Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1085 (2005) 293–298
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Use of non-aqueous capillary electrophoresis for the quality control of commercial saffron samples

M. Zougagh [a], B.M. Simonet [b], A. Ríos [c], M. Valcárcel [a,*]

[a] *Department of Analytical Chemistry, University of Córdoba, Campus de Rabanales, Edificio Marie Curie, E-14071 Córdoba, Spain*
[b] *Department of Chemistry, University of Girona, Campus de Montilivi, E-17071 Girona, Spain*
[c] *Department of Analytical Chemistry and Food Technology, University of Castilla–La Mancha, Campus de Ciudad Real, E-13004 Ciudad Real, Spain*

Received 17 January 2005; received in revised form 10 June 2005; accepted 14 June 2005
Available online 1 July 2005

Abstract

A non-aqueous capillary electrophoresis (NACE) method for quantifying the seven crocin metabolites that are the major biologically active ingredients of saffron was developed. Separation is done by using a fused silica capillary filled with a 12.5 mM H_3BO_3/37.5 mM sodium tetraborate methanolic solution as background electrolyte. The results obtained were compared with the total index "safranal value", widely used as a quality measure of saffron products. The comparison revealed that the proposed NACE method provides useful information not obtained in the safranal value. Infact, samples with a similar safranal value can contain crocin metabolites in different concentrations and relative proportions. This new method is very useful for quality control in commercial saffron samples.

Keywords: Non-aqueous capillary electrophoresis; Quality control; Crocin metabolites; Saffron samples

1. Introduction

Saffron which consists of dried stigmas of *Crocus sativus* L. is a very expensive spice used mainly as herbal medicine for a food colouring and flavouring agent in various regions of the world [1–3]. In Castilla–La Mancha (Spain), which is one of the largest saffron producer regions, saffron products are ranked in three quality grades, namely: Mancha, Río and Sierra [4]. The major biologically active ingredients of saffron are crocin analogues, which are all glycosides of *trans*-crocetin, a carotenoid derivative (see Fig. 1). *Cis*-Crocetin and its glycosides are also present. Crocin gives saffron its albeit at much lower levels. The spice contains the flavonoid derivative, picrocrocin and its a glycone safranal, in small amounts. Safranal, 2,6,6-trimethyl-1,3-cyclohexadien1-carboxaldehyde, is formed during the manipulation and storage of saffron and by the subsequent chemical or enzymatic dehydratation of picrocrocin [5–7]. It is the volatile oil responsible for the characteristic colour and aroma of saffron. Safranal can be determined spectrophotometrically according to ISO 3632 [8] by measuring the absorbance at 330 nm of saffron in aqueous extract [9]. However, safranal is only sparsely soluble in water and some crocins exhibit an absorbance maximum at the previous wavelength that interferes with the analysis [10,11]. In 1996, Spain adopted a SOIVRE method for the quality control of natural saffron [4]. This method, determines total safranal (viz. safranal present in the sample plus safranal formed by picrocrocin hydrolysis), by using a modified version of the method of Corradi and Micheli [9]. This method is labour-intensive and suffers from safranal instability in the distillate, so it warrants improvement for revision of ISO 3632 in order to insure accurate quality control of commercial saffron.

Quality control of saffron involves identifying and quantifying crocin derivatives which are the major biologically active compounds in the spice. Most determinations of saffron components are based on colorimetric measurement [12], thin-layer chromatography (TLC) scanning [13], high-performance liquid chromatography (HPLC) [14,15,9,10]

* Corresponding author. Tel.: +34 957 218616; fax: +34 957 218616.
E-mail address: qa1meobj@uco.es (M. Valcárcel).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.06.041

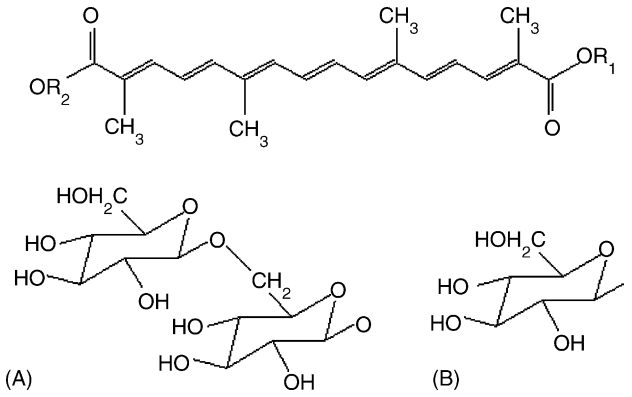

Fig. 1. Structure of some crocin compounds ($R_1 = R_2 = A$; $R_1 = A$ and $R_2 = B$; $R_1 = A$ and $R_2 = H$; $R_1 = B$ and $R_2 = H$) and crocetin ($R_1 = R_2 = H$).

