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Original Article

Sulfur compounds identification and quantification from *Allium* spp. fresh leaves

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ABSTRACT

Background: Pyruvic acid concentration is a critical factor in determining *Allium* spp. pungency. This study was initiated to accurately measure the background pyruvic acid levels in Romanian *Allium* spp. From the pungency point of view, all analyzed plant varieties in this study are considered low pungent cultivars based on the enzymatically produced pyruvate level (between 42 μmol/g and 222 μmol/g fresh wt). Chromatographic analysis was carried out for the different varieties of the most popular fresh leaves (*Allium cepa* var. "Diamant", *Allium cepa* var. "Rubiniu", and *Allium ursinum* L.) in order to identify the sulfur compounds. The thin layer chromatography analysis led to the identification of allicin, with $R_f = 0.377 - 0.47$, as an important sulfur compound. The gas chromatography-mass spectrometry analysis of the leaves' extracts detected disulfides as the major sulfur compounds. Principal component analysis was performed to establish the differences in plant composition. These studies suggest the potential good uses of the fresh leaves of Romanian *Allium* spp. as condiment, ingredient, or preservative in the food industry.

1. Introduction

Allium is the most representative genus of the Liliaceae family, which includes 700 species of widely distributed bulbous perennials and biennials [1,2], and is an economically important genus because of the huge quantities of onions consumed. Nowadays, the *Allium* spp. plants are considered the most important vegetables consumed fresh or in different dishes in Europe, Asia, and America [3]. In Romania, over the past years, significant changes have taken place in the vegetables market. According to the statistical data compiled by the Ministry of Agriculture, in 2010, the production of dried onions was estimated at 362.3 thousand tons, placing it in third place [4]. *Allium* spp. plants are used as common foods and as agents for treatment of many diseases [5] because they contain phytonutrients. In fact, the edible parts of *Allium* spp. plants are used for the treatment and prevention of a large number of

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diseases (e.g., coronary heart disease [6–8], cancer [9,10], obesity, diabetes, disturbances of the gastrointestinal tract, hypercholesterolemia, and inflammatory diseases [11–13]), because they contain volatile sulfur-containing compounds, which have a distinct flavor and are responsible for the pungent odor. A special class of biologically active organosulfuric compounds, S-alk(en)yl-L-cysteine sulfoxides (such as alliin and γ-glutamylcysteines), dominates onion and wild garlic flavor. In the intact cells, the sulfoxides are found in the cytoplasm and in the vacuole. Disruption of plant material results in the hydrolysis of alk(en)yl cysteine sulfoxides, thus creating volatile alk(en)yl-thiosulfinates such as allicin and lipid-soluble sulfur compounds (such as diallyl sulfide, diallyl disulfide), which are the principal flavor compounds (Fig. 1).

These compounds are responsible for the characteristic smell and taste of *Allium* species plants [14] as well as most of their biological properties [5]. The main thiosulfinate compound present in onions and wild garlic extracts is allicin, also called diallyl thiosulfinate [15]. For *Allium* spp., pungency is very important, and the determination of pyruvate, which is formed as a stable primary compound from the enzymatic decomposition of each of the flavor precursors, is a good method for pungency assessment in onion and wild garlic [16,17] (Fig. 1). The health benefits of the bioactive compounds from *Allium* spp. plants have been demonstrated [18–20], so some people commonly consume these plants in an uncooked state. The aim of this study was to identify and quantify the sulfur compounds from Romanian *Allium* spp. leaves, as an important constituent of traditional foods. In order to assess the pungency level, thiosulfinate concentration, chromatographic identification of sulfur compounds, and antioxidant activity of three Romanian *Allium* spp. varieties (white onion, red onion, and wild garlic leaves) were analyzed.

