Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 457 - 467

PHYTOCHEMICAL ANALYSIS OF *ALLIUM FISTULOSUM* L. AND *A. URSINUM* L

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The present study describes the investigation on chemical composition of *Allium fistulosum* L. and *A. ursinum* L. The identification and quantification of polyphenolic compounds was performed by HPLC-UV-MS method, allicin analysis through a LC-MS method, and the presence of five sterols was simultaneously assessed by HPLC-MS-MS. The pattern of phenol carboxylic acids shows the presence of *p*-coumaric and ferulic acids in both species. Isoquercitrin and quercitrin were found only in *A. fistulosum,* so they represent potential taxonomic markers that differentiate the plants. Quercetol and kaempferol were identified before and after hydrolysis in *A. fistulosum*, whereas kaempferol only after hydrolysis in *A. ursinum*. Allicin was identified in all extracts, the higher amounts in *A. ursinum*. β-sitosterol and campesterol were identified in both species, and stigmasterol only in *A. fistulosum*. The results indicate significant differences in chemical composition of *Allium fistulosum* and *A. ursinum*.

(Received December 14, 2012; Accepted March 4, 2013)

Keywords: Allium fistulosum; *Allium ursinum*; Allicin; Polyphenolic compounds; Phytosterols

1. Introduction

The *Allium* L. genus includes more than 400 species that are widespread around the world. The antibacterial and antifungal properties of *Allium* sp. were demonstrated for *A. sativum, A. porrum* [1], *A. cepa* [2], *A. ascalonicum* [3], *A. fistulosum* [4], *A. minutiflorum* [5], *A. neapolitanum* [6], *A. obliquum* [7], *A. senescens* ssp. *montanum* [8], and *A. ursinum* [9]. The ethnobotanical data from Romania mention 32 wild and cultivated species of *Allium* L. [10].

A. sativum (garlic) and *A. cepa* (onion) have a variety of pharmacological effects including chemopreventive activity and tumor cell growth inhibition [11,12]. The antioxidant activity of *Allium* species is due to a variety of sulphur-containing compounds and their precursors, but it is also related to other bioactive compounds: polyphenols, dietary fibers, microelements [12].

The major flavour component of garlic is a thiosulphinate called allicin, which is duly formed when the garlic tissue is damaged due to the hydrolysis product of S-allyl cysteine sulphoxide (alliin) which is specifically produced by the enzyme allinase. For the evaluation of the

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quality of garlic and garlic products, it is important to consider all the precursors and the biological active substances [7,13]. The hypocholesterolaemic activity of garlic has been attributed to diallyl disulphide, a decomposition product of allicin [14]. Antifungal activity is more effective than nystatin (allicin is thought to be the main active component by inhibition of lipid synthesis). In vitro antiviral activity was attributed to allicin and its derivatives, and alliin has antihepatotoxic activity in vitro and in vivo [7,14].

Polyphenols are bioactive substances widespread distributed in natural products, with a great variety of structures. Phenolic compounds have multiple biological properties very well characterised: antioxidant, antimutagenic, antibacterial, antiviral, anti-inflammatory, antithrombotic [15,16,17]. Medicinal plants rich in polyphenols can retard the oxidative degradation of lipids and improve the quality and nutritional value of food [18].

Phytosterols occur in a large segment of plant species; both yellow and green vegetables contain an appreciable quantum. They engage in competitive uptake of the dietary cholesterol in the entire intestinal passage, and have also demonstrated the capability to affect complete blockade in the uptake of cholesterol and also facilitate its subsequent excretion from the body [19]. The most common phytosterols in natural products are β-sitosterol, stigmasterol, and campesterol. Sterols can reduce the atherosclerotic risk and offer protection against cardiovascular diseases [20]. They decrease the risks of breast, prostate and colon cancer [21,22]. Furthermore, phytosterols have anti-inflammatory and immunomodulatory properties [23].

Allium ursinum L. (ramson) is a wild species found in Europe and Northern Asia forests.

