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Evaluation of the colouring strength of saffron spice by UV–Vis spectrometry

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The influence of various steps of the extract preparation procedure on the absorbance readings used for the commercial evaluation of the colouring strength of saffron is described. The study was prompted by the diversity of extraction protocols found in the literature. Extraction period, stage of the extract filtration, filter type and extraction solvents influenced the size and the repeatability of the absorbance measurements. Long extraction periods (24 h) caused loss of the colouring strength. Filtration through narrow pore filters yielded extracts with higher colouring strength. The latter was significantly increased when the extract was filtered prior to final dilution. Alcoholic extracts presented higher colouring strength when compared to aqueous extracts. Derivative spectroscopy was applied to clarify the maxima wavelengths of the extracts. The 'peak to peak' method was used to evaluate the major absorbances in the region 400–500 nm of the second derivative spectrum. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Saffron, the most expensive spice used in the food industry (Oberdieck, 1991), is gaining interest among consumers because of its beneficial properties which are currently being investigated (Abdullaev, 1993). Saffron is distinct for its delicate flavour, bitter taste and the attractive yellow shades of its aqueous or alcoholic extracts. Its colouring properties are attributed mainly to water-soluble carotenoids, the crocins, which are glycosyl esters of 8,8′-diapocarotene-8,8′-dioic acid (crocetin) (Sampathu *et al.*, 1984).

Colour is the major parameter for the quality grading of saffron (ISO, 1980, 1993). Recently, chromatographic methods have been used for a detailed characterisation of saffron pigments (Sujata *et al.*, 1992; Tarantilis *et al.*, 1994). Even so, the colouring strength of a saffron sample estimated by UV–Vis spectrometry and expressed as the specific extinction of an aqueous extract at the λ_{max} of α-crocin is a very convenient approach for the quality control and commercial grading of saffron (Corradi & Micheli, 1979; ISO, 1993).

Different procedures for the preparation of saffron extracts for the spectrometric evaluation of colouring strength are found in the literature (Corradi & Micheli, 1979; ISO, 1980, 1993; Basker & Negbi, 1985; FAO, 1986; Alonso *et al.*, 1990) but little information exists on the factors that may influence the size and repeatability of the readings (Basker & Negbi, 1985). A closer examination of the literature spectrometric (or high-performance liquid chromatographic, HPLC) data shows their dependence on the method of sample preparation, which may be accentuated if various wavelengths are used in the evaluation of the principal saffron carotenoids (Corradi & Micheli, 1979; Himeno & Sano, 1987; Solinas & Cichelli, 1988; Sujata *et al.*, 1992; Tarantilis *et al.*, 1994).

This paper is part of a larger study on the quality control of saffron cultivated systematically in certain areas in Northern Greece. Experiments were conducted to examine the possible sources of analytical variation among data obtained using various extraction procedures. Derivative spectroscopy was used to clarify the wavelength maxima of the extracts and also as a means of evaluating saffron colouring strength. Observations and critical comparisons were made with reference to the methodology proposed by the International Standards Organization (ISO, 1993).

MATERIALS AND METHODS

Chemicals and apparatus

Distilled water and spectroscopic grade solvents (Riedel de Haen) were used in this study. The UV–Vis spectra were recorded by a UV–Vis spectrophotometer (Model 2000; Hitachi, Tokyo, Japan) using quartz cells (1 cm pathlength). Spectra derivatives were obtained by a UV-2101PC Shimadzu scanning spectrophotometer (Shimadzu, Kyoto, Japan) connected to an HP Desk Jet 550C printer.

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Samples

Commercial samples, representative mixtures of saffron stigmas (production year 1994), were used. The samples, kindly donated by the Cooperative de Safran (Crocos, Kozani, Greece), were dried in the air (dark) by the producers. Saffron samples from other countries were also from the same source. Representative subsamples were ground with a pestle and mortar and passed through a 0.4 mm sieve.

Methods

Preparation of saffron extracts

All the following operations took place away from direct sunlight. Repeatabilities were examined for five replicates for each experimental procedure. A flow chart of the methods studied for the preparation of saffron extracts is given in Fig. 1.

