

Characterization of Phytochemicals in Petals of Different Colours From *Viola × wittrockiana* Gams. and Their Correlation With Antioxidant Activity

Monika Skowrya¹, M. Isabel Calvo², M. Gabriela Gallego¹, Nurul A. M. Azman¹ & Maria P. Almajano¹

¹Department of Chemical Engineering, Technical University of Catalonia, Barcelona, Spain

²Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona, Spain

Correspondence: Maria P. Almajano, Department of Chemical Engineering; ETSEIB; Av. Diagonal 647, 08028 Barcelona, Spain. Tel: 34-934-016-686. E-mail: m.pilar.almajano@upc.edu

Received: February, 2014 Accepted: June 22, 2014 Online Published: August 15, 2014

doi:10.5539/jas.v6n9p93

URL: <http://dx.doi.org/10.5539/jas.v6n9p93>

Abstract

A study to establish relationship between the petal colour, extraction solvent, phenolic, flavonoid, anthocyanin content and antioxidant activity in three varieties of *Viola × wittrockiana* (yellow, red and violet) is reported in this article. Identification and quantification of flavonoids and anthocyanins using HPLC-DAD-ESI-MS is also presented. The antioxidant activity was studied by four different analytical assays: the measurement of scavenging capacity against ABTS⁺ and DPPH free radicals, oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP). The violet petals were the most active in all solvents employed for extraction and also showed higher total phenolic, flavonoid and anthocyanin content in comparison with red and yellow ones. Fourteen constituents were identified by HPLC coupled with diode-array detection (DAD) and mass spectrometry (ESI-MS), ten of them were flavonoids and four anthocyanins. The major compounds found in violet pansy were rutin, violanthin and violanin. Aqueous extract of violet pansy can be regarded as a suitable candidate to serve as a radical scavenging agent that could be used in functional foods.

Keywords: *Viola × wittrockiana* extract, phenolic content, antioxidant activity, HPLC-DAD-ESI-MS

1. Introduction

The traditional use of flowers in food has been thoroughly documented. Several ancient world cultures used many species for medical uses and often incorporated them as part of their diet. In Spain the use of flowers in food was not very common until the end of the last century, when they were usually incorporated into dishes, mainly as ornamental elements. This popularity has encouraged their use as additives in food industry due to their well-known potential health benefits, in order to obtain new and attractive functional products (Sanchez-Mata et al., 2012). At that time, studies were performed in order to analyze the consumers' acceptability of flower petals as edible vegetables. Parameters influencing the consumers were analyzed and petal colour obtained the highest influence rate (between 63 and 95 %) (Kelley, Behe, Biernbaum, & Poff, 2001).

Heartsease (*Viola tricolor* L.) is a well-known medicinal plant. Its biological activities are supposed to be related to its antioxidant capacity. Garden pansies (*Viola × wittrockiana* Gams) are common and long cultivated ornamental hybrid plants developed in the 19th century by crossbreeding *Viola tricolor* L. in order to produce more attractive flowers. The plants are known to produce petals of many possible colours. Vukics et al. (2008a) found that both, wild and garden pansies (*V. tricolor* and *V. × wittrockiana*) contained similar amounts of antioxidant substances. Attractive flowers and antioxidant capacity make garden pansy a suitable candidate to be used as a functional food product (Vukics, Kery, & Guttman, 2008).

Violae are rich sources of phytochemical compounds which play important roles in plant metabolism, growth and reproduction, while some of them bring about protection against pathogens, predators and UV radiation. Some of these compounds, such as polyphenols, were shown to cause important nutritional and health benefits in humans (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011). From a nutritional standpoint, polyphenols exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. They have also been linked to oxidative stress disease prevention in Alzheimer, Parkinson, atherosclerosis and various cancers (Feng

& Wang, 2012; Schiffrin, 2010). Studies have demonstrated that dietary polyphenols can act as scavengers of free radicals, inducing, inhibiting or modulating the signal of transduction of antioxidant enzymes or chelating metal ions (Han, Shen, & Lou, 2007). For a complete determination of phenolic content and antioxidant activity, quantitative and qualitative information is needed. Several studies analyzed *Viola tricolor* L. (*Violaceae*) extracts by TLC or HPLC combined with UV detection but results were not specific enough for the identification of flavonoid glycosides. For this reason Papp et al. (2004) examined ethanolic petal extracts by HPLC-DAD and on-line mass spectrometry (ESI-MS or APCI-MS) and five peaks were identified: quercetin, isoquercetin, hyperoside, rutin, and luteolin-7-*O*- β -glucoside (Papp et al., 2004). Vukics et al. (2008c) performed extraction of heartsease herb with chloroform (fraction A) and then with ethanol (fraction B) in an ultrasonic bath. According to their results, the two major pansy flavonoids were violanthin and rutin. In addition, the same authors stated that flowers of heartsease are a potential source of natural antioxidants and that this tissue showed higher antioxidant activity than the herb and leaves. Moreover, a significant correlation between the flavonoid content and antioxidant activity was found (Vukics et al., 2008b). The same authors made a comparative study of antioxidant capacity of garden pansies of three different flower colours and wild pansy (Vukics et al., 2008a). Previous papers had focused on the study of the antioxidant activity of herb and petals of wild and garden pansies but did not identify most of their flavonoids.

The aim of this research was to investigate more deeply the composition of polyphenols, flavonoids and anthocyanins in flowers of *Viola* \times *wittrockiana* and to establish relationships between the colour, the methodology of extraction, polyphenols, flavonoids, total anthocyanins and antiradical activity *in vitro*. Flavonoids and anthocyanins were investigated using HPLC coupled with diode-array detection (DAD) and mass spectrometry (ESI-MS). The differences between three petal colours (yellow, red and violet) using three extraction solvents (water, 50% ethanol and 50% ethanol in acid media) are reported.

2. Material and Methods

2.1 Chemicals and Reagents

All the solvents employed, analytical and HPLC grades were purchased from Panreac (Barcelona, Spain). Standards and reagents such as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox[®]), gallic acid (GA) and rutin were purchased from Sigma-Aldrich Company, Ltd. (Gillingham, UK). Vanillin was obtained from Merck (Darmstadt, Germany). Standards for HPLC-DAD and HPLC-MS analysis were purchased from Extrasynthese (Genay Cedex, France). All other chemicals were purchased from common sources.

2.2 Plant Material and Preparation of Extracts

Three petal colours of garden pansies (*Viola* \times *wittrockiana*), belonging to violet family (*Violaceae*), were analysed: yellow, red and violet. Petals were grown in a greenhouse (Balaguer, Spain). Twelve different tissues of each variant of petal colours were manually collected, dried, and ground to a homogenous powder in January 2012 in collaboration with the company Pàmies Horticoles, S.L., comprising three replicates. The powder was stored protected from light and moisture until the extraction.

Each variant of petal colour was treated with three types of solvent: water, 50% ethanol and 50% ethanol in acid media (hydrochloric acid was added to pH=1.5-2). The extracts were prepared according to the published procedure (Lopez et al., 2008). Furthermore, three different extractions were carried out for each petal colour in order to obtain representative quantitative results. For each extraction, 1.5 g of air-dried and homogenous powder of petals was macerated with 50 mL of each solvent. The mixture was stirred continuously for 24 h at 4 °C. After that, all samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany). Part of the supernatant was introduced in a rotary evaporator until the complete evaporation of the organic solvent, frozen at -80 °C for 24 h and lyophilized for 3 days to provide the sample to be used in the assays. Samples were then weighed and kept protected from light in a dessicator until use. There were no differences in extraction yield among the different extracts. Values ranged between 15 and 20% for all extracts.

