Phenolic compounds profile in wild edible greens from Portugal obtained by HPLC-DAD-ESI/MS

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ABSTRACT

Fruits and vegetables are good sources of a large number of antioxidant compounds; moreover, in some Mediterranean areas traditional wild greens are responsible for a significant percentage of total dietary antioxidant intakes. *Asparagus acutifolius* L. (wild asparagus), *Bryonia dioica* Jacq. (white bryony) and *Tamus communis* L. (black bryony) are important examples of those edible wild greens widely consumed. This study aimed to determine the phenolic profile and composition of edible vernal early shoots in spring of those species. The analysis was performed by high performance liquid chromatography coupled to photodiode array detector and mass spectrometry. Wild asparagus and black bryony revealed glycosides of flavonols as main phenolic compounds, while white bryony showed *C*-glycoslated flavones. Black bryony was the wild green that presented the highest content in phenolic compounds (2197 mg/Kg). Among the eleven flavonols found in this sample, kaempferol glycosides were the main compounds (1759 mg/Kg). In the sample of wild asparagus, quercetin-3-*O*-rutinoside was the main flavonol found (263 mg/Kg). Five flavones and one flavonol were found in the white bryony sample, being apigenin-6-*C*-glucoside-7-*O*-glucoside the major compound (1552 mg/Kg).

Keywords: Wild edible greens; phenolic compounds; HPLC-DAD-ESI/MS.

1. Introduction

A number of naturally occurring compounds in vegetables and herbs have antioxidant properties and, due to their putative protective effects against free radical-related pathologies emerging from the epidemiological studies, natural antioxidants have recently become a major area of research (Trichopoulou, Naska, & Vasilopoulou, 2001). Crude extracts of plant materials rich in phenolic compounds are increasingly of interest in the food industry because they might retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Furthermore, the importance of the antioxidant constituents of plant materials in the maintenance of health is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects (Kähkönen et al., 1999; Loliger, 1991).

Flavonoids represent a large group of phenolic compounds with antioxidant activity that occur naturally in plants and have been linked to reduce the risk of major chronic diseases (Blokhina, Virolainen, & Fagerstedt, 2003; Liu, 2004). The antioxidant properties of these compounds were the first mechanism of action studied, in particular with regard to their protective effect against cardiovascular diseases. Furthermore, flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998), which are possibly involved in DNA damage and tumor promotion (Cerutti, 1985).

Asparagus acutifolius (wild asparagus), *Bryonia dioica* (white bryony) and *Tamus communis* (black bryony) are important examples of wild edible greens traditionally gathered and consumed in the Iberian Peninsula. We had previously reported the nutritional composition and antioxidant properties of the edible portion of the mentioned species: young shoots available during a short period, especially in the case

of both bryonies, because traditional knowledge warns against their toxicity and considers the young shoots the least toxic part (Martins, Barros, Carvalho, & Ferreira, 2011). Nevertheless, their phenolic composition has not been well studied. There are only reports on samples of wild asparagus from Sicily (Salvatore et al., 2005), white bryony from Poland (Krauze-Baranowska & Cisowski, 1995) and black bryony from Turkey (Shaheen, Ali, Ali, Erdemoglu, & Sener, 2009). The present work aims to characterize the phenolic profiles in the edible parts of these species in samples from Portugal.

2. Materials and methods

2.1. Samples

Samples where gathered in 2009 early spring in Bragança, North-eastern Portugal according to local consumers' recommendations. The shoots of asparagus correspond to the young stems that sprout from the underground rhizome, more or less 20 cm long. White bryony shoots are the soft climbing young stems with tendrils and the first 6-8 immature leaves (not yet fully expanded). Black bryony shoots match with the annual, climbing and lengthwise striated young stems of about 20 cm long, with primordia of leaves and spikes (immature floral buds) at the tip.

Morphological key characters described in the book on "Flora Iberica" (Castroviejo, 2005) were used for white bryony identification, and from the book on "Nova Flora de Portugal" (Franco & Afonso, 1994) for asparagus and black bryony determination. Voucher specimens were deposited in the herbarium of the Escola Superior Agrária de Bragança (BRESA). The samples were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and kept in the best conditions for subsequent use.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, France). All the other reagents were of analytical grade purity and were supplied by Lab-Scan (Lisbon, Portugal). All of the phenolic standards were from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Phenolic compounds identification and quantification

Sample preparation. Each sample (1 g) was extracted with 30 ml of methanol:water 80:20 (v:v) for 1h. The extract was filtered through Whatman nº 4 paper. The residue was then re-extracted twice with additional 30 ml portions of methanol:water 80:20 (v:v). The combined extracts were evaporated at 35ºC to remove methanol. The aqueous phase was lyophilized and re-dissolved in 20% aqueous methanol at 20 mg/ml and filtered through a 0.22-µm disposable LC filter disk for HPLC analysis.

