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ScienceDirect
Food Chemistry 107 (2008) 1668–1673
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Flavonol glucosides in *Allium* species: A comparative study by means of HPLC–DAD–ESI-MS–MS

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Received 9 January 2007; received in revised form 4 September 2007; accepted 15 September 2007

Abstract

Cultivars and consumption typologies of some *Allium* species can significantly vary from a chemical point of view and even small differences can be important for their characterization and differentiation. Bulbs of three varieties and four consumption typologies of onion (*Allium cepa* L.) and two varieties of shallot (*Allium ascalonicum* Hort.) were subjected to HPLC–DAD–ESI-MS–MS analysis. Seven flavonol glucosides were identified in all the samples, two of which, quercetin 3,4′-diglucoside and quercetin 4′-glucoside, represent about the 90% of the overall contents. Cultivars and consumption typologies of the *Allium* species under study show significant differences in flavonol contents, from the very low quantity of antioxidant compounds in white onion, about 7 mg/kg against 600–700 mg/kg that were found in red and gold varieties, to the enormous content of flavonols that are present in onions of prompt consumption, where quercetin 4′-glucoside exceeds 1 g/kg and quercetin 3-glucoside is present in a ratio higher then 10:1 with respect to its value in the other onion typologies. Shallots are very rich in the two major flavonols.

Keywords: *Allium cepa* L.; *Allium ascalonicum* Hort.; *Flavonol* glucosides; HPLC–DAD–ESI-MS–MS

1. Introduction

Bulbs from plants of the *Allium* species are extensively employed as food flavoring and appreciated over the years not just for the characteristic taste and smell but also as significant sources of beneficial compounds such as allicin and their derivatives (Block, 1992; Higuchi, Tateshita, & Nishimura, 2003; Xiao & Parkin, 2002) or flavonoid glycosides (Crozier, Lean, McDonald, & Black, 1997; Fattorusso, Iorizzi, Lanzotti, & Taglialatela-Scafati, 2002; Hertog, Hollman, & Venema, 1992). In particular, *Allium* species are rich source of flavonols, among which quercetin 3,4′-*O*-diglucoside and 4′-*O*-glucoside are the major components. Quercetin is known for its antioxidant and free radical scavenging power and its capability in protecting against cardiovascular disease (Clifton, 2004; Gil, Ferreres, & Tomàs-Barberàn, 1999; Ibarra et al., 2002).

Onions (*Allium cepa* L.) and shallots (*Allium ascalonicum* Hort.) occupy a great part of the food market in which they are introduced as a number of cultivars and consumption typologies. Red, white and gold onions represent the most known varieties of this species, but growers distinguish also between freshly-consumer onions and onions for industrial transformation, on the basis of sowing time and technique, harvesting time, bulb size. For instance, freshly-consumer onions, destined for a long storage, have withered external tunics, for a medium-term storage they show still unripe bulbs and finally for a prompt consumption growers offer onions that are harvested before the beginning of the bulb growing, whereas onions destined

Abbreviations: ESI-MS, electron spray mass spectrometry; PCO, prompt consumption onion; MSO, medium-term storage onion; RO, red onion; GO, gold onion; WO, white onion; LOD, limit of detection; LSO, long-term storage onion; ITO, industrial transformation onion; FS, French shallot; IS, Italian shallot; DMF, dimethylformamide.

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doi:10.1016/j.foodchem.2007.09.053

for industrial transformations have usually small dimensions obtained by the use of special techniques: they are produced in day-light conditions, inducing the rapid growing of bulbs which remain of reduced size.

Cultivars and typologies of *Allium* species can vary also from a chemical point of view and even small differences can be significant in their characterization and differentiation. A large part of the literature in these recent years is devoted to a general evaluation of the antioxidant activity in *Allium* species (Fossen, Pedersen, & Andersen, 1998; Nuutila, Puupponen-Pimiä, Aarni, & Oksman-Caldentey, 2003; Stratil, Klejdus, & Kubáň, 2006; Thompson et al., 2005; Yin & Cheng, 1998) but in few papers a comparison of the content of some beneficial compounds, such as flavonoids, has been conducted among different cultivars, typologies and species, whereas the anti-aging and defensive activities of these antioxidant compounds are tightly related to an adequate intake in food (Mullen, Boitier, Stewart, & Crozier, 2004).

