

INFLUENCE OF STORAGE CONDITIONS ON FLAVONOIDS CONTENT AND ANTIOXIDANT ACTIVITY OF SELECTED SHALLOT (*ALLIUM CEPA* VAR. *ASCALONICUM* BACKER) HYBRID CULTIVARS

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Summary

The aim of this study was to determine changes in the content of flavonoids in fleshy scales of shallot bulbs in relation to different compositions of atmosphere during storage. Also antioxidant activity of the bulbs was determined. Bulbs of shallot cvs. Bonilla F₁, Conservor F₁ and Matador F₁ were stored in two storage seasons at the normal atmosphere (NA) and controlled atmosphere (CA) of the 4 compositions: 5% CO₂ + 5% O₂, 5% CO₂ + 2% O₂, 2% CO₂ + 5% O₂, 2% CO₂ + 2% O₂. The temperature of storage was 0-1°C and RH 65%. Before and after 7-month storage the bulbs were examined in respect of flavonoids content and antioxidant activity (AA). Flavonoids were identified in methanolic extracts with HPLC, AA was determined with FRAP and DPPH assays. Three flavonoid compounds were identified in the plants extracts. Quercetin 3,4'-di-O-glucoside and quercetin 4'-glucoside (spiraeoside) were present in the highest amounts. CA conditions influenced content of flavonoids in the bulbs. The highest content after storage was observed at gas composition of 5% CO₂ + 5% O₂. Two major flavonoid compounds, quercetin 3,4'-di-O-glucoside and quercetin 4'-O-glucoside (spiraeoside) showed the increasing tendency, however not all changes were significant. No significant differences were observed between cultivars, but 'Bonilla' F₁ showed slightly higher flavonoids content than the other ones. Investigated cultivars did not differ in respect of AA, measured both with DPPH and FRAP assay. There was no significant influence of storage conditions on DPPH level, while FRAP level was influenced significantly. Bulbs from 5% CO₂ + 2% O₂ composition were characterized by the highest FRAP values and the bulbs stored at normal atmosphere – by the lowest.

key words: shallot, controlled atmosphere storage, quercetin glucosides, antioxidant activity, FRAP, DPPH

INTRODUCTION

Allium vegetables are well known for their biological activity resulting from the presence of sulphur compounds in the bulbs, however other compounds, namely flavonoids and other polyphenols, have been in the area of researchers interest lately. Flavonoids show wide range of biological activity, mainly connected with beneficial effect on cardiovascular system and with their antioxidant activity (Hollman & Katan 1999). Flavonoids show several different ways of antioxidant action, such as free radicals scavenging, breaking the chain of their formation, inhibition of enzymes and bounding the metal ions catalyzing oxidation processes and stimulation of critic gene for glutathione transcription resulting in increased concentration of intracellular glutathione (Moskaug *et al.* 2005). Flavonoids are divided into several groups: flavones, flavonoles, flavanoles, flavanones, isoflavones and anthocyanins. *Allium* vegetables are rich in flavonoles, primarily quercetin, and among the onion varieties the most abundant in quercetin are shallots and red onions (Di Carlo *et al.* 1999; Kyle & Duthie 2006). Several investigations were performed to recognize the changes of flavonoids during storage of shallot bulbs (Horbowicz & Kotlińska 2001, Horbowicz 2006, Tendaj & Mysiak 2010), however the influence of controlled atmosphere storage was not studied. CA storage is recommended in literature for long-term storage of onions and several other vegetable crops, as a method for better keeping

product quality, including its biological value (Bartz & Brecht 2003). The objective of the study was to identify flavonoids in bulbs of hybrid cultivars of shallot (*Allium cepa* var. *ascalonicum* Backer) grown from seeds, examine their antioxidant activity (AA) and influence of storage under different atmosphere compositions on flavonoids content and AA.

