Quantification of Major Flavonoids in Carnation Tissues (*Dianthus caryophyllus*) as a Tool for Cultivar Discrimination

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Z. Naturforsch. **63c**, 161–168 (2008); received September 13/October 30, 2007

One flavone-*C*-glycoside and two flavonol-*O*-glycosides were recognized and isolated as the main flavonoidal components in nine different carnation cultivars, and their chemical structures have been determined by spectroscopic methods, including UV detection, MS and NMR. The distribution of these three compounds in flowers, leaves, stems, young sprouts, and roots of each cultivar was evaluated by a simple HPLC-UV method: the graphic representation of their content in the different tissues allows to identify and characterize unambiguously each considered carnation cultivar. The presented method could be an easy, inexpensive and reliable tool for carnation cultivar discrimination.

Key words: Flavonoids, Carnation, Cultivar Discrimination

Introduction

Among the large number of natural products of plant origin, called secondary metabolites because they are not directly involved in processes of growth and development of the organism that accumulates them, flavonoids play a central role. Their importance to the plant can be ascribed to their effect as physiologically active compounds (Harborne and Williams, 2000; Peer and Murphy, 2006), stress protecting agents (Tattini *et al.*, 2004), attractants or feeding deterrents (Hoffmann-Campo *et al.*, 2001) and, in general, to their significance in plant resistance (Curir *et al.*, 2006; Treutter, 2006).

In carnation (*Dianthus caryophyllus* L.), studies mostly focused on the accumulation of anthocyanins and other flavonoids in flowers, the biosynthesis of which has been extensively studied, and sometimes has been genetically modified in order to obtain new varieties of commercial interest (Fukui *et al.*, 2003; Yoshida *et al.*, 2004). In spite of the fact that in literature studies on carnation secondary metabolites pertain essentially to non-flavonoidal phenolic compounds, flavones and flavonols are particularly abundant in the *Dianthus* genus (Hegnauer, 1964; Harborne, 1977), and they could therefore deserve a greater attention. In effect, the variability in the flavonoid content of different cultivars has been proposed in the past as a method for the identification of azalea varieties (Van Sumere *et al.*, 1984), as well as for the classification of elm (Heimler *et al.*, 1990) and birch (Lahtinen *et al.*, 2006) species. Flavonoid fingerprints of carnation flowers have been likewise employed to recognize different cultivars, although, due to the inaccuracy of the used analytical techniques, it was possible to discriminate only a few genotypes (Ledemé and Jay, 1987). To our knowledge no attempts have been made so far, to measure the levels of diagnostic flavonoid markers in tissues other than flowers, with the aim of crossing and using these data to distinguish precisely a carnation cultivar.

In this paper we describe the isolation and identification of three flavonoid glycosides which are recurrent in carnation plants and the development of an HPLC-UV method to determine them quantitatively and simultaneously. Using this quantitative method, the contents of these compounds in different parts of nine carnation cultivars were determined and compared providing a useful system for the characterization of these and further varieties.

0939–5075/2008/0300–0161 $ 06.00

Materials and Methods

General experimental procedure

UV-vis spectra were recorded on a Hitachi 150–20 spectrophotometer. Low and high resolution FAB mass spectra (glycerol matrix) were measured on a Prospec Fisons mass spectrometer. ^{1}H NMR spectra were recorded on a Varian Unity Inova spectrometer at 500.13 MHz. Chemical shifts were referred to the residual solvent signal (CD_3OD, δ_H 3.31).

Sample preparation

Carnation cultivars America and Esperia were obtained by courtesy of Hybrida (Sanremo, Imperia, Italy); Tiepolo was obtained by courtesy of Santamaria (Sanremo, Imperia, Italy); Harem, Miledy, Roland, Rosa Antico, Raggio di Sole, and Tempest were purchased from Florivivaistica Baratta (S. Stefano, Imperia, Italy). These cultivars were chosen in order to examine different flower colours and different categories (standard and spray carnations). All plants were grown at the CRA-Istituto Sperimentale per la Floricoltura (Sanremo, Imperia, Italy) during 2006. Rooted cuttings were planted in steam-sterilized soil and grown in concrete benches under plastic semi-transparent shelter, following standard cultivation techniques.

