# VOLARIZATION OF SAFFRON INDUSTRY BY-PRODUCTS: BIOACTIVE COMPOUNDS FROM LEAVES

**Lina Smolskaite[1,3], Thierry Talou[1*], Nicolas Fabre[2], Petras Rimantas Venskutonis[3]**

*[1]Universite de Toulouse, INP Toulouse, Laboratoire de Chimie Agro-Industrielle UMR 1010 INRA/INP-ENSIACET, 4 allée Emile Monso 31030 Toulouse, France, *e-mail: talou@cict.fr*
*[2]Universite de Toulouse, UPS Toulouse, Laboratoire Pharmacochimie des Substances Naturelles et Pharmacophores Redox, UMR 152 IRD, 118 route de Narbonne 31062 Toulouse, France*
*[3]Kaunas University of Technology, Department Food Technology, K. Donelaicio G. 73, 44249 Kaunas, Lithuania*

## Abstract

Interest in the development of bioprocesses for the production or extraction of bioactive compounds from natural sources has increased in recent years due to the potential applications of these compounds in food, chemical, and pharmaceutical industries. Obtention of 1kg of spice from saffron stigmas generates 150 000 blooms and 1 500 kg of leaves, which are presently both considered as waste. But due to the biologically valuable compounds like flavonoids, antioxidants which could be found in blossom and leaves of crocus species and could potentially used as functional components for food products and diet supplement. Therefore, the main objective of analysis was proper evaluation of *C. sativus* leaves usability for further valorization based on flavonoids and antioxidants composition and titration. Analysis were performed on dried leaves of *C. Sativus* collected in different parts of the world, regions and provinces in the framework of the European Program CROCUSBANK. Samples of *C. Sativus* were extracted with a in-house developed micro-extraction technique and extracts analyzed by HPLC-UV and by HPLC-MS and spectrophotometer. 8 flavonoids were identified and titrated in *C. sativus* leaves from which 2 (kaempferol-8-C-gluco-6,3-O-diglucoside and kaempferol-8-C-gluco-6-O-glucose) were reported for the first time in saffron. Comparison of flavonoids and antioxidants from samples harvested in different countries states that flavonoid concentrations vary independently of origin, while different cultivation conditions or different picking periods seemed to greatly influence.

**Key words:** *Crocus sativus*, bioactive compounds, HPLC-MS

## Introducton

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products (Kris-Etherton et al., 2002). Most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Hölker et al., 2004). Phenolic compounds comprise flavonoids, phenolic acids, and tannins, among others. Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds. Variations in substitution patterns to ring C in the structure of these compounds result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins. Similarly to the flavonoids, phenolic acids constitute also an important class of phenolic compounds with bioactive functions, usually found in plant and food products. Phenolic acids can be divided in two subgroups according to their structure: the hydroxybenzoic and the hydroxycinnamic acids. The most commonly found hydroxybenzoic acids include gallic, phydroxybenzoic, protocatechuic, vanillic and syringic acids, while among the hydroxycinnamic acids, caffeic, ferulic, p-coumaric and sinapic acids can be pointed out (Bravo, 1998). In the last few years, great attention has been paid to the bioactive compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer and diabetes (Conforti et al., 2009), reduction in risk factors of cardiovascular diseases antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial effects (Balasundram et al., 2006), among others. Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables, plants, agricultural and agro-industrial residues as sources of bioactive phenolic compounds. Saffron with its unique aroma, color, and flavor can by no means be considered a new introduction to 21st century cuisine and medicine. In fact, the history of saffron usage dates back nearly 3000 years,

spanning many continents, civilizations, and cultures (Deo, 2003). Saffron, the highly desirable golden spice, is the dried elongated stigmas and styles of the blue-purple saffron flower (*Crocus sativus,* L.), a member of the Iridaceae (iris) family with origins in the Middle East. At nearly $40–50 per gram, it is the world's most expensive spice. It is estimated that it takes approximately 75,000 crocus blossoms and 1 500 kg of leaves, which are presently considered as waste. But due to the biologically valuable compounds like flavonoids, antioxidants which could be found in blossom and leaves of crocus species and could potentially used as functional components for food and health areas. Therefore, the main objective of analysis was proper evaluation of *C. sativus* leaves usability for further valorization based on flavonoids, antioxidants composition and titration.