MATERIALS AND METHODS

The experiment was carried out in storage seasons 2009/2010 and 2010/2011. For the investigation three hybrid cultivars of shallot: Conservor F₁, Bonilla F₁ and Matador F₁ (Bejo Zaden, Holland) were chosen. Bulbs of 'Bonilla' F₁ are of garlic-like shape, have light brown and yellow dry and fleshy scales. 'Conservor' F₁ and 'Matador' F₁ are characterized by red and brown tints of dry scales, of different intensity, and pink tint of inner scales. 'Matador' F₁ bulbs are of garlic-like shape, with light red-brown dry scales. 'Conservor' F₁ bulbs have red-brown dry scales and elongated, spindle shape. The plants were cultivated from seedlings sowing in the experimental field in Warsaw, on a medium-loam soil. Mature bulbs were harvested in the third week of August and cured in a shed, at ambient temperature, for one month. Bulbs were stored for 7 months in CA cold storage at the temperature between 0 and 1°C, 65% RH.

Five atmosphere compositions were studied: (1) normal atmosphere 0% CO₂ + 21% O₂, (2) 5% CO₂ + 5% O₂, (3) 5% CO₂ + 2% O₂, (4) 2%

CO₂ + 5% O₂, (5) 2% CO₂ + 2% O₂. Because of lack of recommendations for shallot, CA conditions recommended for common onion were chosen. The experiment was established in three analytical replications, with thirty bulbs in each. Each analytical replication was prepared by mixing bulbs from three experimental replications. Chemical analyses were performed before and after storage period. For the analyses good quality, healthy bulbs were chosen. For each cultivar antioxidant activity (AA) was determined and identification of flavonoids by HPLC was performed. To estimate AA two assays were applied: FRAP and DPPH.

Reagents:

Methanol, acetic acid, iron (III) chloride hexahydrate were purchased in Chempur; HPLC grade methanol, acetonitrile, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and ferrous sulfate were obtained from Sigma-Aldrich.

Extracts preparation

5 g of plant fresh material was extracted with 100 ml of methanol, using Büchi B-811 extraction system (modified Soxhlet extractor, Büchi Labortechnik AG, Postfach, Switzerland). After solvent evaporation, the residue was dissolved in 10 ml of methanol.

HPLC analysis:

Extracts prepared as it is described above were filtered with Supelco Iso-Disc™ Syringe Tip Filter Unit, PTFE membrane, diameter 25 mm, pore size 0.20 µm, and subjected to HPLC. Identification and quantification of flavonoids in shallot extracts were performed using a Shimadzu chro-

matograph equipped with autosampler SIL-20, photodiode array detector SPD-M10A VP DAD and Class VP 7.3 chromatography software (Shimadzu, Kyoto, Japan). A modern C-18 reversed-phase column with core-shell technology (Phenomenex Kinetex® 2.6 µm, C18, 100A, 100×4.60 mm i.d.) was used as solid phase. Binary gradient of mobile phase A (deionised water acidified with phosphoric acid 0.1 %) and B (acetonitrile acidified with phosphoric acid 0.1%) was used as follows: 0.01 min, 18% B; 1.4 min, 95% B; 1.5 min, 95% B; 1.51 min, 18% B. The following conditions were applied: injection volume: 1 µl, flow rate 1.2 ml·min⁻¹, oven temperature 30°C, total time of analysis 8 minutes, UV-spectra were recorded between 190 and 450 nm.

Peak identification was confirmed by comparison of retention time and spectral data with adequate parameters of standards purchased from ChromaDex (ChromaDex, Irvine, USA). For quantitation of investigated compounds the 5-point calibration curve method was used. Methanol stock standard solutions were prepared according to the ChromaDex's Tech Tip 0003: Reference Standard Recovery and Dilution. The following concentrations of standards were applied: quercetin 3,4'-di-O-glucoside: 10.0, 20.0, 40.0, 100.0, 200.0 µg·ml⁻¹; quercetin 3-O-glucoside: 49.6, 99.2, 198.4, 496.0, 992.0 µg·ml⁻¹; quercetin 4'-glucoside: 21.4, 42.8, 85.6, 214.0, 428.0 µg·ml⁻¹. The solutions were applied on a column in triplicate. The peak table and spectra library (190-450 nm) of individual compounds were created. De-