Sulfur-containing compounds are responsible for its traditional use in terms of culinary and medicinal purposes. The main cysteine sulfoxides are alliin and isoalliin [24]. Several biological properties of *A. ursinum*, such as antioxidant effects [11], cytostatic [25], and antimicrobial [26] were reported.

A. fistulosum L. (Welsh onion) is one of the cultivated species of *Allium* from Romania. Welsh onion is a perennial species originated from Eastern Asia. Its leaves have nutritional value, and they can be fresh consumed all over the year, still green over the winter [4]. The medicinal properties, especially antifungal and antioxidant were determined, and they are due to sulphur-containing compounds, flavonoids, fatty acids [4,11,27].

To increase our understanding of the pharmacological and nutraceutical properties of *Allium* species, further comprehensive study of its nutrients, especially allicin, polyphenolic compounds and phytosterols, is required.

We employed a rapid, highly accurate and sensitive HPLC method assisted by MS detection for the simultanous determination of polyphenols in plants [28,29,30], and a newly developed LC-CIS-MS/MS method for the quantitative analysis of allicin in natural products [31].

Although numerous studies have been carried out for qualitative and quantitative determination of sterols in natural products, rather limited investigations have been conducted on phytosterols from some *Allium* species [32,33,34].

This is the first report of a simple, accurate and rapid HPLC-MS-MS method for identification and quantification of sterols from *A. fistulosum* and *A. ursinum*. The method is based on a previous published method [35], with some modification: the change of chromatographic column and mobile phase. Because the chemical composition of *A. fistulosum* and *A. ursinum* from Romania has been insufficiently studied, the aim of this work was to bring new data on sulphur-containing compounds, polyphenols and sterols of these two *Allium* species.

2. Experimental

General Apparatus and Chromatographic Conditions: an Agilent 1100 HPLC Series system was used (Agilent Technologies, Darmstadt, Germany), coupled with an Agilent Ion Trap SL mass spectrometer equipped with an electrospray or APCI ion source.

2.1. Chromatographic Conditions for the analysis of polyphenolic compounds

The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump, column thermostat, autosampler and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18

100 x 3.0 mm i.d., 3.5 µm particle); the work temperature was 48 °C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA.

The mobile phase was a binary gradient prepared from methanol and solution of 0.1% acetic acid (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; isocratic elution followed for the next 3 minutes with 42% methanol. The flow rate was 1 mL min-1 and the injection volume was 5 µL.

The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analysed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral mach. The UV trace was used for quantification of identified compounds from MS detection. Using the chromatographic conditions described above, the polyphenols eluted in less than 35 minutes. Four polyphenols cannot be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5–50 µg mL-1 range with good linearity ($R2 > 0.999$) for a five point plot were used to determine the concentration of polyphenols in plant samples [28,29].

2.2. Chromatographic Conditions for the analysis of allicin

The separation of allicin was made using a Synergi Polar 100 mm x 2.0 mm i.d., 4 µm column (Phenomenex, SUA). The mobile phase consisted in 100% ammonium acetate, 1mM in water, isocratic elution, flow 0.6 mL/min. A silver nitrate solution 1mM in water was added post column, with a flow of 10 µL/min. The mass spectrometer operated in positive MRM mode, using an electrospray ion source and nitrogen as nebulising and dry gas. The nebuliser was set at 60 psi, the dry gas flow was 12 L/min at 350ºC. The apparatus was set to record the transition m/z (449+451) > m/z (269; 271; 287; 289), specific to allicin-silver complex. The retention time of allicin in the above described conditions was 0.9 min.