2.3 Antioxidant Assessment

2.3.1 Qualitative Determination on DPPH free Radical Scavenging Capacity by TLC

100 μ g of crude extracts were spotted on silica-gel 60 F₂₅₄ plates (Merck, Germany) and were developed in ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26). Three plates were prepared under same conditions, one for the antiradical test and the others to establish a relation between the antioxidant activity and the nature of the active compounds (Wagner, 1996).

The first plate was sprayed with a methanolic solution of DPPH (2 mg/mL). The second plate was sprayed with vanillin-sulphuric acid (observed at visible) as a general reagent and the third plate was sprayed with natural

products – PEG (view under 366 nm) to detect flavonoids.

2.3.2 Quantitative Determination of Antioxidant Activity

Four different methods were used for the evaluation of the antioxidant activity of the extracts: DPPH radical-scavenging assay (Lopez et al., 2008), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid (ABTS^{•+}) assay (Almajano, Carbo, Jimenez, & Gordon, 2008), Oxygen Radical Absorbance Capacity (ORAC) assay (Almajano, Delgado, & Gordon, 2007) and Ferric Reducing Antioxidant Power (FRAP) method (Skowrya, Falguera, Gallego, Peiró, & Almajano, 2013). Results were expressed as mg Trolox (TE)/g of freeze-dried weight (FDW) extract. All measurements were performed with an automated microplate reader and 96-well plates using a spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France). Absorbance measurements were made in triplicate for each diluted solution.

2.3.2.1 DPPH Assay

The DPPH assay determined the ability of extracts to scavenge the DPPH radical. A solution of DPPH (5.07 mM) in pure methanol was prepared. Appropriate dilutions were made for the study of the samples to allow the fall in DPPH concentration to be in the range 10-90 %. Then, the solution of DPPH and samples (in a concentration of 10% v/v of sample and 90% of the radical) were added to the well of a microplate. Absorbance was measured at 517 nm, every 15 min for 75 min.

2.3.2.2 ABTS Assay

The assay is based on the ability of an antioxidant compound to quench the ABTS^{•+} relative to that of a reference antioxidant such as Trolox. A stock solution of ABTS^{•+} radical cation was prepared by mixing ABTS solution with a potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 16 h before use. The working ABTS^{•+} solution was produced by dilution in 10 mM PBS (pH 7.4) incubated at 30 °C of the stock solution to achieve an absorbance value of 0.7 (± 0.02) at 734 nm. An aliquot of 20 μ L of diluted extract was added to ABTS^{•+} working solution (180 μ L). For the blank and standard curve, 20 μ L of PBS or Trolox solution were used, respectively. Absorbance was measured by means of a UV-vis spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) at 734 nm immediately after addition and rapid mixing (t_0) and then every minute for 15 min. Readings at $t = 0$ min (t_0) and $t = 5$ min (t_5) of reaction were used to calculate the inhibition percentage value for each extract: % inhibition of the sample = $[(t_0 - t_5)/t_0] \times 100 - [\% \text{ inhibition of the blank}]$. A standard reference curve was built by plotting % inhibition value against Trolox concentration (2–32 μ M).

2.3.2.3 ORAC Assay

Diluted extract (40 μ L) was transferred by pipette into each well and then 120 μ L of 1.34 μ M fluorescein working solution in phosphate buffer (13.3 mM) at 37 °C were added to each sample. The plate was placed in a spectrophotometer and incubated at 37 °C. The initial fluorescence was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH, 40 μ L, 30 mM) was then added to each sample well and the fluorescence was measured immediately and every 2 min thereafter for 120 min. For the calibration curve, solutions of Trolox were prepared in the range of 8–58 μ M. The ORAC value for each extract was calculated using a regression equation relating Trolox concentration and the net area under the fluorescence decay curve.

2.3.2.4 FRAP Assay

The FRAP reagent was prepared with acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl, 40 mM) and FeCl₃ (20 mM). The proportions were 10:1:1 (v:v:v), respectively. A suitable dilution of the extract was added to the FRAP reagent (1:30, v:v) and incubated at 37 °C. The assay was performed by means of an automated microplate reader (Fluostar Omega, Perkin-Elmer, Paris, France) with 96-well plates. The absorbance at 593 nm at time zero and after 4 min was recorded. The analysis was performed in triplicate and values were determined from a calibration curve of Trolox (ranging from 2.5 to 33 μ M).

2.4 Total Phenolic (TPC), Total Flavonoid (TFC) and Total Anthocyanin (TAC) Content

To determine the TPC, an originally developed method and improved by Prior et al. was used (Prior, Wu, & Schaich, 2005). Finally, the optical density of the blue-coloured resulting solution was measured at 765 nm using a Hewlett-Packard 8452A diode array spectrophotometer. Gallic acid calibration curve (2-14 mg/L) was used to determine TPC concentrations ($y=0.076x-0.0175$, $r^2=0.9989$). Results are expressed as mg of gallic acid equivalents (GAE)/g of freeze-dried weight.

TFC was determined using the method from German Pharmacopoeia. A known volume of sample reacted with

AlCl_3 20 mg/mL in 5% acetic acid in ethanol in 3:1 ratio. After 30 minutes absorbance was measured at 405 nm. The calibration curve was prepared with rutin (15-90 mg/L) and results are expressed as mg of rutin equivalents (RE)/g of freeze-dried weight ($y=0.008x+0.0246$, $r^2=0.9971$).

The quantification of TAC is based on the spectrophotometric measurement of absorbance at a specific wavelength, 520 nm. Malvidin 3-*O*-glucoside calibration curve (40-300 mg/L) was used to obtain final concentrations and data were expressed as mg of malvidin glucoside equivalents (ME)/g of freeze-dried weight ($y=0.0031x-0.1511$, $r^2=0.9676$).

2.5 HPLC Analysis

Identification of all constituents was performed by HPLC-DAD and MS analysis by comparing the retention time, the UV and MS spectra of the peaks in the extracts.

2.5.1 HPLC-DAD Analysis

HPLC analysis was performed on an Agilent 1200 chromatograph (Agilent Technologies, Germany). Compounds were separated on a C18 reversed-phase column (4.6×250 mm, 5 μm , Phenomenex, Torrance, CA, USA) and detected with a diode array detector (DAD). Injection volume was 10 μL and flow rate was 1 mL/min. Gradient elution was applied with 0.1% formic acid-acidified water (A) and 0.1% formic acid-acidified acetonitrile (B) as solvents. Gradient conditions were as follows: 0 min, 90% A; 4 min, 85% A; 20 min, 80% A; 22 min, 10% A; 24 min, 90% A; 29 min, 90% A. The UV-vis spectra were recorded between 220 and 550 nm.

2.5.2 HPLC-MS Analysis

The HPLC system described above was interfaced with a HP1100 MSD API-electrospray detector (Agilent Technologies, Palo Alto, CA, USA). The column and the chromatographic condition were the same as those used for the HPLC analysis. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from m/z 100 to 1000, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30°C and capillary voltage 3.5 kV. The collision energy was 60 eV.

2.5.3 Validation of HPLC Method and Quantitative Determination of Individual Constituents

The HPLC method was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ) precision, specificity, and accuracy according to the International Conference on Harmonization (ICH) guidelines (Shabir, 2003).