or gas chromatography (GC) [16] assays. HPLC has been widely used in the quality control of saffron because it allows the simultaneous determination of saffron components such as picrocin, its aglycone (HTCC), crocin derivatives and safranal [17,18]. Capillary electrophoresis (CE) has been extensively used as a high-resolution separation technique over the past two decades and can surpass HPLC in terms of simplicity, resolution and economy, it has not yet been used to determine saffron metabolites. In general, saffron metabolites are assumed to neutral. Also, CE is used to separate charged analytes which migrate in a background electrolyte under the influence of an electric field. However, various approaches afford the separation of uncharged compounds. Such approaches use either a complexing agent in an aqueous system, a surfactants (in micellar electrokinetic chromatography (MEKC)) or a non-aqueous solvents (in non-aqueous capillary electrophoresis (NACE)).

Capillary zone electrophoresis (CZE) only allows charged compounds to be separated. However, the migration pattern of the analytes in the capillary can be altered if by chelation with ions in the background electrolyte. One example is the use of a borate buffer to form charged complexes with neutral carbohydrates [19,20]. The separation of flavonoids based on borate complexation of carbohydrate residues has also been reported [21]. In any case, the most widely used capillary electrophoretic technique for the separation of uncharged analytes is MEKC, which was introduced by Terabe et al. in 1984 [22]. In this technique, when a neutral analyte is injected into a micellar medium, a fraction incorporates into the micelles and migrates at the same velocity [23]. The use of non-aqueous solvents is one effective other choice separating uncharged analytes. Organic solvents can facilitate separation through are impossible or too weak in aqueous media. Also, the solubility of both neutral analytes and potentially effective additives for analyte, additive interaction is substantially higher in some organic solvents than in water. Further details can be found elsewhere [24].

This paper discusses available choices for separating neutral saffron compounds and reports a new NACE method for the quality control of commercial saffron with two major advantages over existing HPLC described methods, namely: reagent consumption and higher resolution.

2. Experimental

2.1. Reagents, standards and samples

All chemicals used were analytical reagent grade. The crocin mixture and safranal used were purchased from Fluka (Bushs, Switzerland). A 10 g l^{-1} standard stock solution of each analyte was prepared in chromatographic purity grade methanol (Panreac, Barcelona, Spain) and stored refrigerated in the dark. Standard working solutions (0.1–5 mg ml^{-1}) were prepared on a daily bases by dilution the standard stock solutions with methanol. Sodium tetraborate (Sigma, St. Louis, MO, USA) and boric acid (Panreac, Barcelona, Spain) solutions in methanol, and NaOH (Fluka Chemie, Bushs, Switzerland) solutions in Milli-Q quality water were used to prepare the different electrophoretic solutions. Herbal saffron samples were obtained from Spain local herbal shops and supermarkets.

2.2. Preparation of saffron samples

Saffron samples were powdered to a uniform blend, and 10–20 mg of the bled was treated with 1 ml of methanol. The mixture was magnetically stirred in the dark for 1 h, after which the plant residue, was separated by centrifugation at 2500 rpm for 10 min. Finally, the supernatant was passed through a filter of 0.45 μm pore size, and the clear solution used for CE analysis.

2.3. Instrumentation

CE separation was done on a Beckman Coulter (Palo Alto, CA, USA) P/ACE 5500 capillary electrophoresis system, equipped with a diode array detector and PACE software. A fused-silica capillary, of effective length between inlet and detector of 40 cm (total length 47 cm), and 75 μm i.d. was used for this purpose.