- Method a: cold water extraction according to ISO 3632-1980. One gram of sample was added to 100 ml of distilled water; the mixture was stirred 30 min and allowed to stand for 24 h in the dark (standard solution, 1%). Aliquots of the standard solution (clear supernatant without filtration) were diluted to 0.004% (working solution) with distilled water. Absorbance readings were then measured using quartz cells (1 cm path).
- Method b: cold water extraction according to ISO 3632-2-1993. Sample (0.5 g) was transferred quantitatively into a 1 litre volumetric flask. Water (900 ml) was added and the solution was magnetically stirred for 1 h; the magnetic bar was removed and the solution was made up to the mark (standard solution, 0.05%). An aliquot (20 ml) from the homogenised solution was transferred into a 200 ml volumetric flask and made up to the mark with water (working solution, 0.01%); the dilute extract was filtered through a mebrane filter (0.45 μm) (RC 55; Schleicher & Schuell, Dassel, Germany). Absorbance readings were taken as for method a. Slight modifications for comparison were made by filtration through a Gooch filter no. 4 (5–10 μm) (method b_1) and Whatman paper no. 42 (2–5 μm) (method b_2).
- Method c: cold water extraction plus extract filtration before dilution. Powdered saffron (0.2 g) was extracted with 50 ml of cold distilled water by vigorous shaking at room temperature for 1 h. The extract was made up to the mark (standard solution, 0.4%) and then filtered through a coarse Gooch filter (100–120 μm). Absorbance readings were measured after appropriate dilution (working solution, 0.004%) as for method a.
- Method d: as in method b_1, except that the standard solution aliquot (20 ml) was filterd through a Gooch filter (no. 4) before dilution.
- Method e: water:ethanol, 1:1, v/v, extraction. As in method b, except that water:ethanol, 1:1, v/v, was used as the extraction solvent.
- Method f: water:ethanol, 1:4, v/v, extraction. As in method b, except that the extraction solvent was ethanol 80%, v/v.
- Method g: water:methanol, 1:1, v/v, extraction. As in method b, except that the extraction solvent was a mixture of equal volumes of water and methanol.

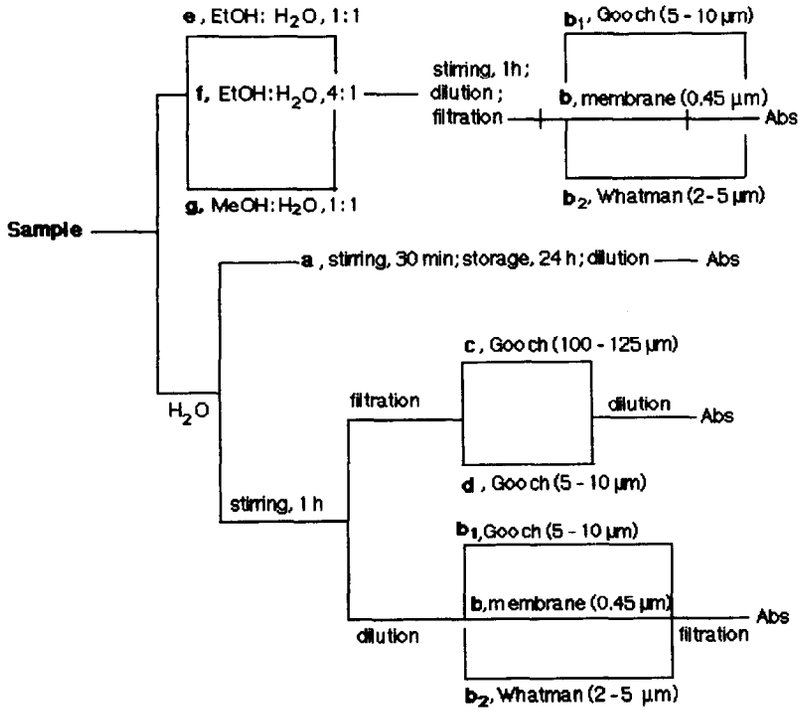

Fig. 1. Flow chart of the methods used for the preparation of saffron extracts.

UV–Vis spectrometry

The UV–Vis spectra of the above extracts were recorded in the region 200–600 nm using matched quartz cells (1 cm path). The colouring strength of the extracts was expressed as $E^{1\%}_{440}$, where $E = A_{440}/C_{(1\text{ g per }100\text{ ml})}$, according to the ISO 3632-2-1993 or as $E^{1\%}_{\lambda max}$ where necessary. Absorbance measurements were related back to the 1% solution. Operating conditions for recording first derivative were: scaling factor, ×10; $\Delta\lambda$, 5 nm. For the second derivative: scaling factor, ×400; $\Delta\lambda$, 20 nm. Quantitative data for second derivatives were expressed as $\Delta E^{1\%}_{\lambda}$ values (e.g. $\Delta E^{1\%}_{442}$), the 'peak to peak' distance of the corresponding peak (minimum–maximum in the side towards longer wavelengths) of the inverted second derivative spectrum (Perkampus, 1992).