**Materials and Methods**

*Plant and extracts.* Leaves of *Crocus sativus* were collected from different countries, regions, provinces and localities in Spain, Castilla-La Mancha University of Agriculture. Raw leaves were dried at temperature of 30 °C for 18 hours. Drying the leaves lost about 50% of their weight. *Crocus stivus* leaves precise locations shown in Table 1. The aqueous-methanolic extract of the leaves was prepared as follows: 5 g of chopped, dried leaves of the plant were extracted with 100 ml of methanol 80% by maceration maceration method. The solvent was then removed under reduced pressure. The plant ingredient concentration in the final extract was adjusted to be 10 g% by adding methanol to the dried extract.

Table 1

***Crocus sativus* leaves sources**

| Code | Country | Region-Province-Locality |
|---|---|---|
| **1** | Azerbaijan | Apsheron – Bilga-Baku |
| **2** | India | Kashmir-Srinagar valley |
| **3** | New Zealand | South Island- Canterbury-Rangiora (Garrymere) |
| **4** | Morocco | Ourzazate-Taliouine |
| **5** | France | Tarn- Midi Pyrene-Andillac |
| **6** | Turkey | Kastamnu |
| **7** | Iran | Khorazan-Torbat Centro |
| **8** | Iran | Khorazan-Torbat Norte |
| **9** | Iran | Khorazan-Torbat Centro |
| **10** | Italy | Sardinia- Caguari-San Gavino |
| **11** | Italy | Tuscany –Toscana-San Gimignamo |
| **12** | Spain | Comunidad- Alicante-Novelda ( La Romana) |
| **13** | Spain | La Rioja -Badaran |
| **14** | Spain | Aragon- Teruel-Estercuel |
| **15** | Spain | Aragon- Teruel-Caminreal |
| **16** | Spain | Aragon- Teruel-Fuentes Claras (las Penuelas) |
| **17** | Spain | Aragon- Teruel-Monreal del Campo |
| **18** | Spain | Aragon- Teruel-Blancas |
| **19** | Spain | Castilla-la Mancha- Toledo-Villafranca de los Caballeros |
| **20** | Spain | Castilla-la Mancha- Toledo-Madrdejos (Comino Viejo) |
| **21** | Spain | Castilla-la Mancha- Toledo-Consuegra |
| **22** | Spain | Castilla-la Mancha- Toledo-Camunas (el Monte) |
| **23** | Spain | Castilla-la Mancha- Cuidad Real-Herencia |
| **24** | Spain | Castilla-la Mancha- Cuidad Real-Herencia |
| **25** | Spain | Castilla-la Mancha- Cuidad Real-La Solana (Camino del Toconal) |
| **26** | Spain | Castilla-la Mancha- Cuidad Real-Membrilla (Camino Hondo) |

Continue of Table1

| Code | Country | Region-Province-Locality |
|---|---|---|
| **27** | Spain | Castilla-la Mancha- Cuenca-Villares del Saz |
| **28** | Spain | Castilla-la Mancha- Cuenca-Ledana |
| **29** | Spain | Castilla-la Mancha- Cuenca-Motilla del Palancar |
| **30** | Spain | Castilla-la Mancha- Albacete-Minaya ( Casa del Cura) |
| **31** | Spain | Castilla-la Mancha- Albacete-Lezuza |
| **32** | Spain | Castilla-la Mancha- Albacete-Nava de Abajo |
| **33** | Spain | Castilla-la Mancha- Albacete-Lezuza (Navamarin) |
| **34** | Spain | Castilla-la Mancha- Albacete-Alcala del Jucar |
| **35** | Spain | Castilla-la Mancha- Albacete-Zulema |
| **36** | Spain | Castilla-la Mancha- Albacete-Tobarra |
| **37** | Spain | Castilla-la Mancha- Albacete-Tarazona de la Manch |
| **38** | Spain | Castilla-la Mancha- Albacete-Madrigueras |
| **39** | Spain | Castilla-la Mancha- Albacete-Minaya |
| **40** | Spain | Castilla-la Mancha- Albacete-El Bonilo |
| **41** | Spain | Castilla-la Mancha- Albacete-Munera (Paraje Vallejo) |
| **42** | Spain | Castilla-la Mancha- Albacete-Munera (Paraje Los Huertos) |
| **43** | Spain | Castilla-la Mancha- Albacete-Munera (Huerta Pantazuelos) |
| **44** | Spain | Castilla-la Mancha- Albacete-Munera (Parcela Huerto Pina) |
| **45** | Spain | Castilla-la Mancha- Albacete-Lezuza (Fuente del Chorro) |
| **46** | unknown | Unknown |

