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Development of an improved procedure for extraction and quantitation of safranal in stigmas of *Crocus sativus* L. using high performance liquid chromatography

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Abstract

A number of methods for the preparation, extraction and HPLC analysis of saffron spice were evaluated to determine a reliable method for quantifying safranal in *Crocus sativus* L. stigma tissues. In vials containing ethanol/water stigma extracts, the concentration of safranal increased significantly over a 6 h time interval. In contrast, vials containing 100% acetonitrile stigma extracts showed no significant increase in safranal concentration over the same time interval. Preparation methods such as heating stigmas at 80°C for 30 min prior to extraction and followed by immediate HPLC analysis increased the concentration of safranal several-fold compared to stigmas which had been prepared by freeze-drying. Preparation of ethanol–water or acetonitrile stigma extracts by drying with a rotary evaporator prior to HPLC analysis eliminated safranal from the extracts.

1. Introduction

Saffron is the world's most expensive spice and apart from its traditional value as a food additive recent studies indicate its potential as an anti-cancer agent (Dufresne, Cormier & Dorion, 1997; Escribano, Alonso, Coca-Prados & Fernandez, 1996; Tarantilis, Polissiou & Manfait, 1994). The value of saffron (dried stigmas of *Crocus sativus* L.) is determined by the existence of three main secondary metabolites: crocin and its derivatives which are responsible for color; picrocrocin, responsible for taste; and safranal responsible for odor (Himeno & Sano, 1987; Sujata, Ravishankar & Venkataraman, 1992; Tarantilis et al., 1994). The amount of these compounds in dried stigma tissues is the most important indicator of quality of this spice. For qualitative and quantitative determination of saffron components in plant extracts, a variety of techniques including HPLC have been used by investigators over the last 10 years (Castellar, Montijano, Manjón & Iborra, 1993; Himeno & Sano, 1987; Hori, Enomoto & Nakaya, 1988; Iborra, Castellar, Canovas & Manjón, 1993; Koyama, Ohmori, Fujioka, Miyagawa, Yamasaki & Kohda, 1988; Sarma, Maesato, Hara & Sonoda, 1990; Sujata et al., 1992; Tarantilis et al., 1994; Tarantilis, Tsoupras & Polissiou, 1995; Visvanath, Ravishankar & Venkataraman, 1990; Zarghami & Heinz, 1971). However, depending on the method of extraction, and HPLC technique used, calculated concentrations of saffron secondary metabolites in stigma tissues varies widely in these reports.

Difficulties encountered in the extraction and biochemical analysis of saffron secondary metabolites may be caused primarily by degradation of these compounds. Crocin and picrocrocin, which are biosynthesised in the cells of the plant, tend to naturally degrade in the cells of the stigmas during flowering, drying, storage, and extraction (Alonso, Varon, Gomez, Navarro & Salinas, 1990; Pfander & Schurtenberger, 1982; Raina, Agarwal, Bhahia & Gaur, 1996; Straubinger, Bau, Eckstein, Fink & Winterhalter, 1998; Tarantilis & Polissiou, 1997;

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Tarantilis et al., 1994, 1995; Tsimidou & Tsatsaroni, 1993). The degree of degradation is dependent on temperature, humidity, light irradiation; the presence of oxygen, peroxide, acid and alkaline solutions as well as the presence of active β-glucosidase (Moromoto, Umezaka, Shoyama, Saito, Nishi & Irino, 1994; Raina et al., 1996; Tarantilis et al., 1995). Degradation of crocin and picrocrocin occurs with the loss of glucose moieties. Water-soluble crocin [$C_{44}H_{64}O_{24}$; digentiobiosyl ester of crocetin; 8,8′-diapo-Ψ,Ψ′-carotenedioic acid bis-(6-*O*-β-D-glucopyronosyl-β-D-glucopyronosyl) ester] degrades via a series of glucosyl esters of crocetins to form water-insoluble crocetin (Tarantilis et al., 1994, 1995). However, water-soluble, colorless picrocrocin [$C_{16}H_{26}O_7$; 4-(β-D-glucopyranosyloxy)-2,6,6- trimethyl-1-cyclohexene-1-carboxaldehyde] degrades to safranal without intermediate steps or through the picrocrocin aglycon (depending on conditions of treatment of stigmas) (Himeno & Sano, 1987; Iborra, Castellar, Canovas & Manjón, 1992a,b, 1993; Zarghami & Heinz, 1971). Safranal [$C_{10}H_{14}O$; 2,6,6-trimethyl-1,3-cyclohexene-1-carboxaldehyde] is the main essential volatile oil responsible for the characteristic saffron odor (Zarghami & Heinz, 1971).

