

The Influence of Extraction Method on the Apparent Content of Bioactive Compounds in Romanian *Allium* spp. Leaves

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Abstract

In order to characterize the composition of bioactive compounds in *Allium* spp., an important source of traditional foods, analyses of their physicochemical properties, total phenols, and flavonoid content were carried out using three of the most popular Romanian leaf varieties (*Allium cepa* var. 'Diamant', 'Rubiniu', and *Allium ursinum* L.). The highest levels of bioactive compounds were detected in the leaves of *Allium ursinum* L., 7.2730 mg QE/kg fresh plant and 27.8884 g GAE/100 g dry basis, respectively. This study suggests potential good uses of the fresh leaves of Romanian *Allium* spp. as condiments, ingredients or preservatives in the food industry.

Keywords: *Allium cepa*, *Allium ursinum*, flavonoids, total polyphenols

Introduction

Allium is the most representative genus of the *Liliaceae* family, which includes 700 species of widely distributed bulbous perennials and biennials (Najjaa *et al.*, 2007; Tepe *et al.*, 2005), and is an important economic genus due to the huge quantities of onions consumed. At present, *Allium* spp. plants are considered the most important vegetables consumed fresh or in different cooked dishes in Europe, Asia, and America (Djurdjevic *et al.*, 2004). In Romania, over the last year, significant changes took place in the vegetable market. In 2010, according to the Ministry of Agriculture, the production of dried onions was estimated at 362.3 thousand tones, and Romania was situated at third place according to statistics (FRD Center Market Report, 2011). *Allium* spp. plants are used as common foods, and for the treatment of many diseases (Lanzotti, 2006), due to their content of phytonutrients. In fact, the edible parts of *Allium* spp. plants are used for the treatment and prevention of a number of diseases: coronary heart disease (Gorinstein *et al.*, 2007; Hiyasat *et al.*, 2009; Siegel *et al.*, 2004), cancer (Sengupta *et al.*, 2004; Shukla and Kalra, 2007), obesity, diabetes, disturbances of the gastrointestinal tract, hyper-cholesterolemia, and inflammatory diseases (Hodge *et al.*, 2002; Kalayarsan *et al.*, 2009; Takahashi and Shibamoto, 2008).

Other researchers (Dini *et al.*, 2008) have reported an increasing interest in *Allium* spp., both by the food industry and by scientists researching vegetables and plants, because of their strong antioxidant properties. These properties are due to many substances, including some vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals, and volatiles compounds. Onion and wild garlic contain polyphenolic compounds. Flavonols and anthocyanins

are the main subclasses of flavonoids present in onions, the latter being found only in red onions (Rodrigues *et al.*, 2009, 2011). The actual number of polyphenols is estimated to be higher because they generally occur as glycosides, and the details of their sugar residues and binding forms show great variety (Miron *et al.*, 2010); so it is important to determine the amounts of polyphenols in plants. Literature reports of polyphenols are difficult to compare due to the use of different extraction solvents and conditions (Santas *et al.*, 2008). Furthermore, onions and the edible portions of wild garlic are important components of the Romanian people's diet, and are commonly consumed uncooked, so the loss of bioactive compounds is minimized, contributing to the health benefits of this food (Gennaro *et al.*, 2002; Santas *et al.*, 2008; Sellappan and Akoh, 2002).

The aim of this study consisted of identifying and characterizing bioactive compounds from Romanian *Allium* spp. leaves, as an important constituent of their traditional foods. For this purpose, three species of plants (white onion, red onion, and wild garlic leaves) were analyzed in order to assess their physicochemical characteristics, and total phenolic and flavonoid contents, as important polyphenolic compounds.

Materials and methods

Plant samples

The most popular varieties of Romanian fresh onion leaves (*Allium cepa* L. var. 'Diamant' (white onion), *Allium cepa* var. 'Rubiniu' (red onion) from the early hybrids group were kindly supplied by the Research and Development Society for Vegetables Buzău, Romania. Fresh *Allium ursinum* L. leaves were collected from the Bacau city

forests in March 2011. All plant samples were free of external damage, hand selected, and chopped in the mortar for the following analyses.