HPLC-DAD-ESI/MS analyses. The phenolic extracts were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2, 3 µm (4.6 mm × 150 mm) column thermostatted at 35°C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% A to 15% B over 5 min, 15–25% C in B over 5 min, 25–35% C in B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 ml/min. Double online detection was carried out in the DAD using 280 nm and

370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an Agilent Technologies MSD Trap XCT detector (Santa Clara, California, USA) equipped with an ESI source and an ion trap mass analyzer, which were controlled by the Chemstation software. Drying gas was nitrogen at flow rate of 80 L/min at 350 ºC. The source voltage was -3500 V, the capillary voltage was -136 V, and skimmer voltage was -40 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500. The MS detector was programmed to perform a series of two consecutive scans: a full scan and an MS–MS scan of the most abundant ion in the first scan, using normalized collision energy of 1 V.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with commercial standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known concentrations (2.5-100 μg/ml) of different standards compounds: apigenin-6-*C*-glucoside (y=246.05x–309.66; R^2=0.9994; LOD 0.19 μg/ml; LOQ 0.63 μg/ml), chlorogenic acid (y=600.27x–763.62; R^2=0.9998; LOD 0.20 μg/ml; LOQ 0.68 μg/ml), isorhamnetin (y=629.14x–2323.4; R^2=0.9967; LOD 0.17 μg/ml; LOQ 0.56 μg/ml), kaempferol 3-*O*-rutinoside (y=175.02x–43.877; R^2=0.9999; LOD 0.13 μg/ml; LOQ 0.43 μg/ml), luteolin-6-*C*-glucoside (y=365.93x–17.836; R^2=0.9997; LOD 0.19 μg/ml; LOQ 0.62 μg/ml), quercetin 3-*O*-glucoside (y=316.48x–2.9142; R^2=1; LOD 0.21 μg/ml; LOQ 0.71 μg/ml), quercetin 3-*O*-rutinoside (y=222.79x–243.11; R^2=0.9998; LOD 0.22 μg/ml; LOQ 0.75 μg/ml), and sinapic acid (y=153.31x–451.69; R^2=0.9919; LOD 0.15 μg/ml; LOQ 0.51 μg/ml). The results were expressed in mg per Kg of fresh weight (fw), as mean ± standard deviation of three independent analyses.

3. Results and discussion

Figure 1 shows the phenolic compounds profile of *Asparagus acutifolius*, *Bryonia dioica* and *Tamus communis* extracts. Data (retention time, λ_{max} in the visible region, molecular ion and main fragment ions observed in MS^2) obtained by HPLC-DAD-MS analysis are presented in **Table 1**.

Four phenolic compounds were detected and identified in the sample of *Asparagus acutifolius* (wild asparagus). Peak 1 showed a UV spectrum similar to sinapic acid with λ_{max} at 330 nm, but eluted at a different retention time. It presented a molecular ion $[M-H]^-$ at *m/z* 355 releasing a unique MS^2 fragment at *m/z* 223 corresponding to sinapic acid; this represents a loss of -132 mass units that could be attributed to a pentose moiety and thus the compound was tentatively identified as a sinapic acid pentoside, which was coherent with its earlier elution (greater polarity) with regard to sinapic acid (15.6 min). The major peak detected in wild asparagus was peak 2, identified as quercetin 3-*O*-rutinoside (rutin) according to its retention time and UV spectrum and mass characteristics compared with an authentic standard. Rutin has also been described as the major flavonoid in cultivated green *triguero* asparagus by Fuentes-Alventosa et al. (2008). Peaks 3 and 4 showed λ_{max} at 346 and 354 nm, and molecular ions at *m/z* 593 and 623, releasing each of them one MS^2 fragment ion at *m/z* 285 and 315, respectively, consistent with the loss of a rhamnosyl-glucoside moiety (-308 amu). UV and mass spectral characteristics allowed identifying them as kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-rutinoside, respectively. These two flavonols were also found in Spanish green *triguero* asparagus (Fuentes-Alventosa et al., 2008). However, no flavonol rutinosides had been reported in samples of *Asparagus acutifolius* from Sicily (Salvatore et al., 2005), where quercetin aglycone was described as the major compound

together with minor levels of isorhamnetin, kaempferol and myricetin and "variously glycosylated" quercetin derivatives, as well as caffeic and protocatechuic acids.