For quantitative analysis six leafy plants from each cultivar were harvested, washed and divided into five different parts: flowers, stems, leaves, young sprouts, and roots. 10 g of fresh tissue from each part were added to 25 mL of an EtOH/MeOH (1:1, v/v) mixture, homogenized and refluxed for 2 h at 80 °C. The suspension was paper-filtered and the solvent evaporated under reduced pressure. The dried extract was re-dissolved in a MeOH/H_2O (1:1, v/v) mixture and paper-filtered to give 10 mL of a clear yellow-brown solution.

HPLC analysis

Carnation extracts were analyzed on a Perkin Elmer chromatographic system that included a binary model lc 200 pump, a rheodyne injector with a 20 μL sample loop and a model 785A UV-vis detector. A PE Nelson NCI900 interface and Turbochrom (version 1.2) computer software were used.

A Supelco Discovery C_{18} column (250 × 4.6 mm i.d.; 5 μm) equipped with a Supelco pre-column (30 × 4.6 mm i.d.) filled with the same stationary phase was employed.

The mobile phase consisted of: (A) 10% MeOH/90% H_3PO_4 (0.2 mM), (B) 90% MeOH/10% H_3PO_4 (0.2 mM). Separation was obtained using an isocratic (62.5% A/37.5% B) elution profile. Flow rate was 0.7 mL/min, UV-vis detection was set at 350 nm, a wavelength appropriate to the UV absorption of flavonoids (Mabry *et al.*, 1970).

Quantitative analysis

Flavonoids **1**–**3** were quantified using the calibration standard method. The calibration curve was established at 5 data points covering the concentration range of each analyte according to the levels expected in plant samples.

A stock solution of each flavonoid was prepared by dissolving the appropriate amount of the substance in methanol at the concentration of 10.0 mg/mL. Calibration solutions of 10.0, 5.0, 2.5, 1.0, and 0.5 μg/mL were prepared by diluting the stock solution with methanol. Triplicate analyses were performed for each concentration. Slope, intercept and other statistics of the calibration line were calculated on a linear regression model using SPSS 13.0 statistics software. Correlation coefficients (R^2) varied between 0.993 and 0.999. Samples prepared as described were injected in the HPLC system after a 5× or 10× dilution. Final quantification results are expressed as mg of compound per g of starting tissue, calculated according to the following formula:

$$\frac{\text{mg}_{\text{flavonoid}}}{\text{g}_{\text{tissue}}} = \frac{\left[\left(\frac{\text{peak area} - \text{intercept}}{\text{slope}}\right) \cdot \frac{\text{MW} \cdot \text{dilution folds}}{\text{injection volume } (\mu\text{L})} \cdot \frac{\text{final extract}}{\text{volume (mL)}} \cdot 100\right]}{\text{extract tissue (g)}},$$

where MW is the molecular weight of the compound under measurement.

*Isolation of compounds **1**–**3***

100 g of fresh carnation stems were extracted as described in sample preparation. Concentrated samples were roughly purified by gel-filtration on a 350 × 28 mm i.d. column filled with Sephadex G-25–80 (Sigma), using distilled water adjusted at

pH 3.5 with HCOOH as mobile phase. Five different fractions were collected; the presence of flavonoids in each fraction was evaluated by TLC using silica TLC plates eluted with a mobile phase composed of: MeOH/hexane/$CH_3COOCH_2CH_3$/H_2O/CH_3COOH (54:20:13:8:5, v/v/v/v/); flavonoids showed an R_f value of about 0.50 and appeared as violet spots under UV light (355 nm). Bands containing flavonoids were eluted and rechromatographed on a column (200 × 28 mm i. d.) filled with silica gel 100 C_{18} reversed phase. Separation was carried out at controlled eluent flux by means of a Gilson model peristaltic pump, using a linear solvent gradient, where A was H_2O (5% HCOOH)/MeOH (85:15, v/v) and B was H_2O (5% HCOOH)/MeOH (20:80, v/v). Compounds **1**, **2** and **3** were collected in this order.