Data analysis

Significance of difference between two means was analysed by *F*-tests at the $P < 0.05$ confidence interval and $(n_1 + n_2 - 2)$ degrees of freedom (Miller & Miller, 1984). Comparison among means of the various extraction methods was carried out by the new Duncan's multiple range test (Finney, 1980).

RESULTS AND DISCUSSION

Standardisation of the extraction procedure

In the recently issued ISO 3632-1-1993, saffron filaments are classified in four categories on the basis of their floral waste, extraneous matter and colouring strength. Major changes are made to the methodology of the sample preparation method b compared with the previous ISO 3632-1980 method a. The method also differs from other procedures reported in the literature. In this work each step of extraction procedure is carefully examined and the factors that may influence inter-laboratory data agreement are studied with respect to the ISO 3632-2-1993 method.

Effect of extraction period

Preliminary experiments have shown that changes in the extraction period affected the spectrometric readings. Long extraction periods, proposed by the ISO 3632-1980 standard, caused significant loss of the colouring strength (Table 1). The degradation of saffron pigments seemed to be fast, so this step of analysis should be considerably shortened. Significant differences among analytical results due to different extraction periods may be of critical importance for the commercial classification of some saffron samples. Indeed, in the recent ISO 3632-2-1993 the extraction period was dramatically reduced and specified 1 h of magnetic stirring.

Effect of filter type

Comparison of the spectrometric data produced by the recommended standard method b and a modification of the ISO-3632-1980 method c showed how important agreement between the investigators on the extraction procedure can be (Table 2). The repeatability of method b was found to be very good (CV% = 0.9) but easily influenced by slight changes in the sample preparation procedure. Instead of the filter type recommended in the ISO standard, filtration through small pore Gooch filters (5–10 μm) or Whatman paper filter (2–5 μm) was used for comparison. Filters up to 10 μm may replace the expensive, disposable membrane filters proposed in the ISO standard. Even so, the repeatability of the measurements according to the standard method b is better than that of the b_1 and b_2 methods. Larger pore filters (100–125 μm) seem to yield extracts with lower colouring strength, which may be due to the presence of larger molecular size water-soluble compounds, such as carbohydrates or proteins.

Effect of filtration stage

The stage of filtration influences the size of the absorption readings. Thus, filtration of the extract before (d) or after (b_1) dilution through a Gooch filter no. 4 yielded significantly different absorbance measurements (Table 3). Filtration after dilution yields extracts with

Table 1. Effect of extraction[a] period on the size and repeatability of absorption readings expressed as $E^{1\%}_{442}$, where $E^{1\%}_{442} = A_{442}/C_{(1\text{ g per }100\text{ ml})}$

Replicate no.	Extraction period	
	24 h	1 h
	Colouring strength as $E^{1\%}_{442}$[b]	
1	195.0	241.9
2	207.5	237.2
3	219.0	240.3
4	208.8	237.5
5	212.8	238.1
Mean	208.6[x]	239.0[y]
SD	8.8	2.0
SE	3.9	0.9

[a]Cold water extraction according to the ISO 3632-1980 method.
[b]λ_{max}.
[x,y]Significantly different ($P < 0.05$).

Table 2. Effect of filter type on the size of absorbance readings expressed as $E^{1\%}_{442}$, where $E^{1\%}_{442} = A_{442}/C_{(1\text{ g per }100\text{ ml})}$

Cold water extraction method	No. of determinations	Colouring strength as $E^{1\%}_{440}$ extraction (±CV%)
b, ISO 3632-2-1993	5	218.5 ± 0.9[x]
b, filter 100–125 μm, dilution	5	191.2 ± 0.8[y]
b_1, as in b, filter (5–10 μm)	5	215.6 ± 1.8[x]
b_2, as in b, filter (2–5 μm)	5	214.5 ± 2.2[x]

[x,y]Means with different superscripts are significantly different ($P < 0.05$).

Table 3. Effect of filtration stage on the size of absorbance readings expressed as $E^{1\%}_{442}$, where $E^{1\%}_{442} = A_{442}/C_{(1\ g\ per\ 100\ ml)}$

Replicate	Extraction method according to ISO 3632-2-1993	
	b_1 (filtration after dilution)	d (filtration before dilution)
	Colouring strength as $E^{1\%}{}_{442}{}^a$	
1	216.2	223.0
2	217.9	219.2
3	215.7	226.0
4	209.7	223.4
5	218.4	219.2
Mean	215.6^x	222.2^y
SD	3.48	2.93
SE	1.6	1.3

[a] λ_{max}.
[x,y] Significantly different ($P < 0.05$).

lower colouring strength. Filtration of the extract before dilution (standard solution, 0.05%) was very difficult through the membrane filter (0.45 μm), but it was easily carried out through the other filters. In our opinion, filtration before dilution is advantageous because the final absorbance readings are taken on a more representative extract of saffron sample pigments.