The quantitative determination of flavonoids and anthocyanins was performed using the external standard method and they were detected at 350 and 525 nm. The calibration curves were obtained with five samples of various concentrations by linear regression analysis. The equation of linear regression for flavonol derivatives was $y=13.28x+3.20$ ($r^2=0.997$) and for anthocyanin derivatives was $y=10.33x+2.53$ ($r^2=0.998$), using rutin and malvidin-3-*O*-glucoside as standards, respectively. Both detection limits were 0.1 $\mu\text{g}/\text{mL}$. The repeatability and reproducibility of the chromatographic separations was verified with several columns, and all chromatographic analyses were reproducible on a second HPLC. Extracts were analysed three times and each extract obtained was injected three times into the HPLC. The amount of flavonoids and anthocyanins in each extract was expressed in mg per gram of dried petals.

2.6 Statistical Analysis

Results were expressed as mean \pm standard deviation. All data were analyzed by the computer application SPSS for Windows (version 15.0, SPSS Inc., Chicago, USA). Statistical differences were evaluated with one-way analysis of variance (ANOVA) and Dunnett *post hoc* test. Differences at the level $P<0.05$ were considered to be significant. Correlations between total phenol contents, total flavonoid contents, total anthocyanin contents and antioxidant activity were established by regression analysis.

3. Results and Discussion

Thin-layer chromatography (TLC) chemical screening is a simple, fast, and low-cost method of identification of chemical ingredients in plant extracts. In order to detect antioxidant activity in water, 50% ethanol and 50% ethanol in acid media extracts, a method based on the reduction of DPPH radical by TLC was carried out. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. DPPH is a free radical stable at room temperature, which produces a violet solution in methanol. In the DPPH free radical scavenging capacity assay by TLC, the extracts that produced yellow spots on the plates were considered to contain antioxidant agents. Some plants are rich in secondary metabolites, such as flavonoids, anthocyanins, phenolic acids and tannins. In all the extracts assayed high antioxidant/antiradical capacities were detected. After studying the plates sprayed

with vanillin/H₂SO₄ and Natural Products reagents PEG it was deduced that the activity might be due mainly to phenolic compounds, flavonoids and anthocyanins.

A number of assays have been described in the literature for the measurement of total antioxidant activity of food, medicinal extracts and pure compounds (Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009). Each method relates to the generation of a different radical, acting through a variety of mechanisms. In this sense, the antioxidant activity of different extracts was evaluated in four different *in vitro* models (Table 1). The four assays gave comparable results for the antioxidant activity measured in extracts. In general, violet petals were the most active with three types of solvent employed.

Significant differences in the antioxidant activity of the extracts were detected depending on the extraction solvent. The 50% ethanol extract was the most active in the yellow and red petals (651.51 µg TE/g and 954.16 µg TE/g, respectively) in DPPH assay. However, the better extraction solvent for violet petals was water (1026.65 µg TE/g). Similar results were revealed in ABTS, ORAC and FRAP assays (Table 1). There was a strong correlation between the values obtained with DPPH, ABTS ORAC and FRAP. Similarly, Ou et al. (2002) reported high correlation of antioxidant activity between the FRAP and ORAC techniques in blueberry fruit, and Awika et al. (2003) observed as well high correlation between ABTS, DPPH, and ORAC among sorghum and its products.

TPC, TFC and TAC of all extracts showed solvent and pH dependence. Variations in the contents of various extracts were attributed to the polarities of the compounds present. The TPC values were in the range from 120.56 mg GAE/g to 465.76 mg GAE/g. In all cases, the TPC content was higher when the extraction solvent was 50% ethanol, followed by 50% ethanol in acid media and water. The TPC values of the violet (465.76 mg GAE/g) and red (464.17 mg GAE/g) petals were similar and significantly higher than in the yellow (287.51 mg GAE/g) ones. Gallego et al. (2013) reported that TPC values for thyme and lavender flowers were 288 and 52 mg GAE/g, respectively. These values are similar or lower than found in the current study. The good correlations between TPC and DPPH[•] radical scavenging activity ($r^2=0.6727$), ABTS^{•+} radical scavenging activity ($r^2=0.8851$), ORAC method ($r^2=0.9234$) and FRAP assay ($r^2=0.7681$) values confirmed that mainly the phenols were responsible for the antioxidant activity of the extracts. These results agreed with previous studies of edible and wild flowers from China like *Rosa rugosa*, *Limonium sinuatum* or *Pelargonium hortorum* (Li et al., 2014).

TFC was also determined and values ranging from 28.20 mg RE/g to 198.57 mg RE/g were found. These results confirmed the previous literature reports indicating that flavonoids represent the main group of phenolic compounds in pansy (Vukics et al., 2008b). The highest TFC value was obtained using water as an extraction solvent in the three colours: yellow (55.91 mg RE/g), red (83.58 mg RE/g) and violet (198.57 mg RE/g), followed by 50% ethanol and 50% ethanol in acid media. The study also showed a high and positive correlation between TFC content and DPPH[•] radical scavenging activity ($r^2=0.6136$), ABTS^{•+} radical scavenging activity ($r^2=0.84$), ORAC method ($r^2=0.7941$) and FRAP assay ($r^2=0.8342$).

Acidified media showed influence on the anthocyanin content and the extraction efficiency was higher at lower pH values. The higher TAC values were seen in violet (38.74 mg ME/g), followed by red (28.72 mg ME/g) and finally yellow (13.98 mg ME/g) petals. However, low correlations were found between total anthocyanin content and antioxidant activity. Therefore, good correlation (TFC and radical scavenging activity) suggested that phenolic compounds, mainly of flavonoid type, play an important role as antioxidants. Variations in the antioxidant capacity of different extracts may be attributed to differences in their individual phenolic contents.

Significant advantages over other chromatographic techniques, such as the potential of being used for qualitative and quantitative analysis with high sensitivity and specificity, have made HPLC-MS a widespread and extremely useful method for phytochemical analysis. This technique combines HPLC, a powerful tool for complex mixture separation, with mass spectrometry which enables a reliable determination of the molecular weight and characterization of ionised compounds.

Table 1. Polyphenol, flavonoid, anthocyanin content and antioxidant activity of different *Viola* × *wittrockiana* extracts. Content of individual flavonoids and anthocyanins were detected in *Viola* × *wittrockiana*