The overall purpose of this paper was to develop a method of extracting and quantifying safranal from freeze or heat-dried stigma tissues of saffron which was reliable and accurate. We had previously experienced difficulty in quantifying the amount of picrocrocin and safranal in samples of saffron stigmas extracted with ethanol and water due to changes in HPLC peak retention times and areas during the HPLC analysis. This led to the hypothesis that degradation of picrocrocin to safranal and other picrocrocin derivatives occurred in ethanol-water solvents in a manner similar to that found by Himeno and Sano (1987). To test this hypothesis we first show that saffron tissue extracts using ethanol–water as a solvent are unstable and that the concentration of safranal and picrocrocin changes with time. The stability of the ethanol–water extracts is then compared to acetonitrile (ACN) extracts. Safranal is more soluble in 100% ACN than it is in ethanol:water 8:2. ACN would preferentially extract safranal from plant tissues, but leave other compounds such as picrocrocin which are ethanol water soluble, in the tissue. This means that the concentration of safranal in ACN extracts should remain stable because picrocrocin is not present and therefore cannot undergo conversion to safranal. The second hypothesis tested is that preparation of fresh saffron by freeze-drying, a non-traditional method, reflects the more accurate concentration of compounds in fresh tissue, whereas heat-drying, the traditional method that may involve drying in an oven at 80°C or by placing stigmas in full sunlight, changes the concentration of safranal in the treated tissue due to conversion of picrocrocin. Subsequent methods for treating the extracts such as rotary evaporation (Sujata et al., 1992) could also have dramatic effects on the concentration of safranal in extracts. There is a report (Sujata et al., 1992) of a method for quantification of safranal where extracts are rotary evaporated prior to HPLC analysis, but since safranal is a volatile oil it seemed that rotary evaporation, even under low heat and vacuum, would result in loss of most or all of the safranal in the sample.

2. Materials and methods

2.1. Chemical standards

Safranal, ca. 88%, was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, USA. Picrocrocin was obtained by preparative TLC of Spanish Saffron (McCormick & Co., Inc., Hunt Valley, Maryland, USA) extract using the method of Iborra et al. (1992a) for isolation and purification. These standards were used to calculate the concentration of safranal in extracts using calibration curves (Waters Millenium 32 software) and to determine the retention time of picrocrocin.

2.2. HPLC instrumentation

Safranal and picrocrocin concentrations were determined by HPLC using a Waters (Millford, Massachusetts, USA) autosampler Model 717, a Model 600 pump, a Model 996m photodiode array (PDA) detector interfaced with NEC power mate Pentium computer and Millennium 2010 software. Ten microlitres of each sample was injected onto a reverse phase Shiseido C_{18} column (5 μm particle size; 120A pore size) (250 mm length × 4.9 mm I.D.). Samples in all experiments were extracted with HPLC grade solvents. Unless otherwise indicated all samples were filtered through a 0.22 μm filter (Millipore, Bedford, Massachusetts, USA) before injection.

2.3. Plant materials

For Experiment 1, dried Spanish saffron (McCormick & Co., Inc., Hunt Valley, MD, USA) was purchased from a local supplier. For Experiment 2, corms of *Crocus sativus* L. were obtained from The Daffodil Mart (Gloucester, Virginia, USA) and planted at the Michigan State University Horticulture Research Center, East Lansing, in early September 1997. Stigmas were collected from flowers in full bloom in the last few days of October 1997.

2.4. Data analysis

Data for both experiments were analyzed using a SAS® statistical software package. Simple linear

regression was used to determine if the increase in safranal concentrations was significant and a *t*-test was performed to compare the amount of safranal from tissue extracted with 80% ethanol in water and 100% ACN. To analyze data in the second experiment an ANOVA was used with a Student–Newman–Keuls comparison of means.

2.5. Comparative study of extraction methods

2.5.1. Extraction and HPLC analysis

For safranal extraction and detection the following methods were used.

