P53: STANDARDIZATION OF SAMPLE PREPARATION CONDITIONS FOR CROCETIN ISOLATION FROM SAFFRON AIDED BY RESPONSE SURFACE METHODOLOGY

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Abstract – The present study aimed at the standardization of sample preparation conditions for the quantitative isolation of crocetin (CRT) from saffron. Response surface methodology (RSM) was employed for the optimization of the first step of the process, i.e. the preparation of a polar saffron extract rich in crocetin sugar esters that represents the most expensive step of the process due to the high price of the starting material.

Keywords: crocetin, saffron, standardization, response surface methodology, acid hydrolysis

1. INTRODUCTION

Saffron apocarotenoids, mainly in the form of crocetin sugar esters (also known as crocins), are reported to exert various biological functions [1] either in the form of extracts or as isolated compounds. The parent molecule, crocetin (CRT) (8,8'-diapocarotene-8,8'-dioic acid) (Fig. 1), a C20-dicarboxylic acid, is not naturally found free but can be liberated in the gastrointestinal tract from bound structures after consumption of the spice or its extracts [2]. Certain of the *in vivo* biological activities (e.g. antioxidant, neuroprotective etc) of saffron have been assigned to crocetin [3,4]. Till now, no systematic studies for the optimization of crocetin isolation process have been reported. The present work aimed at the standardization of the sample preparation conditions for the quantitative isolation of crocetin from saffron [5]. Particular emphasis was paid on the first step of the process that includes the preparation of saffron polar extracts rich in crocetin sugar esters. Optimization of ultrasound assisted extraction of crocetin sugar esters at a preparative scale using response surface methodology (RSM) was focused on saffron:solvent ratio (w/v) and duration of extraction (min). The solvent used, a methanol:water mixture (1:1, v/v), was the optimum one found for ultrasound assisted extraction of crocins from saffron in our previous work [6]. RP-HPLC-DAD and optical microscopy were the means to monitor the extraction process. Precipitation of CRT was then carried out by acid hydrolysis (90 °C, pH = 0.1). The optimum ratio found (1:180, w/v) spares the precious starting material and reduces the major cost of the process. Isolated crocetin could be then encapsulated in proper food grade materials and find applications in food, pharmaceutical and cosmetic industries.

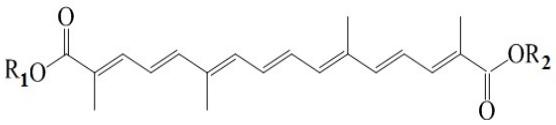

Fig. 1. Chemical structure of crocetin.

2. EXPERIMENTAL

2.1. Samples

Authentic Greek saffron (harvest year 2012) was donated by the Saffron Cooperation of Kozani (Greece). Saffron stigmas were ground using an agate pestle and mortar and passed through a 0.4 mm sieve before analysis.

2.2. Standards, reagents and solvents

Trans-crocetin di-(*β*-D-gentiobiosyl) ester (*trans*-4-GG crocetin ester) was laboratory isolated by semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) according to Kyriakoudi and Tsimidou [5]. Purity of the isolated *trans*-4-GG crocetin ester (97%) was checked (a) chromatographically by RP-HPLC-DAD in the range of 200 – 550 nm and calculated as the percentage of the total peak area at 440 nm and (b) by Nuclear Magnetic Resonance (NMR) spectroscopy, recording the 1H 1D spectra at 300 MHz on a Brucker 300AM spectrometer (Rheinstetten,

Germany). All reagents and solvents used were of the highest purity required. Ultrahigh purity water was produced using a SG Ultra Clear Basic UV system (SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany).

2.3. Crocetin isolation procedure

The experimental procedure that was followed in the present study for the isolation of crocetin from saffron is shown in Fig. 2.