2.3. Chromatographic Conditions for the analysis of phytosterols

Compounds were separated using a Zorbax SB-C18 reversed-phase analytical column (100 x 3.0 mm i.d., 5 µm particle) fitted with a guard column Zorbax SB-C18, both operated at 40ºC. Sterols were separated under isocratic conditions using a mobile phase consisting of 10:90 (v/v) methanol and acetonitrile. The flow rate was 1 ml/min and the injection volume was 5 µl. Mass spectrometry analysis was performed on an Agilent Ion Trap 1100 VL mass spectrometer with atmospheric pressure chemical ionization (APCI) interface. The instrument was operated in positive ion mode. Operating conditions were optimized in order to achieve maximum sensitivity values: gas temperature (nitrogen) 325ºC at a flow rate of 7 l/min, nebulizer pressure 60 psi and capillary voltage -4000 V. The full identification of compounds was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions. To avoid or limit the interference from background, the multiple reactions monitoring analysis mode was used instead of single ion monitoring (e.g. MS/MS instead of MS). The software ChemStation (vA09.03) and DataAnalysis (v5.3) from Agilent, USA were used for the acquisition and analysis of chromatographic data.

2.4. Chemicals

Standards: chlorogenic acid, *p*-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, hyperoside, kaempferol, quercetol, myricetol, fisetin, β-sitosterol, brassicasterol, stigmasterol, campesterol and ergosterol from Sigma (Germany), ferulic acid, sinapic acid, gentisic acid, patuletin, luteolin from Roth (Germany), cichoric acid, caftaric acid from Dalton (USA), allicin from Allicin International (Great Britain). Methanol of HPLC analytical-grade, acetonitrile of HPLC analytical-grade, ammonium acetate of HPLC analytical-grade, silver nitrate of HPLC analytical-grade, chloroform, n-hexane, potassium hydroxide of analytical-grade and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Methanolic stock solutions (100 mg/mL) of the flavonoid standards were prepared and stored at 4˚C, protected from daylight. They were appropriately diluted with double distilled water before being used as working solutions. Methanolic stock solution (4 mg/mL) of allicin was prepared and stored at 4˚C, protected from daylight; it was appropriately diluted with double distilled water before being used as working solution. Chloroformic stock solutions (1 mg/ml) of the phytosterol standards were prepared and stored at 4ºC, protected from daylight. Before being used as working solutions, they were appropriately diluted with acetonitrile. Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system.

2.5. Identification and quantitative determinations

The detection and quantification of polyphenols was made in UV assisted by mass spectrometry detection. Due peak overlapping, four polyphenol-carboxylic acids (caftaric, gentisic, caffeic, chlorogenic) were determined only based on MS spectra, whereas for the rest of compounds the linearity of calibration curves was very good ($R^2 > 0.998$), with detection limits in the range of 18 to 92 ng/mL. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method; retention times were determined with a standard deviation ranging from 0.04 to 0.19 min (Table 1). Accuracy was checked by spiking samples with a solution containing each polyphenol in a concentration of 10 μg/mL. In all analyzed samples the compounds were identified by comparison of their retention times and recorded electrospray mass spectra with those of standards in the same chromatographic conditions.

Table 1. Retention Time (min) of Polyphenolic Compounds

Peak no.	Phenolic compound	t_R±SD	Peak no.	Phenolic compound	t_R±SD
1.	Caftaric acid	3.54±0.05	11.	Rutoside	20.76±0.15
2.	Gentisic acid	3.69±0.03	12.	Myricetin	21.13±0.12
3.	Caffeic acid	6.52±0.04	13.	Fisetin	22.91±0.15
4.	Chlorogenic acid	6.43±0.05	14.	Quercitrin	23.64±0.13
5.	*p*-Coumaric acid	9.48±0.08	15.	Quercetol	27.55±0.15
6.	Ferulic acid	12.8±0.10	16.	Patuletin	29.41±0.12
7.	Sinapic acid	15.00±0.10	17.	Luteolin	29.64±0.19
8.	Cichoric acid	15.96±0.13	18.	Kaempferol	32.48±0.17
9.	Hyperoside	19.32±0.12	19.	Apigenin	39.45±0.15
10.	Isoquercitrin	20.29±0.10			

Note: t_R – retention time; SD – standard deviation.