As part of our ongoing investigation of the chemistry of *Allium* species (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2005) we have now analyzed the flavonol composition of the edible portion of six different varieties and consumption typologies of onion bulbs and two varieties of shallot bulbs, employing the high-performance liquid chromatography–diode array detector (HPLC–DAD) coupled with electron spray mass spectrometry (ESI-MS–MS), a fast and accurate technique that allows quantitative and qualitative analyses of these compounds (Caristi et al., 2003; Caristi, Bellocco, Gargiulli, Toscano, & Leuzzi, 2006; Gattuso et al., 2006; Leuzzi, Caristi, Panzera, & Licandro, 2000). From the comparison of the content of seven flavonol glucosides, present in the *Allium* species under study, significant conclusions can be taken with regard to their characterization and consumption.

2. Materials and methods

2.1. Materials

Prompt consumption onions (PCO) and medium-term storage onions (MSO) were purchased in a local market from April to May 2006, whereas, red (RO), gold (GO) and white (WO) varieties of long-term storage (LSO) onion, onions for industrial transformation (ITO), French (FS) and Italian (IS) shallots were bought from October 2005 to September 2006. The investigation was carried out on 10 samples of each of the *Allium* species under study.

2.2. Reagents and standard solutions

HPLC-grade acetonitrile, methanol, and dimethylformamide (DMF) were supplied by Sigma–Aldrich. Quercetin 4′-*O*-glucoside (spiraeoside) was purchased from Extrasynthèse. The standard was dissolved in DMF (1 mg/mL) and stored at −4 °C. Calibration lines were obtained using DMF solutions of known concentration (10–200 mg/L).

2.3. Preparation of samples

The outer (dry for LSO, FS and IS) layer of onions and shallots was manually removed and the bulb was cut into small pieces. The pieces (10 g) of each type of sample were homogenized with a domestic electric blender after a first extraction in methanol (MeOH, 100 mL) at 4 °C overnight. Homogenized bulbs were stirred in MeOH (2 × 100 mL) for 30 min at room temperature, and the mixture was centrifuged. The three methanolic phases were combined after filtration on paper, reduced to a volume of 10 mL and stored at −4 °C till their use. A part from WO, that was used such as, methanolic extracts (1 mL) were diluted with DMF as follows: MSO, RO, GO, ITO, FS and IS 1:4, and PCO 1:8. The solutions were filtered through ISO-DISC P-34, 3 mm diameter PTFE membrane, 0.45 μm pore size prior to injection. Samples were made of 10 bulbs of each type of onion or shallot.

2.4. Liquid chromatography–mass spectrometry

LC–MS analyses of samples were carried out with a ThermoQuest Model LCQ-DUO equipped with a diode array detector and an ion trap mass spectrometer with electrospray ionization source (ESI), to perform a MS–MS analysis. Separation of flavonoids was performed on a Discovery C18 Supelco column (250 × 4.6 mm, particle size 5 μm) equipped with a guard column Discovery C18 Supelco 20 × 4.0 mm and placed in a column oven set at 30 °C. Injection loop was 20 μL and the flow-rate was 1.0 mL/min. The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5–20% (0–15 min), 20–30% (15–20 min), 30–50% (20–30 min), 50–100% (30–35 min), 100% (35–40 min), 100–5% (40–50 min). UV spectra were recorded between 200 and 450 nm and simultaneous detection by diode array was performed at 350 nm. The detection limit (LOD) was 0.4 mg/kg.

Operating parameters of mass spectrometer were: capillary temperature 250 °C, spray needle voltage set at 4.50 kV, ES capillary voltage +3 and −47 V for positive and negative polarity, respectively. Nitrogen was used as sheath gas with a flow of 50 arbitrary units (arb). Mass analysis was carried out in full-scan mode from 80 to 900 amu, both in positive and negative mode. The negative MS–MS spectra were obtained by applying an optimum of collision energy for different components.