tection wave applied: quercetin 3,4'-di-O-glucoside 254 nm, quercetin 3-O-glucoside 353 nm, quercetin 4'-glucoside 365 nm. The content of the determined compounds was calculated in $\text{mg} \cdot 100 \text{ g}^{-1}$ dry matter (DW). The method was elaborated and validated especially for this purpose in Department of Vegetable and Medicinal Plants (Warsaw University of Life Sciences).

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

DPPH• scavenging activity of examined extracts (prepared as it is described above) was carried out according to Yen and Chen (1995) method. To 1 ml of examined extract and 3 ml of methanol 1 ml of DPPH• methanolic solution ($120 \text{ mg} \cdot \text{ml}^{-1}$) was added. Absorbance was measured on the UV-Vis Shimadzu 1700 PharmaSpec spectrophotometer after 10 minutes at 517 nm. In the blind test extract was replaced by 1 ml of methanol. Trolox in concentrations $0.3\text{--}4.9 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ was used to estimate standard curve.

FRAP (Ferric Reducing/ Antioxidant Power) assay

FRAP was examined according to Benzie and Strain (1996, 1999). FRAP reagent was prepared from 300 mM acetic buffer (pH 3.6), 10 mM TPTZ (4,6-tripyridyl-s-triazine) and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solutions mixed before analysis in 10:1:1 proportion. 3 ml of freshly prepared FRAP reagent was warmed up to 37°C , its absorbance was measured, then $100 \mu\text{l}$ of extract (prepared as it is described above) was added and after 4 minutes of incubation at 37°C change in absorbance caused by reduction of Fe^{3+} to Fe^{2+} in examined sample using UV-

Vis Shimadzu 1700 PharmaSpec spectrophotometer was measured. The total antioxidant capacity of shallot extracts was determined against a standard of known FRAP value, ferrous sulphate ($1000 \text{ } \mu\text{M}$).

Statistical analysis

Numerical data from both seasons were subjected to analysis of variance with Statgraphics Plus 4.1TM software (Statistical Graphics Corp. 1999). Analysis draw a distinction between combinations and therefore Tukey's HSD test was used to compare the means, at the level of significance $P=0.05$. The data presented in tables are means of both seasons of the experiment. Homogenous groups were marked in the tables only in case of significant differences.

RESULTS AND DISCUSSION

Three flavonoids were identified in examined extracts obtained from shallot bulbs: quercetin 3,4'-di-O-glucoside, quercetin 3-O-glucoside (isoquercetin) and quercetin 4'-glucoside (spiraeoside). Quercetin 3,4'-di-O-glucoside and spiraeoside were present in the highest amounts (Table 2 & 3). Bonaccorsi *et al.* (2008) identified 7 flavonol glucosides in onion and shallot bulbs, the main flavonoid constituents of shallots were quercetin 3,4'-di-O-glucoside 4'-glucoside (spiraeoside), just as in this study. Their concentration was also similar to those identified in our study. Quercetin 3,4'-di-O-glucoside and spiraeoside were found also in onion bulbs by Caridi *et al.* (2007) and Rodrigues *et al.* (2011). Lachman *et al.* (2003) found spiraeoside, rutin and