Apigenin 6,8-di-C-β-D-glucopyranoside (**1**): UV (MeOH): λ_{max} = 329.0, 298.0 (sh), 273.0 nm. – HRFABMS (negative ion): found *m/z* = 593.1531 $[M-H]^-$; calcd. for $C_{27}H_{29}O_{15}$ *m/z* = 593.1506. – ^{1}H NMR: δ = 4.98 (1H, d, *J* = 7.5 Hz, H-1 6-*C*-Glc), 5.00 (1H, d, *J* = 7.5 Hz, H-1 8-*C*-Glc), 6.60 (1H, s, H-3), 6.94 (1H, d, *J* = 8.5 Hz, H-3′and H-5′), 7.98 (1H, d, *J* = 8.5 Hz, H-2′and H-6).

Kaempferol 3-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**2**): UV (MeOH): λ_{max} = 322.0, 302.0 (sh), 267.0 nm. – HRFABMS (negative ion): found *m/z* = 755.2076 $[M-H]^-$; calcd. for $C_{33}H_{39}O_{20}$ *m/z* = 755.2034. – ^{1}H NMR: δ = 4.50 (1H, brs, H-1 Rha), 4.75 (1H, brs, H-1 Glc), 5.38 (1H, d, *J* = 7.5 Hz, H-1 3-*O*-Glc), 6.23 (1H, s, H-6), 6.40 (1H, s, H-8), 6.92 (1H, d, *J* = 8.5 Hz, H-3′and H-5′), 8.06 (1H, d, *J* = 8.5 Hz, H-2′and H-6′).

Kaempferol 3-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**3**): UV (MeOH): λ_{max} = 348.0, 302.0 (sh), 266.0 nm. – HRFABMS (negative ion): found *m/z* = 593.1537 $[M-H]^-$; calcd. for $C_{27}H_{29}O_{15}$ *m/z* = 593.1506. – ^{1}H NMR δ = 4.50 (1H, brs, H-1 Rha), 5.18 (1H, d, *J* = 7.5 Hz, H-1 3-*O*-Glc), 6.23 (1H, s, H-6), 6.42 (1H, s, H-8), 6.92 (1H, d, *J* = 8.5 Hz, H-3′and H-5′), 8.06 (1H, d, *J* = 8.5 Hz, H-2′and H-6′).

Results and Discussion

Choice of the best extraction method

Different extraction procedures were tested to assess the one with the highest extraction efficiency: extraction in hot water, reflux extraction in a methanol/ethanol 1:1 (v/v) mixture, starting from dry tissues and starting from fresh tissues. The methanol/ethanol extraction from fresh tissues turned out to be the most efficient approach. Hot water extraction generally produced more sugar-rich extracts which generates difficulties during the analysis step; alcoholic extraction from dry tissues resulted in a yield loss of 15% with respect to starting from fresh tissues. The hydroalcoholic re-dissolution step allowed to get rid of most unpolar porphyrinic substances without loss of flavonoid content. The flavonoid HPLC profiles of the here considered cultivars (see Fig. 1) show that the respective crude extract obtained using the described method appears extremely clean at the UV detection. The reason is: working at 350 nm excludes from the detection of most of the natural organic substances constituting the extract, such as simple phenols and saponins, which could perturb the analysis. In addition, electronic delocalization through the flavonoid skeleton highly increases their extinction molar coefficient and consequently the detectability of this chemical class with respect to other components. Thus, no further purification steps are required before the analysis, so that quantification of flavonoids in carnation is feasible in less than three hours.

Identification of flavonoid constituents

Three main flavonoids (Fig. 2) were isolated by preparative HPLC from nine different carnation cultivars: America, Esperia, Harem, Miledy, Raggio di Sole, Roland, Rosa Antico, Tempest, and Tiepolo. Their structural elucidation was obtained on the basis of NMR and MS data in comparison with those reported in the literature. Compound **1** was identified as an apigenin-*C*-glycoside (vicenin-2) (Endale *et al.*, 2005). The remaining compounds appeared to be kaempferol-*O*-glycosides and were identified as: kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**2**) (Budzianowki, 1990) and kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (nicotiflorin, **3**) (Tomczyk *et al.*, 2002). Structures of vicenin-2 (**1**) and nicotiflorin (**3**) where confirmed by comparison with authentic samples, purchased from Apin Chemicals (Oxon, UK) and Extrasynthese (Lyon, France), respectively.