(i) Three replications per 100 mg of dried stigmas were ground with a pestle using 3 ml 80% (v/v) ethanol in water (Sujata et al., 1992) in centrifuge tubes (15 ml) for 5 min at room temperature and then centrifuged at 8000 rpm for 5 min. The supernatant was decanted and the tissue residues washed with 80% (v/v) ethanol in water for 5 min, and centrifuged as above. The two supernatants from each extraction were combined and immediately analyzed by HPLC using a solvent system of 100% ACN for 6 min at a flow rate of 1.0 ml/min, isocratic with a PDA extraction wavelength of 308 nm for detection of safranal. An isocratic solvent system of 26% ACN in water for 8 min with a flow rate of 1.0 ml/min, PDA extraction wavelength of 250 nm was used for detection of picrocrocin (Iborra et al., 1992a). The concentration of safranal in the ethanol water extracts and HPLC profiles of picrocrocin were determined at this time. Samples, held at room temperature, were then re-injected over periods of 2, 4 and 6 h after extraction and the concentration of safranal determined at each of these time periods.

(ii) Three replications per 100 mg of dried stigmas were each ground with a pestle using 3 ml 100% ACN in centrifuge tubes (15 ml) for 5 min at room tenperature and then centrifuged at 8000 rpm for 5 min. The two supernatants for each extraction were combined and immediately injected in the HPLC using 100% ACN for 6 min at a flow rate of 1.0 ml/min, isocratic, PDA extraction wavelength of 308 nm for the detection of safranal. To determine safranal change with time the samples were injected again over periods of 2, 4 and 6 h after extraction.

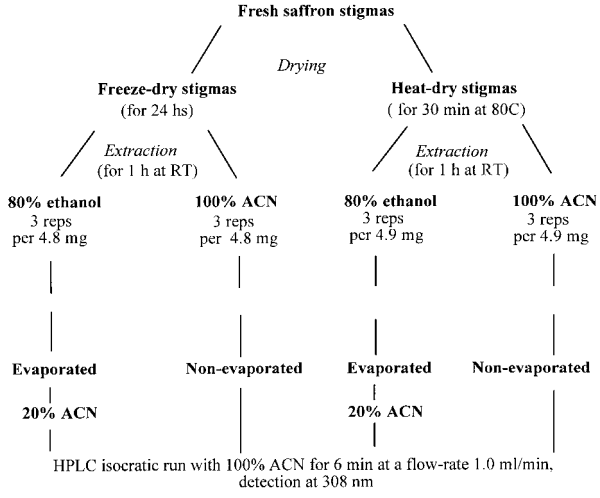
(iii) Exactly the same procedure was followed as in (ii) except that the plant tissue was extracted with 100% ACN for 1 h prior to injection.

2.6. Effects of freeze-drying vs heat-drying of stigma tissues, and rotary evaporation of extracts on the concentration of safranal

Collected fresh mature stigmas were weighed and divided into two equal lots. One of the lots was dried by heating on a sieve (mesh size 1.0 mm^2) at 80°C for 30 min in a lab oven (Himeno & Sano, 1987). The other lot was freeze-dried for 24 h. The heat-dried lot was further divided into six equal subsamples of 4.9 mg each to examine two different extraction techniques (three replications for each extraction). Similarly, the freeze-dried lot was divided into six equal subsamples of 4.8 mg each to examine two different extraction techniques.

2.6.1. Extraction and HPLC analysis

The samples of dried stigmas (heat or freeze-dried) were ground in a microcentrifuge tube with a pestle and extracted for safranal as follows (Scheme 1): tissues were suspended in 1.5 ml 80% (v/v) ethanol in water (Sujata et al., 1992) and intensively shaken for 1 h at room temperature and then centrifuged at 8000 rpm for 10 min. The supernatant was evaporated to dryness using a rotary evaporator (Yamato Scientific, Japan) with the water bath at 40°C. The dried extracts were then redissolved in 1.0 ml of 20% (v/v) ACN in water similar to Sujata et al.; (ii) tissues were suspended in 1.0 ml 100% ACN and intensively shaken for 1 h at room temperature and then centrifuged at 8000 rpm for 10 min. The supernatant was then subjected to HPLC analysis with an isocratic solvent system of 100% ACN for 6 min, flow rate of 1.0 ml/min and PDA extraction wavelength of 308 nm. This experiment permitted quantification of safranal for extracts which had been heat or freeze-dried, and determine the effects of rotary evaporation on safranal concentration (see Fig. 1).



Scheme 1. Drying and extraction procedures followed for HPLC comparison of safranal content between samples.