*HPLC and LC-MS analysis.* The analysis was performed using a Dionex ASI-100 auto injector, P680 HPLC pump and UV Helwett Packard (1100 series) detector (Dionex, CA, USA) with Varian ChromSep reversed-phase column (column HS C18, 100x3 OMM) (Varian, USA). Detection is performed at 350 nm wave length. Injected 20 ml of the extract. Flow rate – 0.65 ml·min$^{-1}$. Elution – gradient. Eluent A - acetonitrile (ACN), eluent B - water with 0.1% TFA (v / v) using the gradient change: 0 min − 3% A (97%. B), 30 min − 20% A (80% B) 35 min 3% A (97%. B), 45 min 3% A (97%. B). Analysis was done at room temperature. Flavonoids identified according the analytes retention times and mass spectrum using a Thermo-Finnigan LCQ DECA Fisher XP MAX ion trap mass spectrometer with electro spray ionization (ECI) and the source of the negative ion mode. Mass Spectrometer pump *Thermo-Fisher Spectra System (TFSP, San Jose, CA, USA) P1000XR*, auto injector *TFSP 6000LP Photodiode Array Detector and a TFSP AS 3000*. Used column Varian Pursuit XRS 5 C18 (250x4.6 mm ID, 5 mm). Flow rate – 1 ml.min$^{-1}$, Injected 10 ml of the sample into the mass spectrometer. Elution used is the same as describe before.

*Antioxidant analysis.* For the determination of total polyphenolics the Folin-Ciocalteau reagent was used. The 0.1% concentration of extracts dissolved in methanol. 1ml prepared extract mixed with 5 ml of Folin-Ciocalteu reagent and 4 ml of 7.5% $Na_2CO_3$ solution. Absorbance was measured spectrophotometrically after 30 minutes at wavelength $\lambda$ = 765 nm. Absorption of the zero point measured using distilled water.The results were expressed as gallic acid equivalents (GAE) in mg g$^{-1}$. Average results were obtained from three parallel determinations.

**Results and Discussion**

*Qualitative Analysis of Crocus sativus leaves.* The chemical composition of crocus leaves samples from 50 different sources was determined using reverse phase LC-MS. The chromatographic conditions employed allowed identification of 8 major components in each sample and a well-resolved baseline separation was obtained. Each component was identified by comparison of its retention time as well as by LC-MS-MS analysis through the detection (*m*/*z*) of its corresponding pseudomolecular ion $(M-H)^-$. Figure 1 shows one representative

chromatogram, one with the highest concentration of kaempferol-8-C-glyc-6.3-O-diglycoside (26.92 RT). This diglycoside is one of the new compound observed in crocus leaves. The peak identification at 350 nm is as follows were: kaempferol-8-C-Glyc-6-O-glycoside (25.80 RT); kvercetin-8-C-glycoside (26.70 RT); kamferol-8-C-Glyc-6.3-O-diglycoside (26.92); luteolin-8.3-c-diglycoside (29.70 RT); kvercetin-3-O-maltotrioside (27.23 RT); orientin (luteolin-8-C-glycoside) (31.42 RT); kaempferol-3-O-sopfobioside (32.12 RT); vitexsin (apigenin-8-C-glycoside)(34.44 RT). According to our analysis different crocus leaves samples did not differ in their chemical composition, but did differ in the concentration of each component.