quercetin as the major flavonoid constituents of onions. Investigated cultivars didn't differ significantly in content of identified flavonoids, however 'Bonilla' F₁ showed a slightly higher mean content of these compounds. This cultivar was also characterized by the highest dry matter content (Table 1). Bulbs of investigated cultivars examined before storage showed bigger differences in flavonoids content, compared to their mean values. Lack of significant differences in average amount of quercetin glycosides between cultivars is a result of their diverse performance in various atmosphere compositions. According to Horbowicz and Kotlińska (2001), and Bonaccorsi *et al.* (2008) vegetatively propagated populations of shallot examined after harvest shows differences in flavonoids content. Also many studies performed on common onion reveal differences between cultivars in respect to flavonoids content (Patil *et al.* 1995, Prakash *et al.* 2007, Bonaccorsi *et al.* 2008, Beesk *et al.* 2010, Rodrigues *et al.* 2011). There is a lack of information about influence of CA conditions on flavonoids changes in shallot bulbs during storage. In our study increase of quercetin 3,4'-di-O-glucoside was observed during the storage in all atmosphere

compositions (Table 2). Quercetin 3-O-glucoside (isoquercetin) and quercetin 4'-O-glucoside (spiraeoside) contents were significantly higher only in the case of 5% CO₂ + 5% O₂ (2) variant (Table 3 & 4). Horbowicz and Kotlińska (2001) observed decrease of flavonols in shallot bulbs after 8 months of storage in a cold store. However, in the case of shorter period of curing, increase of flavonols during the first two months of storage, followed by further decrease and repeated increase at the end of storage period, was observed. Increase of quercetin content in onions at the beginning of storage period under normal atmosphere was reported by Patil *et al.* (1995), especially for higher temperature, but onions stored in CA (99% N₂ + 1% O₂) showed no significant changes during storage. Increase of spiraeoside and rutin was also reported by Lachman *et al.* (2003) during 36 weeks of storage at 4°C and 22°C. Content of quercetin in bulbs of two Polish onion cultivars, Błońska and Sochaczewska, was higher after five months of storage in a cold store than after curing, however during the storage period some drops were visible, depending on cultivar and month of storage (Horbowicz 2001).

Table 1. Dry matter content in shallot bulbs in relation to storage conditions and the cultivar, means of the two seasons of the experiment (%)

Cultivar (B)	Before storage	Storage (A)					Means
		Storage conditions					
		1	2	3	4	5	
Conservor	14.0cde	8.86a	12.9c	14.1cde	13.0c	13.3cd	12.7A
Matador	13.4cd	9.93ab	13.3cd	13.4cd	13.4cd	13.1cd	12.7A
Bonilla	16.4e	15.3cde	15.2cde	16.9e	15.9de	12.6bc	15.4B
Means	14.6C	11.4A	13.8BC	14.8C	14.1BC	13.0B	
LSD _{0.05} AxB		2.33					

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Means which differ significantly according to HSD Tukey's test at P < 0.05 are marked with different letters (The main effects are marked with capital letters, interactions with small letters).

Table 2. Quercetin 3,4'-di-O-glucoside content in shallot bulbs in relation to storage conditions and the cultivar, means of the two seasons of the experiment (mg·100 g⁻¹ DW)

Cultivar (B)	Storage (A)						Means
	Before storage	Storage conditions					
		1	2	3	4	5	
Conservor	251	853	842	687	512	353	583
Matador	329	586	866	752	476	364	562
Bonilla	335	515	766	770	593	531	585
Means	305B	651AC	825A	736A	527ABC	416BC	
LSD _{0.05} AxB	n. s.						

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Note: see Table 1

Table 3. Quercetin 3-O-glucoside (isoquercetin) content in shallot bulbs in relation to storage conditions and the cultivar, means of the two seasons of the experiment (mg·100 g⁻¹ DW)

Cultivar (B)	Storage (A)						Means
	Before storage	Storage conditions					
		1	2	3	4	5	
Conservor	19.4	9.79	28.1	16.5	12.7	20.7	17.9
Matador	14.0	15.7	36.7	23.4	15.3	10.2	19.2
Bonilla	26.1	20.8	34.7	22.6	14.4	12.6	21.9
Means	19.9AB	15.5A	33.2B	20.8AB	14.1A	14.5A	
LSD _{0.05} AxB		n. s.					