	Yellow			Red			Violet		
	H ₂ O	50% EtOH	50% EtOH (pH=2)	H ₂ O	50% EtOH	50% EtOH (pH=2)	H ₂ O	50% EtOH	50% EtOH (pH=2)
TPC (mg/g)	120.56 ± 4.48 ^d	287.51 ± 4.05 ^e	257.07 ± 7.59 ^e	419.28 ± 4.75 ^b	464.17 ± 8.06 ^a	352.56 ± 10.04 ^b	395.90 ± 6.24 ^b	465.76 ± 9.72 ^a	442.52 ± 14.35 ^b
TFC (mg/g)	55.91 ± 1.19 ^e	35.79 ± 2.43 ^e	28.20 ± 0.07 ^e	83.58 ± 1.60 ^b	46.11 ± 0.40 ^d	42.84 ± 0.14 ^d	198.57 ± 3.27 ^a	45.35 ± 0.40 ^d	41.20 ± 1.85 ^d
TAC (mg/g)	4.11 ± 0.13 ^e	9.66 ± 0.42 ^d	13.98 ± 0.95 ^d	21.50 ± 0.52 ^e	23.50 ± 0.68 ^e	28.72 ± 0.92 ^b	19.24 ± 0.75 ^e	17.64 ± 0.27 ^e	38.74 ± 5.12 ^a
DPPH (μmol/g)	357.83 ± 1.20 ^d	651.51 ± 4.31 ^e	552.32 ± 1.80 ^e	928.19 ± 2.26 ^b	954.16 ± 2.61 ^b	555.81 ± 3.97 ^e	1026.65 ± 1.33 ^a	961.88 ± 1.64 ^b	652.31 ± 2.76 ^e
ABTS (μmol/g)	519.79 ± 2.50 ^e	998.30 ± 2.59 ^e	906.06 ± 6.47 ^d	1100.00 ± 2.04 ^e	1178.23 ± 4.82 ^b	1098.39 ± 1.83 ^e	1482.78 ± 1.17 ^a	1103.94 ± 2.26 ^b	1200.00 ± 6.31 ^b
ORAC (μmol/g)	791.90 ± 0.31 ^e	2232.47 ± 1.11 ^d	2152.28 ± 2.69 ^d	3057.20 ± 0.58 ^e	3590.09 ± 2.57 ^b	2758.74 ± 1.42 ^e	3863.65 ± 0.63 ^a	3744.59 ± 0.93 ^b	3640.80 ± 0.83 ^b
FRAP (μmol/g)	418.34 ± 3.94 ^d	503.60 ± 4.52 ^e	228.39 ± 6.03 ^e	880.69 ± 2.04 ^b	953.30 ± 2.75 ^a	719.45 ± 3.30 ^b	989.30 ± 0.60 ^a	758.66 ± 1.17 ^b	400.72 ± 1.63 ^d
F1 (mg/g)	3.63 ± 0.04 ^b	2.85 ± 0.02 ^b	-	4.49 ± 0.12 ^a	-	1.54 ± 0.35 ^e	-	-	-
F2 (mg/g)	-	-	-	10.18 ± 0.89 ^a	8.94 ± 0.87 ^a	13.16 ± 2.02 ^a	-	-	-
F3 (mg/g)	5.60 ± 0.14 ^d	3.68 ± 0.05 ^d	7.32 ± 0.11 ^d	30.14 ± 2.33 ^b	17.27 ± 1.21 ^e	17.36 ± 1.67 ^e	107.96 ± 4.36 ^a	26.17 ± 1.87 ^b	21.30 ± 2.79 ^b
F4 (mg/g)	-	-	-	-	-	-	37.66 ± 1.88 ^a	6.76 ± 0.42 ^b	8.80 ± 0.61 ^b
F5 (mg/g)	1.80 ± 0.02 ^d	1.43 ± 0.01 ^d	1.72 ± 0.15 ^d	3.56 ± 0.36 ^e	1.84 ± 0.02 ^d	1.72 ± 0.03 ^d	32.34 ± 2.45 ^a	7.38 ± 0.70 ^b	5.24 ± 0.29 ^b
F6 (mg/g)	3.09 ± 0.01 ^b	2.44 ± 0.09 ^e	4.75 ± 0.07 ^b	6.71 ± 1.43 ^a	3.30 ± 0.07 ^b	3.62 ± 0.06 ^b	8.55 ± 0.11 ^a	2.36 ± 0.03 ^c	1.92 ± 0.02 ^e
F7 (mg/g)	40.81 ± 2.87 ^a	24.12 ± 1.49 ^c	10.98 ± 0.99 ^d	33.61 ± 3.01 ^b	19.55 ± 2.01 ^d	16.18 ± 1.02 ^d	4.36 ± 0.09 ^e	1.37 ± 0.01 ^e	1.26 ± 0.01 ^e
F8 (mg/g)	-	-	-	2.65 ± 0.21 ^a	-	1.80 ± 0.01 ^a	-	-	-
F9 (mg/g)	-	-	-	1.88 ± 0.13 ^a	-	-	-	-	-
F10 (mg/g)	-	-	-	0.76 ± 0.01 ^a	-	-	-	-	-
Total	54.94 ± 1.16 ^e	34.53 ± 1.58 ^e	24.84 ± 1.32 ^e	79.34 ± 3.49 ^b	42.01 ± 2.31 ^d	40.66 ± 2.16 ^d	190.87 ± 5.09 ^a	44.05 ± 1.03 ^d	38.53 ± 1.72 ^d
A1 (mg/g)	2.96 ± 0.02 ^d	4.58 ± 0.11 ^c	4.69 ± 0.20 ^e	7.47 ± 1.05 ^b	8.80 ± 0.92 ^b	11.40 ± 1.74 ^a	7.01 ± 0.77 ^b	8.56 ± 0.46 ^b	11.58 ± 1.02 ^a
A2 (mg/g)	0.40 ± 0.01 ^f	2.08 ± 0.06 ^e	1.99 ± 0.13 ^e	5.17 ± 1.00 ^e	6.89 ± 0.54 ^b	7.72 ± 0.83 ^b	3.49 ± 0.04 ^d	0.78 ± 0.00 ^f	9.89 ± 1.01 ^a
A3 (mg/g)	0.65 ± 0.01 ^e	2.83 ± 0.09 ^e	4.39 ± 0.27 ^b	5.75 ± 0.99 ^b	4.39 ± 0.08 ^b	7.25 ± 0.05 ^a	3.74 ± 0.22 ^e	4.27 ± 0.70 ^b	7.74 ± 0.81 ^a
A4 (mg/g)	-	-	2.72 ± 0.34 ^e	3.03 ± 0.72 ^e	3.62 ± 0.17 ^e	4.74 ± 0.34 ^b	4.88 ± 0.31 ^b	3.67 ± 0.52 ^c	9.07 ± 1.00 ^a
Total	4.02 ± 0.57 ^e	9.49 ± 1.04 ^d	13.81 ± 1.94 ^e	21.43 ± 2.75 ^b	23.70 ± 2.13 ^b	31.11 ± 2.52 ^a	19.13 ± 1.37 ^b	17.30 ± 1.51 ^b	38.29 ± 2.71 ^e

TPC: total polyphenol content; TFC: total flavonoid content; TAC: total anthocyanin content.

Data expressed as means ± SD of triplicate analyses. Values in the same line with different letters present significant differences $p < 0.05$.

Components of red, yellow and violet garden pansy, extracted with three different solvents were identified and quantified by comparing retention times, ultraviolet and mass spectra and mass fragmentation data with those described in existing literature. In total, 14 constituents were identified by HPLC-DAD-ESI-MS. In Figure 1, the HPLC-DAD chromatograms of the yellow, red and violet petals are presented. Data concerning the identification of the peaks are shown in Table 2, where the retention time, UV-Vis absorptions and electrospray ionisation mass spectrometry in negative and positive ion mode of all the compounds detected are reported.