Effect of extraction solvent

Some investigators prepare the extracts in alcoholic solutions. In order to study the effect of sample solvent on absorbance readings, mixtures of water:ethanol, 1:1, v/v (e), water:ethanol, 1:4, v/v (f) and water:methanol, 1:1, v/v (g) were used. These solvents have been used by some investigators for the extraction of saffron colouring matter (Visvanathan *et al.*, 1990; Sujata *et al.*, 1992; Narasimhan *et al.*, 1992; Tarantilis *et al.*, 1994). Comparison of means by the new Duncan multiple range test for absorbance readings at 442 nm (maximum wavelength for aqueous extracts) and at λ_{max} for each extract are presented in Table 4. Better comparison was made for measurements taken at the wavelength maxima for each extract. It seems that the alcoholic moiety is important for a better extraction of the colouring matter of saffron. Higher colouring strength values were obtained with the water:methanol extraction system. The data indicate that the polar carotenoids of saffron are not freely soluble in cold water. Extraction with warm water (Basker & Negbi, 1985) or better with water:alcohol mixtures should be considered as alternative extraction systems in a future revision of the ISO standard.

Absorbance spectra of saffron aqueous or alcoholic extracts

The solvent change affected the wavelength maxima of saffron extract spectra. Less polar solvents exhibit a hypsochromic effect to all the spectra peaks (Fig. 2). Despite general agreement among investigators about the characteristic wavelength maxima of an aqueous or alcoholic saffron extract spectrum in the region 200–600 nm, there is a dispute in their interpretation. For example, three maxima at about 257, 330 and 440 nm are used in the ISO 3632-1-1993 specification standard to estimate safranal, picrocrocin and crocin, respectively. Safranal and its precursor picrocrocin are responsible for the aroma and the bitter taste of saffron and are proposed as secondary quality criteria of this precious spice. However, according to other investigators (Kuhn & Winterstein, 1934; Buchecker & Eugster, 1973; Himeno & Sano, 1987; Tarantilis *et al.*, 1995), picrocrocin is expected to absorb at about 250.5 nm and safranal at about 308 nm in aqueous (or alcoholic) extracts. Absorbance at about 257 nm is attributed to glycosidic bonds of crocins and absorbance at about 330 nm is related to *cis*-crocin (Speranza *et al.*, 1984; Manitto *et al.*, 1987). Recent findings using HPLC separation of saffron extract components and their detection at different wavelengths (Sujata *et al.*, 1992; Tarantilis *et al.*, 1994) support our opinion that maxima at 257 nm and 330 nm should be used as colour indices rather than as quality criteria for the aroma and bitterness of saffron. A future revision of the ISO specification standard should consider this fundamental piece of information. In this study, elucidation of the spectra wavelength maxima shown in Fig. 2 was performed by derivative spectroscopy.

First derivative spectra

The first derivative spectra of saffron extracts presented improved resolution of overlapped peaks and the characteristic broad absorption band around 440 nm (Fig. 3, α) displayed a fine structure (Fig. 3, β). Absorption maxima were recorded with greater precision as zero crossing points (Table 5).

Table 4. Effect of extraction solvent on the size of colouring strength expressed as $E^{1\%}$ (mean value ± CV%)

Extraction method according to ISO 3632-2-1993	No. of determinations	Colouring strength at:	
		442 nm	$\lambda_{max}{}^a$
b, cold water	5	212.6 ± 0.9^x	212.6 ± 0.9^x
e, water:ethanol, 1:1, v/v	5	222.2 ± 0.9^y	224.2 ± 0.9^y
f, water:ethanol, 1:4, v/v	5	213.5 ± 1.9^x	223.8 ± 2.0^y
g, water:methanol, 1:1, v/v	5	228.4 ± 4.0^y	234.5 ± 0.9^z

[a] λ_{max}: e, 439.0 nm; f, 436 nm; g, 439 nm.
[x,y,z] Means with different superscripts are significantly different ($P < 0.05$).