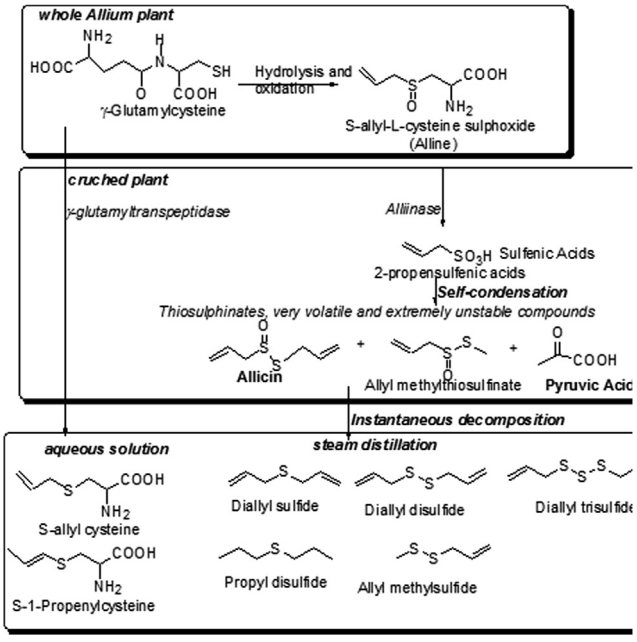

Fig. 1 – Enzymatically produced sulfur compounds and pyruvic acid in *Allium* species.

2. Materials and methods

2.1. Plant samples

The most popular varieties of Romanian fresh onion leaves [*Allium cepa* L. var. "Diamant" (white onion), *A. cepa* var. "Rubiniu" (red onion)] from early hybrid groups were kindly supplied by the Research and Development Society for Vegetables Buzău, Romania. Fresh *Allium ursinum* L. leaves were collected from the forests of Bacău City in March 2011. All plant samples were free of external damages, hand selected, and chopped in the mortar before analysis.

2.2. Chemicals and materials

Vanillin and silica gel 60 F254 plates were supplied by Merck (Darmstadt, Germany). Toluene, glacial acetic acid, dichloromethane, and methanol were purchased from Lach-Ner s.r.o (Brno, Czech Republic). In addition, 0.0125% 2,4-dinitro-phenyl hydrazine (DNPH; with 95% purity), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ethyl acetate were obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water (TKA Smart 2 Pure UV/UF system; Niederelbert, Renania-Palatinat, Germany) was used for the experiments.

2.3. Thin layer chromatography identification of volatile sulfur compounds

The TLC (thin layer chromatography) pattern of various extracts can vary according to the extraction methods. The Wagner and Bladt method [21] was adopted to obtain the dichloromethane extracts of fresh samples of *A. cepa* var. "Diamant" (white onion leaves), *A. cepa* var "Rubiniu" (red onion leaves), and *A. ursinum* (wild garlic leaves), and spotted on Silica gel 60 F254 plates. After impregnation in the developing system with toluene/ethyl acetate (10:3), the silica gel plates were dried at 105°C and immersed in the detection system solvent (1 g vanillin to 100 mL glacial acid reagent). Sulfur compounds were visible as yellow, green, and brownish spots, and identified using retention factor (R_f) values.

2.4. Volatile sulfur compounds extraction

Fresh leaves (20 g; *A. cepa* var. "Rubiniu" and *A. ursinum* L.) were subjected to hydrodistillation using the Clenvenger apparatus. The fresh hydrodistillates were immediately analyzed by gas chromatography-mass spectrometry (GC-MS), and the antioxidant activity was determined.

2.5. GC-MS

Analysis was run on a Thermo Scientific ITQ-900 system (Trace GC Ultra/ITQ 900, Thermo, Milano, Italy) coupled with an ionic trap and fitted with a TG-XLB-MS capillary column with 0.25 μm film thickness, 30 m length, and 0.25 mm inner diameter, using helium as gas carrier with a flow rate of 1 mL/minute. The operating conditions were as follows: injector port temperature 250°C, split ratio 20:20, detector

temperature 270°C, and program temperature from 40°C to 100°C with 4°C/minute, from 100°C to 150°C with 4°C/minute and from 150°C to 270°C with 10°C/minute.