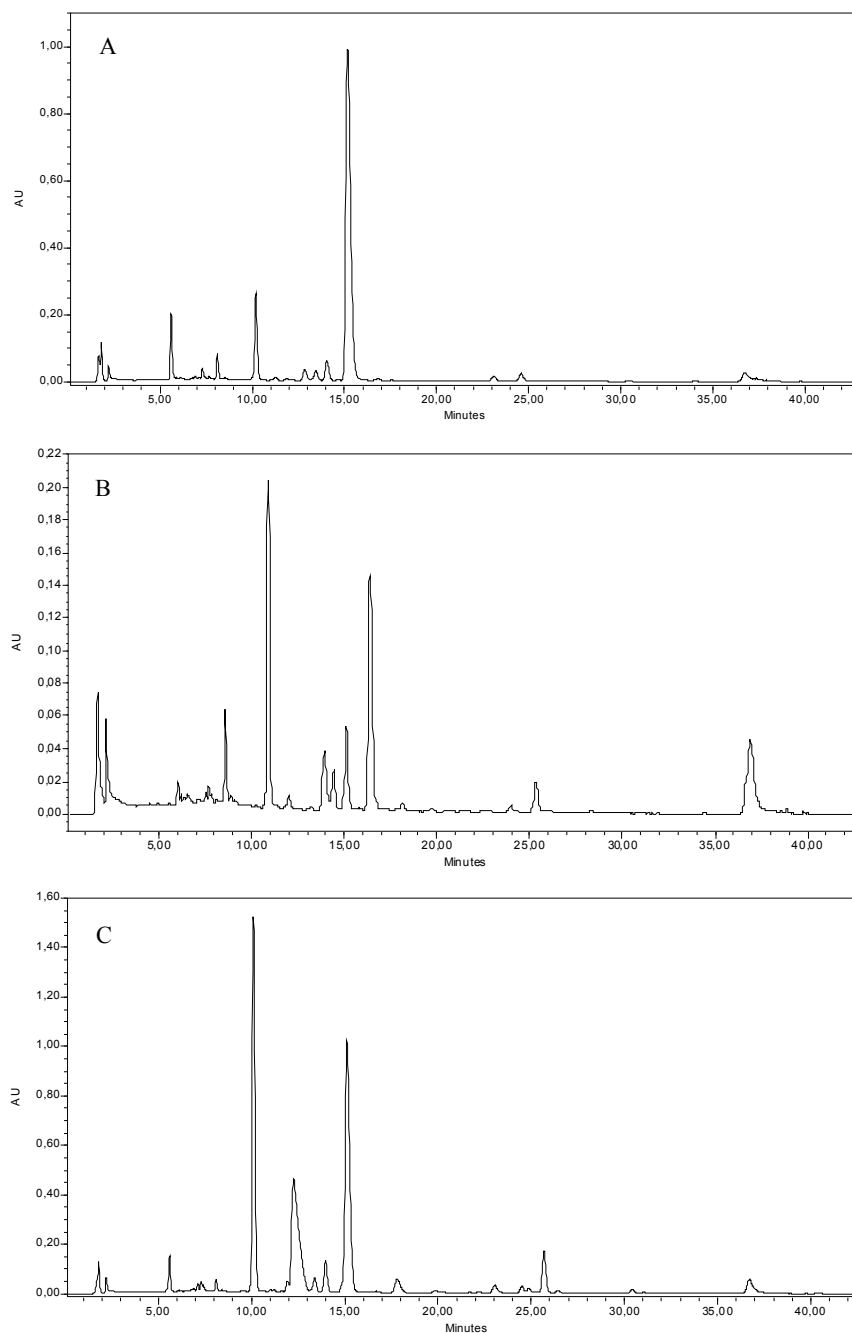


Figure 1. Chromatographic profiles, acquired at 355 nm, of *V. wittrockiana* extracts (A: yellow, B: red, C: violet)

Table 2. Retention time, UV-vis absorption data, MS fragmentation and name of the main compounds detected in *Viola × wittrockiana*

No.	Rt	UV (nm)	<i>m/z</i>	Negative	Positive	Identification	Literature reference	
F1	5.6	255.6, 352.9	756	755 [M-H] ⁻ , [M-H-Glu] ⁻ , [M-H-Glu-Rha] ⁻ , [A-H] ⁻	593 447 301	779 [M+Na] ⁺ , 757 [M+H] ⁺ , 303 [A+H] ⁺	Quercetin-3- <i>O</i> -di-rhamnosyl-glucoside	Karioti et al. (2011)
A1	6.1	288, 312sh, 529	919	935 [M-2H+H ₂ O] ⁻ , [M-Glu] ⁻ , [M-Glu-Cm] ⁻ , [M-Glu-Cm-CH ₂] ⁻	755 609 563	757 [M-Cm] ⁺ , 465 [M-Cm-Rha-Glu] ⁺ , 303 [A] ⁺ ,	Delphinidin-3-(4''- <i>p</i> -coumaroyl)-rutinoside-5-glucoside (violananin)	Hase et al. (2005), Karioti et al. (2011), Zhang et al. (2011)
A2	8.0	312sh, 528	933	949 [M-2H+H ₂ O] ⁻ , [M-Glu] ⁻ , [M-Glu-Cm] ⁻ , [M-Glu-Cm-CH ₂] ⁻	769 623 577	761 [M-Cm] ⁺ , 479 [M-Cm-Rha-Glu] ⁺ , 317 [A] ⁺ ,	Petunidin-3-(4''- <i>p</i> -coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F2	8.1	266, 344	740	739 [M-H] ⁻ , [M-H-Glu] ⁻ , [M-H-Glu-Rha] ⁻ , [A-H] ⁻	577 431 285	763 [M+Na] ⁺ , 741 [M+H] ⁺ , 287 [A+H] ⁺	Kaempferol-3- <i>O</i> -di-rhamnosyl-glucoside	Karioti et al. (2011)
A3	8.4	312sh, 520	903	919 [M-2H+H ₂ O] ⁻ , [M-Glu] ⁻ , [M-Glu-Cm] ⁻ , [M-Glu-Cm-CH ₂] ⁻	739 593 547	741 [M-Cm] ⁺ , 449 [M-Cm-Rha-Glu] ⁺ , 287 [A] ⁺	Cyanidin-3-(4''- <i>p</i> -coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F3	10.2	255.6, 354.1	610	609 [M-H] ⁻ , [M-H-Rha] ⁻ , 301 [A-H] ⁻	463 301	633 [M+Na] ⁺ , 611 [M+H] ⁺ , 303 [A+H] ⁺	Quercetin-3- <i>O</i> -rhamnosyl-glucoside (rutin)	Karioti et al. (2011), Papp et al. (2004), Vukics et al. (2008b,c)
A4	10.7	311sh, 535	947	963 [M-2H+H ₂ O] ⁻ , [M-Glu] ⁻ , [M-Glu-Cm] ⁻ , [M-Glu-Cm-CH ₂] ⁻	783 637 591	775 [M-Cm] ⁺ , 493 [M-Cm-Rha-Glu] ⁺ , 331 [A] ⁺ ,	Malvidin-3-(4''- <i>p</i> -coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F4	12.4	255.6, 352.9	626	625 [M-H] ⁻ , [M-H-Rha] ⁻ , [M-H-Glu] ⁻ , 285 [A-H] ⁻	463 447 285	649 [M+Na] ⁺ , 627 [M+H] ⁺ , 287 [A+H] ⁺	Luteolin-6- <i>C</i> -rhamnosyl-8- <i>C</i> -glucoside	Present study
F5	13.5	266.2, 341.0	610	609[M-H] ⁻ , [M-H-Rha] ⁻ , [M-H-Glu] ⁻ , 269 [A-H] ⁻	447 431 269	649 [M+Na] ⁺ , 611 [M+H] ⁺ , 271 [A+H] ⁺	Apigenin-6- <i>C</i> -rhamnosyl-8- <i>C</i> -glucoside (violanthin)	Vukics et al. (2008b,c)
F6	14.3	271.0, 335.0	626	625 [M-H] ⁻ , [M-H-Glu] ⁻ , 269 [A-H] ⁻	447 269	649 [M+Na] ⁺ , 627 [M+H] ⁺ , 271 [A+H] ⁺	Apigenin-6,8-di- <i>C</i> -glucoside (vicenin-2)	Vukics et al. (2008a)
F7	15.2	266.6, 354,1	448	447 [M-H] ⁻ , 269 [A-H] ⁻		471 [M+Na] ⁺ , 449 [M+H] ⁺ , 271 [A+H] ⁺	Apigenin-8- <i>C</i> -glucoside (orientin)	Vukics et al. (2008a)
F8	17.8	266.4, 348.7	448	447 [M-H] ⁻ , 269 [A-H] ⁻		471 [M+Na] ⁺ , 449 [M+H] ⁺ , 271 [A+H] ⁺	Apigenin-6- <i>C</i> -glucoside (isoorientin)	Vukics et al. (2008a)
F9	23.0	255.6, 348.1	464	463 [M-H] ⁻ , 301 [A-H] ⁻ ,		487 [M+Na] ⁺ , 465 [M+H] ⁺ , 303 [A+H] ⁺	Quercetin-3- <i>O</i> -glucoside (isoquercitrin)	Karioti et al. (2011), Papp et al. (2004)
F10	25.6	265.1, 331.4	432	431 [M-H] ⁻ , 269 [A-H] ⁻		455 [M+Na] ⁺ , 433 [M+H] ⁺ , 271 [A+H] ⁺	Apigenin-7- <i>O</i> -glucoside (apigetrin)	Karioti et al. (2011)