2.4. Operating conditions

The background electrolyte solution was a 25:75 mixture of a 50 mM H_3BO_3 in methanol and 50 mM $Na_2B_4O_7$ also in methanol. Separation was accomplished by using an applied voltage of 16 kV and a capillary temperature of 20 °C. The current thus produced was above 40 μA. Detection was performed at 245, 335 and 435 nm. The capillary was conditioned by rinsing with Milli-Q water for 5 min, 0.1 M NaOH for 5 min, methanol for 5 min and the running buffer for 5 min once a day. Before each analysis, the capillary was flushed with running buffer for 10 min. Samples were introduced into the capillary under a pressure of 3.5 kPa for 8 s.

3. Results and discussion

The quality control of saffron involves the determination and quantification of neutral crocin compounds. However, separating neutral compounds directly by CZE its impossible. For this reason, the neutral crocin compounds were separated as borate complexes, in micellar media (MEKC) and organic solvents (NACE) The three approaches used to this end are discussed. An NACE is proposed for the quality control of commercial saffron samples. The influence of the variables affecting electrophoretic separation is described below.

3.1. Separation of crocin analytes

Based on previous HPLC studies, saffron samples usually contain six different compounds. Crocin compounds contain a neutral carbohydrate residue in their structure (Fig. 1). We should note that these compounds posses no acid or basic groups that can be charged by changing the pH. In fact, the crocin compounds were found to collapse in two-three peaks at pH 8–10 adjusted with a 50 mM borate solution. This was probably a result of a low stability of the borate complexes. In fact the migration time was close to that of the electroosmotic flow, consistent with a low charge. In order to examine the behaviour of these compounds at lower pH values, a mixed phosphate-borate solution was used as BGE. Based on the obtained results, analyte-borate interactions were negligible below pH, where the analytes migrated at the electroosmotic velocity. In order to increase borate-analyte interactions and hence resolution, we explored the use of organic modifiers such as methanol and acetonitrile. A single peak was obtained observed in the electropherogram with acetonitrile as modifier. Therefore, methanol was to be preferred for this purpose. In general, increasing the amount of in the background electrolyte, increased borate-analyte interactions was observed, and hence in longer migration times that clearly departed from that of the electroosmotic flow. The best results were obtained by using a high methanol concentration (60%). However, even with such as high proportion of methanol, crocin exhibited only four peaks because resolution can be also be improved via the applied voltage, we also examined its effect voltage above 15 kV produced an unstable current in the capillary and irreproducible migration times. Also resolution was scarcely improved by operating at 15 kV.

Anionic and cationic micelles were tested to separate crocin compounds. Only three, partially resolved peaks, were obtained by using a solution of 50 mM sodium dodecyl sulphate (SDS) in 30 mM ammonium acetate at pH 5.0 as BGE. Resolution was optimed at pH 8.5, which allowed four peaks to be resolved and quantified. The use of a cationic surfactant such as cetyltrimethylammonium bromide (CTAB) was discarded because it provided worse results.

Because the separation was based on the partitioning of analytes between the micellar and non-micellar phase, the effect of adding borate was also studied. A new equilibrium involving the different borate complexes was thus introduced that might result in improve a resolution. However, the results were similar to those obtained in the absence of borate. This is consistent with the result with CZE results and confirms the low stability of these complexes. The low resolution achieved with MEKC was due to the rather weak analyte-micelle interactions. The carbohydrate residue probably hindered penetration of the analytes into the micelles lowing to its high polarity and the resulting ability to form hydrogen bonds with water. In fact, the migration time was only slightly increased by presence of micelles and continued to be of similar to that of the electroosmotic flow.

As alternative the separation of these compounds in non-aqueous medium was studied. Similarly to CZE, NACE can only separate charged analytes. The main advantage of using an organic solvent is that it ensures strong interactions between additive and the analytes. For this reason, we used a solution of borate in methanol as BGE. One important effect to be considered is that the organic solvent affects the acid-base properties of analytes. Thus, depending on the particular solvent, the pK_a of a chargeable compound can be many orders of magnitude different. In this case, protonation might affect the borate-analyte interactions and as charges of the complexes. Two different solutions were therefore required. One consisted of 50 mM H_3BO_3 in methanol and the other of 50 mM sodium borate also in methanol. In order to study the effect of acid and bases, the two solutions were mixed in variable proportions. As can be seen in Fig. 2, the best resolution was obtained by mixing 25% of the 50 mM boric acid solution with 75% of the 50 mM borate solution. It should be noted that these conditions enabled the resolution of 7 peaks for their commercial crocin standard. One of the advantages of non-aqueous systems, in this context, is their low current, which occasionally allows one to increase the electrolyte salt concentration or to use stronger electric fields. We therefore examined the effect of the total concentration of salt and the applied voltage. The former was studied over 10–100 mM range. All solutions contained boric acid and borate in the same proportion as above. A concentration of 50 mM was adopted because higher salt levels resulted in current instability during the electrophoretic separation. The effect of the voltage was investigated over the range from 6 to 22 kV. In general, the migration times of all crocin compounds decreased with increasing voltage. A voltage of 16 kV thus chosen as a compromise between running time, current level and peak resolution. Above 50–60 μA, the BGE had to be reviewed too frequently. Moreover, high voltages and currents can lead to bubble formation through solvent evaporation and result in current interruptions.