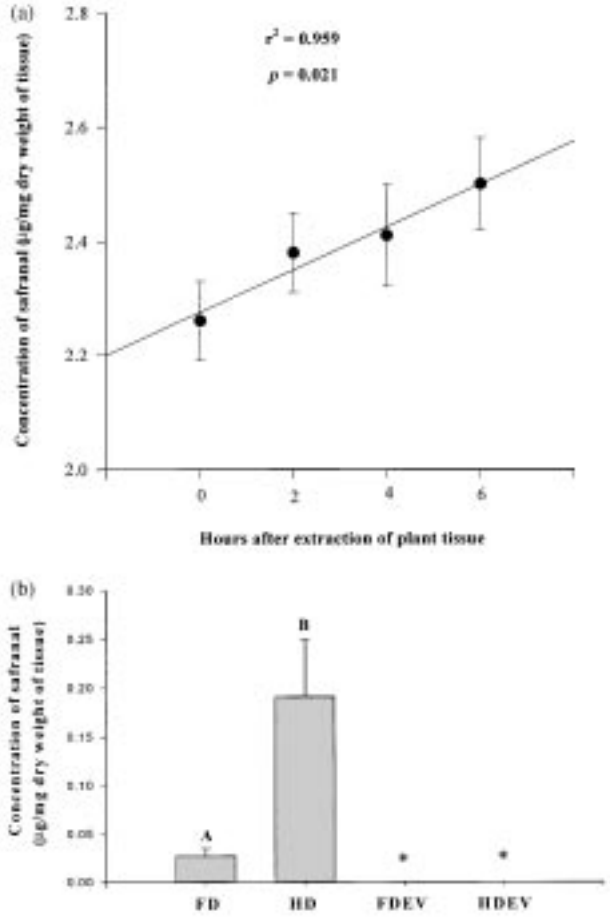


Fig 1. (a) Change in concentration of safranal with time for a 100 mg sample of stigmas extracted with 80% ethanol in water. Repeated injections made from the same vials. HPLC method was 100% acetonitrile, 1.0 ml/min, isocratic, column RP C18 Shiseido 250×4.9 mm, extraction $\lambda = 308$ nm. (b). Comparison of safranal concentration from fresh saffron stigmas. FD = freeze-dried, then extracted with 100% acetonitrile immediately prior to injection; HD = heat-dried at 80°C for 30 min, then extracted immediately with 100% acetonitrile prior to injection; FDEV = freeze-dried, extracted with 80% ethanol, rotary evaporated to dryness and re-dissolved in 20% acetonitrile in water prior to injection; HDEV = heated 80°C for 30 min, rotary evaporated to dryness and redissolved in 20% acetonitrile in water prior to injection; * = not detected. Letters which are different above bars indicate significant differences.

To confirm the presence or absence of safranal in the extracts and to compare the HPLC profiles and retention times of safranal with some of those in the literature (Sujata et al., 1992) a representative sample of extract which had been rotary evaporated prior to resuspension and HPLC injection was spiked with 200 µl of standard safranal solution (10 µg/ml). The solvent system was isocratic 76% (v/v) ACN in water for 15 min at a flow rate of 0.5 ml/min and a PDA extraction wavelength of 308 mm (Sujata et al., 1992). Similarly a sample which had not been rotary evaporated prior to HPLC analysis was spiked with standard safranal solution and analyzed as above.

3. Results and discussion

3.1. Comparative study of extraction methods

This experiment was conducted to test the hypothesis that conversion of picrocrocin to safranal and other secondary metabolites in extracts containing ethanol and water caused changes in peak retention times and areas, making quantification of these compounds problematic. We also wanted to determine if extraction with 100% acetonitrile would allow an accurate quantification of free safranal in stigma tissues.