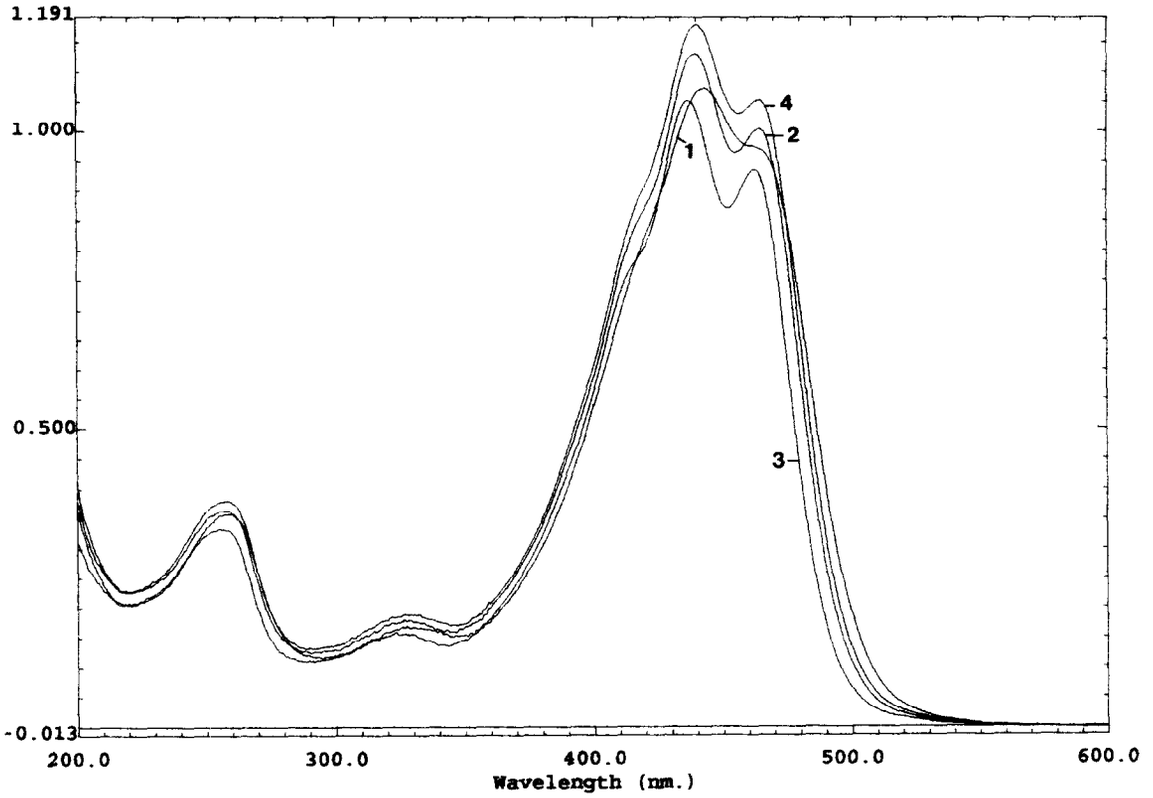

Fig. 2. Typical absorption spectra of saffron extracts obtained according to methods: 1, method b; 2, method e; 3, method f; 4, method g.

