The experiments conducted for optimization of metabolite extraction and HPLC analysis were repeated three times. Tissues from the corm, cataphylls, leaves, petals and stamen of saffron were extracted following the optimized protocol, and their HPLC fingerprints were compared with that of stigma. HPLC profiles of 1 to 6 years old stigma were also examined to study the HPLC fingerprint stability over time. The standardized protocol for metabolite extraction and authentication by HPLC fingerprinting was validated using market samples, which were identified as authentic or inauthentic by pharmacognostic and DNA-barcoding methods.

Statistical analysis

The efficiency of extraction of the metabolites from saffron as measured by peak area in the HPLC chromatograms was expressed as mean ± standard error of the mean. One-way ANOVA and Bonferroni's multiple comparison tests were used for statistical testing of significance.

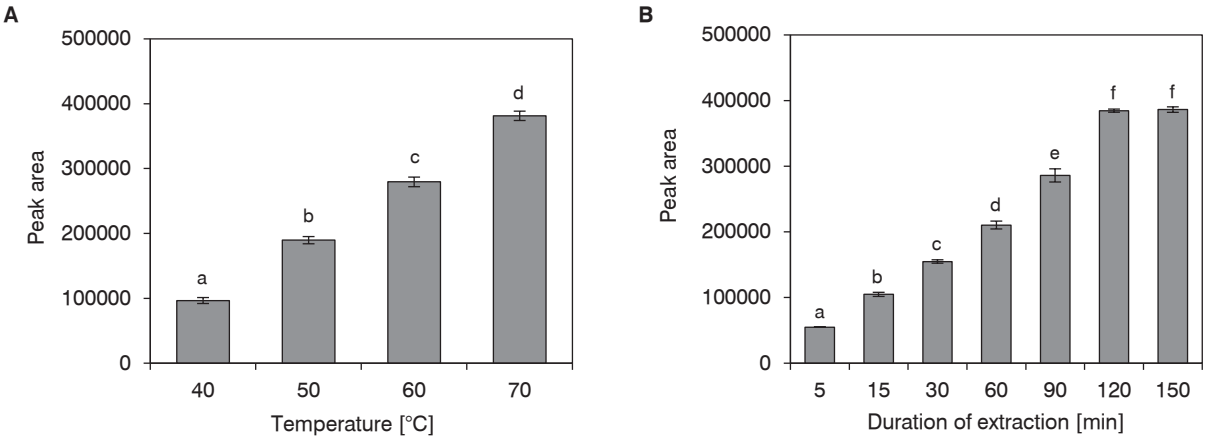

Fig. 2. Extraction of metabolites from saffron.

A – extraction at different temperatures, B – extraction for different durations at 70 °C.
Different letters above columns indicate statistically significant difference at $p < 0.01$.

RESULTS AND DISCUSSION

The saffron plants used as reference were authenticated by the Botanical Survey of India (Coimbatore, India). Authentication of the 25 market samples of saffron using pharmacognostic and DNA barcoding revealed that 20 were authentic and five were inauthentic. These samples were then used for validating the HPLC fingerprinting-based authentication method, which was developed in this study. For developing an HPLC-based analytical method for routine authentication of the market samples, it is essential to have a robust method for the extraction of the metabolites from the saffron stigma.

Based on the previous reports, we chose acetonitrile as the solvent for extraction because it was reported that safranal was more soluble in acetonitrile than in ethanol, and it did not convert picrocrocin to safranal [13], which is crucial for developing a dependable method to authenticate saffron. Extraction of saffron was performed using various amounts of samples in various volumes of acetonitrile. It was found that 5 mg of sample extracted in 100 µl of acetonitrile was sufficient for analytical purposes. Considering the boiling point