The identification of sterols was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions. To avoid or limit the interference from background, the multiple reactions monitoring analysis mode was used instead of single ion monitoring (e.g. MS/MS instead of MS). The software ChemStation (vA09.03) and

DataAnalysis (v5.3) from Agilent, USA were used for the acquisition and analysis of chromatographic data. Linearity of calibration curves was very good ($R^2 > 0.998$), with detection limits in the range of 69 to 3312 ng/mL for ergosterol, 62 to 2952 ng/mL for brassicasterol, 59 to 2808 ng/mL for campesterol, 136 to 6528 ng/mL for stigmasterol, and 132 to 6336 ng/mL for β-sitosterol.

The calibration curve of allicin standard was linear between 18-864 μg/mL.

2.6. Plant material and preparation of extracts

Fresh *Allium fistulosum* L. herba (A1), *A. ursinum* L. leaves (A2), and *A. ursinum* L. flowers (A3) were used for extraction with 70% ethanol (Merck, Bucuresti, Romania) in Mycology Laboratory of Babes-Bolyai University, Cluj-Napoca, Romania, by modified Squibb's repercolation method [36]. Briefly, three successive applications of the same menstruum were repercolated to the plant material. In each percolator, plant material (150 g in the first, 90 g in the second, 60 g in the third percolator) was moistened with the menstruum, macerated for two days and then percolated at a rate of about 4 to 6 drops per minute for each 100 g of raw material. The first percolated fractions from each percolator were saved and the next fractions were poured in the next percolator. Then, saved fractions (60 ml from the first one, 90 ml from the second one and 150 ml from the third one) were mixed and the resulting extract was 1:1 (w:v) [4,7].

All plants were identified and voucher specimen (CL 659761 and CL 659750) was deposited at the Herbarium of "A. Borza" Botanical Garden, "Babes-Bolyai" University of Cluj-Napoca, Romania.

In order to obtain more accurate data on flavonoid glycosides and aglycones concentration, each sample was analyzed before and after acid hydrolysis. 2mL extractive solution was treated with 2 mL 2M hydrochloric acid and 0.2 mL ascorbic acid solution 100 mg/mL, and the mixtures were heated at 80˚C on a water bath for 30 min, ultrasonicated for 15 min, and heated for another 30 min at 80˚C. During the heating, 1mL methanol was added to the extraction mixture every 10 min, in order to ensure the permanent presence of methanol. The mixtures were centrifuged at 4000 rpm and the solutions were diluted with distilled water in a 10 mL volumetric flask and filtered through a 0.45 μm filter before injection.

3. Results and discussion

3.1. The analysis of polyphenols

A high performance liquid chromatographic (HPLC) method has been developed for the determination of nineteen phenolic compounds (eight phenolic acids, four quercetin glycosides, and seven flavonol and flavone aglycones) from natural products. The simultaneous analysis of different classes of polyphenols was performed by a single pass column, and the separation of all examined compounds was carried out in 35 minutes. In order to obtain more accurate data on flavonoid glycosides and aglycones concentration, and to estimate the nature of hydrolysed compounds, each sample was analyzed before and after acid hydrolysis.

The concentrations of identified polyphenolic compounds in all samples before and after acid hydrolysis are presented in Table 2. The HPLC Chromatogram of non-hydrolysed sample of *A. fistulosum* (A1 N) is presented in Figure 1, and the HPLC Chromatogram of hydrolysed sample of *A. fistulosum* (A1 H) is presented in Fig. 2.

Table 2. The Content in Polyphenolic Compounds of Allium species (µg/100g vegetal product)
Note: N – non-hydrolysed sample; H - hydrolysed sample.