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Note: see Table 1

Table 4. Quercetin 4'-O-glucoside (spiraeoside) content in shallot bulbs in relation to storage conditions and the cultivar, means of the two seasons of the experiment ($\text{mg} \cdot 100 \text{ g}^{-1} \text{ DW}$)

Cultivar (B)	Storage (A)						Means
	Before storage	Storage conditions					
		1	2	3	4	5	
Conservor	195	586	384	256	446	296	361
Matador	407	378	651	369	385	375	428
Bonilla	349	460	588	556	460	442	476
Means	317A	475AB	541B	394AB	430AB	371AB	
LSD _{0.05} AxB				n.s.			

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Note: see Table 1

The examined cultivars didn't show significant differences in their antioxidant activity, measured with DPPH assay. FRAP assay showed 'Matador F₁' to be cultivar with the lowest AA, what is a result of AA decrease during storage in most of examined atmosphere compositions. DPPH radical scavenging activity and FRAP of examined extracts showed no differences before and after storage of shallot bulbs (Table 5 & 6). According to other authors, AA of onions varies, what may be a result of different methods of extraction and applied assays of AA measurement (Nuutila *et al.* 2003). Pellegrini *et al.* (2003), who tested the FRAP and TEAC (Trolox Equivalent Antioxidant Capacity) of over 30 vegetable species, classified onion as a vegetable of medium antioxidant capacity. Lower AA than common onion showed garlic bulbs (Nuutila *et al.* 2003). According to Leelarungrayub *et al.* (2006) AA of garlic was similar to AA of shallot. By contrast, in the

study carried out on several onion cultivars, shallot exhibited the highest phenolic content and also the highest AA (Yang *et al.* 2004). Shallot showed also higher radical scavenging activity than onion and garlic in the study of Chaitharghyuthi *et al.* (2009). Ismail *et al.* (2004) examined AA of shallot, spinach, swamp cabbage, cabbage and kale using β -carotene bleaching assay. Among the investigated vegetables, shallot showed the highest AA, both for fresh and thermal treated bulbs.

Since flavonoids are known to be strong antioxidants, relationship between their contents in shallot bulbs and AA values was calculated (Table 7). Moderate relationship between quercetin 3,4'-di-O-glucoside, quercetin 3-O-glucoside and FRAP was found, however all identified flavonoids showed relatively weak or no relationship with DPPH value, which can be explained by differences in operating principles of these assays (Bartoň *et al.* 2005).

Table 5. Antioxidant activity of shallot bulbs in relation to storage conditions and the cultivar, measured with DPPH assay, means of the two seasons of the experiment (mg TE·100 g⁻¹ DW)

Cultivar (B)	Storage (A)						Means
	Before storage	Storage conditions					
		1	2	3	4	5	
Conservor	230	215	186	202	209	177	203
Matador	201	174	169	171	175	191	180
Bonilla	206	320	182	145	172	250	212
Means	212	236	179	173	185	206	
LSD _{0.05} AxB	n. s.						

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Note: see Table 1

Table 6. Antioxidant activity of shallot bulbs in relation to storage conditions and the cultivar, measured with FRAP assay, means of the two seasons of the experiment (mmol Fe²⁺·100 g⁻¹ DW)

Cultivar (B)	Storage (A)						Means
	Before storage	Storage conditions					
		1	2	3	4	5	
Conservor	3.93	2.46	5.79	7.28	4.88	4.14	4.75AB
Matador	3.96	3.19	4.27	3.82	3.44	3.85	3.76A
Bonilla	4.29	4.26	4.96	4.35	4.26	5.85	4.66B
Means	4.06	3.30	5.01	5.15	4.19	4.62	
LSD _{0.05} AxB				n. s.			

