HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-DAD ANALYSIS OF APO-CAROTENOIDS PRESENT IN THE SAFFRON (*Crocus sativus* L.) FLOWER STIGMA

R.M.T.N. Perera [1], J.A.S.U. Gunawardena [1], U.G. Chandrika [2], D.D.D.H. Alwis [1*]

[1]Faculty of Natural Sciences, The Open University of Sri Lanka
[2]Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenapura, Sri Lanka

INTRODUCTION

Saffron is a high-value spice obtained from the dried stigmas of Saffron (*Crocus sativus L.*) flowers. It is mainly used as a food additive due to its gentle aroma and attractive colour. Apart from these, therapeutic significances like being anti-cancer, an anti-oxidant and an anti-depressant have also been reported elsewhere(de Castro & Quiles-Zafra, 2020). Carotenoids, monoterpene aldehydes, monoterpenoids and flavonoids are the major phytochemical constituents present in the saffron stigmas (Dhiman & Kharkwal, 2020). Among those secondary metabolites, major bioactive compounds found are apocarotenoids like crocin, crocetin and picrocrocin, and an aldehyde called safranal. Crocins are glycosides of the crocetin, which imparts a red-orange colour to the stigma, and water-soluble pigments. Crocetin is a dicarboxylic acid that consists of two terminal carboxylate groups at the two ends of the backbone of the terpenoid structure. Glycosylation of crocetin with D-glucose, D-gentiobioses and various other sugars lead to form crocins and make it more hydrophilic than other carotenoids. Compounds such as crocin give the colour of saffron while a specific bitter taste is brought by the monoterpene glycoside picrocrocin and the aroma is due to safranal (Dhiman & Kharkwal, 2020).

Carotenoids consist of conjugated tetraterpenoid hydrocarbon structures and are lipid soluble pigments. However, crocins belonging to the carotenoid family are unusual because these are water soluble. Hydrophilicity of the crocin is due to the presence of the terminal glycosyl units. Almost 30% of the dry matter of saffron is composed of crocins (C) and it is a mixture of C-1 or a-C (where gentiobiosyl presence in both ends), C-2 (tricrocin – one end contains gentiobiosyl and other end contain glucosyl), C-3 (one end contains gentiobiosyl), C-4 (one end contain glucosyl) and C-5 (both ends contain glucosyl) or dicrocin. Crocetin can be obtained when the crocins are hydrolysed by diluted acids. The conjugated polyene system of apocarotenoid allows the absorption of visible light with wavelengths between 400-500 nm resulting in the ground state, S_0 to singlet excited state, S_2 which is an allowed transition (M.M. Mendes-Pinto, 2013). This relationship between chromophore and light absorption properties are widely employed in the identification and quantification of saffron carotenoids and also to understand light-harvesting and photo-protective actions (Britton, 1995). Picrocrocin (l_{max} at 250 nm) and safranal (l_{max} at 310 nm) show maximum absorption in the UV region whereas crocin absorbs in the visible region (l_{max} at 433 nm) of the UV-vis spectrum. Although there is much-published literature on saffron analysis, no standardised carotenoid extraction method for saffron was found. Furthermore, no recorded analysis was found for crocins and crocetin in the commercial saffron samples available in Sri Lanka. The following study was performed to analyse carotenoids present in a commercial saffron sample obtained from a local supplier in Sri Lanka.

METHODOLOGY

Samples and chemicals

Saffron samples were purchased from a local supplier in Sri Lanka. Analytical grade absolute ethanol was used for the extraction of carotenoids, and HPLC grade water and acetonitrile for HPLC analysis were purchased from Lionchem Pvt (Ltd).

Sample preparation

The extraction of carotenoids present in Saffron stigma

Saffron stigmas (0.0209 g) were finely ground, and it was extracted with cold absolute ethanol (300ml for 2 hours). The extract was filtered and the filtrate was rotary evaporated to concentrate. The concentrated extract was dried by purging nitrogen, resulting in a residue, which was stored at -20 °C.

The extraction of Crocetin from crocin

The residue (0.0109 g) obtained above was hydrolysed with 0.1 M Hydrochloric acid (1 ml) for 1 hour at 95 °C. The solid thus obtained was subsequently washed with distilled water. This was centrifuged at 4000 rpm for 10 minutes. The precipitate was collected and transferred to a sample vial, purged of Nitrogen and stored at -20 °C.