For samples extracted with 80% ethanol in water a gradual and significant increase in safranal concentration was observed (Fig. 1a). However, for samples extracted with 100% ACN for 10 min ($r^2 = 0.424$, $P = 0.349$) or 1 h ($r^2 = 0.60$, $P = 0.225$) no significant increase in the amount of safranal over the same time period (6 h) was observed. We also found that samples extracted with 80% ethanol in water appeared to have three to four times the amount of safranal as samples extracted with ACN (t-test, $P = 0.0029$). It is well known that in basic solutions picrocrocrocin is converted to safranal (Himeno & Sano, 1987) and also that rapid enzymatic conversion of picrocrocin to safranal can occur (Iborra et al., 1992a; Raina et al., 1996). This increase is not due to the fact that ethanol and water are better extraction solvents since we observed that standard safranal begins to precipitate in 100% ethanol solutions upon the addition of even small amounts of water (data not shown). It is, therefore, likely that the higher level of safranal observed in ethanol water extractions is due to rapid enzymatic conversion of picrocrocin to safranal during the extraction process. Acetonitrile selectively extracts safranal, which is completely soluble in 100% ACN, but leaves the water and ethanol soluble compounds like picrocrocin in the tissues. Therefore, during extraction and HPLC analysis the concentration of safranal remains stable over time, because enzymatic activity is inhibited by ACN, and because picrocrocin is not extracted from the tissue. Use of 100% ACN as an extraction solvent and HPLC sovent system allows for reliable quantification of at least one secondary metabolite, safranal, over time.

Unfortunately, we could not reliably quantify picrocrocin over the same time period as safranal. The HPLC profile of picrocrocin changed dramatically over time (Fig. 2a,b). After 4 h it appeared that the picrocrocin peak had completely converted into another, unknown, compound (Fig. 2b, peak 3). Therefore, quantification of some compounds from saffron using ethanol/water as an extraction solvent is problematic, especially if extraction times are long and many samples need to be analysed in a single HPLC experiment.

3.2. Effects of freeze-drying vs heat-drying of stigma tissues, and rotary evaporation of extracts on concentration of safranal

In this experiment we tested the hypothesis that heating-drying stigmas affects the concentration of safranal, and also that rotary evaporation of extracts at any point prior to HPLC injection results in a loss of safranal. Safranal was not detected in fresh tissues we examined (data not shown). Similarly, Himeno and Sano (1987) found safranal (50% ethanol extract) only at trace levels in their tissue cultures of fresh stigma-like structures. Drying, especially heating, promotes conversion of picrocrocin to safranal (Himeno and Sano, 1987;