Sample	A1 N	A1 H	A2 N	A2 H	A3 N	A3 H
p-Coumaric acid	11.05	302.23	109.11	369.03	34.92	69.66
Ferulic acid	499.01	1636.56	40.16	163.38	38.89	52.97
Sinapic acid	-	57.56	-	-	-	-
Isoquercitrin	29.14	-	-	-	-	-
Rutoside	-	-	-	-	-	-
Quercitrin	73.22	-	-	-	-	-
Quercetol	101.97	123.68	-	-	-	-
Luteolin	-	-	-	-	-	-
Kaempferol	153.94	171.38	-	252.76	-	1839.33
Apigenin	-	-	-	-	-	-

p-Coumaric acid and ferulic acid were identified in all ethanolic extracts. *A. fistulosum* (A1) was the richest species in ferulic acid (499.01 µg/100g before, and respectively 1636.56 µg/100g after hydrolysis). *A. ursinum* leaves (A2) contain higher quantities of *p*-coumaric and ferulic acids then *A. ursinum* flowers (A3), both before and after hydroysis. Sinapic acid was present only in *A. fistulosum* after hydrolysis. The pattern of flavonoids indicates large differences between the two *Allium* species, they can be used as potential taxonomic markers in order to distinguish the plants: isoquercitrin and quercitrin were identified only in *A. fistulosum* (29.14 µg/100g and respectively 73.22 µg/100g). *A. fistulosum* contains quercetol before (101.97 µg/100g) and after hydrolysis (123.68 µg/100g).

Kaempferol was present in both non-hydrolysed (153.94 µg/100g) and hydrolysed (171.38 µg/100g) sample of *A. fistulosum*, and only in hydrolysed extracts of *A. ursinum* leaves (252.76 µg/100g) and *A. ursinum* flowers (1839.33 µg/100g).

The simultaneous determination of wide range of polyphenolic compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by mass spectrometry detection, and the comparative study showed large differences between the two *Allium* species.

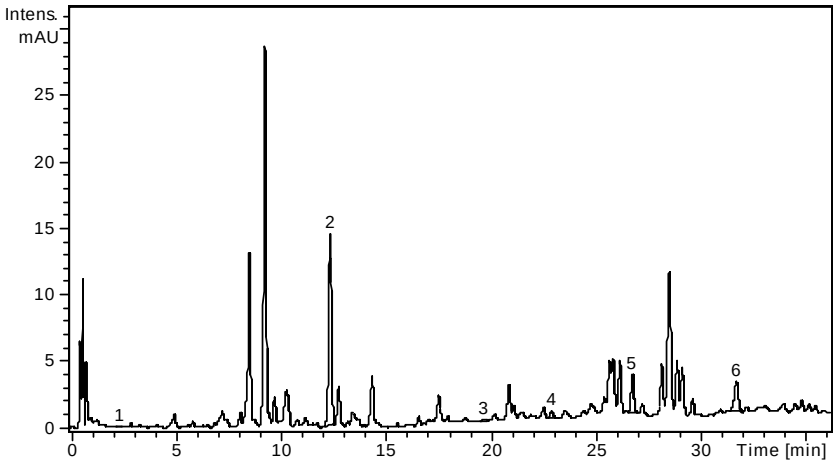

Fig 1. The HPLC Chromatogram of non-hydrolysed sample of A. fistulosum (A1 N).

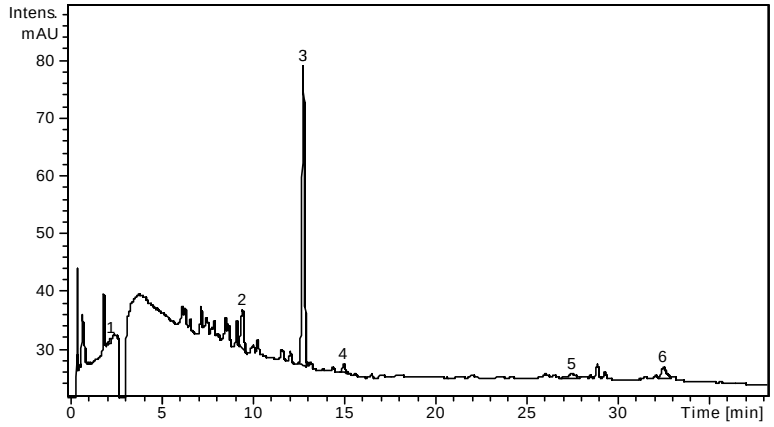

Fig. 2. HPLC Chromatogram of hydrolysed sample of A. fistulosum (A1 H).

