



APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Analysis of Polyphenols in Saffron Petal Extracts with UHPLC/UV/MS

Introduction

Saffron is one of the most expensive spices by weight and is savored around the world. It is derived from the flowers of the crocus plant *Crocus sativus*, which is

mostly cultivated in the Mediterranean and Middle East regions. Saffron has recently received additional focus for its naturally-containing carotenoids and polyphenols, both of which have been reported to have nutraceutical/medicinal value, particularly for their antioxidant and anti-inflammatory characteristics¹.

With the above-mentioned interest in mind, this particular work focused on the characterization of the major polyphenolic compounds that are naturally present in saffron petal extract, using UHPLC (ultra-high pressure liquid chromatography) and both UV/Vis and ESI-MS (electrospray ionization mass spectrometer) detectors. The UV/Vis detector was used for the initial detection of the chromatographically-separated analytes while the complimentary ESI-MS was primarily used for analyte identification/confirmation.

Experimental

Solvents, Standards and Samples

All solvents and diluents used were HPLC grade.

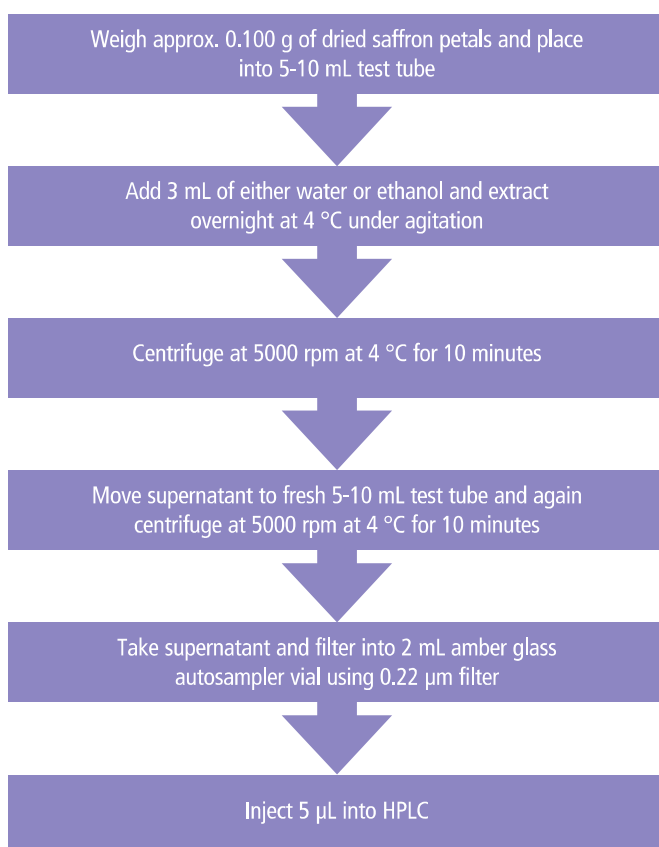
Formic acid, used as a solvent modifier, was obtained from Sigma-Aldrich®, St. Louis, MO.

All standards were obtained from Polyphenols Laboratories AS, Sandnes, Norway. All standards were diluted in 90:10 methanol: water with 0.1 % formic acid.

Saffron petal extracts were obtained from Agenzia per lo Sviluppo, Azienda Speciale CCIAA, L'Aquila, Italy.

Sample Preparation

The saffron petal extracts were prepared using the following procedure:



Hardware/Software

For all chromatographic separations, a PerkinElmer Flexar™ UHPLC system was used, including an FX-15 pump, FX UHPLC autosampler, UHPLC UV/Vis detector, Flexar SQ 300 MS detector, vacuum degasser and column oven. All instrument control, analysis and data processing was done via PerkinElmer Chromera™ software.

Method Parameters

The HPLC method and SQ 300 MS detector parameters are shown in Table 1 and Table 2, respectively.

Table 1. HPLC Method Parameters.

HPLC Conditions					
Column	Zorbax RRHD Extended C18 3x150 mm, 1.8 µm				
Mobile Phase	A = water with 0.1 % formic acid B = methanol with 0.1 % formic acid				
	Step	Time [min]	A%	B%	Curve
	0	4	95	5	Isocratic
	1	12	35	65	-1.5 (convex)
	2	2	20	80	1 (linear)
Analysis Time	14 min				
Flow Rate	0.45 mL/min.				
Oven Temp.	25 °C				
Detection	1) Flexar UV/Vis; Wavelength: 270 nm 2) Flexar SQ 300 MS				
Injection Volume	5 µL				

Table 2. SQ 300 MS Conditions.