The main identified compounds in the sample of *Bryonia dioica* (white bryony) were *C*-glycosylated flavonoids, which showed a fragmentation pattern different from those of the *O*-glycosides. The identification of those compounds was confirmed by mass analysis and data available in literature for some species of the Cucurbitaceae family (Krauze-Baranowska & Cisowski, 1994; Krauze-Baranowska & Cisowski, 1995). Peak 1 showed λ_{max} at 348 and a molecular ion $[M-H]^-$ at *m/z* 609 that generated three fragment ions in the MS^2 analysis: *m/z* 447 ($[M-162]^-$, loss of a glucoside moiety), *m/z* 357 ($[M-162-90]^-$) and *m/z* 327 ($[M-162-120]^-$). Losses of 120 amu and 90 amu, corresponding to cross-ring cleavages in the sugar unit, were demonstrated as characteristic of *C*-flavonoids by Becchi & Fraisse (1989). The peak was identified as luteolin-6-*C*-glucoside-7-*O*-glucoside (lutonarin), based on its fragmentation pattern and data reported by Krauze-Baranowska & Cisowski (1994, 1995), which identified a variety of flavone *C*-glycosides using HPLC and NMR in different species of the Cucurbitaceae family such as *Bryonia alba*, *Bryonia dioica* and *Lagenaria siceraria*. Peak 2 was the major compound in white bryony and presented a molecular ion $[M-H]^-$ at *m/z* 593 that released three MS^2 fragment ions at *m/z* 431 ($[M-162]^-$), *m/z* 341 ($[M-162-90]^-$) and *m/z* 311 ($[M-162-120]^-$). This fragmentation pattern was identical to peak 1, which allowed identifying peak 2 as apigenin 6-*C*-glucoside-7-*O*-glucoside (saponarin), also described as the major compound in samples of white bryony from Poland (Krauze-Baranowska & Cisowski, 1995).

Peak 3 showed a molecular ion $[M-H]^-$ at *m/z* 447 giving place to three MS^2 fragment ions, a major one at *m/z* 357 $[M-90]^-$, and other two at *m/z* 327 $[M-120]^-$ and at *m/z* 429 $[M-18]^-$. The fragmentation pattern was characteristic of *C*-glycosylated flavones at C-

6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-6 according to the data reported by Ferreres, Silva, Andrade, Seabra, & Ferreira (2003) regarding fragmentation of flavone *C*-glycosides. Thus, the peak was identified as luteolin-6-*C*-glucoside, which was further confirmed by comparison with an authentic standard. Similar MS^2 behaviour was found for peak 4 ($[M-H]^-$ at *m/z* 431) that was identified as apigenin 6-*C*-glucoside, also confirmed by comparison with a standard.

Peak 5 ($[M-H]^-$ at *m/z* 577) showed a UV spectrum characteristic of a kaempferol derivative. The MS^2 analysis gave two ions at *m/z* 431 ($[M-146]^-$) and *m/z* 285 ($[M-146-146]^-$), corresponding to the successive losses of two rhamnosyl moieties, which allowed assigning it as a kaempferol dirhamnoside. The position of substitution of the sugar residues could not be definitely concluded, although the successive losses of the two moieties and the relative abundances of the two fragment ions (100% for *m/z* at 431, and 26% for *m/z* at 431) suggested that they were located at different positions on the aglycone. This assumption was also supported by the observations of Ferreres, Llorach, & Gil-Izquierdo (2004) that found a similar fragmentation pattern for flavonoid 3,7-diglycosides. Thus, peak 5 could be tentatively identified as kaempferol 3,7-di-*O*-rhamnoside.