Chemicals and materials

Gallic acid, quercetin, vanillin, and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Petroleum ether, toluene, glacial acetic acid, and dichloromethane were purchased from Lach-Ner s.r.o (Brno, Czech Republic). Ethanol was purchased from Tunic Prod (Bucharest, Romania). Solutions of 7.5% sodium carbonate, 2.5% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$, and 10% sodium carbonate were freshly prepared. Ultrapure water (TKA Smart 2 Pure UV/UF system, Niederelbert, Germany) was used for the experiments.

Determinations of moisture, ash, oil, pH and titratable acidity

The recommended methods were adopted to determine the levels of moisture, ash and oil (AOAC, 2000). The moisture content was determined by drying around 5.0 g of the samples, at 105°C, to a constant weight. Oil content was determined by the Soxhlet method (Extraction Unit 6, SER 148 model, VELP Scientifica, Italy). Crude fat was obtained by exhaustively extracting 10 g of each sample in a Soxhlet apparatus using petroleum ether (boiling point range 40-60°C) as the solvent (Dini et al., 2008). The ash content was determined by incinerating 5 g of fresh leaves at 550-600°C until a homogeneous white ash without black points was obtained. The pH and titratable acidity were determined in accordance with Roldán-Marín et al. (2009). After determination of pH, the solution was titrated with 0.1 N NaOH to pH 8.1, and the results were expressed as percentage of citric acid [g of citric acid/per 100 g of plant (db)].

Extract preparation by conventional maceration (CM) and ultrasound-assisted extraction (UAE)

Fresh plant leaves were extracted with 70% ethanol at a ratio between fresh plants: ethanol of 1:5. The mixture was left for 12 days macerating at room temperature (20°C) in dark conditions. The solids were then separated by vacuum filtration through a ceramic filter with a porosity of 40 µm, and the extracts were stored at 4°C until their use in the following analysis. To avoid degradation of bioactive compounds, an ultrasonic bath (Transsonic T310, Elma, Singen, Germany) was used as an ultrasound source (frequency 35 kHz) (Aspé and Fernández, 2011). The ultrasound-assisted extraction was performed under ambient conditions (20-25°C) for 15 min in cycles over two hours. The crude extracts were stored at 0-4°C until analysis.

Determination of total polyphenol content (TP)

The total polyphenol content was determined by a spectrophotometric method, using gallic acid as a standard, according to the method described by the International Organization for Standardization ISO14502-1 (Anesini et

al., 2008; Miron et al., 2010). In a short description of the method, 0.1 mL of each extract was transferred into glass tubes, then 5 mL of Folin-Ciocalteu's reagent diluted 1/10 in distilled water, and 4 mL of a sodium carbonate solution (7.5% w/w) were added. All the experiments were run in duplicate, and allowed to stand at room temperature for one hour before the absorbance was determined at 765 nm against a blank containing distilled water. A standard curve of gallic acid (ranging from 10 to 50 µg gallic acid/mL and $R^2 = 0.9988$) was used to express the concentration of polyphenols in samples as gallic acid equivalents (GAE) in g/(100 g dry basis of material).

Estimation of total flavonoid content

The total flavonoid content in the extracts was determined by a spectrophotometric method based on the formation of complex flavonoid-aluminium with an absorptivity maximum between 420-430 nm (Bozin et al., 2008; Jia et al., 1999). Briefly, 1 mL of each extract was separately mixed with 1 mL of 2.5% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 2 mL of 10% sodium acetate solution, and 70% ethanol to a total volume of 10 mL. The experiments were run in duplicate, and after incubation at room temperature for 30 minutes, the absorbance of the reaction mixtures was measured at 420 nm against a water standard. The flavonoid content values were determined from a standard curve prepared with quercetin (ranging from 10 to 50 µg/mL final volume and $R^2 = 0.9732$) and expressed as mg quercetin equivalents (QE)/kg plant.