The MS conditions were as follows: ionization voltage 70 eV, ion source temperature 200°C, scan range *m*/z 50–450. The qualitative identification of the sulfur compounds was based on computer matching with Wiley9 library and by comparison with data in the literature.

2.6. Determination of pyruvic acid

The enzymatically produced pyruvic acid content was estimated using the dinitrophenyl hydrazine (DNPH) reagent [17,22] and was calculated using the equation:

$$P_E = P_T - P_C, \quad [1]$$

where P_T is the total pyruvic acid (μmol/g fresh wt) and P_C is the control level of endogenous pyruvic acid (μmol/g fresh wt). For a total pyruvic acid determination (P_T), around 1 g of the selected plants was homogenized for 2 minutes in an ultrasound bath with distilled water. The homogenate was allowed to stand for 15 minutes at room temperature, and then quantitatively filtrated through a Whatman cellulose filter paper. Endogenous pyruvic acid was also measured in conjunction with the total pyruvic acid. This background amount of pyruvic acid is subtracted from the total pyruvic acid to obtain the enzymatically produced pyruvic acid. A certain amount of pyruvic acid exists endogenously in these plants. Pyruvic acid is a major product formed via glycolysis and other metabolic processes. The heat provided in the microwave step denatures the enzyme, *alliinase*, and therefore stops the reaction. The control level of endogenous pyruvic acid (Pc) was determined after the microwave treatment (Microwave oven Panasonic NN-E202W model; power to 1000 W) of the plant sample during 30 seconds to deactivate *alliinase*, wherein 1 g of the microwave-treated plant was ultrasound homogenized for 2 minutes in distilled water and allowed to stand at room temperature for 15 minutes, and then filtrated. For each type of filtrate, 2 mL clarified filtrate was taken, and to this 1.0 mL of 0.0125% DNPH [prepared in 2 N HCl (hydrochloric acid)] was added. The reaction mixture was placed in a water bath at 37°C for 15 minutes. After removing the samples from the water bath, 5.0 mL of 0.6 N NaOH was added. The absorbance was recorded at 515 nm. An extended standard curve was constructed for pyruvic acid (ranging from 0.05 μmol/mL to 1 μmol/mL), and the pyruvic acid levels in samples were determined by directly comparing the absorbance rates with those on the curve.

2.7. DPPH assay

Antioxidant activity was measured by the free radical DPPH as previously described [2,23,24]. The samples (0.5 mL) were mixed with 1 mL of 0.0035% of DPPH solution in methanol, and 95% MeOH was added to a final volume of 4 mL. The absorbance of the resulting solutions and the blank (with the same chemicals, except sample) was recorded after 1 hour at room temperature. The disappearance of DPPH was measured spectrophotometrically at 515 nm. The results, expressed as a percentage, were calculated using the following equation:

$$\text{DPPH scavenging effect (RSC) } (\%) = [(A_0 - A_1)/A_0] \times 100, \quad [2]$$

where A_0 is the absorbance of the control reaction (containing all reagents except the extract) and A_1 is the absorbance of the analyzed extract.

2.8. Statistical analysis

The experimental results were analyzed using principal component analysis (PCA) with full cross-validation. PCA constitutes the most basic statistical method of all multivariate data analysis, and involves decomposing one "data matrix" into a structural part (model) and a "noise" part (error). The main purpose of all multivariate data analyses 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 evaluate the experimental results for pyruvic acid, thiosulfinate concentrations, and antioxidant activity for all the studied plant species.