Peak 6 presented a molecular ion $[M-H]^-$ at *m/z* 739 that yielded three MS^2 fragment ions, one major fragment at *m/z* 431 ($[M-308]^-$, loss of a rhamnosyl-hexoside moiety), and two minor ones at *m/z* 341 ($[M-308-90]^-$ and 311 $[M-308-120]^-$ characteristics of flavone *C*-glycosides. The similarity of this fragmentation pattern with that obtained for peak 4 (apigenin 6-*C*-glucoside) allowed tentatively identify peak 6 as apigenin 6-*C*-glucoside-*O*-rhamnosyl-hexoside. The high relative abundance of fragment ion at *m/z* 431 (100%) and the absence of intermediate ions after the cleavage of bonds 0 and 2 in the sugar residue, would indicate that the interglycosidic bond was 1→6 rather than

1→2 more labile and that should give rise to an additional fragment from the cleavage of the rhamnosyl-hexose linkage (Cuyckens, Rozenberg, Hoffmann, & Claeys, 2001).

Eleven peaks in the sample of *Tamus communis* (black bryony) were identified as phenolic compounds. The molecular ion ($[M-H]^-$ at *m/z* 355) and UV spectrum (λ_{max} at 328 nm) of peak 1 revealed a chlorogenic acid that was identified as 3-*O*-caffeoylquinic acid by comparison with a commercial standard. The rest of peaks corresponded to flavonol derivatives, with MS fragmentation patterns typical of flavonol *O*-glycosides. Peaks 2, 4, and 7-11 were assigned to kaempferol derivatives according to their UV spectra and the main fragment released in MS^2 (*m/z* at 285), whereas peaks 3, 4 and 6 were associated to quercetin derivatives.

Peak 8 showed a molecular ion $[M-H]^-$ at *m/z* 577 that generated two MS^2 fragment ions at *m/z* 431 ($[M-146]^-$, loss of a rhamnose moiety), and at *m/z* 285 $[M-146-146]^-$, loss of two rhamnose moieties). The successive loss of the two sugar moieties pointed out to a different location of each sugar in the aglycone. Furthermore, if they were linked as a disaccharide only one MS^2 fragment ion would have been released. A compound with similar chromatographic and spectral characteristics was found by Shaheen et al. (2009) in a sample of *Tamus communis* from Turkey and fully characterized by NMR as kaempferol 3,4'-di-*O*-rhamnoside. The second major peak (4) presented a molecular ion $[M-H]^-$ at *m/z* 593 that yielded MS^2 fragments at *m/z* 447 (loss of a rhamnose moiety), 431 (loss of an hexose moiety), and 285 (loss of rhamnose + hexose moieties). As for peak 8, the formation of fragments derived from the respective losses of each sugar moiety pointed to they were located at different positions on the aglycone, and thus the compound was tentatively identified as a kampferol *O*-rhamnoside-*O*-hexoside, although the precise position of location of each could not be concluded. Peak 7 presented a molecular ion $[M-H]^-$ at *m/z* 563. Its MS^2 gave two fragment ions, a major

fragment at *m/z* 431 ([M-132]$^-$, loss of a pentose moiety), a minor one at *m/z* 285 ([M-132-146]$^-$, loss of rhamnosyl-pentoside moiety), corresponding to kaempferol. Similarly, the successive losses of the two sugar moieties and the relative abundances of the two fragment ions suggested that they were located at different positions on the aglycone, although it was also not possible to conclude about the precise position. Thus, the compound was tentatively assigned as kaempferol *O*-rhamnoside-*O*-pentoside. Similar reasoning was applied for the characterization of the rest of peaks, whose tentative identities are indicated in **Table 1**.

Table 2 shows the results of the quantification of the phenolic compounds detected in the samples analysed. Black bryony was the wild green with the highest content of total flavonoids (2014 mg/Kg) and total phenolics (2197 mg/Kg), which is in agreement with a previous study of our research group that reported black bryony as having the highest antioxidant activity and the highest total phenolics and flavonoids, measured by spectrophotometric assays (Martins et al., 2011).

Despite a few previous studies have been published dealing with the phenolic composition of the herein analysed wild greens (Krauze-Baranowska & Cisowski, 1995; Salvatore et al., 2005; Shaheen et al., 2009), this is the first report on samples from Portugal. Furthermore, some new compounds were identified in these wild greens, such as sinapic acid pentoside and isorhammetin-3-*O*-rutinoside in wild asparagus, kaempferol-dirhamnoside and apigenin-*C*-hexoside-*O*-rhamnosyl–hexoside in white bryony, and all the eleven compounds identified in black bryony. The observed differences in the same species could be due to their different origin and therefore different climatic and soil conditions. Moreover, the plant materials used for analysis were also different; while in the present study young shoots without leaves full expanded were used according to traditional edible uses of these species, other authors

used aerial parts which include already expanded stems and leaves. Furthermore, differences could also be due to the extraction methodologies including solvent, time and temperature of extraction applied to each sample.