1 - normal atmosphere (control); 2 - 5% CO₂ + 5% O₂; 3 - 5% CO₂ + 2% O₂; 4 - 2% CO₂ + 5% O₂; 5 - 2% CO₂ + 2% O₂

Note: see Table 1

Table 7. Correlation coefficients between identified flavonoids content in shallot bulbs and antioxidant activity measured with DPPH and FRAP assays

Chemical compound	DPPH	FRAP
Quercetin 3,4'-di-O-glucoside	0.209	0.536*
Quercetin 3-O-glucoside (Isoquercetin)	0.364*	0.563*
Quercetin 4'-O-glucoside (Spiraeoside)	0.333*	0.428*

* correlation significant at P < 0.05

CONCLUSIONS

Three flavonoids were identified in shallot bulbs in this study, and quercetin 3,4'-di-O-glucoside and spiraeoside were present in the highest amounts. Two assays used to measure AA of shallot extracts gave different results. Results of FRAP assay showed moderately strong relationship with flavonoids content in the bulbs. Atmosphere composition during storage of the bulbs influenced flavonoids content and AA measured with FRAP for the bulbs. Increase of quercetin 3,4'-di-O-glucoside, isoquercetin and spiraeoside and AA (FRAP) in some atmosphere compositions was observed. There were no significant differences in flavonoids content and AA between bulbs of investigated shallot cultivars.

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WPLYW WARUNKÓW PRZECHOWYWANIA
NA ZAWARTOŚĆ FLAWONOIDÓW I AKTYWNOŚĆ PRZECIWUTLENIAJĄCĄ
WYBRANYCH ODMIAN MIESZAŃCOWYCH SZALOTKI
(*ALLIUM CEPA* VAR. *ASCALONICUM* BACKER)

Streszczenie

Celem pracy było zbadanie zmian w zawartości flawonoidów w łuskach mięsistych cebul szalotki w zależności od składu gazowego atmosfery podczas przechowywania. Badano również aktywność antyoksydacyjną cebul. Cebule szalotki odmian 'Bonilla' F₁ 'Conservor' F₁ i 'Matador' F₁ przechowywano w dwóch sezonach przechowalniczych w atmosferze normalnej (NA) oraz kontrolowanej (CA) o czterech składach gazowych: 5% CO₂ + 5% O₂, 5% CO₂ + 2% O₂, 2% CO₂ + 5% O₂, 2% CO₂ + 2% O₂. Temperatura przechowywania była utrzymywana na poziomie 0-1°C, a wilgotność względna powietrza 65%. Przed przechowywaniem i po 7 miesiącach przechowywania oznaczano zawartość flawonoidów i aktywność antyoksydacyjną (AA). Flawonoidy identyfikowano w ekstraktach metanolowych przy pomocy HPLC. AA określano przy pomocy metod FRAP oraz DPPH. W ekstraktach zidentyfikowano cztery związki z grupy flawonoidów. W największych ilościach występowały 3,4'-di-O-glucozyd kwercetyny oraz 4'-glucozyd kwercetyny (spiraeozyd). Warunki CA wpłynęły na zawartość flawonoidów w cebulach. Największą zawartość stwierdzono po przechowaniu w atmosferze o składzie 5% CO₂ + 5% O₂. Dwa główne związki flawonoidowe, 3,4'-di-O-glucozyd kwercetyny oraz 4'-glucozyd kwercetyny, wykazywały tendencję wzrostową, jednak nie we wszystkich obiektach zmiany były istotne. Nie stwierdzono istotnych różnic między odmianami, jednak cebule odmiany Bonilla F₁ wykazywały tendencję do wyższej zawartości flawonoidów niż cebule pozostałych odmian. Cebule badanych odmian nie różniły się istotnie pod względem AA mierzonej obu metodami. Nie stwierdzono wpływu składu atmosfery na AA mierzoną metodą DPPH, natomiast wpływ ten był istotny przy użyciu metody FRAP. Cebule przechowywane w atmosferze o składzie 5% CO₂ + 2% O₂ charakteryzowała wyższa AA mierzona metodą FRAP, natomiast przechowywane w NA – najniższa.