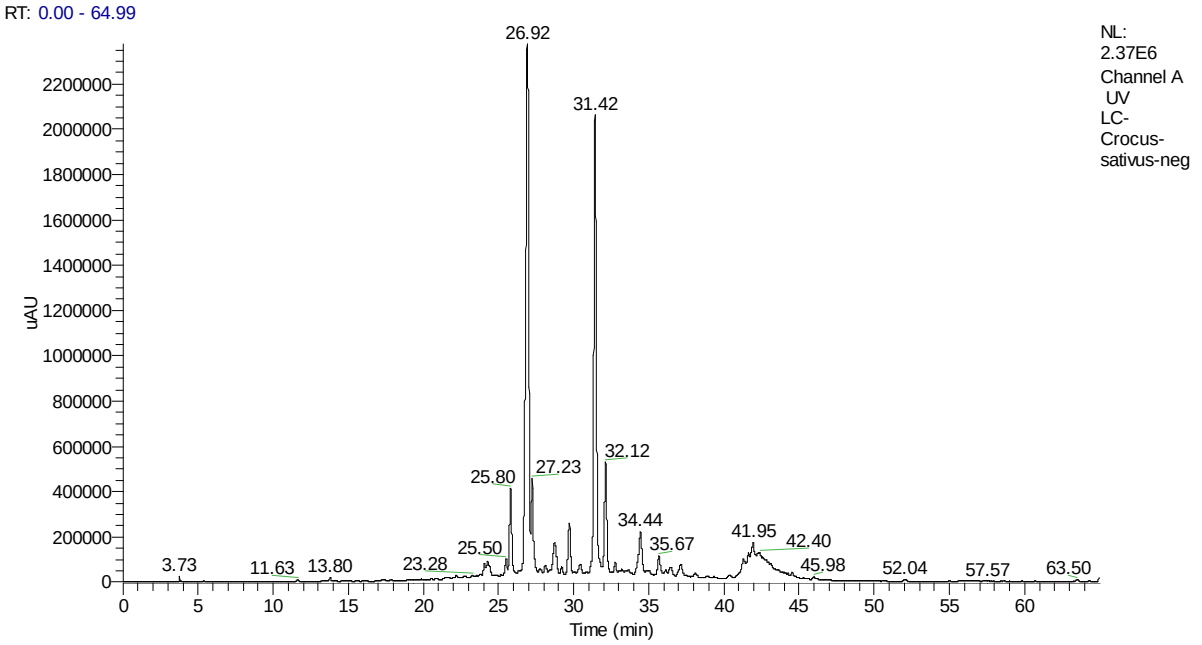
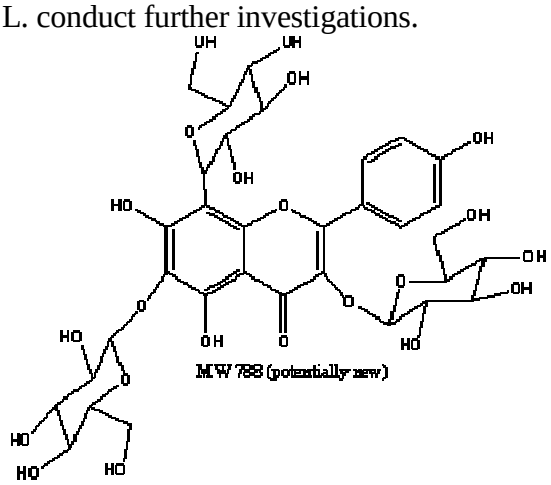
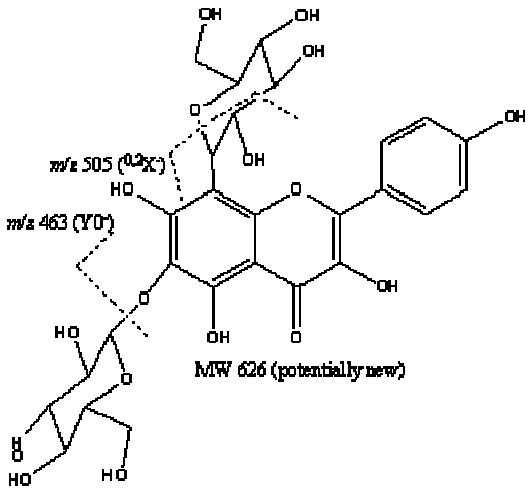