HPLC-DAD-ESI-MS analyses evidenced the presence of 10 flavonoids (Table 2, Figure 1). Their UV spectra exhibited two major absorption maxima in the region of 240-400 nm. HPLC-MS and HPLC-UV data indicated the presence of complex flavonol and flavone glucosides. Compounds **F1**, **F3**, and **F9** were derivatives of quercetin, whereas **F5**, **F6**, **F7**, **F8** and **F10** revealed the presence of apigenin. Fragmentation patterns of

compounds **F2** and **F4** suggested the presence of kaempferol and luteolin derivatives, respectively.

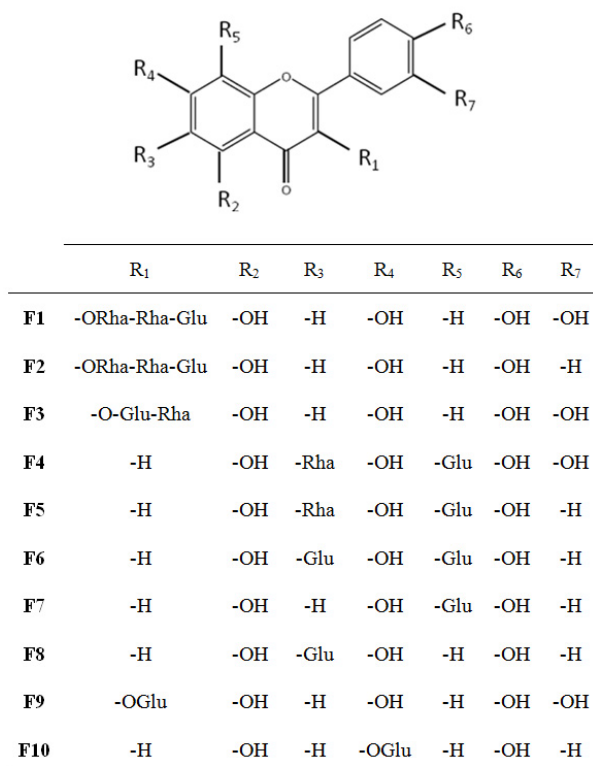


Figure 2. Chemical structures of flavonoids identified from *Viola × wittrockiana*

Molecular masses were higher than 400 Da and the characteristic ions of sugar losses (146 Da and 162 Da for deoxyhexose and hexose units, respectively) indicated the presence of one or more sugar units in their structure.

The high retention times of 15.2, 17.8, 23.0 and 25.6 min of less polar compounds **F7**, **F8**, **F9** and **F10** in combination with their mass data and literature references indicated the presence of one sugar unit. The MS spectra of peaks **F9** and **F10** showed the lack of the fragment ions characteristic of sugar losses (162 Da) for glucose units, which suggested that both compounds were flavonoid *O*-glucosides. Similarly, compounds **F7** and **F8** are flavonoid *C*-glucosides [M-H-178]. These results suggested the identity of the compounds as orientin (**F7**), isoorientin (**F8**), isoquercitrin (**F9**) and apigetrin (**F10**), which is compatible with literature references for *Viola × wittrockiana* and other species of *Viola* (Karioti, Furlan, Vincieri, & Bilia, 2011; Papp et al., 2004).

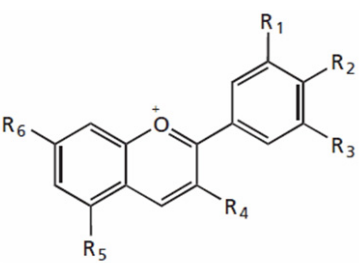
Fragmentation patterns for compounds **F3**, **F4**, **F5** and **F6** showed the presence of a second sugar. Compound **F3** was identified, based on the presence of molecular ion at m/z 610 [M]⁺ in MS spectra, UV spectra and several literature references (Karioti et al., 2011; Papp et al., 2004; Vukics et al., 2008b, 2008c) as rutin, compound **F5** as violanthin and compound **F6** as vicenin-2.

Compound **F4** was identified as luteolin-6-*C*-rhamnosyl-8-*C*-glucoside. The structural elucidation was determined first of all on the basis of the UV spectra (λ_{max} =255.6, 352.9 nm). Furthermore, the MS spectrum (m/z 626 [M]⁺, 625 [M-H]⁻, 463 [M-H-Glu]⁻, 447 [M-H-Rha]⁻, 285 [A-H]⁻) was similar to that of the compound **F5**. The difference of 16 u in the molecular ion was assigned to one additional hydroxyl group at C3' of the ring B of the flavonoid. Since this constituent was observed for the first time in *Viola sp.*, it would be necessary to characterize it completely by 1D and NMR spectroscopy after isolation and purification.

On the other hand, compounds **F1** and **F2**, at earlier retention times (5.6 and 8.1 min), displayed molecular ions at m/z 756 and 740 respectively, due to the presence of a third sugar. Compound **F1** was identified as quercetin-3-*O*-rhamnorhamnoglucoiside and compound **F2** as kaempferol-3-*O*-rhamnorhamnoglucoiside, based on the data obtained from the literature from *V. odorata* (Karioti et al., 2011).

The red and violet petals indicated the presence of anthocyanins, which are glycosides and acylglycolides of anthocyanidins, according to the hydroxyl or methoxyl substitutions in the basic skeleton. In this type of compounds, acid media are generally preferred in HPLC analyses, as they enhance anthocyanin stabilisation. In this case, the possible presence of labile flavonoids with more than two saccharides, as well as the fact that under acid conditions hydrolysis of labile acyl groups may occur, did not permit to conduct the analyses in pH lower than 2.5.

Four anthocyanins (**A1**, **A2**, **A3** and **A4**) were tentatively identified by comparing retention time, MS and UV-vis spectrum (Figure 2). All of them showed a typical chromatogram of the anthocyanins with selective wavelength (500–550nm absorption). The chromatographic and spectral data are summarised in Table 2. These identifications were confirmed by the scientific literature (Karioti et al., 2011).



	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
A1	-OH	-OH	-OH	-O-Glu- <i>p</i> -CmRha	-OGlu	-OH
A2	-OH	-OH	-OCH ₃	-O-Glu- <i>p</i> -CmRha	-OGlu	-OH
A3	-OH	-OH	-H	-O-Glu- <i>p</i> -CmRha	-OGlu	-OH
A4	-OCH ₃	-OH	-OCH ₃	-O-Glu- <i>p</i> -CmRha	-OGlu	-OH

Figure 3. Chemical structures of anthocyanins identified from *Viola × wittrockiana*

Anthocyanins derived from aglycones cyanidin, delphinidin, petunidin and malvidin were identified, according to the presence of a series of fragment signals at m/z 287, 303, 317 and 331 in MS spectra, respectively. The shoulder absorption at 290–340 nm showed that compounds were acylated with aromatic acids (Wrolstad, Durst, & Lee, 2005).