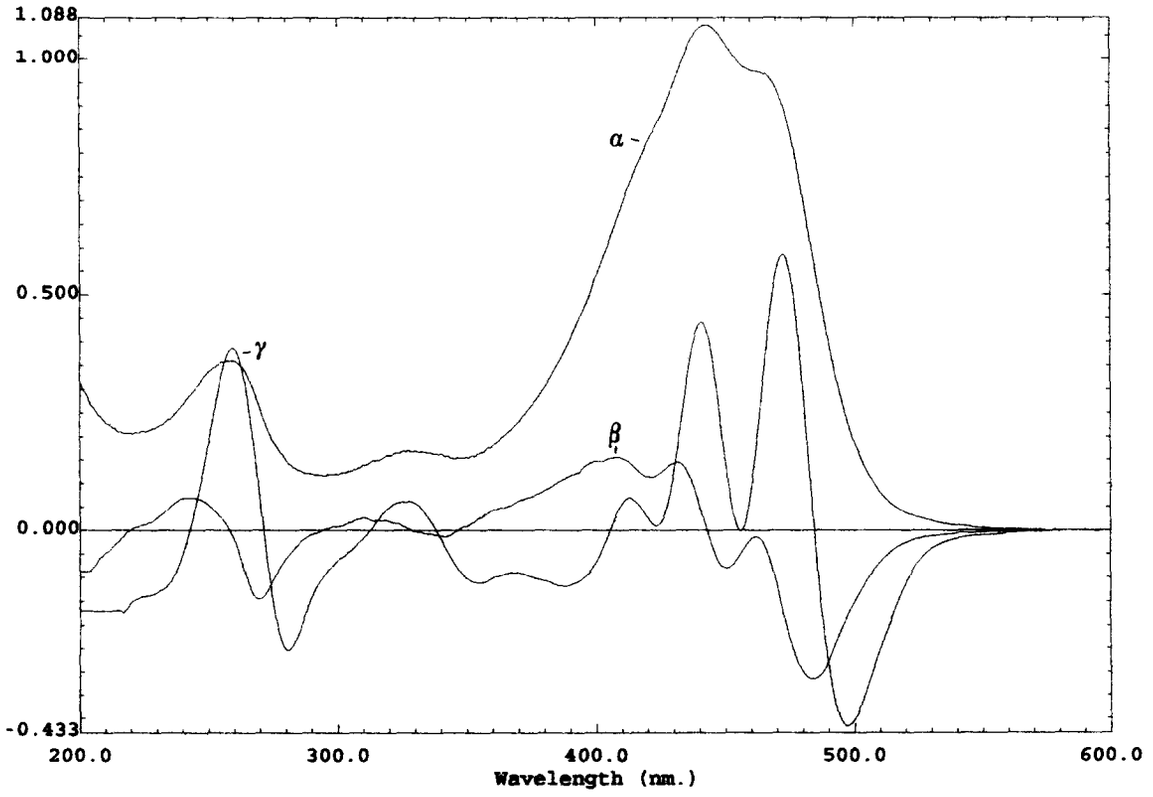

Fig. 3. Absorption spectra of an aqueous saffron extract obtained according to method b: α, overall; β, first derivative; γ, second derivative (inverted).

Second derivative spectra

The inverse of the second derivative spectrum was more suitable for quantitative evaluation of the colouring strength (Fig. 3, γ) compared to the first derivative spectrum. Vertical distances between a maximum and an adjoining minimum in a derivative spectrum is proportional to the solute concentration and may be used for quantification (Berzas Nevado *et al.*, 1993). The 'peak to peak' method was used to evaluate the major absorbances in the region 400–500 nm (Perkampus, 1992). ΔE_1 and ΔE_2, as defined in Fig. 4, were subsequently used for quantitation.

Table 5. Wavelength maxima of saffron extracts as elucidated using derivative spectroscopy

Extraction method	Wavelength maxima (nm)			
b, cold water	–	442.0	328.0	257.5
e, water:ethanol, 1:1, v/v	463.5	439.0	326.5	256.5
f, water:ethanol, 1:4, v/v	462.0	436.5	325.0	256.5
g, water:methanol, 1:1, v/v	463.5	439.5	327.5	257.0

Application

Spectra were taken for 20 authentic saffron samples extracted according to method (b). The samples were from various places of origin (Greece, Iran, Spain), harvesting periods (1989–1994) or storage conditions

