Comparison of Different Extraction Procedures and HPLC Methods to Detect Crocins in Saffron

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Abstract

Isolation of crocins isomers has been studied in terms of extraction, focusing in the solvent variable (methanol or water) and its characterisation by two different chromatographic methods. The use of methanol either on the extraction procedure or in the HPLC analysis produces crocins re-esterification. Though, methanol may increase crocins extraction yield, but then it would be impossible to know the real ratio between crocins in saffron.

INTRODUCTION

The quality of saffron is certified in the international trade market following the ISO 3632 Normative since 1993. The most important parameter is colouring strength, calculated from UV-Vis measurements at 440 nm in aqueous extracts of this spice. Such measurements are related with the total crocins content. Such compounds are glycosides of the carotenoid trans-crocetin which is a dicarboxylic acid carotenoid.

The European community is interested on the so called "product traceability" and for example crocins as biomarkers of saffron colour are being studied. This information may be useful not only for a quality control process but to define characteristics of certain saffron origins. However, isolation and characterisation of crocins isomers is not an easy process, because they are very sensitive to light, humidity, and high temperatures. There have been many attempts to study its extraction, focusing in the solvent variable. Acetone and ethanol and ethanol-water solutions in different proportions were the first ones to be study (Kakimura and Nakazato, 1985). Other researches (Himeno and Sano, 1987; Tarantilis et al., 1995; Li et al., 1999; Lozano et al., 1999) have also tested methanol-water systems and water as a sole solvent (Iborra et al. 1992; Asimiadis et al. 1998; Alonso et al., 2001).

Some analytical methods have been developed to separate, identify and quantified the crocins. Techniques such us thin-layer chromatography (TLC) (Visvanath et al., 1990; Sujata et al., 1992) is commonly used in ISO normative, but with no doubt is high performance liquid chromatography (HPLC) the most effective technique for the analysis of crocins and relate compounds in saffron extracts (Himeno and Sano, 1987; Visvanath et al., 1990; Sujata et al., 1992; Tarantilis et al., 1995; Li et al., 1999; Lozano et al., 1999; Alonso et al., 2001).

The aim of this study was to discuss different extraction and HPLC-DAD methods; especially the ones appeared recently in the bibliography that permitted simultaneous detection and identification of saffron crocins. From the two main group of methodologies, the one that uses methanol as extractant or in the mobile phase (Lozano et al., 1999) and those ones that use acetonitrile (Alonso et al., 2001) will be studied.

MATERIALS AND METHODS

Materials

Spanish saffron samples of category I were analysed. Methanol (Merck) and acetonitrile (Merk) was of chromatographic purity. Water was double distilled and purified through Elgastat UHQII System. Membranes of cellulose acetate (Whatman) of

0,45 μm were used to filtrate saffron extracts.

Sample Preparation and HPLC Conditions

1. Alonso et al. Method (2001). Saffron was grounded until a 95% pass through a 500 μm sieve. A sample of 400 mg was suspended for 1 hour in 200 ml water previously bubbled with helium. The whole process is carried out in darkness and at room temperature. The resulted mixture was filtered through 0.45μm membrane.

A Perkin-Elmer HPLC instrument (Norwalk, USA) composed of an LC 410 pump, an autosampler LC ISS 200 410 joined to a diode array detector 1100 of Hewlett-Packard was used. The wavelength was set at 250, 330, 440. The column used was a Hewlett-Packard C_{18} Nucleosil 5 μm (200 x 4 mm i.d.). The gradient profile was as follows: 80% A + 20% B (5 min., flow rate 0.8 ml/min) to 25% A + 75% B in 15 min (flow rate 0.8 ml/min), then to 100% B in 3 min (flow rate 0.8 ml/min) and 10 min with 100% B (flow rate 1 ml/min); where A: Water and B: Acetonitrile. The oven temperature was set at 25º C and injection volume at 10 μl.

2. Lozano et al. Method (1999). Saffron was grounded until a 95% pass through a 500 μm sieve. A sample of 400 mg was suspended in 200 ml of methanol-water (50%, v/v) and stirred during 1 hour in darkness at 25 ºC. The extract was filtered through 0.45μm membrane.

The HPLC equipment is the one mentioned before. The column used was a Water C_{18} Nova-Pack 5 μm (4,6×150 mm i.d.). Solvents employed for elution were: The gradient profile was as follows: 80% A + 20% B to 30% A + 70% B in 50 min; where A: Water and B: Methanol. The flow rate 1.2 ml/min and the oven temperature was set at 25 ºC and injection volume at 10 μl.