Violanin (**A1**) was assigned to a peak with retention time of 6.1 min and showed a characteristic pseudomolecular ion in the positive ion mode at m/z 919 and a peak at m/z 303 (corresponding to the delphinidin aglycone). UV maximum was at 312 nm due to the presence of the coumaroyl moiety. Other characteristic ions at m/z 757 [M-162]⁻, m/z 465 [M-308-146]⁻, were observed and attributed to the loss of coumaroyl and coumaroyl–rutinosyl moieties.

Peaks **A2**, **A3** and **A4** had similar mass spectra patterns as **A1**. Based on their retention times and literature references, they were identified as petunidin-3-(4'-*p*-coumaroyl)-rutinoside-5-glucoside ($t_R=8.0$ min), cyanidin-3-(4'-*p*-coumaroyl)-rutinoside-5-glucoside ($t_R=8.4$ min) and malvidin-3-(4'-*p*-coumaroyl)-rutinoside-5-glucoside ($t_R=10.7$ min), respectively. Several anthocyanins were detected at earlier times but the content was very low and their identification was impossible.

In Table 1, the results of the quantitative analyses are reported. Concerning flavonoids, rutin (**F3**), violanthin (**F5**), vicen-2 (**F6**) and orientin (**F7**) were presented in all extracts. The major flavonoid found was **F3** (107.96±4.36 mg/g), followed by **F5** (32.34±2.45 mg/g) in aqueous extract of violet petals. The same results had been presented by Vukics et al. (2008c). Moreover, Goncalves et al. (2012) reported a similar content of rutin in *Viola tricolor* flowers in ethyl acetated and butanol fractions (33.70 and 143.57 mg/g, respectively).

Luteolin-6-*C*-rhamnosyl-8-*C*-glucoside (**F4**) was detected only in violet pansy. The content was 4–6-fold higher in aqueous (37.66±1.88 mg/g) than 50% ethanol (6.76±0.42 mg/g) or 50% ethanol in media acid (8.80±0.61 mg/g) extract. In the same way, kaempferol-3-*O*-di-rhamnosyl-glucoside (**F2**), isoorientin (**F8**), isoquercetrin (**F9**)

and apigetrin (**F10**) appeared only in red pansy. The good correlation found between quercetin and luteolin derivatives (**F1**, **F3**, **F4**, **F9**) and antioxidant activity assays ($r^2 > 0.9$) suggests that these compounds may be partly responsible for this activity. This confirms the importance of the presence of hydroxyl groups in *ortho* position in relation to this activity, which has previously been described by numerous authors (Casado et al., 2011).

The four previously identified anthocyanins (**A1**, **A2**, **A3**, **A4**) were detected and quantified in all extracts analyzed, with the exception of malvidin-3-(4'-*p*-coumaroyl)-rutinoside-5-glucoside (**A4**), which could not be quantified in water and 50% ethanol extract of yellow pansy (Table 1).

In all extracts anthocyanin content was pH dependent, and therefore the best results were obtained when the solvent for extraction was 50% ethanol in acid media.

Violanin (**A1**) was the major anthocyanin in the three different coloured petals, highlighting its high content in red (11.40 mg/g) and violet (11.58 mg/g) in comparison with yellow (4.69 mg/g) petals. The good correlation found between violanin (**A1**) and antioxidant activity assays ($r^2 = 0.877$) suggests that this compound was also involved in the antioxidant activity. Delphinidin derivatives were reported to have relatively strong antiradical activity among various anthocyanins (Kahkonen & Heinonen, 2003).

Anthocyanins are a class of compounds responsible for red, blue and violet colours of plants. However, this class of compounds is highly unstable and easily susceptible to degradation. The stability of anthocyanin colour can be improved by copigmentation, where the anthocyanin molecule reacts with other natural plant components directly or through weak interactions, resulting in an enhanced and stabilized colour (Di Meo, Sancho Garcia, Dangles, & Trouillas, 2012, Deng et al., 2013). A wide range of different molecules has been found to act as copigments. The most studied group of copigments is flavonoids, of which flavones, flavonols, flavanones, and flavanols have been under profound examination. Several complex formations (Figure 3) through intermolecular interactions between delphinidin derivatives and quercetin derivatives have been described (Osawa, 1982).

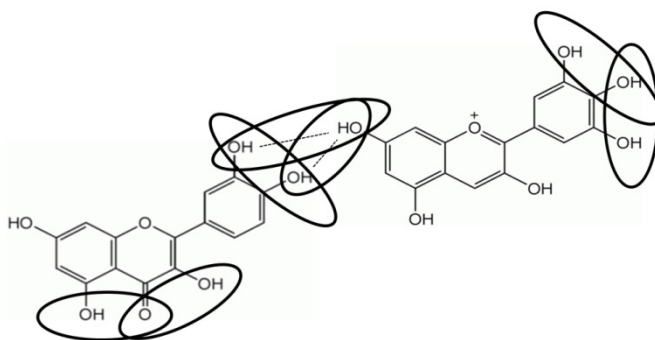


Figure 4. *ortho*-dihydroxyphenil moiety and complex formations through intermolecular interactions between delphinidin and quercetin

Therefore, this copigmentation phenomenon could demonstrate the high antioxidant activity detected in aqueous extract of violet pansy, taking into consideration several factors, such as: i) high content in rutin and violanin (quercetin and delphinidin derivatives, respectively), ii) the *ortho*-dihydroxyphenil moiety in both types of compounds, iii) complex between anthocyanins and flavonoids, which are soluble in water solution.

4. Conclusion

This study has proved the antioxidant activity of extracts from pansy. The extracted amount of phenolics, flavonoids and anthocyanins, as well as their antioxidant activity, depends on both the extraction solvent and petal colour. In this way, the use of a solution of ethanol 50% results in the highest quantity of phenolics. The phenolic content in violet and red petals was similar and significantly higher than in yellow petals. Flavonoids represented the main group of phenolic compounds in pansy. The flavonoid content of violet petals was significantly higher than that of red and yellow material. Acidified media showed influence in anthocyanin content and the extraction was more efficient at lower pH values. Chromatographic analysis with UV spectra recorded on-line gave useful complementary information and HPLC coupled to mass spectrometry (LC-MS)

provided additional structural information about the potentially active metabolites. In total, 14 constituents were identified by HPLC-DAD-ESI-MS, 10 of them were flavonoids (quercetin and apigenin derivatives, mainly) and 4 anthocyanins. Concerning flavonoids, rutin, violanthin, vicen-2 and orientin were presented in all extracts. The major flavonoid found was rutin, followed by violanthin in aqueous extract of violet petals. Violanin (delphinidin glucoside) was the major anthocyanin in the three pansy colours, with the highest values measured in red and violet petals. In conclusion, the copigmentation phenomenon between violanin and rutin evidences the high antioxidant activity detected in aqueous extract of violet pansy. Our results indicate that aqueous extracts of violet pansy can be used in functional foods as potential radical scavenging agents.

Acknowledgements

The authors would like to thank Pàmies Horticoles for kindly supplying us the fresh plants. The authors also thank Laura Bover and Martina Albir for technical support.