Interface	ESI
Polarity	Negative
Scan	TIC (200-800 amu)
Drying Gas Temperature	320 °C
Drying Gas Flow Rate	12 L/min
Nebulizer Gas Pressure	85 psi
Capillary Exit	-300 eV
Skimmer	-30 V

Results and Discussion

Figures 1 a/b and 2 a/b show the UV chromatogram ($\lambda = 270$ nm) and the ESI-MS TIC (total ion chromatogram) for both ethanolic and aqueous saffron extracts, respectively. As can be seen, using either extraction procedure, the chromatograms showed the presence of many analytes, demonstrating that either of the two extraction procedures is equally effective. A number of these analytes were expected to be free or glycosylated flavonoids.

Some of the analytes were identifiable through the combination of chromatographic retention times and mass-spectrum analysis, while others, to be properly identified, would require a more thorough literature review and a comparison with pure standards.

Being particularly interested in the discrimination between two flavonoids having similar MWs, we focused our analysis on kaempferol sophorose and quercetin rutinoside, two glycosylated compounds, both having a parent MW of 610. The UV chromatographic results of a 2 ppm standard mix of quercetin rutinoside and kaempferol sophorose are shown in Figure 3.

Using the before-mentioned chromatographic conditions employed, the peak of quercetin rutinose had a retention time of about 5.20 minutes, while that of kaempferol-3O-sophorose had a retention time of 4.75 minutes. These align quite well with labeled peaks in Figures 1 a/b and 2a/b. However, for more definitive identification, more information is needed. This is where the MS spectral results play a key role.

As these two compounds have very similar MWs, the basic mass spectrum analysis and their identification via just the molecular ion weight can be misleading. Taking advantage of both the information in an on-line database (www.phenol-explorer.com) and ChemSketch, also available on the web, it was possible to analyze the structure of molecules having similar molecular weight (MW), develop an understanding of how they fragment and, then, derive the mass spectrum that would be expected by ESI-MS.

As shown in Figures 4a and 5a, by molecularly analyzing both quercetin rutinose and kaempferol-3O-sophorose, fragmented at the 3O-glycosidic bond or between the two glucose molecules, it allowed us to determine the most likely ESI-MS fragmentations and, thereby, target fragments with corresponding weights in the actual mass spectral results.

Thereupon, for both quercetin rutinose and kaempferol sophorose, respectively, Figures 4 b/c and 5 b/c show the MS spectral results for both ethanolic and aqueous extracts. These results closely matched the expected fragmentations derived from ChemSketch. The combined chromatographic and mass spectral results allowed for easy interpretation and clear confirmation of the targeted analytes.

Similarly, using the same analytical procedure, it is possible to identify other polyphenols present in the chromatographic separation. By way of example, the expected fragmentations and the mass spectral results obtained for sophorose quercetin and kaempferol rutinose via both ethanolic and aqueous extracts are shown in Figures 6 and 7, respectively.

Combined, these results provided a definitive characterization of the targeted analytes present in the two saffron extracts. Such composite information is often crucial in the qualitative analysis of complex molecules, such as polyphenols.

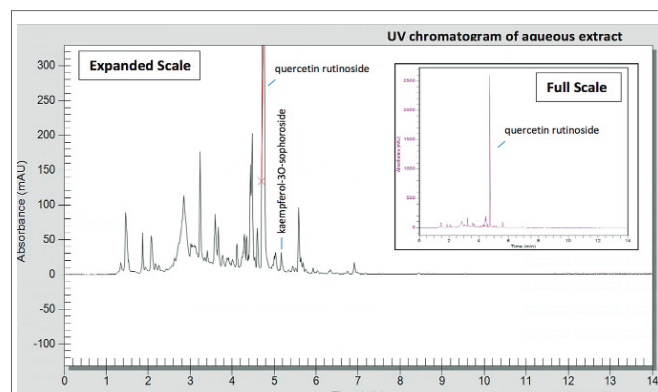


Figure 1a. UV chromatogram of the ethanolic extract of saffron petals; UV at 270 nm.