The stability of the BGE solution was studied in several consecutives analysis. By using vials containing 4 ml of BGE we found the same solution to be usable for eight analyses. Pressurizing the system at 3.5 kPa on both sides of the capillary end was found to raise such as a to 12.

A diode array detector was used to determine the maximum absorbance of the analytes. Crocins 1, 2 and 3 exhibited

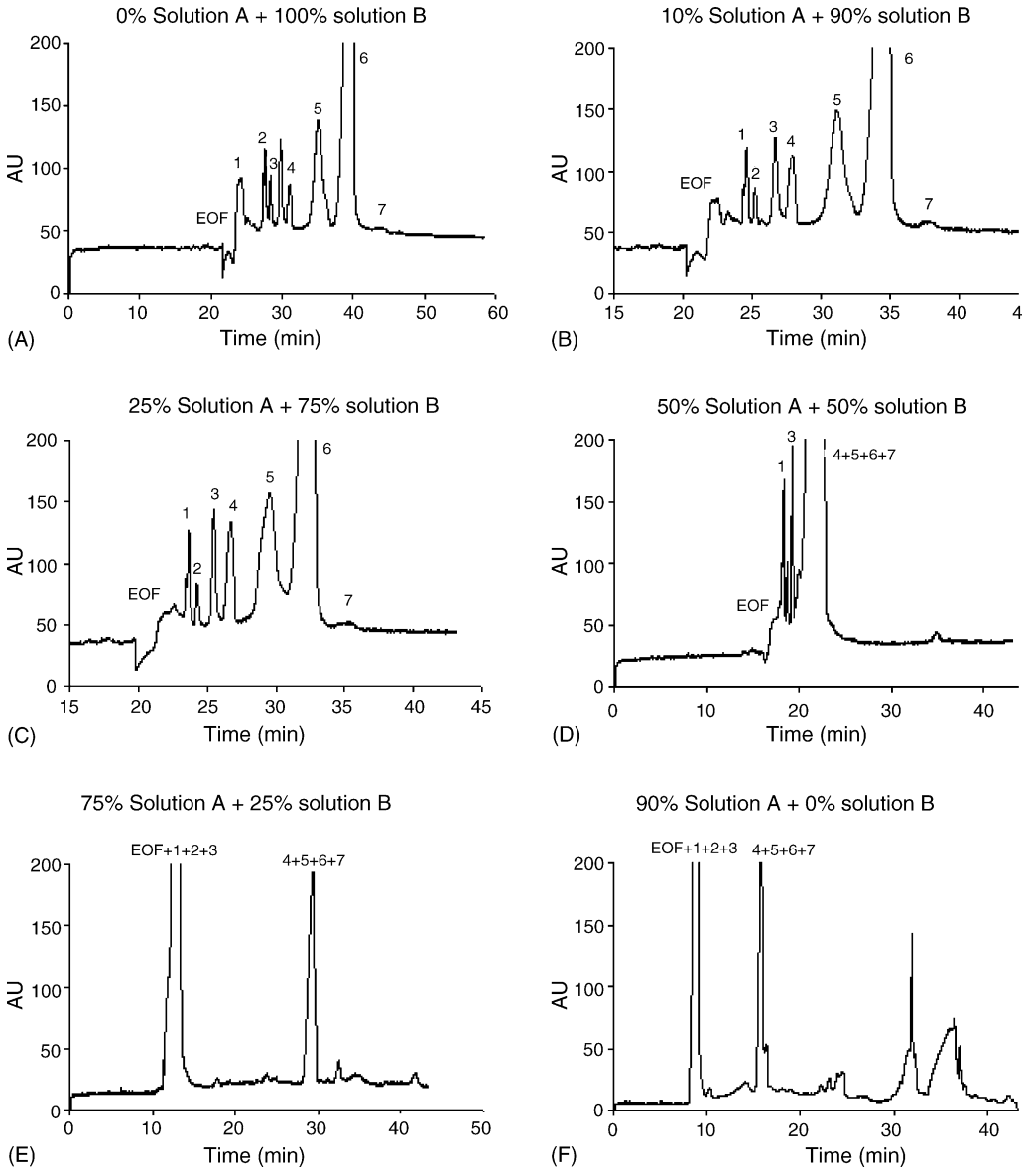

Fig. 2. (A–F) Influence of the buffer pH on peak resolution (λ = 245 nm). Crocin standard concentration: 5 mg ml^{-1}. Peaks: (1) crocin 1, (2) crocin 2, (3) crocin 3, (4) crocin 4, (5) crocin 5, (6) crocin 6 and (7) crocin 7. Solution A: 50 mM H_3BO_3 in methanol. Solution B: 50 mM $Na_2B_4O_7$ in methanol.

present an absorbance maximum at 335 nm, crocins 4 and 5 one at 435 nm, and crocins 6 and 7 one at 245 nm.

3.2. Performance of the NACE method

Calibration graphs were obtained by using crocin standard solutions of concentrations over the 0.1–5 mg ml^{-1} range. Each point in the calibration graph represented to the mean value obtained from three separate area measurements. The proposed method allows crocins to be determined at different concentration levels (see Table 1). The limits of detection and quantification were calculated as $3{\cdot}S_{y/x}$/sensitivity and $10{\cdot}S_{y/x}$/sensitivity, respectively. The corresponding regression equation and other characteristic parameters analytical for the determination of these crocin analytes are shown in Table 1. Eleven replicate analyses were performed on the standard solution (5 mg ml^{-1} crocin) in order to evaluate the precision of the method for each compound. The relative standard deviation was less than 7.3% in all cases.