The obtained information should help consumers and food technologists to know about the interest of incorporating these traditional wild greens in contemporary diets, as potential sources of antioxidants.

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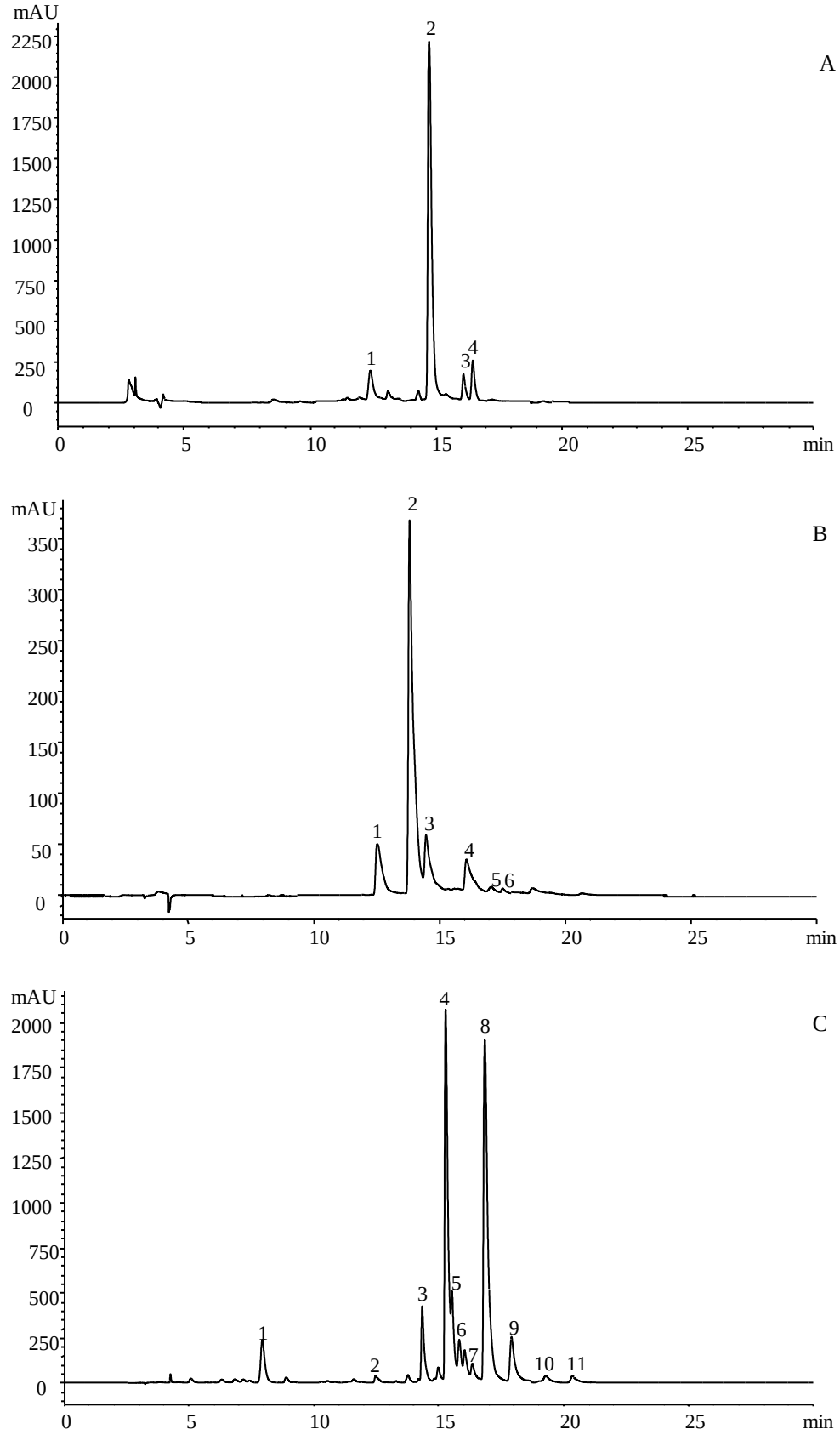