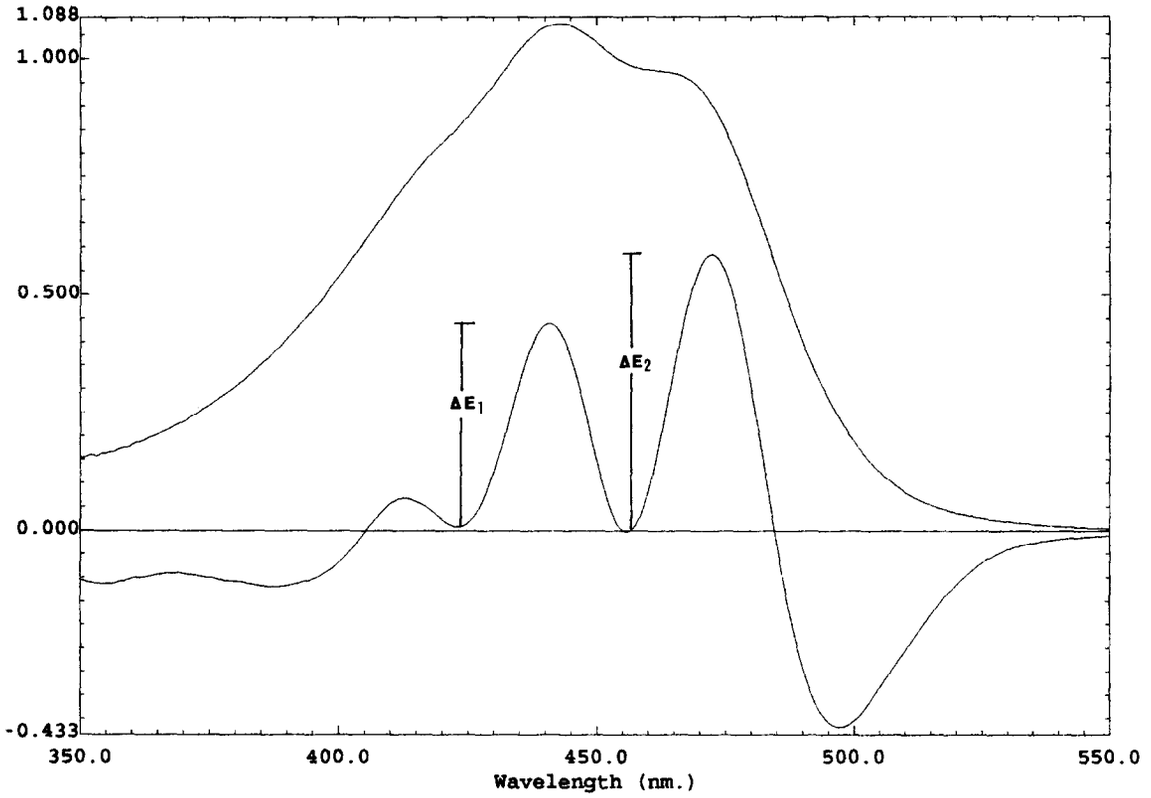

Fig. 4. Definition of ΔE_1 and ΔE_2 in the second derivative spectrum of an aqueous saffron extract: ΔE_1 = 442.5 nm (min) −440.5 nm (max); ΔE_2 = 455.5 nm (min) – 472.0 nm (max).

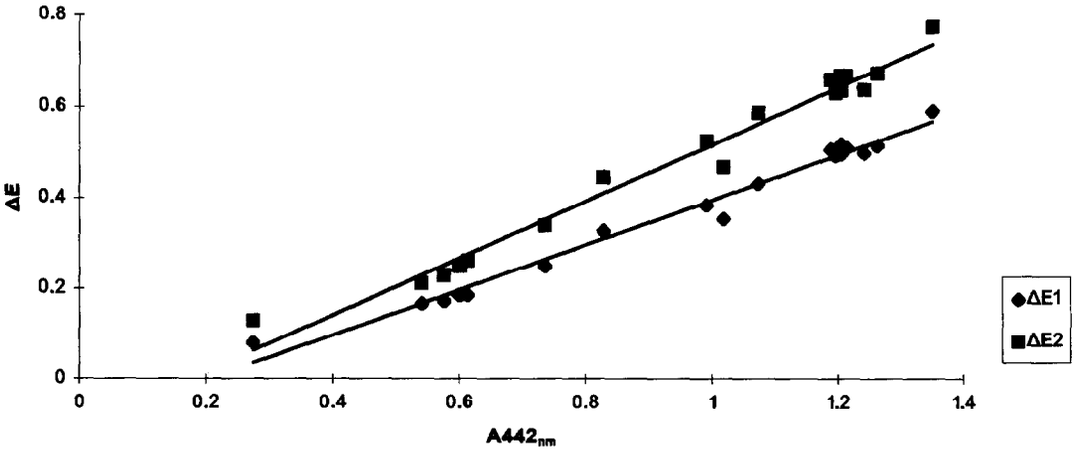

Fig. 5. Regression line for comparing evaluation of saffron colouring strength using absorption readings at 442 nm and ΔE values.

(controlled or not controlled relative humidity and temperature). Absorption maxima were 442.0 nm, 328.0 nm and 257.5 nm for all samples. Absorbance readings recorded using UV–Vis spectrometry correlated well with ΔE_1 ($r = 0.941$) and ΔE_2 ($r = 0.895$), respectively (Fig. 5). ΔE_1 and ΔE_2 may be useful for a better evaluation of saffron colouring strength using standard solutions of suitable chromophores: for example, potassium dichromate solutions which were proposed in the past for visual comparison of intensities with those of saffron aqueous extracts (ISO, 1980). The latter is under investigation in our laboratory.

CONCLUSION

Different extraction protocols influence absorbance readings, which in turn may influence the quality grading of saffron. Standardisation of methodology will reduce analytical variation and improve interlaboratory data agreement. The merits of the 3632-2-1993 ISO standard methodology are presented and future improvements are proposed. Derivative spectroscopy, which was used in this work to resolve overlapping peaks in the spectra, may offer an alternative quantitative approach for the evaluation of colouring strength of commercial saffron samples.

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REFERENCES

Abdullaev, F. I. (1993). Biological effects of saffron. *BioFactors*, **4**, 83–86.

Alonso, G. L., Varon, R., Gomez, R., Navarro, F. & Salinas, M. R. (1990). Autoxidation in saffron at 40°C and 75% relative humidity. *J. Food Sci.*, **55**, 595–596.