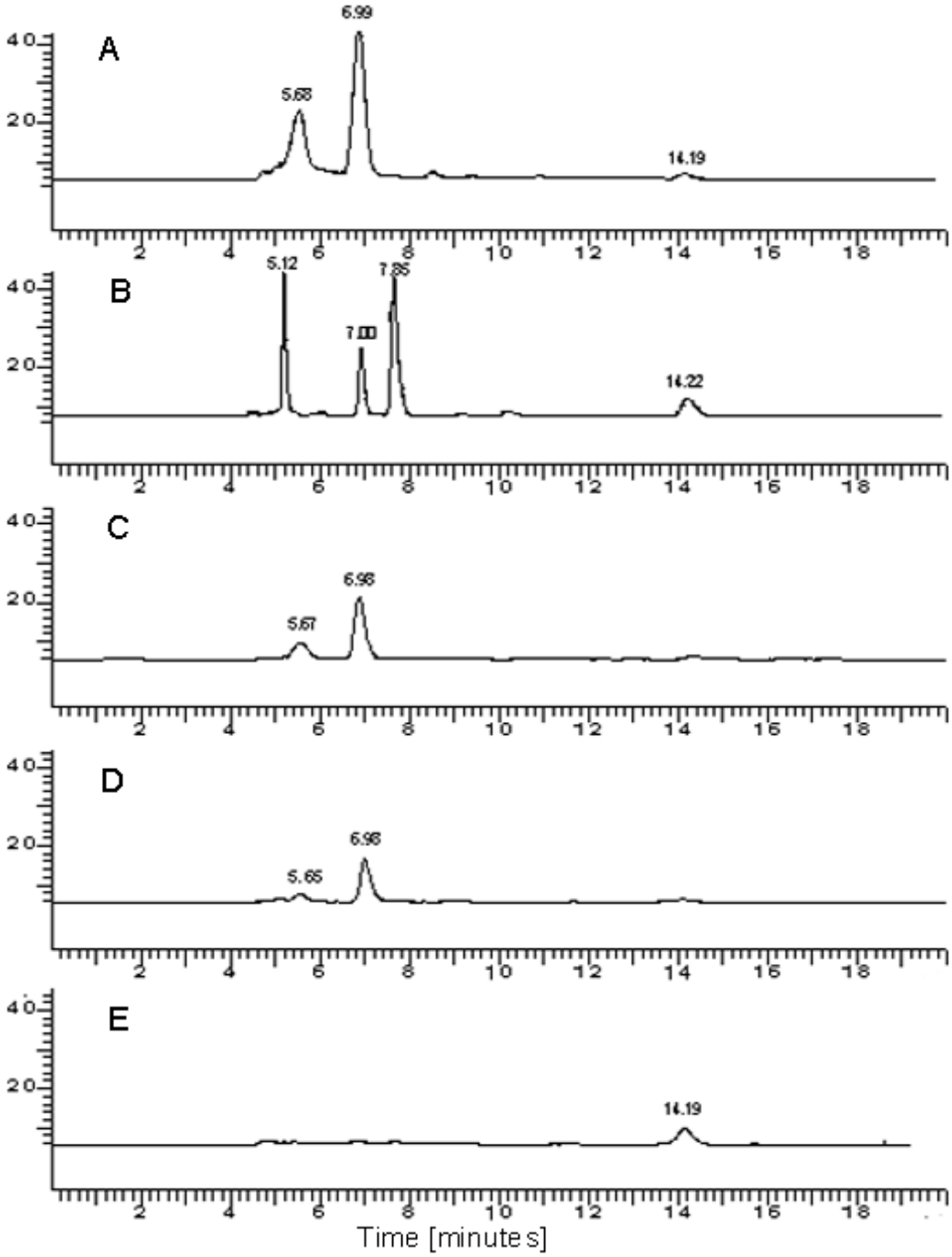

Fig. 1. HPLC profile of different parts of carnation cultivar America acquired using UV detection at 350 nm, showing: (A) young sprouts; (B) flowers; (C) leaves; (D) stems; and (E) roots. $R_t = 5.67 \pm 0.02$ (compound **1**); 6.99 ± 0.02 (compound **2**); 14.20 ± 0.05 (compound **3**).