3. Results and discussion

3.1. TLC identification

The TLC method was applied for identification of sulfur compounds. Fresh extracts showed brown or dark yellow zones, with these colors being specific for thiosulfinate compounds. After treatment with the detection system (Fig. 2), *A. ursinum* (wild garlic leaves) mainly showed yellow-brown (R_f = 0.477–0.151), yellow, or dark yellow (R_f = 0.129) spots. Extracts of *A. cepa* var. "Rubiniu" (red onion leaves) showed brown and yellow-brown zones with R_f values of 0.488 and 0.213, respectively. Moreover, the extract of *A. cepa* var. "Diamant" is distinguishable by the characteristic yellow-brown and gray-yellow zones with R_f values of 0.377 and 0.209, respectively. The R_f values of 0.377–0.47 allowed the conclusion that the method is adequate to identify allicin. In agreement with other reports [21], all the leaf extracts also presented other compound-specific zones (dark green) for chlorophyll with an R_f range of 0.547–0.436, and ajoens or cepaenes with a low R_f range between 0.056 and 0.078. The brown zones at the solvent front are attributable to sulfides such as allyl disulfide. The brown zones from the starting line may be attributable to degradation products.

3.2. Volatile sulfur compounds analysis

A. cepa var. "Rubiniu" and *A. ursinum* L. fresh hydrodistillate were analyzed to identify the sulfur compounds. The identified sulfur compounds and their mass spectral data are listed in Table 1 [25–27] (*A. cepa* var. "Rubiniu" hydrodistillate) and Table 2 [27] (*A. ursinum* L. hydrodistillate), respectively.

The identified components are disulfides with different radicals (allyl, methyl, and propyl) that are specific for *Allium* varieties leaves, and they are mainly presented in hydrodistillates. This aspect is very important because

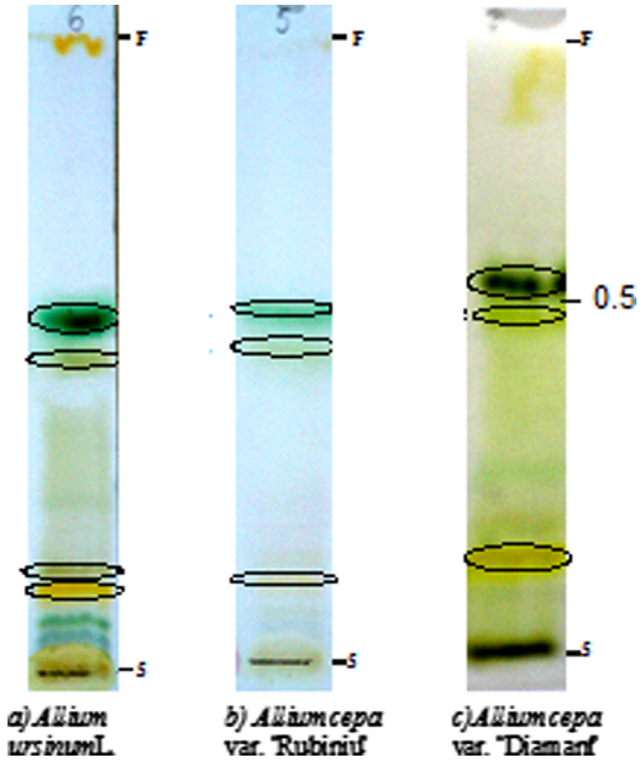

Fig. 2 – Thin layer chromatography chromatograms for dichloromethane extracts of the fresh leaves (brown zones at the solvent front—sulfides; brown or dark yellow zones—thiosulfinates; dark green—chlorophyll).

these compounds can be produced during the cooking process [14].

In comparison, in the *A. ursinum* L. hydrodistillate, based on mass spectrum analysis and mass fragmentation at *m/z* 119.8, 1.3-dithiane was identified (Table 2).