**Figure1. LC-MS/MS crocus leaves chromatogram**

Figure 2 describes structural formulas of two new glucosydes which were observed first time in Crocus leaves. Currently, in order to clarify the existence of new flavonoids *Crocus sativus* L. conduct further investigations.




Kaempferol-8-C-glyc-6,3-O-diglycoside




Kaempferol-8-C-gluco-6-O-glucose

**Figure 2. Glucosydes**

*Quantitative analysis of Crocus sativus leaves.* Figure 3 shows the concentration of kaempferol-8-C-glyc-6.3-O-diglycoside compound detected in the 50 tested samples. The results indicate that the differences might be due to the origin of the sample, to the dissimilar

drying processes possibly involving varied time periods, as well as to storage conditions. Spain, Aragon region crocus leaves had the highest total concentration of diglycoside (45.99%) followed in order by the Azerbaijan, Marocco and Spain Castilla la Mancha region. As well as the Spain, Castilla la Mancha region (9.73%) showed the lowest. Spanish leaves had low and high concentration of components in comparison to the sample analysed from La Mancha, Spain. This variation could be the result of different drying processes used, or the time and conditions under which the plant product was packed and stored in each country, all of which could affect the concentration of kamferlo diglycoside as they are thermally labile and photochemically sensitive components.

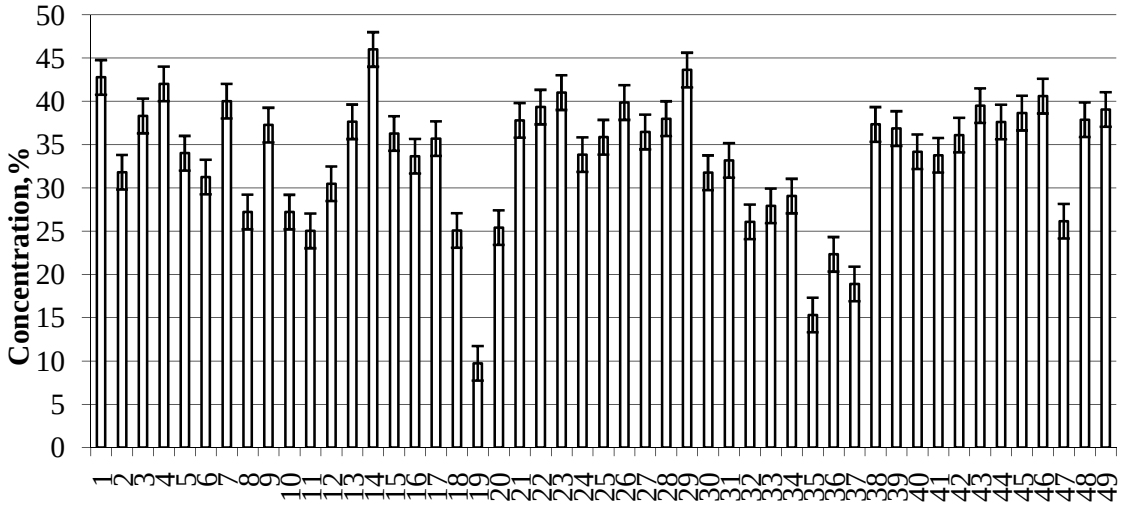



**Figure 3. Kaempferol-8-C-glyc-6.3-O-diglycoside content in *Crocus sativus* leaves**

*Total polyphenolic content.* The analysis and comparison of 50 extracts, observed different amounts of total phenolic compounds. In all extracts shown in Figure 4. The highest content of phenolic compounds was characterized in crocus leaves from Spain, Castilla-La Mancha region, in Albacete province of San Pedro locality (17%).