HPLC analysis

Samples (0.003g from each) were dissolved in 1.5 ml of absolute ethanol from which 10 µl was injected to the HPLC system (Agilent 1260 infinity with Agilent 1260 diode array detector). All the pigments were analysed using Varian, Pursuit C-18 reversed phase column (250 mm × 4.6 mm, 5 µm). The mobile phase was a linear gradient from 20% to 80% acetonitrile in water in 20 minutes, with gradient elution from 0-60 minutes. The solvent flow rate was 0.5 ml/min. Eluted compounds were simultaneously detected by DAD at 440 nm. (Figure 1 (b)). Peaks that appeared in the 200-300 nm region were not detected after the hydrolysis of crocins (Figure 1 (b)).

HPLC chromatogram of saffron constituents

The HPLC chromatograms of apo-carotenoids extracted from saffron stigmas, and their acid hydrolysed products and respective photodiode array spectra are shown in Figure 2 and 3, respectively. The analysis was done at 440 nm and it was observed that there are 5 types of crocins that were detected in the saffron sample. Peaks eluted at 14.421 min and 15.495 min are the two major types of crocin that are more polar in nature compared to other types of crocins. It has been reported in literature that the sugar moieties present in these crocin are most probably D-glocosyl and gentiobiosyl (Dhiman & Kharkwal, 2020). The peak that appeared at 16.547 min is another type of crocin that shows the same carotenoid characteristic fine structure, which exists in minute quantities of Saffron stigmas. Therefore, it was confirmed that there are 5 different types of crocins present in this sample (Hadizadeh et al., 2010). Among these five types, the two major peaks probably represented by 1 and 2 are C-3 and C-2 crocins, respectively.

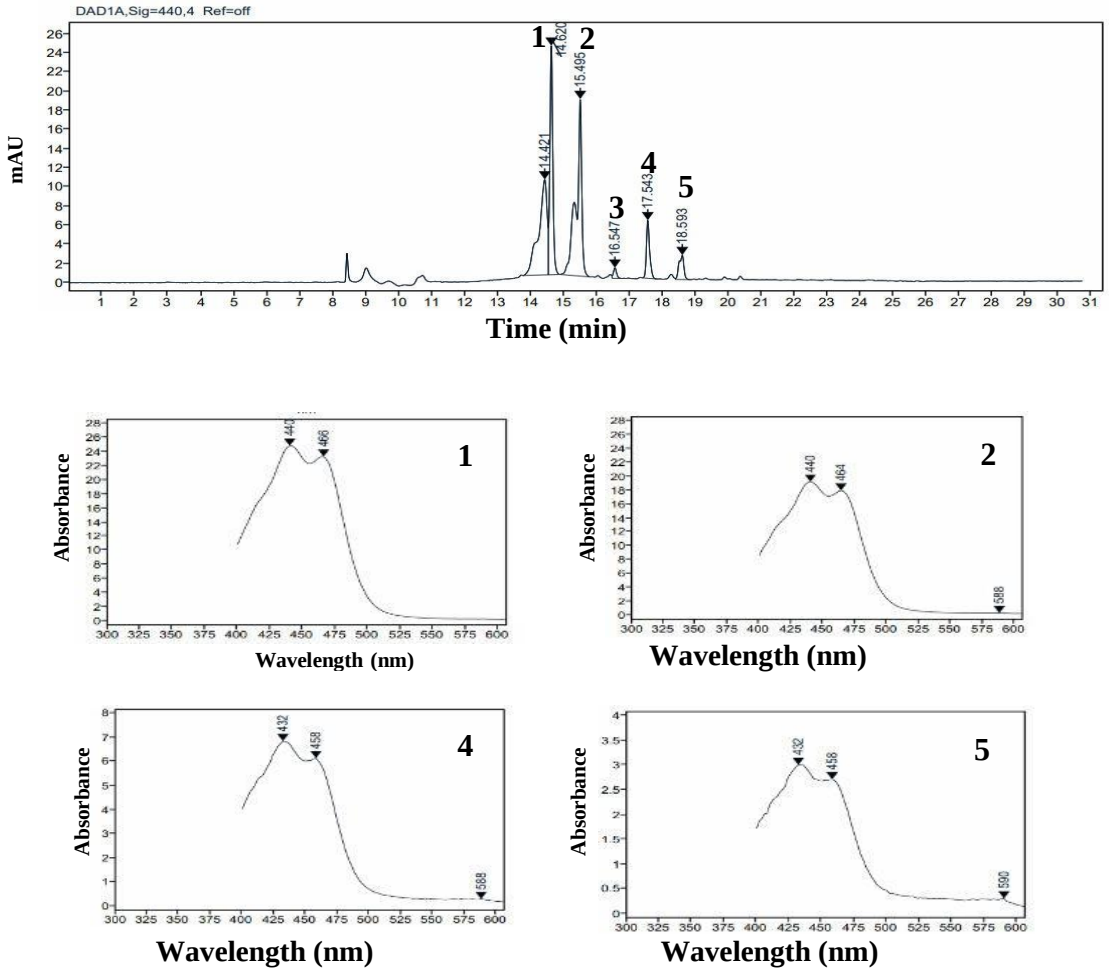
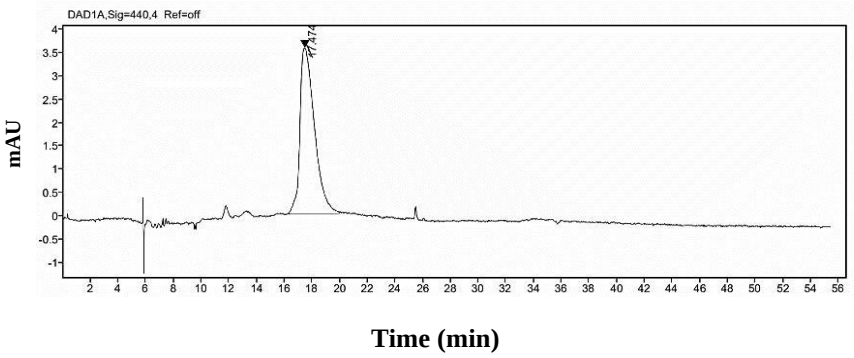