Basker, D. & Negbi, M. (1985). Crocetin equivalent of saffron extracts. Comparison of three extraction methods. *J. Assoc. Publ. Anal.*, **23**, 65–69.

Berzas Nevado, J. J., Rodriquez Flores, J. & Villasenor Llerena, M. J. (1993). Simultaneous determination of tartrazine and sunset yellow by derivative spectrophotometry and ratio spectra derivative. *Talanta*, **40**, 1391–1396.

Buchecker, R. & Eugster, C. H. (1973). Absolute Konfiguration von Picrocrocin. *Helv. Chim. Acta*, **56**, 71–124.

Corradi, C. & Micheli, G. (1979). Determinazione spettrofotometrica del potere colorante, amaricante ed odoroso dello zafferano. *Boll. Chim. Farm.*, **118**, 553–562.

FAO (1986). *Food Analysis: Quality, Adulteration and Tests of Identity*. Food and Agricultural Organization of the United Nations, Rome, p. 251.

Finney, D. J. (1980). *Statistics for Biologists*. Chapman & Hall, London, pp. 103–107.

Himeno, H. & Sano, K. (1987). Synthesis of crocin, picrocrocin and safranal by stigma-like structures proliferated *in vitro*. *Agric. Biol. Chem.*, **51**, 2395–2400.

ISO (1980). Saffron specification, ISO-3632-1980(E). International Organization for Standardization, Geneva.

ISO (1993). Saffron (*Crocus sativus* Linnaeus): (a) ISO-3632-1-1993 Part 1: Specification; (b) ISO-3632-2-1993(E) Part 2: Test methods. International Organization for Standardization, Geneva.

Kuhn, R. & Winterstein, A. (1934). Uber die Konstitution des Picro-crocins und seine Beziehung zu den Carotin-Farbstoffen des Safrans. *Ber. Dtsch. Chem. Ges.*, **67**, 344–357.

Manitto, P., Speranza, G., Monti, D. & Gramatica, P. (1987). Singlet oxygen reactions in aqueous solution. Physical and chemical quenching rate constants of crocin and related carotenoids. *Tetrahedron Lett.*, **28**, 4221–4224.

Miller, J. C. & Miller, J. N. (1984). *Statistics for Analytical Chemistry*. Ellis Horwood, Chichester, pp. 82–118.

Narasimhan, S., Chand, N. & Rajalakshmi, D. (1992). Saffron: quality evaluation by sensory profile and gas chromatography. *J. Food Qual.*, **15**, 303–314.

Oberdieck, R. (1991). Ein Beitrag zur Kenntnis und Analytik von Saffron (*Crocus sativus* L.). *Dtsch. Lebensm. Rundsch.*, **87**, 246–252.

Perkampus, H. H. (1992). *UV–Vis Spectroscopy and Its Applications*. Springer-Verlag, Berlin, pp. 88–107.

Sampathu, S. R., Shivashankar, S. & Lewis, Y. S. (1984). Saffron (*Crocus sativus* Linn.)—cultivation, processing, chemistry and standardization. *CRC Crit. Rev. Food Sci. Nutr.*, **20**, 123–157.

Solinas, M. & Cichelli, A. (1988). Analisi HPLC dei composti responsabili del colore e dell'aroma dello zafferano. *Ind. Aliment.*, **XXVII**, 634–640.

Speranza, G., Dada, G., Manitto, P., Monti, D. & Gramatica, P. (1984). 13-*cis*-Crocin: a new crocinoid of saffron. *Gazz. Chim. Ital.*, **114**, 189–192.

Sujata, V., Ravishankar, G. A. & Venkataraman, L. V. (1992). Methods for the analysis of the saffron metabolites crocin, crocetins, picrocrocin and safranal for the determination of the quality of the spice using thin-layer chromatography, high performance liquid chromatography and gas chromatography. *J. Chromatogr.*, **624**, 497–502.

Tarantilis, P. A., Polissiou, M. & Manfait, M. (1994). Separation of picrocrocin, *cis–trans* crocins and safranal of saffron using HPLC with photodiode-array detection. *J. Chromatogr.*, **664**, 55–61.

Tarantilis, P. A., Tsoupras, G. & Polissiou, M. (1995). Determination of saffron (*Crocus sativus* L.) components crude plant extract using high performance liquid chromatography–UV-visible photodiode-array detection–mass spectrometry. *J. Chromatogr.*, **699**, 107–117.

Visvanathan, S., Ravishankar, G. A. & Venkataraman, L. V. (1990). Induction of crocin, crocetin, picrocrocin and safranal synthesis in callus cultures of saffron—*Crocus sativus* L. *Biotechnol. Appl. Biochem.*, **12**, 336–340.