3.3. *Pyruvic acid, thiosulfinate, and antioxidant activity from* Allium *spp. leaves*

The pyruvic acid that universally exists in the plant tissue and is an indicator of pungency in onion plants [17] was determined. Estimation of the pungency level, which is an important attribute, enables the classification of onion leaves and bulbs. Pungency was quantitatively determined by measuring the enzymatically produced pyruvic acid. All the analyzed plant samples can be considered low pungent cultivars because of the concentration of enzymatically produced pyruvic acid (42.6–222.422 μmol/g fresh wt). Calculation for thiosulfinate compounds is based on $^1/_2$ mol thiosulfinate for each mole of pyruvic acid produced. The antioxidant activity of some *Allium* species is attributed to sulfur-containing constituents and their precursors [2]. Because of its stability (in radical form) and the simplicity of the assay, the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) is one of the most commonly used substrates for the fast evaluation of antioxidant activity [23].

Statistical analysis indicates that two PCs account for 99.89% of the data variation. All three variants of *Allium* were very well defined as different plant types owing to their different chemical properties.

PC1 was given by the pyruvic acid and thiosulfinate concentrations found in fresh plants. Furthermore, pyruvic acid and thiosulfinate concentrations were very well correlated to each other, but negatively correlated with plant species (Figs. 3 and 4).

PC2, which explains almost 37% of the data variation, was given by the DPPH scavenging activities of the plant hydrodistillates. DPPH scavenging activity was uncorrelated with the other variables. We observed that all three types of plants were very different from each other according to their pyruvic acid content, thiosulfinate concentrations, and DPPH scavenging activity.

A. ursinum L. and *A. cepa* var. "Diamant" have similar concentrations of pyruvic acid and thiosulfinate, but were different in terms of antioxidant activity. We observed that *A. ursinum* L. leaves (with % RSC = 32.41 ± 0.777) presented the highest value, whereas that of *A. cepa* var. "Diamant" showed the weakest antioxidant activity (with % RSC = 19.63 ± 0.535). *A. cepa* var. "Rubiniu" presented an antioxidant activity of % RSC = 28.01 ± 0.815. *A. ursinum* L. has the highest amounts for all analyzed parameters.

A. cepa var. "Rubiniu" contains the lowest amounts of pyruvic acid and thiosulfinate concentrations. These differences can be attributed to the natural qualitative and quantitative variability in the raw material. Therefore, *A. ursinum* L. leaves are a natural source of good protection against the oxidative damage that occurs in our body; unfortunately, they are available in fresh form only during spring.

Outliers have not been detected. The Hotellings T2 statistic, one of the most powerful outlier diagnostics used in

Table 1 – Sulfur compounds identified in *Allium cepa* var. "Rubiniu" hydrodistillate.

No.	Sulfur compound	Retention time, t_r (min)	Mass spectral data (relative abundance, %)	Probability, %
1.	Allyl methyl disulfide[a,b]	10.71	120 (M*, 100), 105 (1.6), 80 (9.3), 79 (17.4), 73 (8.2), 71 (7.4), 64 (12.9)	70
2.	Methyl propyl disulfide[a,c]	17.35	122 (90), 80 (M*, 100), 41(45), 43(38), 45(25), 64 (15)	48.64
3.	2.4-Dimethyl thiophene[a,b]	18.21	111(M*, 100), 97 (40), 112(78)	74,7
4.	Dipropyl disulfide[a,c]	23.05	150 (37), 108 (24), 43(M*, 100), 41 (22), 27 (15)	43.5
5.	1.5-Dithiocane[a]	25.17	148 (60), 106 (M*, 100), 41 (35), 45 (30), 46 (20), 47 (14), 73 (20), 74 (14)	56.2

[a] Identified by mass spectral data in the Wiley date base library stored in the gas chromatography-mass spectrometry system.
[b] Identified by mass spectral data and compared with data reported in the literature [25,26].
[c] Identified by mass spectral data compared with data reported in the literature [27].

Table 2 – Sulfur compounds identified in hydrodistillate from wild garlic leaves.