The detection limit Each sample was tested three times and gave superimposable chromatograms.

3. Results and discussion

Bulbs of red (RO), gold (GO) and white (WO) onion (*A. cepa* L.), that represent the most common varieties of long-term storage onions (LSO) on the market, bulbs of three consumption typologies, the medium-term storage onion (MSO), the prompt consumption onion (PCO) and

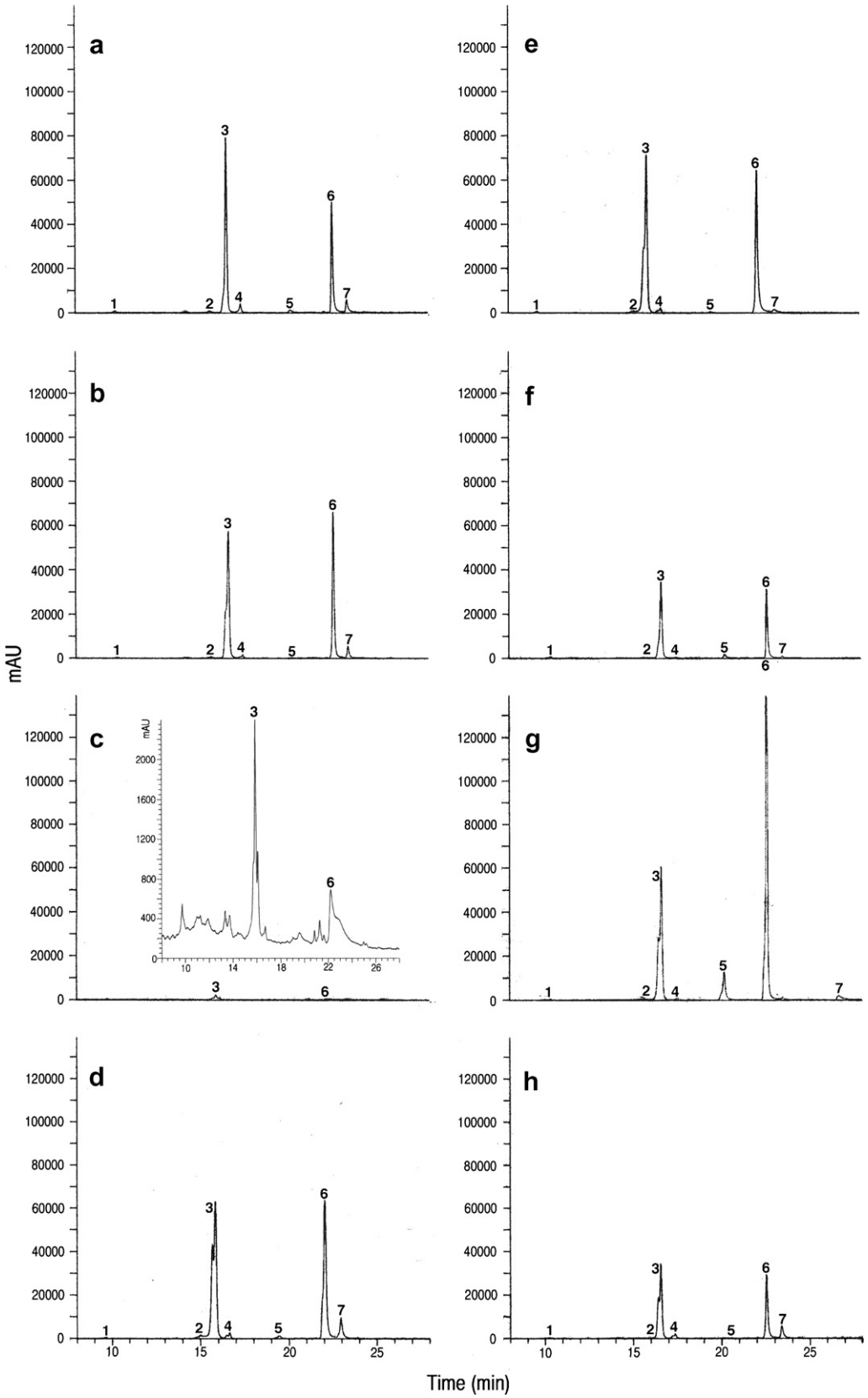

Fig. 1. Chromatograms at 350 nm of RO (a), GO (b), WO (c), FS (d), IS (e), MSO (f), PCO (g), ITO (h). Components **1**–**7** were identified as: quercetin 3,7,4′-triglucoside **1**; quercetin 7,4′-diglucoside **2**; quercetin 3,4′-diglucoside **3**; isorhamnetin 3,4′-diglucoside **4**; quercetin 3-glucoside **5**; quercetin 4′-glucoside **6**; isorhamnetin 4′-glucoside **7**.

the industrial transformation onion (ITO), bulbs of French (FS) and Italian (IS) shallot (*A. ascalonicum* Hort.), were extracted with MeOH and the extracts of each of the varieties and typologies were subjected to HPLC–DAD–ESI-MS–MS analysis that allowed the simultaneous acquisition of DAD chromatograms at 350 nm, UV, ESI-MS and ESI-MS–MS spectra. Following an already adopted methodology (Bonaccorsi et al., 2005), seven flavonol glucosides were identified in all the samples, two of which, quercetin 3,4′-diglucoside and quercetin 4′-glucoside, represent about the 90% of the overall contents, as shown in Fig. 1, were the typical chromatograms of each of the varieties and consumption typologies of the *Allium* species under study are reported. Numbers **1**–**7**, in the chromatograms of Fig. 1, identify the seven flavonol components, the names of which are reported in the legend. All the samples were diluted before injection, a part from the white onion samples that were analyzed such as, since they were found poor in flavonol glucosides, and the PCO samples that needed further dilution for obtaining results comparable with those of the other samples. In the window of Fig. 1c a rescaled chromatogram of the white onion is shown.