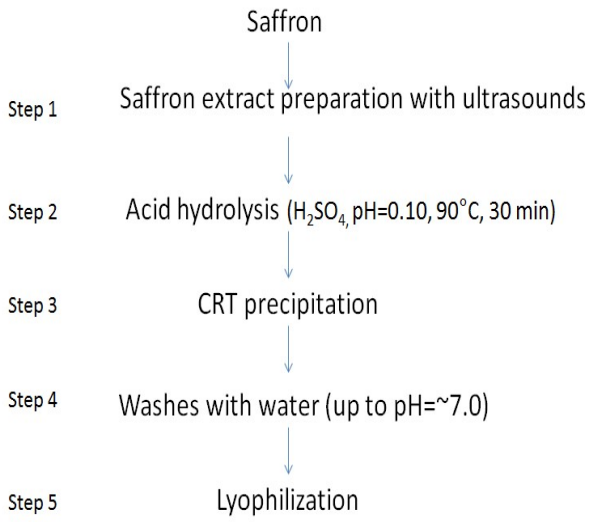

Fig. 2. Flow diagram of crocetin (CRT) isolation.

The effect of saffron:solvent ratio (w/v) (X1) and of the duration of the extraction process (min) (X2) on the recovery of crocetin sugar esters was examined using an unblocked full factorial central composite design (CCD). Each factor (X1 and X2) had five equally spaced levels in the design, coded as -a, -1, 0, +1, +a, where a stands for the distance of a star point to the center point in a CCD and -1, +1 and 0 correspond to the low, high and middle levels of the X1 and X2 factors. The CCD included 13 runs. As dependent variable the total crocetin sugar esters content (g/100 g dry material) was selected.

The extraction procedure (step 1) was as follows: Appropriate amount of ground saffron (0.011-0.11 g) was transferred into a 50 mL Falcon tube and 20 mL of a methanol:water mixture (1:1, v/v) were added. Extraction was carried out with the aid of an ultrasonic processor (Ultrasonic Homogenizers HD 2070, Berlin, Germany). The duty cycles (active intervals, s) were set at 0.2 and the amplitude was 10%. The immersion depth of the probe was 20 mm and temperature was kept at 15 ± 0.5 °C in a thermostated water bath. After the end of the extraction process, the extract was centrifuged (4500 rpm, 5 min, 4 °C). The supernatant was collected and examined by RP-HPLC-DAD as previously described in detail by Kyriakoudi and Tsimidou [5]. Quantification of crocetin sugar esters was accomplished with a proper calibration curve of in-house isolated *trans*-4-GG crocetin ester. The solid residue was examined by optical microscopy using a Zeiss Axiolab reflection light microscope equipped with a camera. In each case, test samples were prepared in triplicate and a large number of microscopic fields (> 50) were examined.

The next step in the experimental procedure involved the precipitation of crocetin (steps 2 & 3) from the saffron extract that was prepared under the optimum conditions. The precipitation of crocetin was carried out by acid hydrolysis of crocetin sugar esters. In particular, 0.11 g of saffron sample were extracted with a methanol:water mixture (1:1, v/v) with the aid of ultrasounds for 29 min. The extract was then centrifuged at 4100 g for 15 min at 4 °C. Methanol was removed by evaporation and the aqueous supernatant was acidified with the addition of H_2SO_4 to pH 0.10, heated at 90 °C for 30 min, cooled and centrifuged under the same conditions. The efficiency of the hydrolysis of crocetin esters was monitored by Thin Layer Chromatography (TLC) using petroleum ether:acetic acid (1:1, v/v) as the development system and by RP-HPLC-DAD of the hydrolysate. Unhydrolyzed crocetin sugar esters were removed with repeated washes with deionized water (step 4). The obtained crocetin was then lyophilized (step 5). Its purity and identity were confirmed by UV-Vis spectroscopy, RP-HPLC-DAD, FT-IR spectroscopy and NMR spectroscopy.

3. RESULTS AND DISCUSSION

3.1. Optimization of sample preparation conditions

In the present study, particular emphasis was given on the preparation of a saffron extract rich in crocetin sugar esters (step 1), which is the most expensive step due to the high price of the starting material. In this view, this step was optimized in terms of saffron:solvent ratio and duration of extraction to spare the precious starting material. A preliminary trial examining the highest saffron:solvent ratio found in literature (i.e. 1:18

w/v) [4] revealed only limited extraction of these colorants from the tissues after examination of the latter with optical microscopy as illustrated in Fig 3. For this reason, the selected range regarding the saffron:solvent ratio was 1:180-1:1800 w/v. The duration of the extraction process was in the range 5-30 min to diminish the possibility of degradation of crocetin sugar esters. As solvent, a mixture of methanol:water (1:1, v/v) that was the optimum one found for the ultrasound assisted extraction of crocetin sugar esters from saffron [6] was used. The pulsed mode of sonication was adopted at a predetermined value of duty cycles (0.2 s).