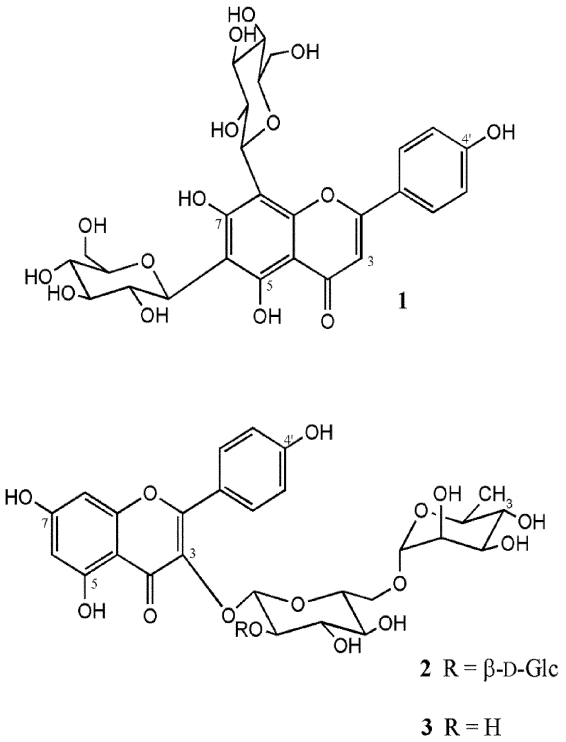

Fig. 2. Chemical structures of compounds **1**–**3** isolated from carnation.

Flavonoid distribution within different cultivars

The flavonoid differences within the cultivars can be resumed as follows: America, Esperia, Harem, and Miledy resulted to be the cultivars richest in flavonoids among those assayed, Raggio di Sole and Roland the poorest, while Rosa Antico, Tiepolo, and Tempest showed to contain intermediate amounts of flavonoids (Table I). The presence of the three flavonoids was attested in each cultivar, except for Raggio di Sole, in which compound **2** was not detected at all, and Roland and Tiepolo, in which compound **3** was missing. Major concentrations of flavonoids were found in flowers, leaves and sprouts, while stem and root tissues were generally poor in flavonoids.

As expected, many compounds different from those assayed, were detected in the flowers. At least two or three extra flavonoids, different from the previous ones, were detected in each cultivar. In Tiepolo, HPLC analysis of the flowers provided 7 extra flavonoid peaks. The presence of these compounds only in the flowers suggests a specific function related with this part of the plant, such as pigmentation and durability of flowers. Since these flavonoids appear to be specific for a cultivar and not ubiquitous for every carnation cultivar, they were considered useless for our purposes and were not taken into account in this article.

The observed differences in the contents of compounds **1**–**3** suggest that the described procedure could be applied for developing a reliable method to discriminate among different cultivars. The fact that we focused attention just on these three compounds makes the elaboration of data and their comparison easier. In fact, the specificity of every cultivar could be obtained by crossing the quantitative data regarding the same three compounds in five different parts of the plant, without taking into account the presence of peculiar compounds, which could not be considered as a general parameter. The graphical representation showed in Fig. 3 confirms this hypothesis. The flavonoid profile for each cultivar looks significantly different from all the others assayed. Even if we consider two cultivars of which the flavonoid profile looks similar, such as Esperia and Harem, at least a couple of differences, in this case flowers and roots contents of **3**, renders unmistakable the discrimination between them. Since compounds **1**–**3** turned out to be ubiquitous in the assayed cultivars, it is likely that at least two out of them are detectable in further carnation cultivars.

To our knowledge, this is the first study that reveals the presence of a number of identical flavonoids in different carnation varieties, and quantifies them. As observed in previous studies, flavonoids in carnation are constitutive, and their concentration in tissues is not significantly related with elicitation by a pathogen infection (Higuera and De Gòmez, 1996). In addition, measurements obtained with the procedure here described, repeated in different periods of the year, revealed that concentration fluctuations of the three compounds in a specific cultivar, are absent or negligible (±7%).