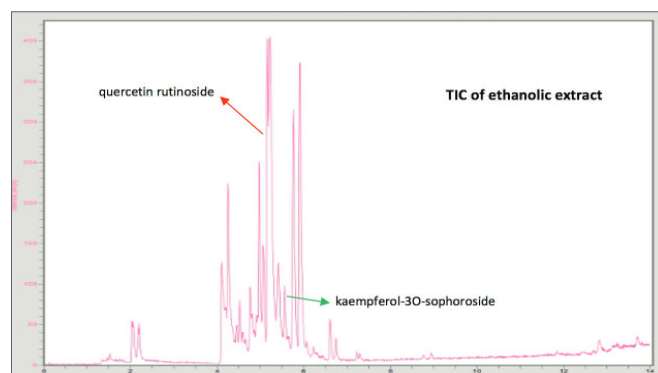


Figure 1b. TIC (total ion chromatogram) of the ethanolic extract of saffron petals. The mass spectra of the highlighted peaks are shown in detail in Figures 5 and 6.

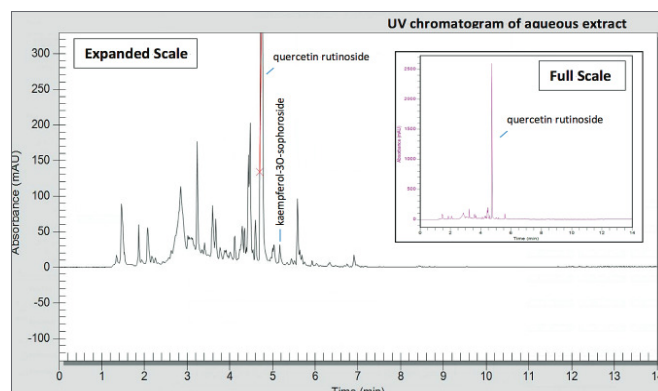


Figure 2a. UV chromatogram of the aqueous extract of saffron petals; UV at 270 nm.

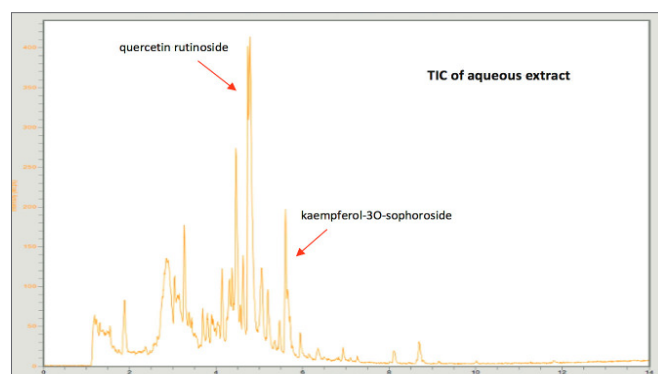


Figure 2b. TIC chromatogram of the aqueous extract of saffron petals.

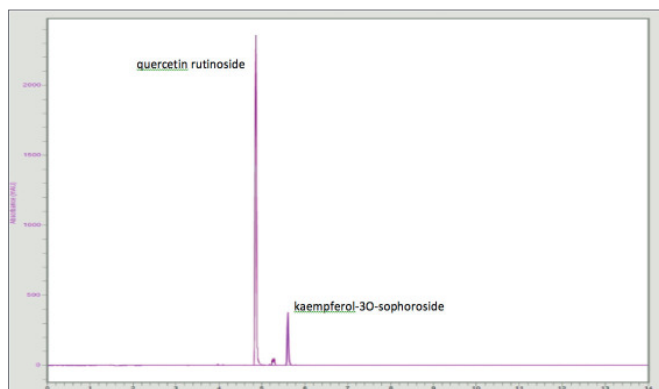


Figure 3. Chromatogram of quercetin rutinoside and kaempferol-3O-sophoroside standard mix (2 ppm each); UV at 270 nm.