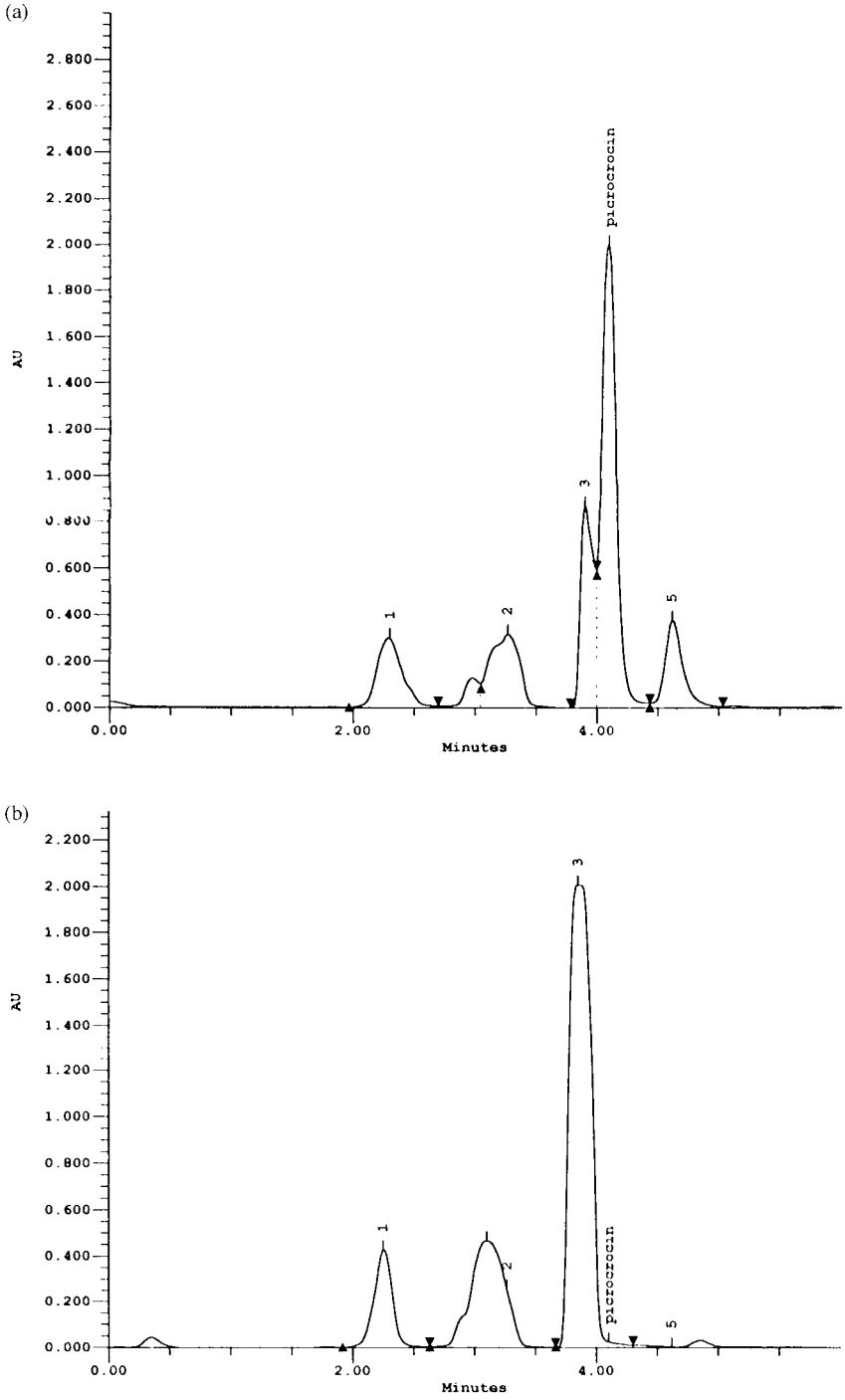


Fig. 2. (a) HPLC profile of stigmas extracted with 80% ethanol in water. Injected immediately after extraction. HPLC system 1.0 ml/min, 26% ACN in water, extraction $\lambda = 250$ nm. (b). HPLC profile of the same extract as in Fig. 2a after standing 4 h at room temperature. Note loss of picrocrocin peak and peak 5, and increase in size and area of peak 3. HPLC conditions the same as in Fig. 3a

Raina et al., 1996; Sampathu, Shivashankar & Lewis, 1984). Theoretically, freeze-drying the plant tissues should reduce enzymatic conversion of picrocrocin to safranal. Thus, the stigmas obtained from the field were either freeze or heat-dried for comparison. Fresh stigmas which had been freeze-dried had significantly less safranal than did stigmas which had been heat-dried (Fig. 1b).

We also did not detect safranal in extracts we had rotary evaporated to dryness and then redissolved in