3.3. Analytical uses

The proposed method was used to analyse natural saffron samples. Table 2 shows the results obtained for five different types of natural saffron samples, and Fig. 3 a typical electropherogram for a real sample. As can be seen in Table 2, not all crocin metabolites were found in the saffron samples. Also the relative proportions of the crocin metabolites differed among samples. Thus, the metabolite crocin 6 was present at a concentration significantly higher than the others.

Table 1
Analytical figures of merit for the proposed method

Crocins	$y = a + bx$	$S_{y/x}$	R	Precision RSD (%)	LOD	LOQ
Crocin 1	$a = -23265.1 \pm 5225.8$; $b = 11586.5 \pm 178.1$	7927	0.997	3.4	2.1	6.8
Crocin 2	$a = -11199.9 \pm 2832.6$; $b = 11508.3 \pm 379.8$	3870.7	0.998	6.5	1.0	3.4
Crocin 3	$a = -39945.7 \pm 10630.4$; $b = 11413.9 \pm 318.4$	15370.3	0.997	4.3	4.0	14
Crocin 4	$a = -99595.9 \pm 34744.7$; $b = 11353.6 \pm 113.0$	45554.6	0.991	7.3	12	40
Crocin 5	$a = -173432.2 \pm 70540.7$; $b = 11323.2 \pm 163.7$	113543.5	0.997	2.5	30	100
Crocin 6	$a = -1243383.0 \pm 207277.8$; $b = 11531.2 \pm 261.6$	348529.5	0.998	5.3	91	302
Crocin 7	$a = -31459.9 \pm 26476.2$; $b = 11929.8 \pm 4178.9$	9788.6	0.999	4.8	2.5	8.2

a: Intercept; b: slope; R: correlation coefficient; $S_{y/x}$: standard deviation of residuals; RSD: relative standard deviation of the peak area ($n = 11$); LOD: limit of detection; LOQ: limit of quantification. Concentrations, LOD and LOQ, are expressed in $\mu g\,ml^{-1}$.