3.2. The analysis of allicin

In the literature there are analytical methods reporting detection of allicin in UV at 220nm [37,38], but frequent interferences may appear at this wavelength because the lack of selectivity, which can lead to measurement errors.

The sulphur-containing compounds have the ability to form adduct complexes with some transitional metals. The complex has an electric charge and it can be analyzed by mass spectrometry with electrospray ionization. In order to obtain selectivity in quantitative determination of allicin by LC-MS, we used the adduct complex formed by allicin and the silver ion for quantification [31]. The peak of allicin was observed at R_T=0.9 min (Figure 3).

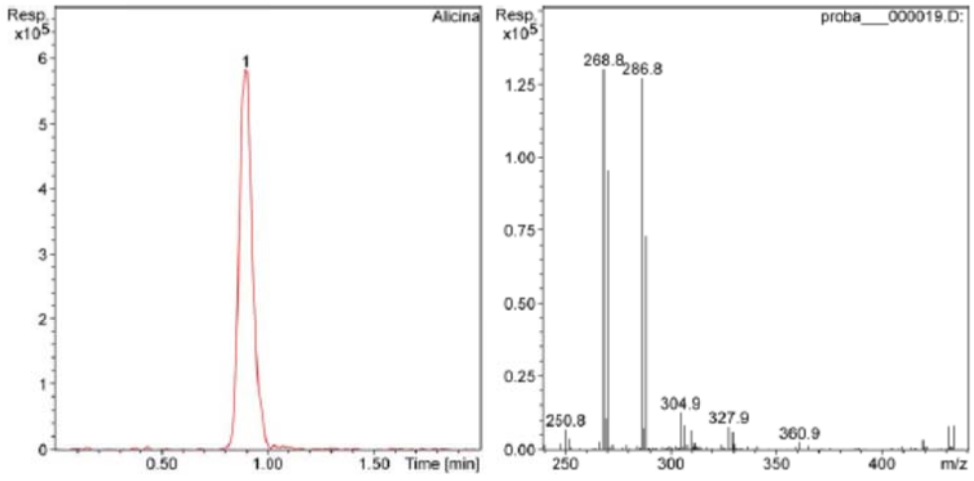

Fig. 3. The LC-MS chromatogram of allicin from A. fistulosum extract (a) and MS-MS spectra of allicin (b)

The results obtained for quantification of allicin in *A. fistulosum* (A1) , *A. ursinum* leaves (A2), and *A. ursinum* flowers (A3) are presented in Table 3. The *A. fistulosum* extract prepared at room temperature (R extracts) have a lower allicin content than the extract prepared by heating at 60°C (C extracts), because higher temperature extraction can favor the transformation of alliin into allicin.

Table 3.The content in allicin (mg/100 g vegetal product) of Allium species extracts

Vegetal product	Allicin (R extracts)	Allicin (C extracts)
A. fistulosum herba (A1)	5.275	896.2
A. ursinum leaves (A2)	1.965	-
A. ursinum flowers (A3)	175.614	-

Note: R – prepared at room temperature; C – prepared by heating.

Allicin was determined in all extracts obtained at room temperature, the higher amounts in *A. ursinum* flowers (A3). The allicin content was higher in extract obtained by heating than in those prepared at room temperature, because alliin and its derivatives were transformed into allicin in the working conditions (896.2 mg/100g in *A. fistulosum*).

3.3. The analysis of phytosterols

In the proposed chromatographic conditions, retention times of the five analysed sterols were: 3.2 min for ergosterol, 3.9 min for brassicasterol, 4.9 min for stigmasterol and campesterol (co-elution), and 5.7 min for β-sitosterol. The ions monitorized in the MS method are presented in Table 4. Because in the ionization conditions all sterols have lost a water molecule, the ions detected by the spectrophotometer are always in the form $[M\text{-}H_2O+H]^+$.