No.	Compounds name	Retention time, t_R (min)	Mass spectral date (relative abundance), %
1.	Dimethyl trisulfide (DMTS)[a,b]	23.47	126 (M*, 100); 79 (42), 47 (30), 46 (20), 64 (20), 80 (16), 128 (14)
2.	*cis*-Methyl propenyl disulfide	19.79	120 (M*, 100); 72 (56), 75 (39), 80 (28), 87 (12)
3.	Dimethyl disulfide (DMS)[b]	11.51	94 (M*, 100); 79(64), 45(68), 47(28), 61(20)
4.	1.3-Dithiane[c]	19.45	119.8 (M*, 100); 86.9 (34), 72.9 (50), 63.8 (38)
5.	*trans*-Methyl-propenyl sulfide[b]	7.69	73 (M*, 100); 88 (98), 45 (M*, 100), 39 (50), 47 (30), 72 (17)
6.	Methyl propyl disulfide[b]	17.49	122 (70); 80 (M*, 100), 43 (69), 41 (52), 45 (40), 47 (20), 64 (15), 94 (10)

[a] Identified by mass spectral data in the Wiley date base library stored in the gas chromatography-mass spectrometry system.
[b] Identified by mass spectral data compared with data reported in the literature [27].
[c] Detected only in *Allium ursinum* L. hydrodistillate.

multivariate analysis problems, did not detect outliers at the 5% significance level. Furthermore, the explained variance in validation follows the general pattern, indicating the absence of outliers.

4. Conclusion

TLC analysis allowed the identification of allicin, the main thiosulfinate compound that is formed by the action of the enzyme *alliinase* on alliin. Pyruvic acid determination is an indirect measure of total thiosulfinate present in disrupted plant tissue; however, it is lacking in specificity and cannot provide any differentiation of the individual alk(en)yl thiosulfinates. Further chromatographic and biological studies are needed to identify the constituents and precisely evaluate their biological activities and mechanisms. There seems to be an urgent need to validate available research data using evidence-based methodology to reach decisive conclusions.

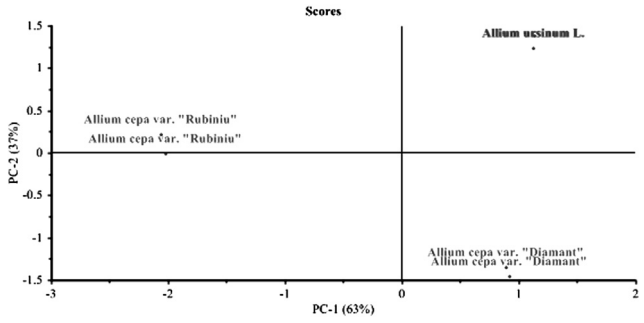

Fig. 3 – Scores plot of the analyzed *Allium* fresh plant extracts.

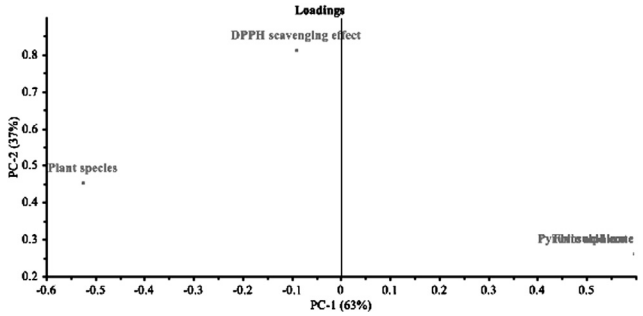

Fig. 4 – Loading plot of the variables used to describe *Allium* fresh plant extracts.

Our study may be considered a new report on the identification and characterization of bioactive compounds in fresh leaves of Romanian *Allium* spp. as natural and important food sources, especially during the spring season. PCA analysis allowed us to validate the experimental results and to distinguish the *Allium* plant species based on their chemical composition.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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