Noteworthy, in most of the chromatograms shown in Fig. 1 the peak related to component **3** splits into two parts that exhibited the same UV, ESI-MS and ESI-MS–MS spectra. ESI-MS and ESI-MS–MS analyses showed, in both cases, a fragmentation pattern in line with their diglycoside nature, and characteristic of quercetin aglycone (Fabre, Rustan, de Hoffman, & Quetin-Leclercq, 2001; Justensen, 2000). The UV absorption peaks were, in both cases, centered at 254 nm (band II) with a shoulder at 264 nm and at 344 nm (band I). Their superimposition excludes the possibility of two constitutional isomers, since different positions of the glucose residues would certainly produce differences in the hypsochromic shift of band I (Mabry, Markham, & Thomas, 1970). We cannot exclude the possibility of two conformational isomers of the same molecule, that, for some reasons, present a barrier of interconversion higher than 30 °C (the column temperature). This possibility is under study and anyway allows us to consider both peaks as part of a unique component, the quercetin 3,4′-diglucoside.

Fig. 2 shows the negative ESI-MS–MS spectra of quercetin 3,4′-diglucoside and quercetin 4′-glucoside (Components **3** and **6** in Fig. 1, respectively) that have been identified as the two major flavonols in all the varieties and typologies of *Allium* species under study. Negative ESI-MS–MS analysis was carried out on the pseudomolecular ion at *m/z* 625 $[M-H]^-$ and showed a fragmentation pattern in line with the diglucosyl nature of component **3** (Fig. 2a), whereas the same analysis conducted on ion at *m/z* 301 $[M-H-162-162]^-$ provided fragments that are typical of quercetin aglycone (Fig. 2c) (Fabre et al., 2001; Ding et al., 2006). Component **6** exhibited the negative ESI-MS–MS spectrum shown in Fig. 2b, carried out on the ion at *m/z* 463 $[M-H]^-$, where the fragmentation pattern is consistent with the loss of a glycosyl moiety and