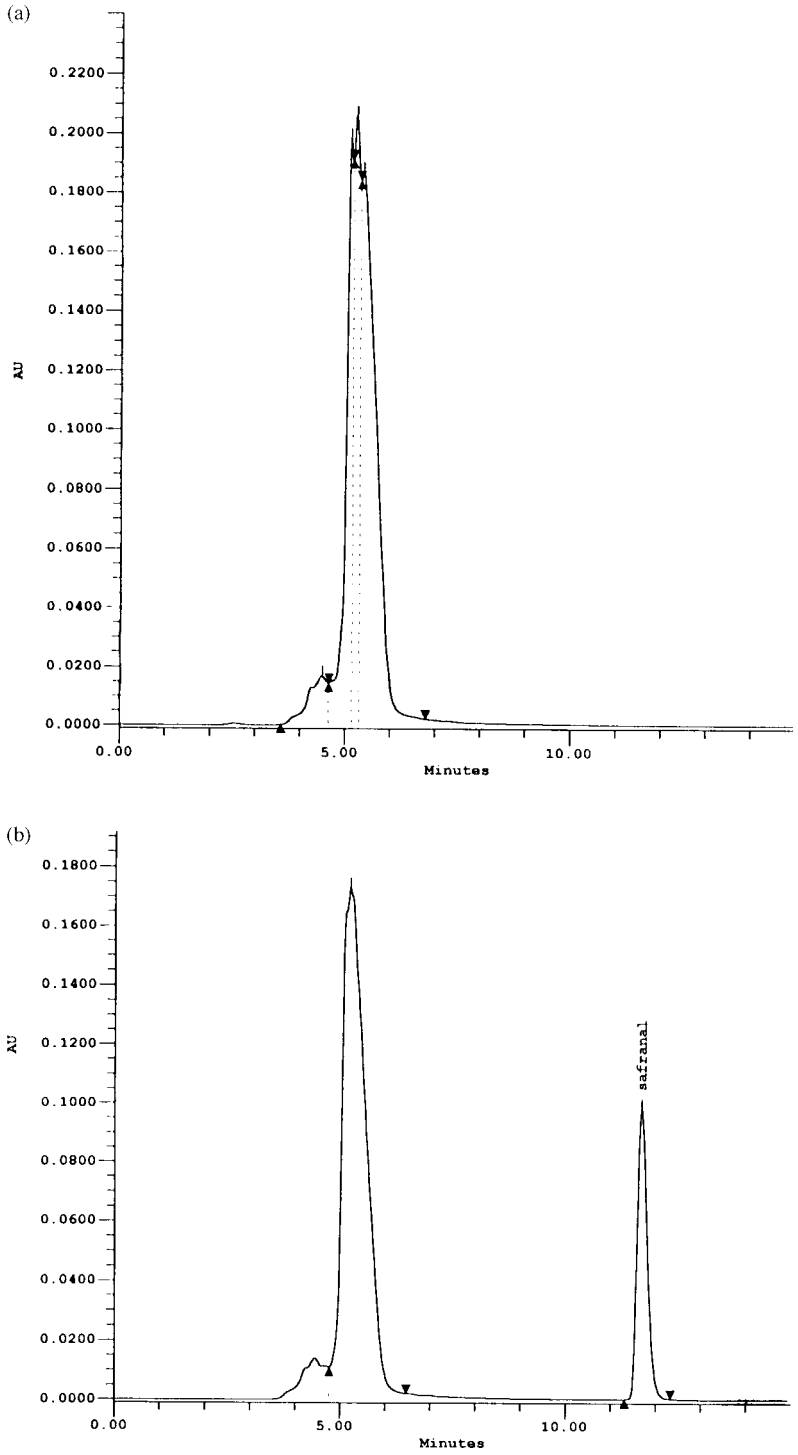


Fig. 3. (a) A sample of stigmas dried by heating at 80°C for 30 min and then extracted with 80% ethanol in water, rotary evaporated to dryness and then re-suspended in 20% acetonitrile in water prior to injection after Sujata et al. (1992) HPLC conditions 76% acetonitrile in water, isocratic, column RP C18 Shiseido 250×4.9 mm, $\lambda = 308$ nm also after Sujata et al. (1992). (b) Same sample injected and HPLC conditions as in Fig. 3a, but sample was spiked with 200 μl of 10 μg/ml safranal standard. Note safranal peak at 12, not 6 min.