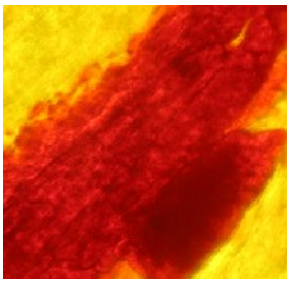

Fig. 3. Observation of saffron tissues by optical microscopy after extraction at saffron:solvent ratio 1:18 (w/v).

Statistical analysis of the experimental results of the 13 runs showed that the factor saffron:solvent ratio (X1) had a positive and significant effect (at 95% confidence level) on the recovery of crocetin sugar esters. The duration of sonication (X2) showed non significant effects on the total crocetin esters content even though shorter extraction time was not found to favour their extraction.

The optimum extraction conditions were found to be saffron:solvent ratio; 1:180 (w/v) and duration of sonication; 30 min. Under these conditions, the mean experimental value of total crocetin sugar esters (34.8±0.4 g/100 g dry material, *n*=3) fitted well with the predicted one (35 g/100 g dry material). Most of the crocetin sugar esters were found to be extracted from the tissues without the need for a second or a third extraction cycle (Fig. 4).

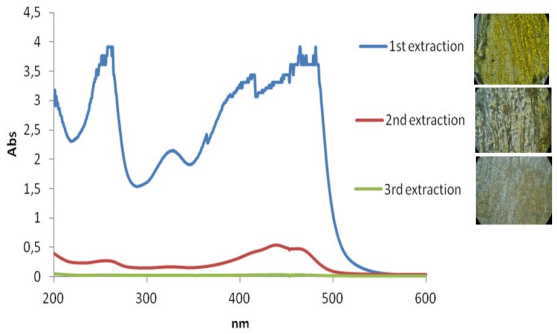

Fig. 4. Zero order UV-Vis spectra of extracts after 1st, 2nd and 3rd successive extraction under the optimum conditions; dilution 1:10 v/v. Insert: Observation of saffron tissues by optical microscopy after each extraction.

3.2. Crocetin isolation

Methanol was removed from the saffron extract prior to acid hydrolysis in order to avoid the possibility of re-esterification of crocetin, as traces of methyl esters of crocetin were detected in the RP-HPLC profile of the hydrolysate in its presence (Fig. 5).

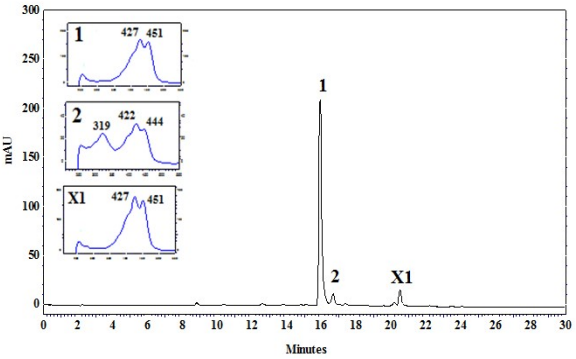

Fig. 5. RP-HPLC-DAD profile of the hydrolysate in the presence of methanol at 440 nm. Peaks 1 and 2, *trans*- and *cis*- isomers, respectively; peak X1, methyl-ester of crocetin.

The precipitated crocetin, after repeated washes with water, was then lyophilized. The identity of the isolated crocetin was confirmed by UV-Vis spectroscopy, RP-HPLC-DAD, FT-IR spectroscopy and NMR spectroscopy (Fig. 6). The purity of the obtained crocetin, calculated using RP-HPLC, was 98%. Crocetin was a mixture of the *trans*- and *cis* isomers with the former being the predominant one (89%).