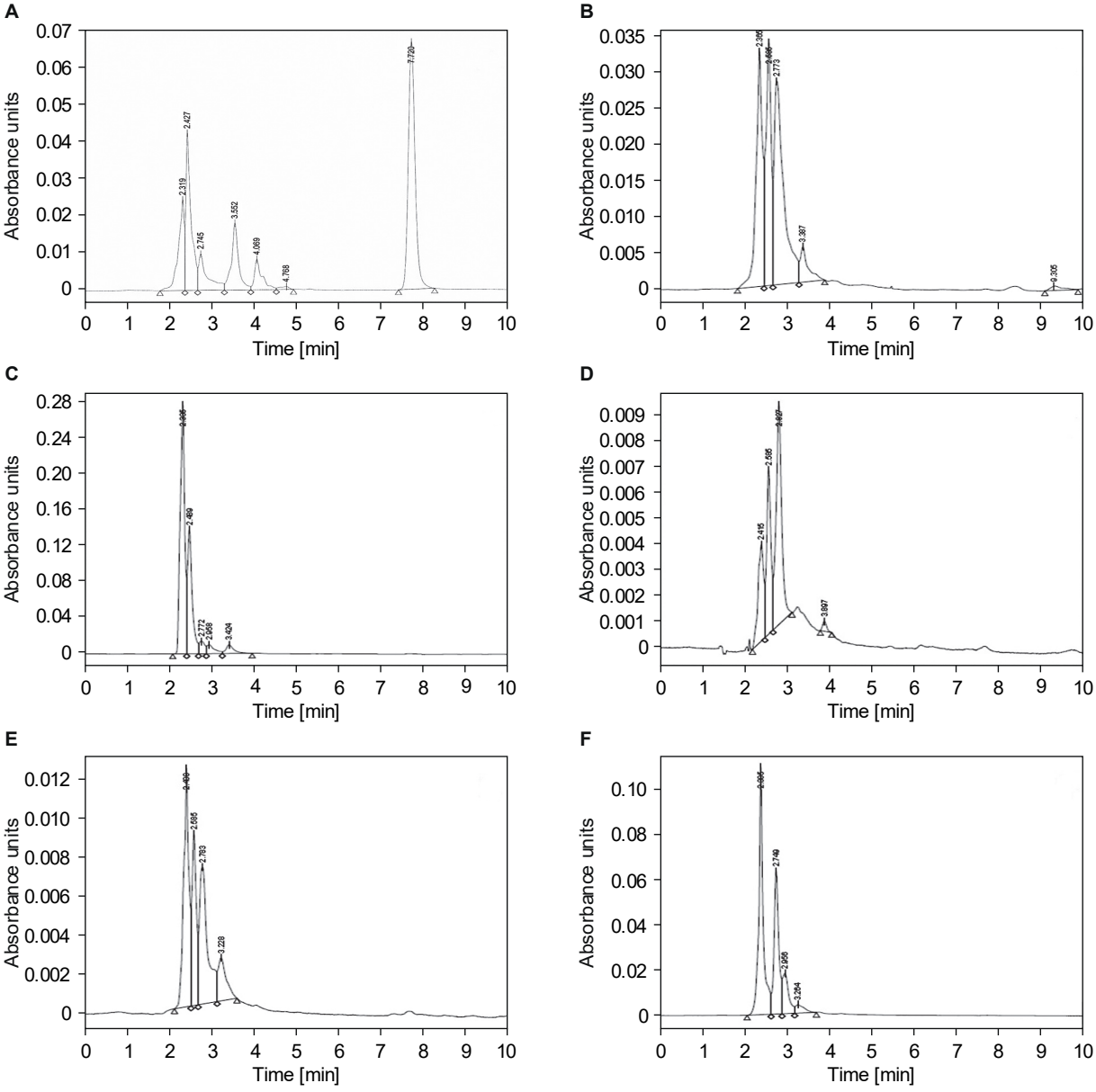

Fig. 3. HPLC profiles of various parts of the saffron plant.