In conclusion, we have developed a simple method for the simultaneous detection of three specific flavonoids, constitutive of carnation species. The results achieved show that accumulation of these molecules in different parts of the plant is characteristic for the cultivar; so that this kind of analysis can be proposed as a method for distinguishing different carnation varieties.

Table I. Contents of compounds **1**–**3** in different parts of nine carnation cultivars.

Cultivar		Content [mg compound/g tissue][a]		
		1	**2**	**3**
America	Flowers	–	0.684 ± 0.036 L	0.381 ± 0.066 I
	Leaves	0.466 ± 0.014 M	1.191 ± 0.087 R	0.074 ± 0.012 D
	Sprouts	0.666 ± 0.160 N	1.589 ± 0.083 S	0.032 ± 0.022 ABC
	Stems	0.042 ± 0.005 AB	0.326 ± 0.067 G	0.004 ± 0.002 A
	Roots	–	–	0.141 ± 0.04 EF
Esperia	Flowers	–	1.598 ± 0.113 S	0.799 ± 0.008 L
	Leaves	0.289 ± 0.013 H	0.709 ± 0.056 N	0.032 ± 0.015 ABC
	Sprouts	0.235 ± 0.047 G	0.747 ± 0.038 M	0.017 ± 0.006 A
	Stems	0.032 ± 0.003 AB	0.086 ± 0.014 CDE	0.008 ± 0.001 A
	Roots	–	–	0.065 ± 0.004 BCD
Harem	Flowers	–	2.003 ± 0.164 T	0.125 ± 0.012 E
	Leaves	0.380 ± 0.060 I	0.933 ± 0.203 P	–
	Sprouts	0.185 ± 0.036 EFG	0.523 ± 0.046 I	–
	Stems	0.020 ± 0.002 AB	0.134 ± 0.009 E	–
	Roots	0.001 ± 0.001 A	0.002 ± 0.001 A	–
Miledy	Flowers	–	2.680 ± 0.211 U	0.240 ± 0.029 H
	Leaves	0.451 ± 0.086 LM	0.817 ± 0.0731 N	–
	Sprouts	0.401 ± 0.028 IL	0.885 ± 0.050 O	–
	Stems	0.022 ± 0.007 AB	0.061 ± 0.007B CD	0.008 ± 0.001 A
	Roots	–	–	0.062 ± 0.005 BCD
Raggio di Sole	Flowers	0.005 ± 0.002 A	–	0.802 ± 0.055 L
	Leaves	0.195 ± 0.040 FG	–	0.178 ± 0.10 G
	Sprouts	0.127 ± 0.002 CD	–	0.259 ± 0.027 H
	Stems	0.006 ± 0.001 A	–	0.087 ± 0.004 D
	Roots	–	–	0.070 ± 0.010 CD
Roland	Flowers	–	0.312 ± 0.022 G	–
	Leaves	0.149 ± 0.012 DEF	0.043 ± 0.004 ABCD	–
	Sprouts	0.074 ± 0.003 BC	0.015 ± 0.005 AB	–
	Stems	–	0.037 ± 0.003 ABC	–
	Roots	0.003 ± 0.001 A	0.008 ± 0.001 AB	–
Rosa Antico	Flowers	–	0.570 ± 0.040 IJ	0.008 ± 0.001 A
	Leaves	0.227 ± 0.016 G	0.750 ± 0.048 M	–
	Sprouts	0.198 ± 0.009 FG	1.023 ± 0.096 Q	–
	Stems	0.018 ± 0.004 AB	0.094 ± 0.008 DE	–
	Roots	0.004 ± 0.001 A	0.012 ± 0.001 AB	0.029 ± 0.006 AB
Tempest	Flowers	–	0.251 ± 0.036 F	0.029 ± 0.066 AB
	Leaves	0.192 ± 0.011 FG	0.404 ± 0.011 H	0.002 ± 0.001 A
	Sprouts	0.133 ± 0.007 DE	0.525 ± 0.064 I	0.004 ± 0.001 A
	Stems	0.011 ± 0.001 AB	0.027 ± 0.012 ABC	–
	Roots	–	–	0.165 ± 0.005 FG
Tiepolo	Flowers	–	1.161 ± 0.084 R	–
	Leaves	0.200 ± 0.008 FG	0.601 ± 0.078 JK	–
	Sprouts	0.115 ± 0.014 CD	0.625 ± 0.060 K	–
	Stems	0.012 ± 0.002 A	1.192 ± 0.088 R	–
	Roots	–	–	–