References

- Almajano, M. P., Carbo, R., Jimenez, J. A. L., & Gordon, M. H. (2008). Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry*, *108*(1), 55-63. <http://dx.doi.org/10.1016/j.foodchem.2007.10.040>
- Almajano, M. P., Delgado, M. E., & Gordon, M. H. (2007). Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate. *Food Chemistry*, *101*(1), 126-130. <http://dx.doi.org/10.1016/j.foodchem.2006.01.009>
- Awika, J. M., Rooney, L. W., Wu, X. L., Prior, R. L., & Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *Journal of Agricultural and Food Chemistry*, *51*(23), 6657-6662. <http://dx.doi.org/10.1021/jf034790i>
- Casado, R., Landa, A., Calvo, J., Garcia-Mina, M. J., Marston, A., Hostettmann, K., & Calvo, M. I. (2011). Anti-inflammatory, antioxidant and antifungal activity of *Chuquiraga spinosa*. *Pharmaceutical Biology*, *49*(6), 620-626. <http://dx.doi.org/10.3109/13880209.2011.577436>
- Deng, J., Chen, S., Yin, X., Wang, K., Liu, Y., Li, S., & Yang, P. (2013). Systematic qualitative and quantitative assessment of anthocyanins, flavones and flavonols in the petals of 108 lotus (*Nelumbo nucifera*) cultivars. *Food Chemistry*, *139*(1-4), 307-12. <http://dx.doi.org/10.1016/j.foodchem.2013.02.010>
- Di Meo, F., Garcia, S. J. C., Dangles, O., & Trouillas, P. (2012). Highlights on anthocyanin pigmentation and copigmentation: A matter of flavonoid pi-stacking complexation to be described by DFT-D. *Journal of Chemical Theory and Computation*, *8*(6), 2034-2043. <http://dx.doi.org/10.1021/ct300276p>
- Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., & Merillon, J. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, *57*(5), 1768-1774. <http://dx.doi.org/10.1021/jf803011r>
- Feng, Y., & Wang, X. (2012). Antioxidant therapies for Alzheimer's disease. *Oxidative Medicine and Cellular Longevity*, 472932. <http://dx.doi.org/10.1155/2012/472932>
- Gallego, M. G., Gordon, M. H., Segovia, F. J., Skowrya, M., & Almajano, M. P. (2013). Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions. *Journal of the American Oil Chemists' Society*, *90*(10), 1559-1568. <http://dx.doi.org/10.1007/s11746-013-2303-3>
- Gonçalves, A. F. K., Friedrich, R. B., Boligon, A. A., Piana, M., Beck, R. C. R., & Athayde, M. L. (2012). Anti-oxidant capacity, total phenolic contents and HPLC determination of rutin in *Viola tricolor* (L.) flowers. *Free Radicals and Antioxidants*, *2*(4), 32-37. <http://dx.doi.org/10.5530/ax.2012.4.6>
- Han, X., Shen, T., & Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences*, *8*(9), 950-988. <http://dx.doi.org/10.3390/i8090950>
- Kahkonen, M. P., & Heinonen, M. (2003). Antioxidant activity of anthocyanins and their aglycons. *Journal of Agricultural and Food Chemistry*, *51*(3), 628-633. <http://dx.doi.org/10.1021/jf025551i>
- Kaisoon, O., Siriamornpun, S., Weerapreeyakul, N., & Meeso, N. (2011). Phenolic compounds and antioxidant activities of edible flowers from Thailand. *Journal of Functional Foods*, *3*(2), 88-99. <http://dx.doi.org/10.1016/j.jff.2011.03.002>
- Karioti, A., Furlan, C., Vincieri, F. F., & Bilia, A. R. (2011). Analysis of the constituents and quality control of viola odorata aqueous preparations by HPLC-DAD and HPLC-ESI-MS. *Analytical and Bioanalytical Chemistry*, *399*(4), 1715-1723. <http://dx.doi.org/10.1007/s00216-010-4473-2>

- Kelley, K. M., Behe, B. K., Biernbaum, J. A., & Poff, K. L. (2001). Consumer and professional chef perceptions of three edible-flower species. *HortScience*, *36*(1), 162-166.
- Li, A. N., Li, S., Li, H. B., Xu, D. P., Xu, X. R., & Chen, F. (2014). Total phenolic contents and antioxidant capacities of 51 edible and wild flowers. *Journal of Functional Foods*, *6*, 319-330. <http://dx.doi.org/10.1016/j.jff.2013.10.022>
- Lopez, V., Akerreta, S., Casanova, E., Garcia-Mina, J. M., Cavero, R. Y., & Calvo, M. I. (2008). Screening of Spanish medicinal plants for antioxidant and antifungal activities. *Pharmaceutical Biology*, *46*(9), 602-609. <http://dx.doi.org/10.1080/13880200802179634>
- Osawa, Y. (1982). *Copigmentation of anthocyanins* In P. Markakis (Ed.). New York: Academic Press.
- Ou, B. X., Huang, D. J., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *Journal of Agricultural and Food Chemistry*, *50*(11), 3122-3128. <http://dx.doi.org/10.1021/jf0116606>
- Papp, I., Apati, P., Andrasek, V., Blazovics, A., Balazs, A., Kite, P. J., ... Kery, A. (2004). LC-MS analysis of antioxidant plant phenoloids. *Chromatographia*, *60*, S93-S100. <http://dx.doi.org/10.1365/s10337-004-0348-z>
- Prior, R. L., Wu, X. L., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*(10), 4290-4302. <http://dx.doi.org/10.1021/jf0502698>
- Sanchez-Mata, M. C., Cabrera Loera, R. D., Morales, P., Fernandez-Ruiz, V., Camara, M., Diez Marques, C., & Tardio, J. (2012). Wild vegetables of the mediterranean area as valuable sources of bioactive compounds. *Genetic Resources and Crop Evolution*, *59*(3), 431-443. <http://dx.doi.org/10.1007/s10722-011-9693-6>
- Schiffrin, E. L. (2010). Antioxidants in hypertension and cardiovascular disease. *Molecular Interventions*, *10*(6), 354-362. <http://dx.doi.org/10.1124/mi.10.6.4>
- Shabir, G. A. (2003). Validation of high-performance liquid chromatography methods for pharmaceutical analysis - understanding the differences and similarities between validation requirements of the US food and drug administration, the US pharmacopeia and the international conference on harmonization. *Journal of Chromatography A*, *987*(1-2), 57-66. [http://dx.doi.org/10.1016/S0021-9673\(02\)01536-4](http://dx.doi.org/10.1016/S0021-9673(02)01536-4)
- Vukics, V., Kery, A., & Guttman, A. (2008a). Analysis of polar antioxidants in heartsease (*Viola tricolor* L.) and garden pansy (*Viola x wittrockiana* Gams.). *Journal of Chromatographic Science*, *46*(9), 823-827.
- Vukics, V., Toth, B. H., Ringer, T., Ludanyi, K., Kery, A., Bonn, G. K., & Guttman, A. (2008b). Quantitative and qualitative investigation of the main flavonoids in heartsease (*Viola tricolor* L.). *Journal of Chromatographic Science*, *46*(2), 97-101.
- Vukics, V., Kery, A., Bonn, G. K., & Guttman, A. (2008c). Major flavonoid components of heartsease (*Viola tricolor* L.) and their antioxidant activities. *Analytical and Bioanalytical Chemistry*, *390*(7), 1917-1925. <http://dx.doi.org/10.1007/s00216-008-1885-3>
- Wagner, H. & Bladt, S. (Ed.). (1996). *Plant drug analysis: A thin layer chromatography atlas* (2nd ed.). Berlin: Springer.
- Wrolstad, R. E., Durst, R. W., & Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology*, *16*(9), 423-428. <http://dx.doi.org/10.1016/j.tifs.2005.03.019>

Copyrights

Copyright for this article is retained by the author(s), with first publication rights granted to the journal.

This is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).