Table 2
Analysis of natural saffron samples

Analytes	Wavelength (nm)	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	Sample 4 (%)	Sample 5 (%)
Crocin 1	335	0.014 ± 0.001	n.d	n.d	n.d	n.d
Crocin 2	335	n.d	n.d	n.d	n.d.	n.d
Crocin 3	335	0.023 ± 0.001	n.d	n.d	n.d	n.d
Crocin 4	435	0.063 ± 0.002	0.0450 ± 0.0002	0.0556 ± 0.0009	n.d	n.d
Crocin 5	435	0.86 ± 0.07	0.45 ± 0.02	0.26 ± 0.02	0.20 ± 0.02	0.103 ± 0.006
Crocin 6	245	7.5 ± 0.4	5.3 ± 0.4	3.8 ± 0.1	2.9 ± 0.1	1.81 ± 0.09
Crocin 7	245	0.028 ± 0.002	0.024 ± 0.001	0.023 ± 0.001	0.0225 ± 0.0002	0.0220 ± 0.0009
ISO 3632 method, safranal value	330	58.17	56.81	55.75	29.27	33.15
Soivre method, safranal value	317.5	3.64	3.56	3.44	1.92	1.80

Values are expressed as mean ± standard deviation ($n = 3$).

The total concentration of crocin also differed among samples. As can be seen in Fig. 4, when crocin related compounds are analysed by HPLC, following the method described in ref. [18], the crocin 1 is the most abundant analogue. The comparison of the spectral peaks obtained by HPLC with those obtained by CE pointed out that spectrum of peak 1 in HPLC correspond to peak 6 in CE; peak 2 in HPLC corresponds to peaks 1 in CE; peak 3 in HPLC corresponds to peak 2 in CE and peak 4 in HPLC corresponds to peak 5 in CE.

We also determined the "safranal value" according to ISO 3632 [8] and using the SOIVRE method [4]. The ISO method is based on measurement of the absorbance at 330 nm of an aqueous extract of 20 mg of saffron extracted with 200 ml water at room temperature in the darkness for 24 h. SOIVRE method involves alkaline hydrolysis of saffron, distillation in a vapour stream and measurement of the absorbance of the distillate at 317.5 nm. Finally, the total index called the "safranal value" was calculated from "$E = ABS_{330} v/(100 \times p)$" (where ABS_{330} is the measured absorbance at 330 nm; v, the volume of H_2O added in ml; and p the weight of the saffron sample in g) for the ISO method and as four times the measured absorbance at 317.15 nm for the SOIVRE one. This total index has been proposed as a measure of quality of natural saffron. As can be seen in Table 2 the safranal value for samples 1, 2 and 3 were roughly 1.5 times higher than those found for samples 4 and 5. As can be also seen from Table 2, samples with a similar safranal value exhibited a different concentration and distribution of crocin metabolites. Therefore, the safranal value, which is widely used as a total index, does not suffice to establish the quality

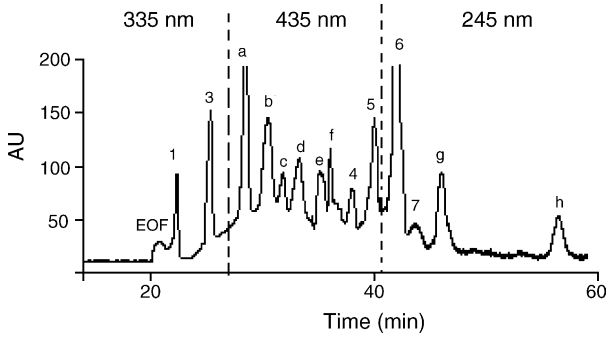

Fig. 3. Electrophoregrams for natural saffron samples. Peaks: (1) crocin 1, (2) crocin 2, (3) crocin 3, (4) crocin 4, (5) crocin 5, (6) crocin 6 and (7) crocin 7. (a–h) Not identified in this work.