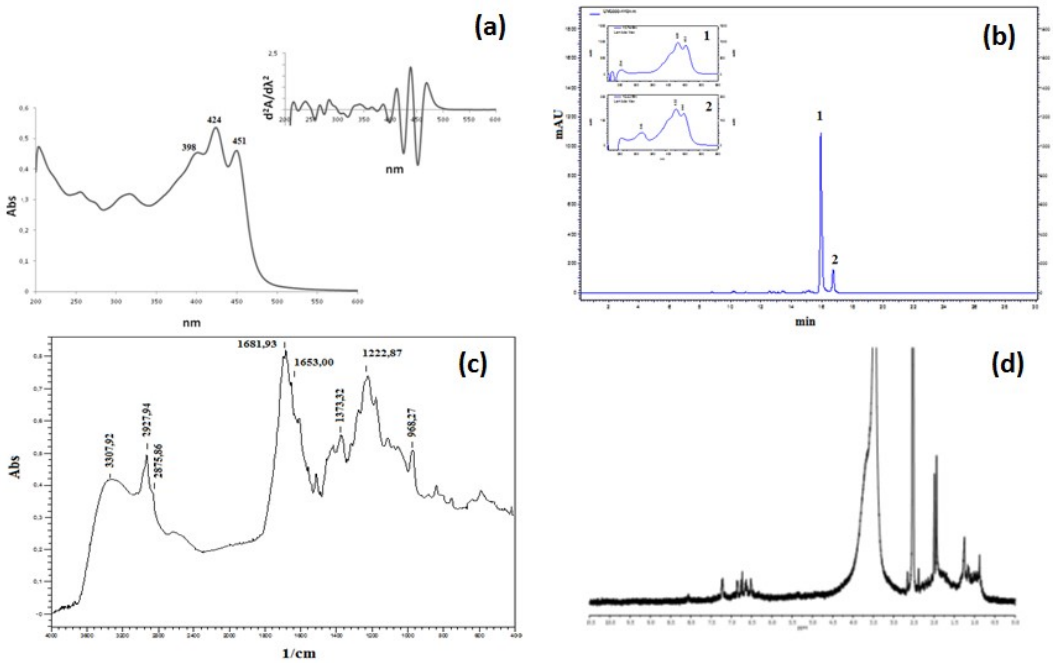

Fig. 6. (a) Zero order spectrum of crocetin. Insert: 2nd order derivative spectrum. (b) RP-HPLC-DAD profile of crocetin at 440 nm; peaks 1 and 2, *trans*- and *cis*-isomers, respectively. Insert: the UV-Vis spectra of the peaks 1 and 2. (c) FT-IR spectrum of crocetin (4000-400 cm^{-1}). (d) ^{1}H NMR spectrum of crocetin (in DMSO-d_6).

4. CONCLUSIONS

The optimum saffron:solvent ratio found in the present study spares the precious starting material and reduces the major cost of the crocetin isolation process. Isolated crocetin could be then encapsulated in proper food grade materials and find applications in food, pharmaceutical and cosmetic industries due to increased stability.

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REFERENCES

[1] C. Ulbricht, J. Conquer, D. Costa, W. Hollands, C. Iannuzzi, R. Isaac, J.K. Jordan, N. Ledesma, C. Ostroff, J.M.G. Serrano, M.D. Shaffer, M. Varghese "An evidence-based systematic review of saffron (*Crocus sativus*) by the natural standard research collaboration" Journal of Dietary Supplements vol. 8 pp. 58-114, 2011.

[2] A. Asai, T. Nakano, Takahashi M., Nagao A. "Orally administered crocetin and crocins are absorbed into blood plasma as crocetin and its glucuronide conjugates in mice" Journal of Agricultural and Food Chemistry vol. 53, pp. 7302-7306, 2005

[3] A.S. Ahmad, M.A. Ansari, M. Ahmad, S. Saleem, S. Yousuf, M.N. Hoda, F. Islam "Neuroprotection by crocetin in a hemi-parkinsonian rat model" Pharmacology Biochemistry and Behavior vol. 81, pp. 805-813, 2005

[4] S.A. Ordoudi, C.D. Befani, N. Nenadis, G.G. Koliakos, M.Z. Tsimidou "Further examination of antiradical properties of *Crocus sativus* stigmas extract rich in crocins" Journal of Agricultural and Food Chemistry vol. 57, pp. 3080-3086, 2009.

[5] A. Kyriakoudi, M.Z. Tsimidou "A food-grade approach to isolate crocetin from saffron (*Crocus sativus* L.) extracts" Food Analytical Methods, vol. 8, pp. 2261-2272, 2015

[6] A. Kyriakoudi, A. Chrysanthou, F. Mantzouridou, M.Z. Tsimidou "Revisiting extraction of bioactive apocarotenoids from *Crocus sativus* L. dry stigmas (saffron)" Analytica Chimica Acta vol. 755, pp. 77-85, 2012.