Modification. Changes have been carried out on Lozano elution programme, the other extraction and chromatographic parameters are maintained. The new elution programme was defined as: 80% A + 20% B to 30% A + 70% B in 50 min then to 10% A + 90% B in 5 min and 10 min with 90% B; where A: Water and B: Methanol.

RESULTS AND DISCUSSION

Important differences were observed in terms of crocins absorbance, after analysing the same saffron extract by the two selected methods (Figure 1A and 1B). Within Alonso et al. (2001) method the peak area of the crocins was twice the one identified by Lozano et al. (1999). This behaviour it cannot be explained by differences on crocins absortivity due to were dissolved in different solvents. The reasonable hypothesis is that some crocins maybe has been re-esterified with methanol. The fact of using an alcoholic solvents is not appropriate owing to the lability of the glycosyl esters towards hydrolysis and possible esterifications (Pander et al., 1982; Morimoto y col., 1994; Tarantilis y col., 1994) and also many lipids are extract interfering in the fractions to be analysed, so the use of water as a sole extract is more adequate than using any other water-alcohol solutions (Iborra et al. 1992; Assimiadis et al., 1998; Alonso et al., 2001). Due to the oxidation carotenoid structure, water for extraction needs to be free of oxygen (Alonso et al., 1990; 2001).

In case the mentioned re-esterification takes place, then the resulting compounds would have higher polarity than the natural crocins, showing higher retention time. For this reason, Lozano´s method was modified by inserting two more steps with higher percentage of methanol. It was observed that a new group of compounds (tr 50-60 min) appeared (Figure 1C) with an UV-Vis spectra similar to crocins ones. Such polar compounds are not present in the starting aqueous extract. Definitely, the use of methanol as extractant or in the mobile phase produces crocins re-esterification. Some authors propose methanol to get better extraction yield, but then it would be impossible to know the real ratio between crocins in saffron.

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Figures

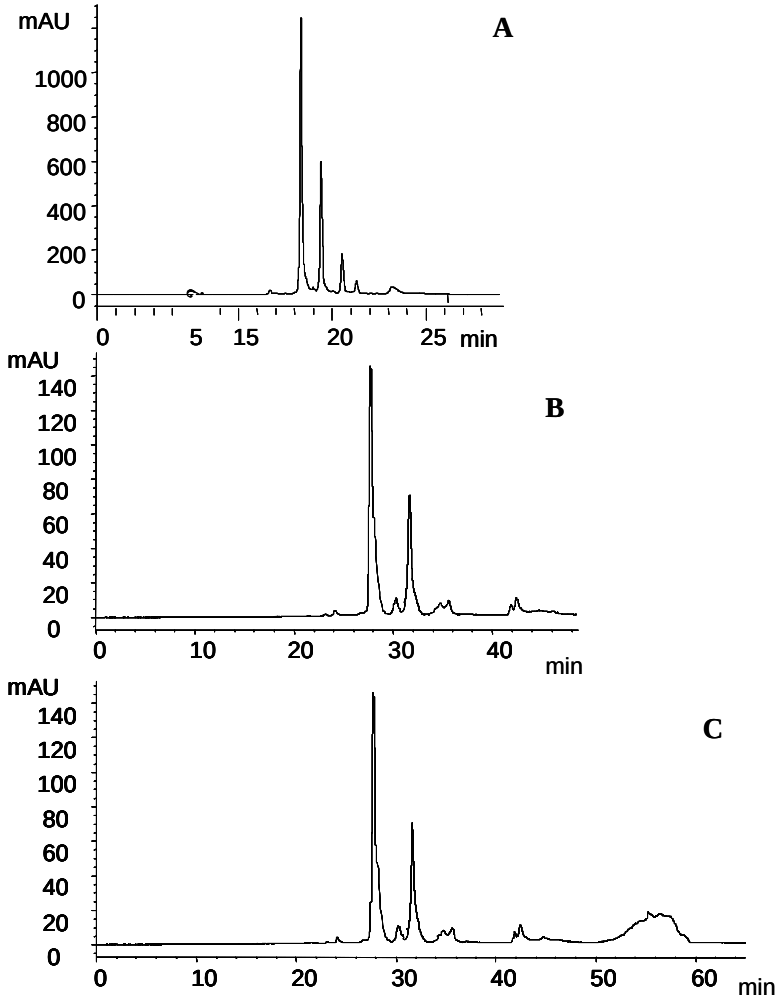

Fig. 1. Chromatograms obtained following A) Alonso et al. (2001) methodology B) Lozano et al. (1999) and C) Lozano et al. (1999) modification.