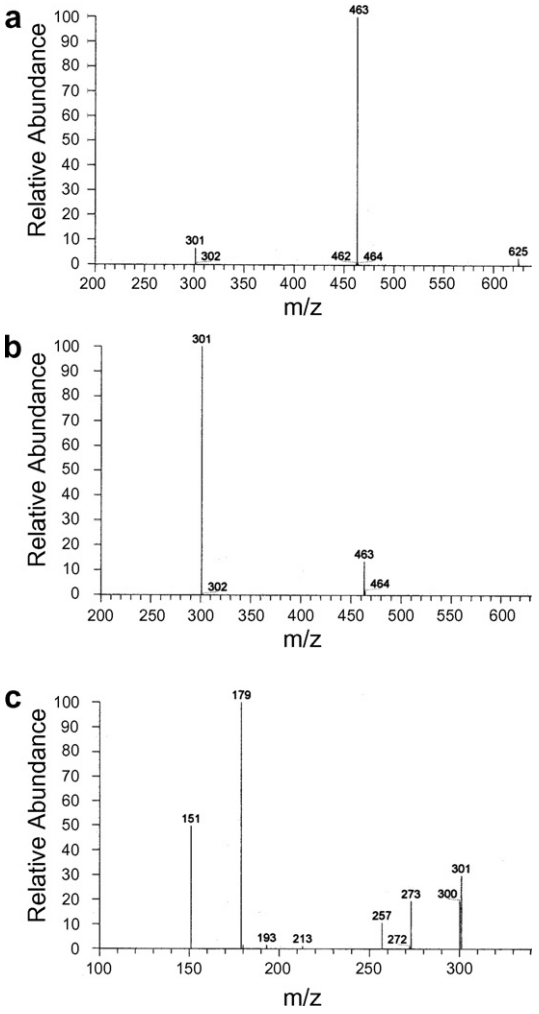

Fig. 2. Negative ESI-MS–MS spectra of: quercetin 3,4′-diglucoside on ion at *m/z* 625 $[M-H]^-$ (a) ; quercetin 4′-glucoside on ion at *m/z* 463 $[M-H]^-$ (b); quercetin aglycon ion *m/z* 301 $[M-H-162-162]^-$ (c).

therefore with the monoglucosylated nature of this component.

The content of flavonols, in the samples under study, is reported in Table 1 and Table 2 and is expressed as quercetin 4′-glucoside. Table 1 shows the range of values of these antioxidant components in the most common varieties of long-term storage onion (LSO) and shallot bulbs. Amazingly, values of the white onion are not comparable with those of the other onion varieties, as easily deducible also from Fig. 1c. In WO the content of the two major flavonols, quercetin 3,4′-diglucoside and quercetin 4′-glucoside, is about 7 mg/kg against 600–700 mg/kg that were found in RO and GO varieties. Shallots are the richest *Allium* species in the two major flavonols, quercetin 3,4′-diglucoside being nearly doubled with respect to its content in the onion varieties. Furthermore, in the French shallot the content of isorhamnetin 4′-glucoside (Component **7** in Fig. 1d) ranges between 57.5 and 69.5 mg/kg against about the 40 mg/kg in red and gold onions and just the 22 mg/kg in Italian shallot. Gold onion shows a very low content of the minor flavonol glucosides except for isorhamnetin

Table 1
Range values of flavonol glucosides (mg/kg fresh weight) in long-term storage onions (LTO) and shallots

	RO[a]	GO[b]	WO[c]	FS[d]	IS[e]
Quercetin 3,7,4′-triglucoside	4.1–6.8	1.8–2.9	<LOD	5.2–6.8	5.6–6.2
Quercetin 7,4′-diglucoside	5.7–7.9	3.1–4.1	<LOD	5.5–7.5	0.8–2.1
Quercetin 3,4′-diglucoside	321–368	288–323	4.0–5.2	531–605	516–572
Isorhamnetin 3,4′-diglucoside	20.4–23.5	6.3–8.6	<LOD	16.5–23.5	17.5–21.3
Quercetin 3-glucoside	18.5–22.6	0.8–1.2	<LOD	13.6–15.0	6.0–10.4
Quercetin 4′-glucoside	221–240	296–312	2.0–2.6	478–509	393–452
Isorhamnetin 4′-glucoside	39.8–45.1	33.2–36.8	<LOD	57.5–69.5	18.5–23.7

[a] RO = red onions.
[b] GO = gold onions.
[c] WO = white onions.
[d] FS = French shallot.
[e] IS = Italian shallot.