[a] Mean value ± SD (n = 3). Values with the same letter in the same column are not statistically different for P = 0.05 according to the Duncan test.

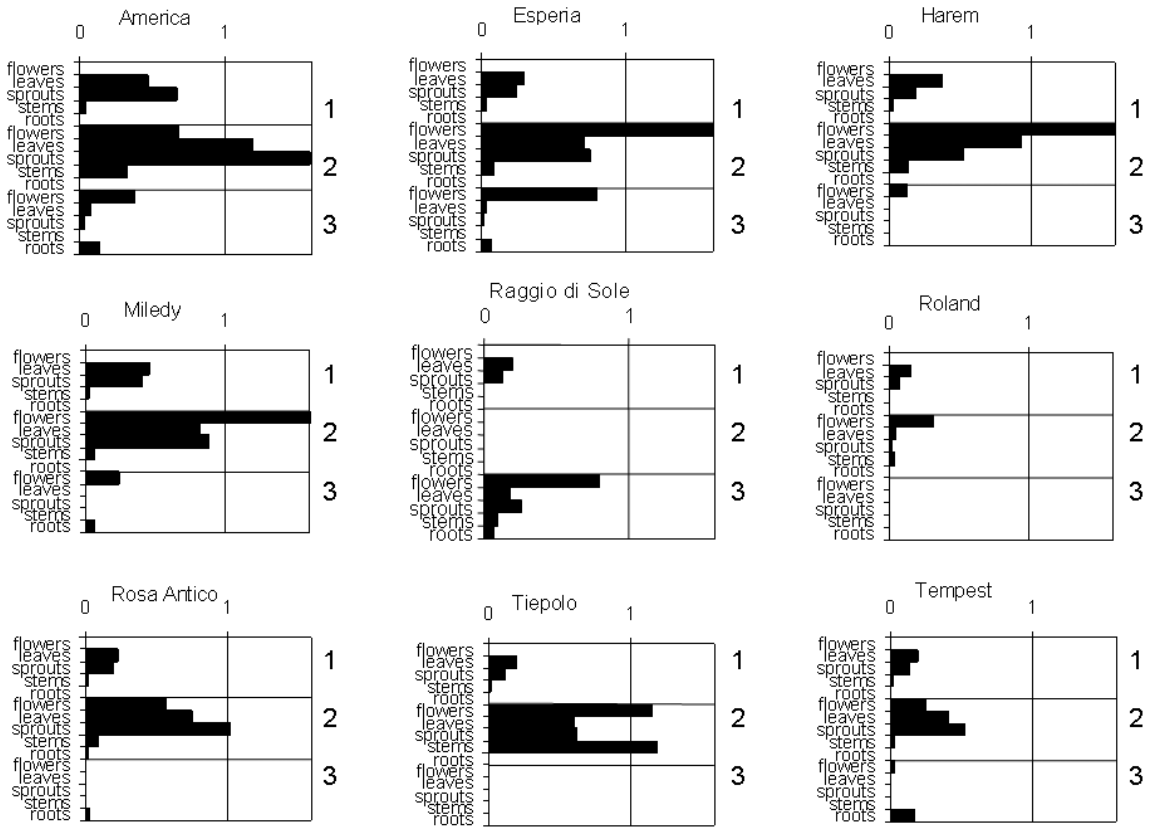

Fig. 3. Graphical representation of analysis results. For each cultivar, the content of compounds **1**–**3** measured in each kind of tissue is denoted by a horizontal bar. Values on *x*-axes go from 0 to 1.6 and are expressed in milligrams of flavonoid per gram fresh carnation tissue.

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