Statistical analysis

The experimental results were analysed using Principal Component Analysis (PCA) with full cross-validation. PCA constitutes the most basic statistical method of all multivariate data analyses, and involves decomposing one "data matrix" into a structural part (model) and a "noise" part (error). The main propose of all multivariate data analysis is to decompose the data in order to detect and model "hidden phenomena". PCA was assessed using the Unscrambler X 10.1 software version from CAMO Software AS (Oslo, Norway). PCA was used to determine the influence of extraction method and type of *Allium* spp. on the total polyphenol and flavonoid content of the extracts obtained.

Results and discussion

Physicochemical properties of raw plant material

This study is based on the fact that, in Romania during to the spring season, an increased consumption of onion and wild garlic leaves as dietary supplements occurs. Thus, it was also took into account the growing interest in the development of agronomical area of the raw materials selected. Therefore, particular attention was given to the initial physical and physicochemical characteristics presented in Tab. 1. For *Allium cepa* varieties, the pH values were between 5.7-5.9. Other reports have present for some Span-

ish onion variety the same values for pH (Roldán-Marín *et al.*, 2009). The crude fat content may suggest, in the case of the *Allium ursinum* and *Allium cepa* varieties, the possibility of using them to obtain essential oils useful for making dishes more flavor, or for encapsulation/formulation processes.

Bioactive composition of onion and wild garlic leaves (total polyphenols and quercetin content)

The bioactive compositions of three varieties of *Allium* spp. leaf extracts were analyzed for total phenolic and flavonoid contents using different methods of extraction. It has been reported that onion is one of the major sources of dietary flavonoids present as glycosides or as aglycones (Corzo-Martínez *et al.*, 2007). Sellappan and Akoh (2002) identified the major flavonoid compound in onion as quercetin, present in conjugated form as quercetin 4'-O- β -glucopyranoside, quercetin 3,4'-O- β -diglycopyranoside, and quercetin 3,7,4'-O- β -triglycopyranoside.

The bioactive compounds from different *Allium* spp. plants have also been confirmed by other literature reports. Some authors previously identified 3,4'-O-diglucoside and 4'-O-monoglucoside as the major quercetin derivatives of the mature red onion bulb; these components account for about 93% of the total flavonols (Pérez-Gregorio *et al.*, 2011). Rodríguez *et al.* (2009) affirmed that different treatments (curing and cooking) of onion bulbs could influence their flavonol and anthocyanin levels, and demonstrated that intense microwave cooking treat-

ment caused flavonol losses of 16% and 18% for quercetin 3,4'-diglucoside and quercetin 4'-glucoside, respectively.

In order to investigate the impact of different extraction methods on the content of bioactive compounds, selected materials from each plant type were obtained using two different types of extracts: conventional maceration, and ultrasound-assisted extraction. The amount of total polyphenolics varied widely with the extraction method and source of plant materials. The highest total polyphenolic content (Fig. 1), with a conventional maceration extract from *Allium ursinum* L. leaves, was determined to be 27.8884 g GAE/100 g dry basis.

The level of total polyphenols was much higher (57.74%) with a 'Rubiniu' variety extract obtained by conventional maceration, and 58.64% higher with ultrasound-assisted extraction, respectively. Gökçe Fuat *et al.* (2010) reported that the red onion had a higher equivalent gallic acid content than the white onion (2.2 mg GAE/g). Significant differences in flavonoid concentrations (Fig. 2) were obtained between varieties. Most studies have reported higher levels of flavonoids in red onion than in other onion varieties (Lin and Tang, 2007; Rodrigues *et al.*, 2011) but the analysis were done on bulbs.