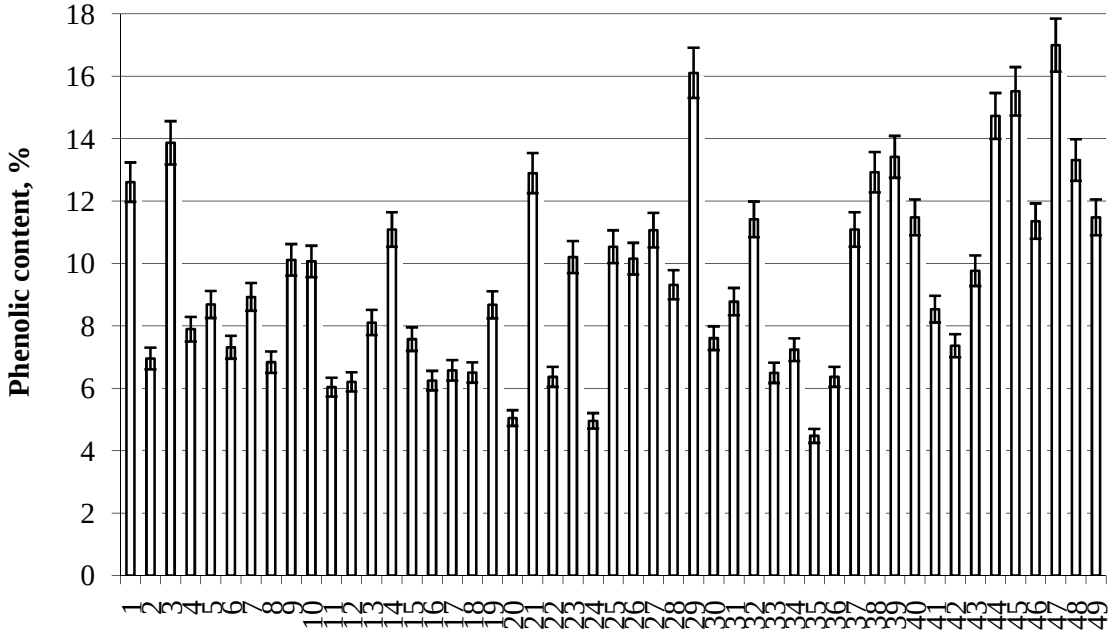



**Figure 4. Total plyphenolic content in *Crocus sativus* leaves**

The lowest amount of phenolic compounds in Crocus sativus leaves were in extract from Spain, Castilla-La Mancha region, in Albacete province, Zulema locality (4.48%) Total phenolic compounds in all extracts from different sources were average between 8.55%−9.49%. Influence of phenolic compounds content in saffron, grown in the same country, but in another province or locality may have different growing conditions, plant material preparation time and harvest time.

## Conclusions

1. Plants produce a wide variety of bioactive compounds with significant applications in the health and food areas (Sarikaya and Ladisch, 1999). Such compounds include a variety of flavonoids, phenolic acids, lignans, sallicylates, stanols, sterols, glucosinolates, among others (Hooper and Cassidy, 2006).
2. In fact, plants are considered to be excellent sources of phenolic compounds with very interesting nutritional and therapeutic applications (Li et al., 2008). Among these compounds, a strong correlation between antioxidant activity and the total phenolic content in the plants has been observed, suggesting that phenolic compounds could be the major contributor of their antioxidant capacity (Li et al., 2008).
3. Phenolic compounds are widely distributed in plants, usually being found in higher concentrations in leaves and green steams (Bennett and Wallsgrove, 1994). These compounds are considered natural defense substances, and their concentration in each plant may be influenced by several factors including physiological variations, environmental conditions, geographic variation, genetic factors and evolution (Figueiredo et al., 2008).
4. The large biodiversity of plants existent provides a great exploration field for researches on bioactive phenolic compounds and their biological properties (Shetty and McCue, 2003).

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