A – stigma, B – stamen, C – petals, D – leaves, E – cataphylls, F – corm.

of acetonitrile (81.6 °C), the extraction of saffron was conducted at various temperatures from 40 °C up to 70 °C. The efficiency of extraction increased as the temperature increased and the maximum efficiency was observed at 70 °C (Fig. 2A). When the extraction time was varied between 5 min and 150 min at this extraction temperature, the highest level of extraction was reached in 120 min (Fig. 2B). Based on these results, it was concluded that a sufficient quantity of metabolites to authenticate saffron by HPLC could be directly extracted without grinding or any other processing from 5 mg of saffron in 100 µl of acetonitrile at 70 °C during 2 h. The metabolites extracted from saffron were separated by HPLC on reversed phase using various acetonitrile concentrations and flow rates during 30 min. Based on the results (data not shown), 70% acetonitrile, 1.0 ml·min^{-1} and 10 min were chosen as the optimum mobile phase, flow rate and run time, respectively.

From the HPLC profiles of saffron extracts, six peaks were selected to constitute an HPLC fingerprint, which can be used to authenticate the market samples of saffron. The first peak of the HPLC fingerprint eluted at 2.3 min and the second to fifth peak eluted within 4 min (retention time 2.5,

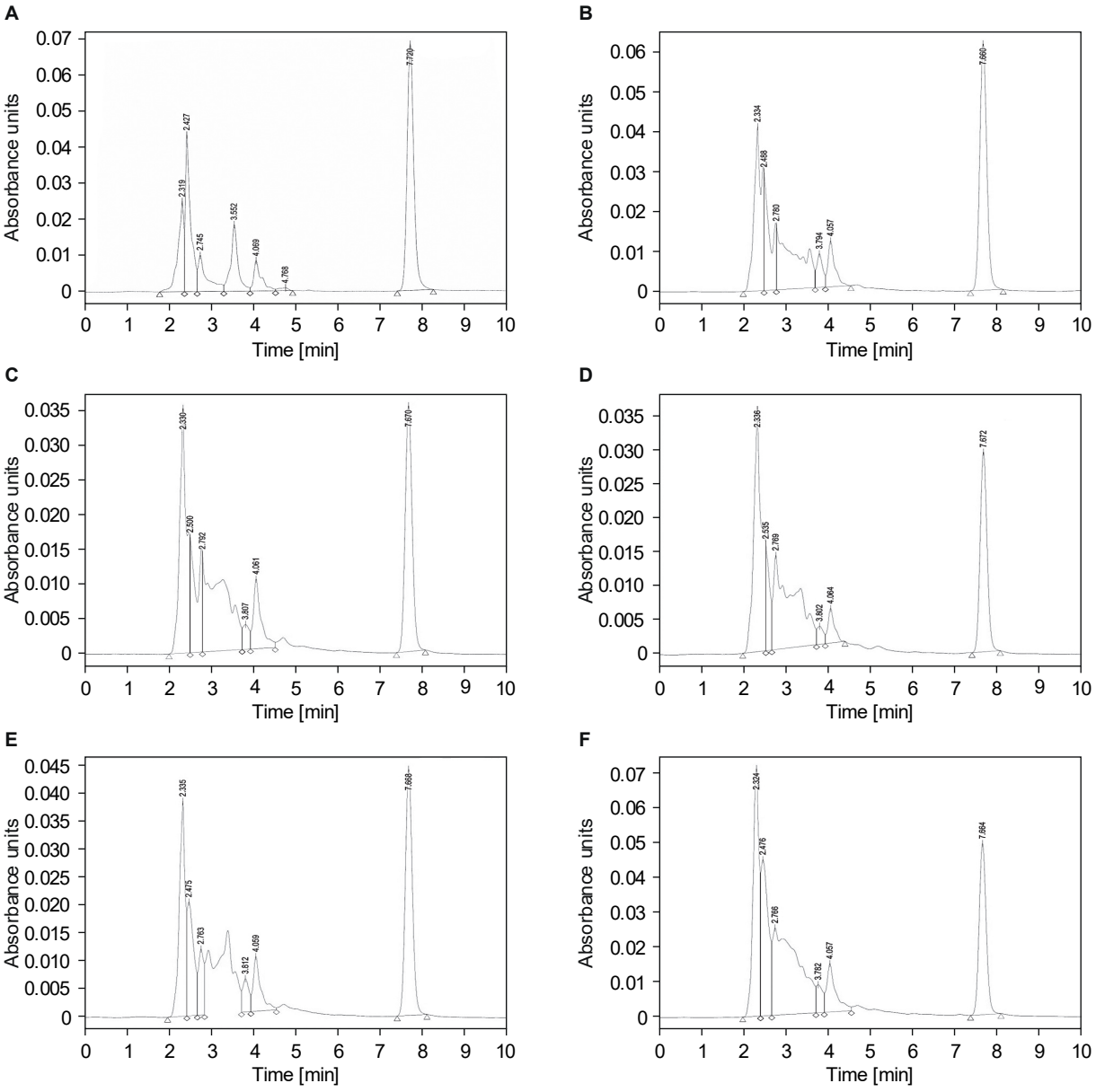

Fig. 4. HPLC fingerprinting of market samples of saffron that were stored at room temperature for various time.

A – 1 year, B – 2 years, C – 3 years, D – 4 years, E – 5 years, F – 6 years.