Figure 2: Reversed phase HPLC chromatogram of the apo carotenoids of the saffron stigmas and photodiode array spectra of crocins.

Figure 3 shows the HPLC chromatogram of crocetin, which shows only one prominent peak (at RT 17.474 min). Hence, it was confirmed that all types of crocin present in the sample were converted into crocetin by the acid hydrolysis process. Therefore, 100% pure crocetin was obtained by the acid hydrolysis of crocin.



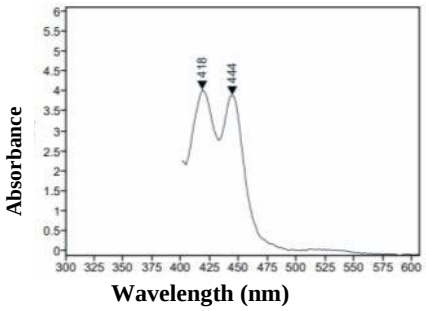


Figure 3: Reversed phase HPLC chromatogram of apo-carotenoid obtained after acid hydrolysis of crocin and photodiode array spectra.

The main aim of this research study was to separate the pure crocetin to study further their efficiency of using crocetin as a dye sensitiser and their photodegradation studies. It was proved by the HPLC chromatogram that different types of crocins, having D-glocosyl and gentiobiosyl as sugar moieties, were completely hydrolysed to dicarboxylic Crocetin during the acid digestion. The photodiode array detector was also confirmed by the l_{max} value (418 nm), spectral fine structure and III/II value.

CONCLUSION

Spectral data revealed that the Saffron stigma contained Crocin, Picrocrocin and Safranal. Five types of crocin were present in the sample. 100% pure Crocetin can be obtained by the acid hydrolysis of crocin.

REFERENCES

De Castro, M. D. L., & Quiles-Zafra, R. (2020). Appropriate use of analytical terminology – examples drawn from research on saffron. *Talanta Open*, *2*(June), 100005. Dhiman, N., & Kharkwal, H. (2020). Biosynthesis and Derivatization of the Major Phytoconstituents of Saffron. In *Saffron* (pp. 83–92). Elsevier.

M.M. Mendes-Pinto, E. Sansiaume, H. Hashimoto, A.A. Pascal, A. Gall, B. Robert, Electronic absorption and ground state structure of carotenoid molecules, J. Phys. Chem. B 117 (2013) 11015–11021.

Hadizadeh, F., Mohajeri, S. A., & Seifi, M. (2010). Extraction and purification of crocin from saffron stigmas employing a simple and efficient crystallization method. *Pakistan Journal of Biological Sciences*, *13*(14), 691–698.

Jafari, S.-M., Tsimidou, M. Z., Rajabi, H., & Kyriakoudi, A. (2020). Chapter 16 - Bioactive ingredients of saffron: extraction, analysis, applications. In A. Koocheki & M. B. T.-S. Khajeh-Hosseini (Eds.), *Woodhead Publishing Series in Food Science, Technology and Nutrition* (pp. 261–290).

Lozano, P., Castellar, M. R., Simancas, M. J., & Iborra, J. L. (1999). A quantitative high-performance liquid chromatographic method to analyse commercial saffron (Crocus sativus L.) products. *Journal of Chromatography A*, *830*(2), 477–483.

ACKNOWLEDGEMENT

Research funding by The Open University of Sri Lanka is gratefully acknowledged by the authors.