Figure 1. Individual chromatograms of the studied wild greens, A- *Asparagus acutifolius*; B- *Bryonia dioica*; C- *Tamus communis*, recorded at 370 nm.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, relative abundances of fragment ions, and tentative identification of phenolic compounds in the studied wild edible greens.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2(*m/z*), (%)	Tentative identification
			Asparagus acutifolius		
1	12.3	330	355	223(100)	Sinapic acid pentoside
2	14.7	356	609	301(100)	Quercetin 3-*O*-rutinoside
3	16.1	346	593	285(100)	Kaempferol 3-*O*-rutinoside
4	17.3	354	623	315(100),300(15)	Isorhamnetin 3-*O*-rutinoside
			Bryonia dioica		
1	12.4	348	609	447(100),357(6),327(24)	Luteolin-6-*C*-glucoside-7-*O*-glucoside
2	13.7	336	593	431(100),341(10),311(41)	Apigenin-6-*C*-glucoside-7-*O*-glucoside
3	14.3	350	447	429(34),357(100),327(80)	Luteolin-6-*C*-glucoside
4	16.0	336	431	413(13),341(48),311(100)	Apigenin-6-*C*-glucoside
5	17.4	346	577	431(100),285(26)	Kaempferol 3,7-di-*O*-rhamnoside
6	17.8	332	739	431(100),341(9),311(32)	Apigenin-*C*-hexoside-*O*-rhamnosyl–hexoside
			Tamus communis		
1	7.9	328	353	191(100)	Chlorogenic acid
2	12.5	346	609	447(100),285(11)	Kaempferol-*O*-dihexoside
3	14.3	355	609	463(28),447(100),301(12)	Quercetin-*O*-rhamnoside-hexoside
4	15.2	347	593	447(49),431(100),285(76)	Kaempferol -*O*-rhamnoside-hexoside
5	15.5	352	447	301(100)	Quercetin-*O*-rhamnoside
6	15.8	352	593	447(100),301(13)	Quercetin *O*-dirhamnoside
7	16.3	346	563	431(100),285(39)	Kaempferol-*O*- rhamnosyl-*O*-pentoside
8	16.8	344	577	431(100),285(25)	Kaempferol 3,4'-di-*O*-rhamnoside
9	17.9	346	447	285(100)	Kaempferol-*O*-hexoside
10	19.2	346	417	285(100)	Kaempferol-*O*-pentoside
11	20.3	345	431	285(100)	Kaempferol-*O*-rhamnoside

Table 2. Quantification of the phenolic compounds found in the studied wild edible greens.

Phenolic compound	Quantification (mg/Kg, fw)
Asparagus acutifolius	
Sinapic acid pentoside	53.2 ± 1.9
Quercetin 3-*O*-rutinoside	262.7 ± 12.9
Kaempferol 3-*O*-rutinoside	21.9 ± 4.2
Isorhamnetin 3-*O*-rutinoside	16.2 ± 2.1
Total flavonoids	300.9 ± 19.2
Total phenolic compounds	354.1 ± 21.1
Bryonia dioica	
Luteolin-6-*C*-glucoside-7-*O*-glucoside	155.9 ± 15.4
Apigenin-6-*C*-glucoside-7-*O*-glucoside	1551.7 ± 67.0
Luteolin-6-*C*-glucoside	279.0 ± 3.4
Apigenin-6-*C*-glucoside	318.4 ± 41.5
Kaempferol 3,7-di-*O*-rhamnoside	82.6 ± 3.6
Apigenin-*C*-hexoside-*O*-rhamnoside hexoside	24.7 ± 0.1
Total flavonoids	2412.2 ± 123.5
Tamus communis	
Chlorogenic acid	183.0 ± 3.2
Kaempferol dihexoside	16.8 ± 1.6
Quercetin rhamnoside hexoside	103.3 ± 7.2
Kaempferol rhamnoside hexoside	697.3 ± 13.2
Quercetin rhamnoside	90.9 ± 4.3
Quercetin dirhamnoside	60.8 ± 5.8
Kaempferol-*O*-rhamnosyl-*O*-pentoside	107.1 ± 12.6
Kaempferol 3-4'-di-*O*-rhamnoside	834.9 ± 18.1
Kaempferol hexoside	86.8 ± 2.0
Kaempferol pentoside	6.5 ± 0.6
Kaempferol rhamnoside	9.9 ± 0.3
Total flavonoids	2014.3 ± 60.4
Total phenolic compounds	2197.3 ± 63.6