The results indicate that ultrasound-assisted extraction is a good method for obtaining the highest flavonoid content, for all the raw materials used. Consequently, the total flavonoid content was lower in the white variety 'Diamant', than in the red variety 'Rubiniu', ranging from 5.4867 mg QE/kg fresh plant to 6.0304 mg QE/kg fresh plant. Instead, the highest flavonoid content was identified in *Allium ursinum* L. leaves, 7.2730 mg QE/kg fresh plant. The present results are in agreement with other reports that indicate a higher quercetin content in red onions than in yellow or white onions (Nemeth and Piskula, 2007). Pérez-Gregorio *et al.* (2010) determined the flavonol and anthocyanin concentrations in different varieties of red and white onions. They found that flavonols (quercetin 7,4-diglucoside, quercetin 3,4-diglucoside, quercetin 3-glucoside, quercetin 4-glucoside) were the predominant polyphenolic compounds. White onion cultivars had the lowest total flavonol content (89.3 ± 38.5 and 101.0 ± 18.9 mg quercetin/kg fresh weight for 'Braca da Póvoa' and the hybrid 'SK409'), whereas red onions presented the highest levels of flavonols, with 280.2 ± 41.5 and 304.3 ± 81.2 mg quercetin/kg fresh weight for 'Vermelha da Póvoa' and 'Red Creole', respectively.

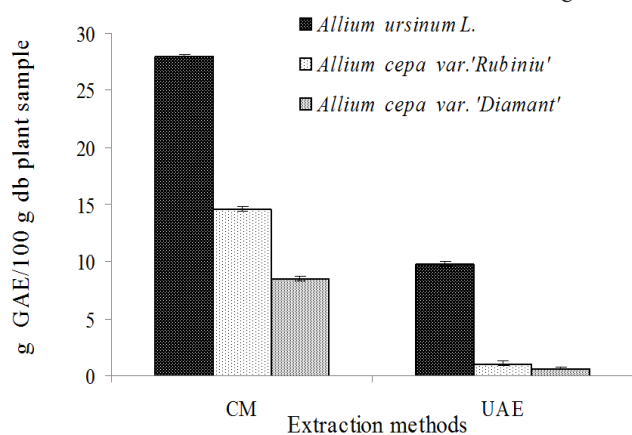


Fig. 1. Polyphenol content of onion and wild garlic leaf extracts. (*Data expressed as means \pm standard deviations of duplicate analyses of each extraction method; CM-conventional maceration; UAE-ultrasound-assisted extraction; db-dry basis)

Tab. 1. Initial physical and physicochemical characteristics of onion and wild garlic leaves

Characteristic*	<i>Allium cepa</i> var. 'Diamant'	<i>Allium cepa</i> var. 'Rubiniu'	<i>Allium ursinum</i> L.
dry matter, %	5.23204 \pm 0.088672	6.48318 \pm 0.0253	8.95453 \pm 0.26351
moisture, %	94.76795 \pm 0.0724	93.5168 \pm 0.0051	91.04546 \pm 0.47269
ash content, % (db)	9.32582 \pm 0.02075	9.08414 \pm 0.1116	8.77613 \pm 0.0902
crude fat content, %	2.3944 \pm 0.2354	2.48053 \pm 0.0869	3.61355 \pm 0.0778
pH	5.93 \pm 0.0565	5.75 \pm 0.0353	6.235 \pm 0.16263
titratable acidity g acid citric/100 g plant (db)	0.8884 \pm 0.10784	1.19856 \pm 0.0521	0.86734 \pm 0.0019

*Values are the mean of two independent determinations \pm standard deviations; db: dry basis

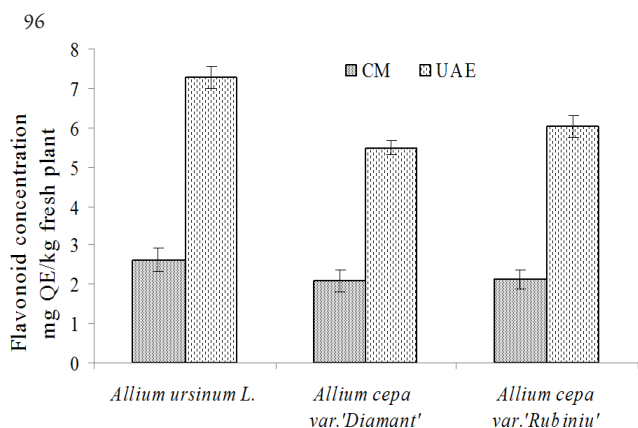


Fig. 2. Flavonoid contents of wild garlic, white, and red onion leaf extracts. (*Error bars represent standard deviations for two duplicate determinations)