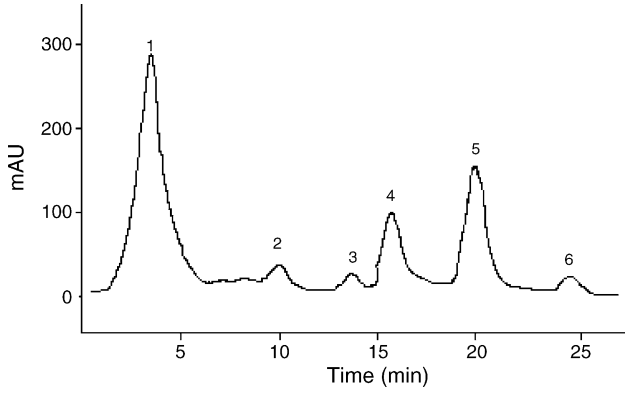

Fig. 4. Chromatogram of a crocin standard obtained following the method described in [18].

of saffron and should be completed with the information provided by the proposed method.

4. Conclusions

The proposed NACE method is a simple, effective choice for the identification and determination of seven crocin metabolites, and hence for assessing saffron quality. The NACE method surpasses the RP-HPLC analysis, not only in expeditiousness but also in ease operation. Also, it uses relatively low volumes of electrolyte in electrophoretic run.

Although the method is mainly intended for the separation and quantification of the main crocin metabolites present in natural saffron samples, it additionally allows the separation of other analytes (viz. those giving peaks a–h in Fig. 3).

Acknowledgement

Spain's Ministerio de Ciencia y Technologia (MCyT) is gratefully acknowledged for funding this work with Grant CTQ2004-01220.

References

[1] S.C. Nair, B. Panikkar, K.R. Panikkar, Cancer Lett. 57 (1991) 109.
[2] S.C. Nair, S.K. Kurumboor, J.H. Hasegawa, Cancer Biother. 10 (1995) 257.
[3] D. Basker, M. Negbi, Econ. Bot. 37 (1983) 228.
[4] Parámetros de Calidad en el Azafrán, Laboratorios del Soivre (Boletín Técnico Informativo), Dirección General de Comercio Exterior, Ministerio de Economía y Hacienda, Lekro Print, Madrid, Spain, 1996.
[5] P.C. Curró, G. Micali, L. Lanuza, Res. Chim. 6 (1986) 331.
[6] H. Pfander, H. Schutenberger, Phytochemistry 21 (1982) 1039.
[7] J.L. Iborra, M.R. Castellar, M. Cánovas, A. Manjón, J. Food Sci. (57) (1992) 714.
[8] ISO 3632-1&2, Saffron (*Crocus sativus* L.), The International Organisation for Standardization, first ed., Switzerland, 1993.
[9] C. Corradi, G. Micheli, Boll. Chim. Farm. 118 (1979) 553.
[10] P.A. Tarantilis, M. Polissiou, M. Manfait, J. Chromatogr. A 664 (1994) 55.
[11] P.A. Tarantilis, G. Tsoupras, M. Polissiou, J. Chromatogr. A 699 (1995) 699.
[12] N. Bizzozero, A. Cami, G. Micheli, Ind. Aliment 38 (1999) 125.
[13] R.L. Jin, Z.P. Qiao, S.D. Zhou, Y.L. Ye, J.X. Zhou, J. Nanking Coll. Pharm. 17 (1986) 247.
[14] H. Pfander, M. Rychener, J. Chromatogr. 234 (1984) 443.
[15] M.R. Castillar, H. Montijano, A. Manjón, J.L. Iborra, J. Chromatogr. 648 (1993) 187.
[16] V. Sujata, G.A. Ravishankar, L.V. Venkataraman, J. Chromatogr. 624 (1992) 497.
[17] P. Lozano, M.R. Castellar, M.J. Simancas, J.L. Iborra, J. Chromatogr. A 830 (1999) 477.
[18] N. Li, G. Lin, Y.W. Kwan, Z.D. Min, J. Chromatogr. A 849 (1999) 349.
[19] H. Schwaiger, P.J. Oefner, C. Huber, E. Grill, G.K. Bonn, Electrophoresis 15 (1994) 941.
[20] S. Suzuki, S. Honda, Electrophoresis 24 (2003) 3577.
[21] P.G. Pietta, P.L. Mauri, L. Zini, C. Gardana, J. Chromatogr. A 680 (1994) 175.
[22] S. Terabe, K. Otsuka, K. Ichikawa, T. Suchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[23] M. Molina, M. Silva, Electrophoresis 23 (2002) 3907.
[24] M.L. Riekkola, M. Jussila, S.P. Porras, I.E. Valkó, J. Chromatogr. A 892 (2000) 155.