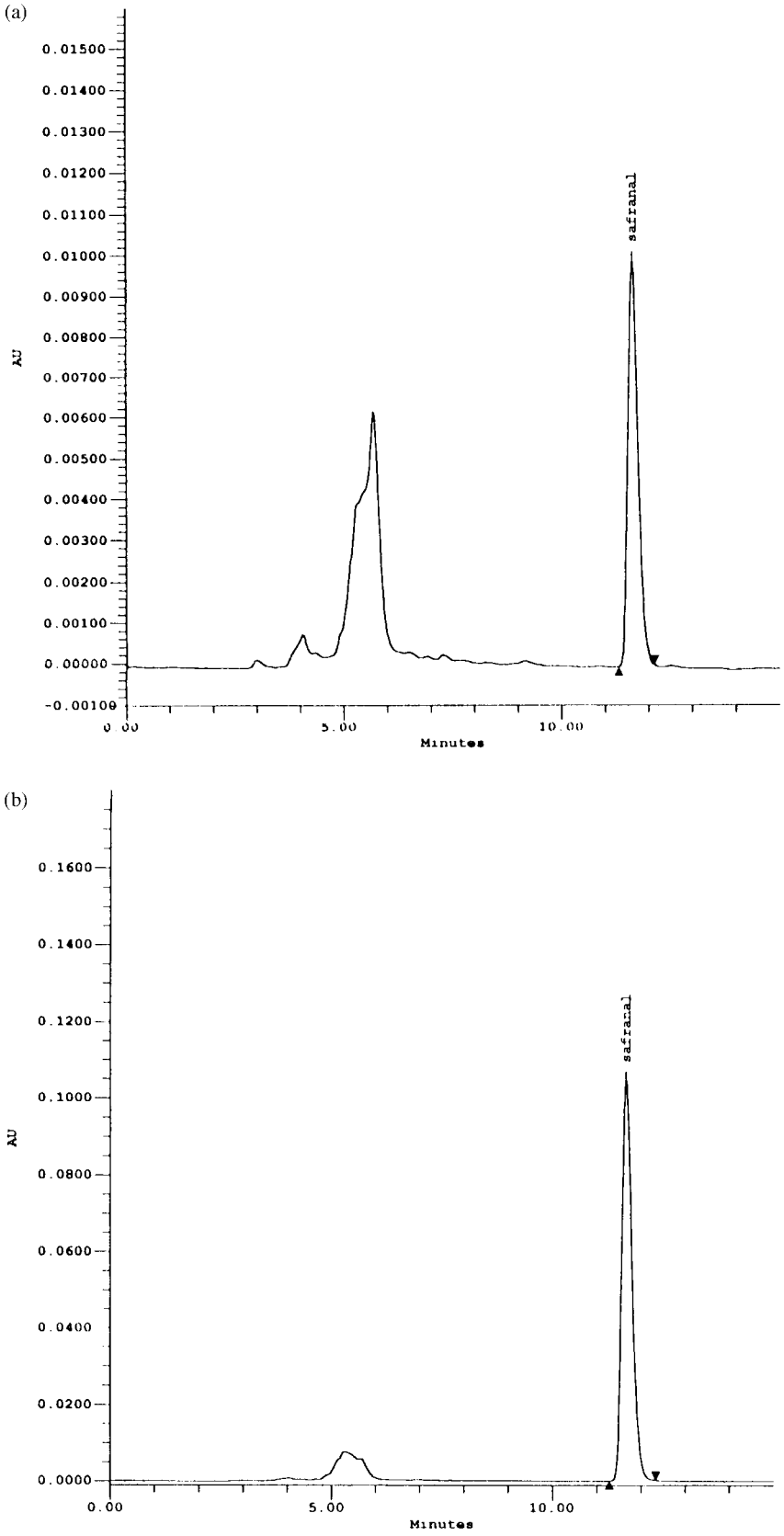


Fig 4. (a) A sample of stigmas dried by heating at 80°C for 30 min, extracted with 100% acetonitrile then directly injected onto the column with no rotary evaporating. HPLC method as in Fig. 3a. (b) Same sample as in Fig. 4a with same HPLC method but spiked with 200 μl of safranal standard at 10 μg/ml.

solvent prior to HPLC analysis (using a 100% ACN solvent system). According to the standard safranal injections used to calculate the calibration curve, a safranal peak at approximately 5–7 min retention time should have been observed using this solvent system, but was not.

The HPLC solvent system was then changed to 76% acetonitrile in water, 1.0 ml/min, isocratic (Sujata et al., 1992) and one of the rotary evaporated samples that had been injected previously was re-injected (Fig. 3a). Three unresolved peaks at 5.0–6.0 min were observed using this solvent system. To test for the presence of safranal, the vial was spiked with 200 μl of standard safranal at 10.0 μg/ml (Fig. 3b). Safranal was not observed to be one of the unresolved peaks with a retention time of 5.0–6.0 min but appeared at almost 12.0 min in the HPLC run. Therefore, using the solvent system of Sujata et al. (1992) and our own, we did not observe a safranal peak for rotary evaporated samples. Sujata et al. (1992) did not indicate what the water temperature was in their procedure, but it is difficult to see how a volatile oil such as safranal could remain in extracts which had been completely dried under a vacuum even without heating.

Finally, a sample which had been extracted with 100% acetonitrile but had not been rotary evaporated, was injected using the HPLC solvent method given in Sujata et al. (1992) (Fig. 4a). In the non-rotary evaporated sample a peak at almost 12.0 min retention time was obtained which was the same retention time previously obtained with this method for safranal. To be completely certain that this was not a mis-identification, the same sample was spiked with 200 μl of standard safranal at 10.0 μg/ml and yielded a large increase in the size of the peak at 12.0 min indicating the presence of safranal (Fig. 4b). We, therefore, propose that rotary evaporating samples prior to HPLC injection removes all safranal from the sample, and that using the solvent system of Sujata et al. (1992), with a 250 mm column, the peak for safranal has a retention time of approximately 12.0 min. Sujata et al. used a column which was shorter than our own (150 mm) and, therefore, safranal would be expected to have a shorter retention time. However, it is uncertain that the peak they identified as safranal is actually safranal in extracts which had previously been rotary evaporated to dryness prior to HPLC injection.

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