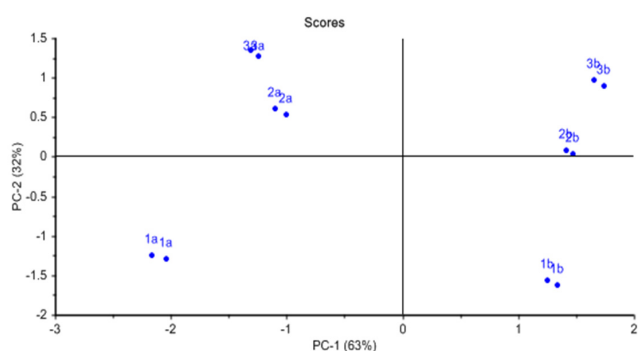


Fig. 3. Score plot after PCA of the individuals in the plane defined by the two first PCs. 1-*Allium ursinum L.*; 2-*Allium cepa var. 'Diamant'*, 3-*Allium cepa var. 'Rubiniu'*; a-conventional maceration; b-ultrasound-assisted extraction

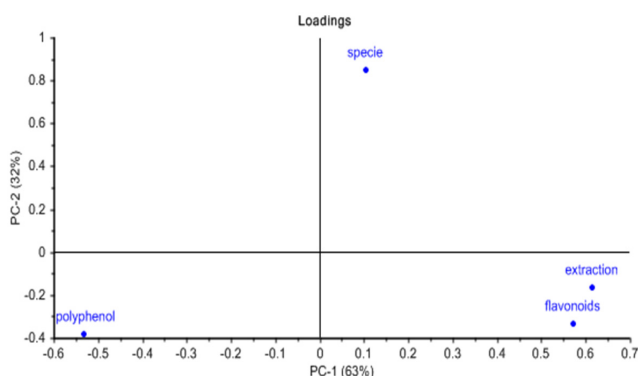


Fig. 4. Loading plot after PCA with full cross-validation of the analysed samples

A principal component analysis (PCA) with full cross-validation was carried out in order to evaluate the influence of plant species and extraction method on the concentrations of bioactive compounds. PCA is a linear transformation of a set of original data to a set of uncorrelated components in such a way that only a few of the resulting variables account for the majority of the variability observed in the original data. Therefore, a reduction in dimensionality is achieved with minimal loss of information (Calvo et al., 2010). Visualization of the results of PCA is usually achieved by plotting pairs of the first few principal components (PCs). In this study, Principal Component

1 (PC1) and Principal Component 2 (PC2) represented 94.93% of the system variance. Principal component 2 (PC2) is the species analysed, but the most important influence (63%), as indicated by Principal Component 1 (PC1), represented the extraction method used in the experiment; that was of significant importance for the variations in concentrations of bioactive compounds, total polyphenols, and flavonoids. Fig. 3 and 4 represent the score plots (maps of analysed samples) and loading plots (plot of variables) for all the *Allium* spp. and extraction methods used in the experiment.

It can also be observed that the extraction method significantly influenced the type of bioactive compounds extracted. Using the conventional maceration extraction method (range 1), higher amounts of polyphenols were extracted, while after the ultrasound-assisted extraction method (range 2), concentrations of flavonoids were much higher. The type of Romanian *Allium* spp. did not influence the overall amount of bioactive components extracted.

Conclusions

The obtain results showed that *Allium ursinum* leaves contained the highest amounts of oil (3.61%) and dry matter (8.95%), and pH (6.235). Comparatively with *Allium cepa var. 'Diamant'*, all physicochemical properties are much higher in the *Allium cepa var. 'Rubiniu'*, with 27.59% dry matter and 31.30% oil content. The high crude fat content suggests that wild garlic is a good source of aromatic oils in steam distillation processes. In conclusion, and considering the harvest period of the vegetal products, the polyphenol content of the analyzed extracts is influenced by the type of processing method the samples undergo. The results obtained were statistically validated and compared with data in the scientific literature. This study may be considered a new report about the identification and characterization of bioactive compounds from Romanian fresh *Allium* spp. leaves as natural and important sources that are consumed, especially, during the spring season.

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