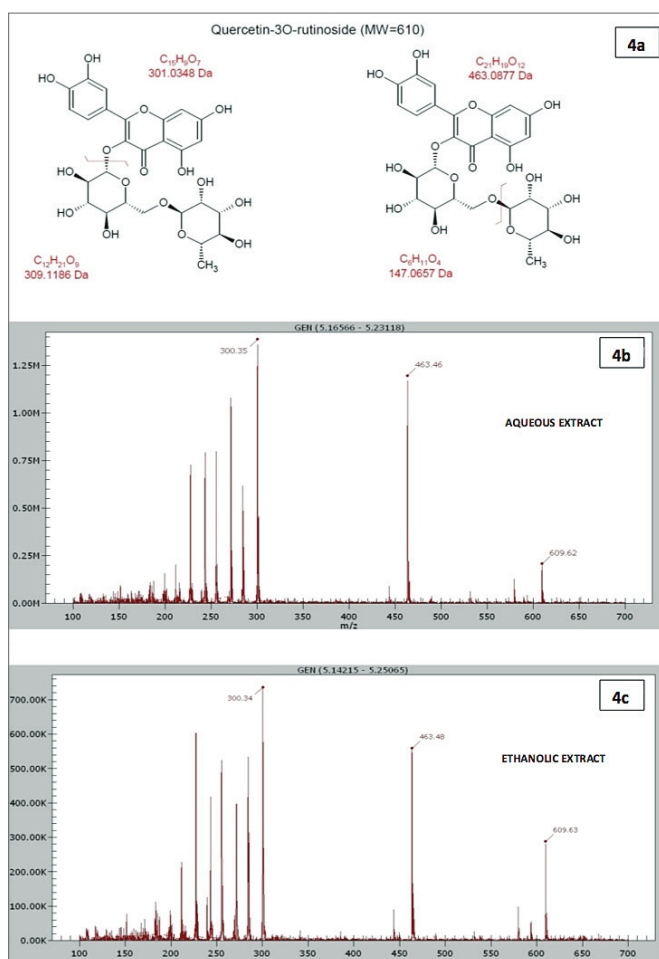


Figure 4. Figure 4a shows the most likely ESI-MS fragmentations for quercetin-3O-rutinoside. Figures 4b and 4c show the actual mass spectra obtained from the chromatographic analysis of the aqueous and ethanolic extracts, respectively.

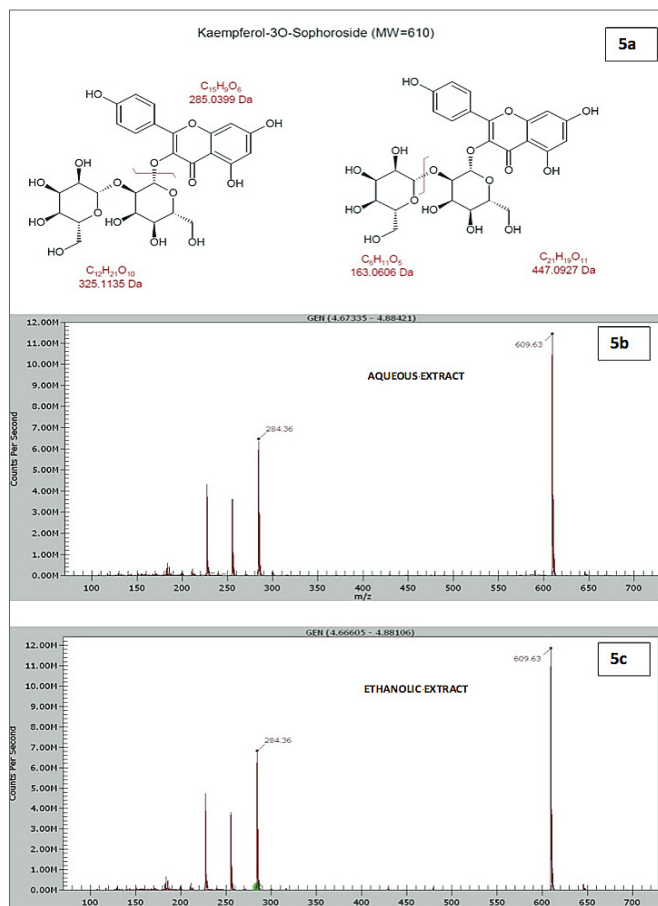


Figure 5. Figure 5a shows the most likely ESI-MS fragmentations for kaempferol-3O-sophoroside. Figures 5b and 5c show the actual mass spectra obtained from the chromatographic analysis of the aqueous and ethanolic extracts, respectively.