Table 2
Range values of flavonol glucosides (mg/kg fresh weight) in consumption typologies of red onions

	LSO[a]	MSO[b]	PCO[c]	ITO[d]
Quercetin 3,7,4′-triglucoside	4.1–6.8	2.1–2.9	3.2–3.9	1.5–2.4
Quercetin 7,4′-diglucoside	5.7–7.9	1.4–1.7	6.0–7.5	1.2–1.9
Quercetin 3,4′-diglucoside	321–368	181–205	580–612	179–193
Isorhamnetin 3,4′-diglucoside	20.4–23.5	1.8–2.5	3.9–5.1	9.1–11.3
Quercetin 3-glucoside	18.5–22.6	13.5–18.2	171–195	1.2–1.8
Quercetin 4′-glucoside	221–240	168–187	1318–1420	165–182
Isorhamnetin 4′-glucoside	39.8–45.1	6.2–7.5	16.3–18.4	40.1–44.5

[a] LSO = long-term storage onions.
[b] MSO = medium-term storage onions.
[c] PCO = prompt consumption onions.
[d] ITO = industrial transformation onions.

4′-glucoside. In particular, quercetin 3-glucoside ranges between 0.8 and 1.2 mg/kg against the 10–20 mg/kg of the other *Allium* species.

In Table 2 the range values of flavonol content in some consumption typologies of red onion are reported. For a significant comparison of these values we have chosen to reintroduce into Table 2 the values of the red onion variety, representing the flavonol content of LSO. The four consumption typologies of red onion show significant differences in flavonol content, the PCO typology being by far the richest in quercetin 3,4′-diglucoside, quercetin 3-glucoside and quercetin 4′-glucoside. In particular, in PCO, this last component exceeds 1 g/kg and quercetin 3-glucoside (Component **5** in Fig. 1g) is present in a ratio higher then 10:1 with respect to its value in the other onion typologies. Although differences in the production, the onions for industrial transformations (ITO) do not show strong differences in flavonol content with respect to the onions of medium-term storage (MSO), a part from the contents of isorhamnetin 4′-glucoside (Component **7** in Fig. 1h) and quercetin 3-glucoside (Component **5** in Fig. 1h) that are higher in ITO.

The histogram illustrates the total content in flavonols of the *Allium* species under study (Fig. 3). Values were obtained by the addition of mean values of the seven detected flavonol components for each species. Cultivars and consumption typologies of onion revel significant differences in flavonol contents: from the very low quantity

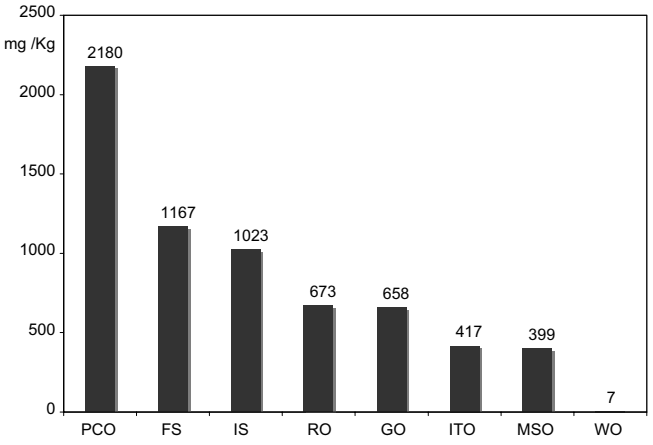

Fig. 3. Total content (mg/kg fresh weight) in flavonols (mean values) of *Allium* species under study.

of antioxidant compounds in WO, either in comparison with the other two varieties of RO and GO or in the general contest, to the exceeding content of flavonols that are present in PCO with respect to the other analyzed *Allium* species. PCO are the richest consumption typology of onion in flavonol components but they suffer of a minor diffusion on the food market with respect to LSO, that are the most common typologies of onions, and MSO. There are not significant differences between the two varieties of shallot, FS and IS, that in each case show high content of flavonols.

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