Table 4. Characteristic ions of standard sterols in full scan

Compound	Retention time (min)	M	$M\text{-}H_2O$	$M\text{-}H_2O+H^+$
Ergosterol	3.2	396	378	379
Brassicasterol	3.9	398	380	381
Stigmasterol	4.9	412	394	395
Campesterol	4.9	400	382	383
β-Sitosterol	5.7	414	396	397

The specific ions of the five standard sterols (379 for ergosterol, 381 for brassicasterol, 395 for stigmasterol, 383 for campesterol and 397 for β-sitosterol) have been fragmented, and based on the fragments from the MS spectrum the extracted chromatograms of each compound were drawn.

This method of analysis (also called MS-MS) is highly specific compared to the screening method, where only the intensity of the main ion is recorded, and an isomeric compound - with the same molecular weight - can give a false positive signal. Based on analysis of fragments of the MS spectrum, which are specific to each structure separately and are not the same for different isomers, the MS-MS method will only detect the compound of interest without other interferences. Moreover, because the intensity of ions in the mass spectrum is proportional to the concentration of the substance in the sample, the method can also be applied for quantitative determination.
In order to quantify the five sterols from *Allium* species extracts, we have constructed the extracted chromatograms for each compound, taking into account the intensity of major ions in the mass spectrum (Table 5).

Table 5. Ions from MS spectra of the standard sterols used in quantification

Compound	Specific ions for identification Ion [M-H_2O+H^+] > Ions from spectrum
Ergosterol	379> 158.9; 184.9; 199; 213; 225; 239; 253; 295; 309; 323
Brassicasterol	381> 201.3;203.3;215.2;217.3;241.2;255.3;257.4;271.1;297.3;299.3
Stigmasterol	395> 255; 297; 283; 311; 241; 201
Campesterol	383> 147; 149; 161; 175; 189; 203; 215; 229; 243; 257
β-Sitosterol	397> 160.9; 174.9; 188.9; 202.9; 214.9; 243; 257; 287.1; 315.2

Calibration curves were obtain from standard solutions at different concentration levels, selected as representative of the range of concentration in the sample. Regression analysis of various concentrations of standard solutions (0.08-8 μg/mL) gave good correlation coefficients for the calibration curves of sterols. Concentrations of phytosterols in *Allium* species extracts are presented in Table 6.

Table 6. The content in sterols (μg/100 g vegetal product) of Allium species extracts

Phytosterol	*A. fistulosum* (A1)	*A. ursinum* leaves (A2)	*A. ursinum* flowers (A3)
β-Sitosterol	6533.8	8.5	442.2
Campesterol	76.6	0	20
Stigmasterol	55.4	0	0
Ergosterol	0	0	0
Brassicasterol	0	0	0

β-sitosterol and campesterol were identified in *A. fistulosum* and *A. ursinum* flowers; the richest species in both compounds was *A. fistulosum* (6533.8 μg/100 g vegetal product, and respectively 76.6 μg/100 g vegetal product), and stigmasterol was found only in *A. fistulosum* (55.4 μg/100 g vegetal product). This is the first report for determination of phytosterols content in *A. fistulosum* and *A. ursinum*.

4. Conclusions

We analyzed the polyphenols from *Allium fistulosum* L. and *A. ursinum* L., and we completed the literature data with new information concerning the polyphenolic substances from *Allium* species. The simultaneous determination of wide range of polyphenolic compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by mass spectrometry detection. The content in allicin was also determined, showing the transformation of alliin and its derivatives into allicin by heating. The analysis of phytosterols from the two *Allium* species was performed for the first time, and we quantified β-sitosterol, campesterol, and stigmasterol in ethanolic extracts. The comparative study showed large differences between *Allium fistulosum* L. and *A. ursinum* L., both qualitative and quantitative.

Acknowledgements

This work was supported by CNCSIS-UEFISCSU, project number PNII – IDEI code 2272/2008.

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