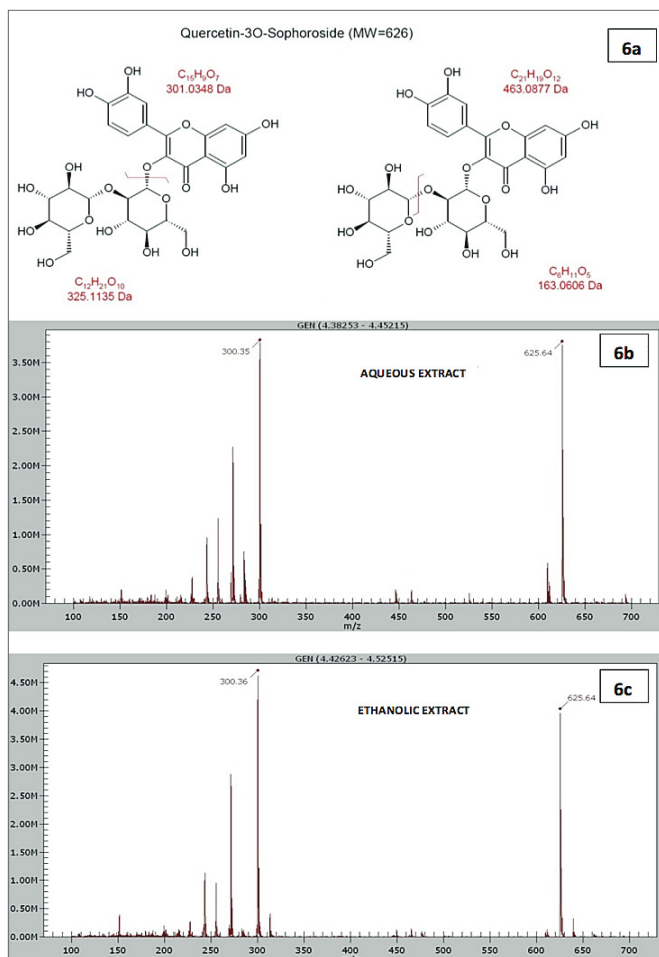


Figure 6. Figure 6a shows the most likely ESI-MS fragmentations for quercetin-3O-sophoroside. Figures 6b and 6c show the actual mass spectra obtained from the chromatographic analysis of the aqueous and ethanolic extracts, respectively.

Conclusion

Combining chromatographic results, projected fragmentation ions (via software, such as ChemSketch) and mass spectral results allowed for the definitive identification of four polyphenols naturally found in saffron petal extract. This was based on the chromatographic retention times of standards, the mass of the molecular ion and the primary fragment ions that were obtained.

Regarding quercetin rutinose and kaempferol sophoroside, the results clearly demonstrate the ability of definitively associating the resulting spectra with one of the two flavonoids, even though both have the same parent MW.

For quercetin sophoroside and kaempferol rutinose, the corresponding fragment ions obtained from these molecules provided clear confirmation of compound identity.

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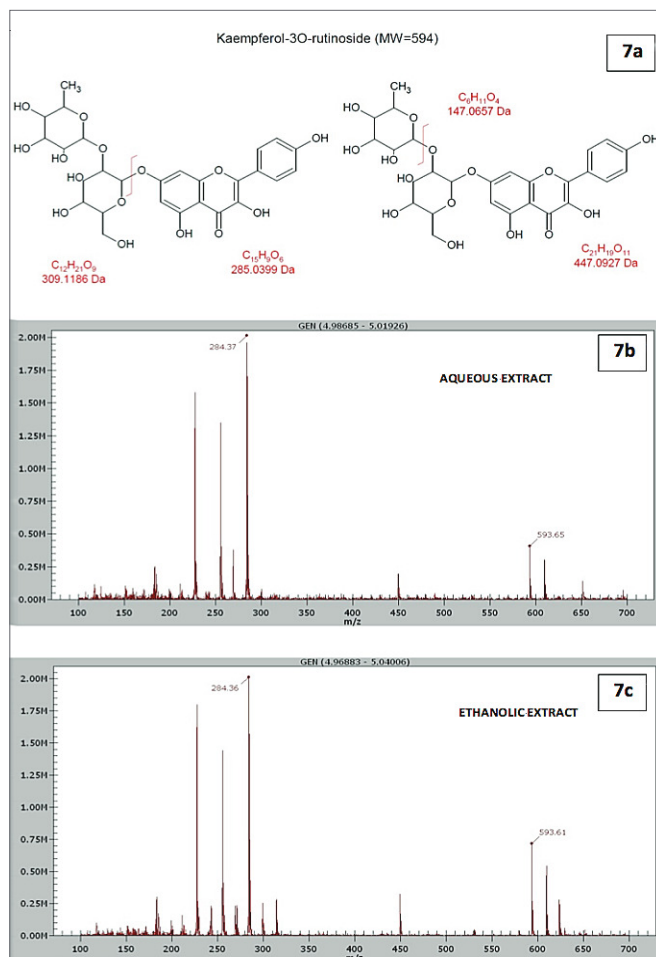


Figure 7. Figure 7a shows the most likely ESI-MS fragmentations for kaempferol-3O-rutinoside. Figures 7b and 7c show the actual mass spectra obtained from the chromatographic analysis of the aqueous and ethanolic extracts, respectively.

In summary, this analytical approach allows for the conclusive identification of primary polyphenols in saffron petal extract.

Acknowledgements

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References

1. Saffron: A Natural Potent Antioxidant as a Promising Anti-Obesity Drug, Antioxidants (ISSN 2076-3921), 2013, 2, 293-308