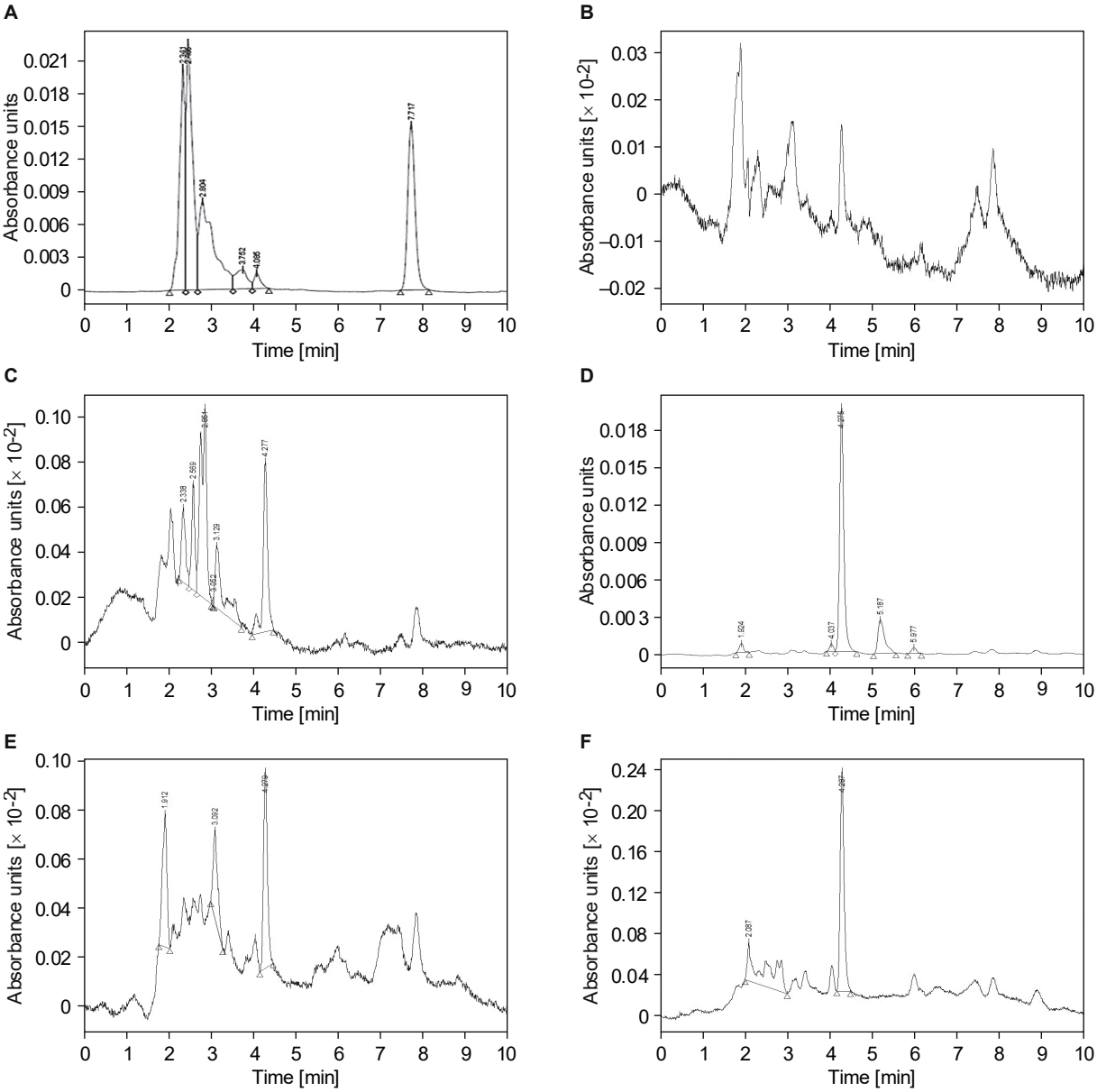

Fig. 5. Authentication of saffron samples by HPLC fingerprinting.

A, B, C, D, E – authentic saffron samples, F – non-authentic saffron sample.

2.7, 3.7 and 4.0 min). The sixth peak was safranal, which eluted at 7.7 min.

As only the stigma from the flowers of saffron plants is used as a spice, it is essential to make sure that the observed HPLC fingerprint is specific to saffron stigma. The metabolites from the other parts of the saffron plants, such as corm, cataphylls, leaves, petals or stamen of the saffron plant, were extracted using the optimized protocol, and their HPLC profiles were compared with those of saffron stigma. Although four peaks of the HPLC fingerprint of saffron stigma were also present in the other plant parts, the HPLC fingerprint as such was specific to the stigma (Fig. 3).

The HPLC fingerprint metabolites should be stable in the samples while on the market. Therefore, to determine the HPLC fingerprint stability over time, we compared the HPLC fingerprints of 1–6 years old market samples stored at room temperature. It was found that the saffron HPLC fingerprints were identical in all the samples, which indicated that the metabolites of the HPLC fingerprints were stable for at least six years of storage at room temperature (Fig. 4). This means that HPLC fingerprinting could be reliably used to authenticate the market samples of saffron that are stored

at room temperature for at least up to six years after harvest.

Twenty-five market samples of saffron, which were authenticated by using pharmacognostic and DNA barcoding methods, were used to evaluate the efficiency of the HPLC fingerprinting method. Extraction of metabolites and HPLC analysis were performed under identical conditions for the authentic (n = 20) and non-authentic samples (n = 5). It was observed that the HPLC profiles of all the non-authentic samples were entirely different from those of authentic saffron (Fig. 5). Therefore, it was established that, combined with the simple extraction protocol developed in the present study, HPLC fingerprinting can be used for rapid and reliable authentication of the market samples of saffron.

CONCLUSION

Picrocrocin, crocin and safranal are used as chemical markers to identify saffron. Each marker has its limitations and the currently available protocols for the extraction of these marker compounds from saffron are cumbersome for routine authentication of the market samples. The extraction protocol reported in this study proved to be simple, cost-effective and environmentally friendly, as it required substantially smaller quantities of the sample and organic solvents. The HPLC fingerprint of the extracted metabolites was specific to saffron, stable at room temperature, and successfully identified all non-authentic saffron samples among the market samples tested.

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