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Research Article

Low oxygen concentrations affecting antioxidant activity and bioactive compounds in coloured rice

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Abstract

Apart from nutritional values, coloured rice is being recognized as a potential source of antioxidants and bioactive compounds and this has seen increased interest from the food industry to find better ways to preserve them. The aim of this study was to determine the effect of low oxygen storage (0, 5 and 10% O_2) on antioxidant activity and bioactive compounds (polyphenol and total anthocyanins) on coloured rice during four months storage and to investigate the relationship between them. The antioxidant activity increased as the storage time increased and the highest antioxidant activity was observed in both samples stored at 0% O_2. Total anthocyanin contents were retained under low O_2 concentrations while polyphenol contents significantly declined during storage. After four months storage, minimum losses of free and soluble conjugated phenolic contents were observed in the rice samples from 0% O_2 storage, whereas minimum loss of insoluble bound phenolics was detected in rice samples stored at 5% O_2. However, total phenolics content did not significantly differ among the tested oxygen concentrations. Antioxidant activity was significantly, but weakly, correlated with bound and total phenolics content. Rice stored at 0% O_2 can preserve antioxidant activity resulting in maintaining the minimum losses of bioactive compounds during storage. These research findings may be useful as a guideline for rice growers and traders to prevent the losses of bioactive compounds and antioxidant activity during storage.

Keywords: polyphenol, phenolics, anthocyanins, low oxygen storage, Thailand, Myanmar.

Introduction

Rice remains a staple food for the majority of the world's population due to it being an excellent source of carbohydrates, a good source of energy, low in fat and salt and no cholesterol. There are many different kinds of rice including white rice and several varieties of coloured rice. The most common type of rice has a white pericarp (>85%), while coloured rice has either a green, black or red pericarp. The black and red rice cultivars are mainly planted in South Asia and other countries such as Italy, Greece and the United States [1]. Pigments of rice cultivars vary greatly in content and distribution. Coloured rice cultivation increased through the advent of genetic engineering in the early 1970's [2]. Rice with a coloured pericarp has long been consumed in Japan and China and is considered to be a healthy food. The health functions of black rice have been assumed in traditional Chinese medicine for centuries [3]. There is great interest in the antioxidative and radical-scavenging properties of coloured rice cultivars because of their potential to provide and promote human health by reducing the concentration of reactive oxygen species and free radicals [3-5].

Khao Mali Daeng, or Red Hom Mali, rice is a well known cultivar in Thailand. It is widely cultivated and consumed due to its good taste and nutritional value. Because this variety is unpolished, it retains many of its nutrients, vitamins and minerals. Khao Hom Nil, or black fragrant rice, is a special line of rice developed by researchers from the private sector and Kasetsart University in Thailand. Khao Hom Nil has been shown to contain more protein than meat and also has high mineral content, in particular, iron, zinc, calcium and potassium.

Storage induced changes in physiochemical, physiological and functional properties of rice and these changes affect the rice quality [6]. Storage conditions after harvest may also have a large impact on the bioactive compounds [7]. The quality of stored grain depends on various treatments applied to the grains during storage period such as aeration, drying and fumigation. The quality also depends largely on storage atmospheres [8]. Oxygen concentration of the storage atmosphere is believed to have an important effect on rice quality [9, 10]. Oxygen may cause degradation of anthocyanins by a direct oxidation mechanism and/or by indirect oxidation whereby oxidized constituents of the medium react with the oxygen [11]. However, oxygen deficient atmosphere is able to suppress insect pest and microorganism populations in rice during storage [10]. To date, no information is available regarding the effects of low oxygen concentration on the changes of bioactive compounds and antioxidant activity in coloured rice. This information, when available, will be useful for designing rice packaging that would maximize the shelf life and nutritional value of coloured rice. The aim of this study was to investigate the effects of low oxygen concentration on antioxidant activity and bioactive compounds of the black rice (Khao Hom Nil) and the red rice (Red Hom Mali) during storage.

Materials and Methods

Grain samples

The rice cultivars Red Hom Mali and Black fragrant Khao Hom Nil harvested in 2007 were purchased from a local market in Thailand. The moisture content of the rice samples was reduced to 7% by drying at 110°C for 16 hours to prevent microbial growth during the experiment. The experiment was conducted from April to August 2008 at the Division of Postharvest Technology, King Mongkut's University of Technology Thonburi, Thailand.

Chemicals and reagents

2,2-diphenyl-1-picryhydrazyl (DPPH), Folin-Ciocalteu's reagent and β-carotene were purchased from Sigma-Aldrich, U.S.A. Sodium carbonate and ferulic acid were obtained from Ajax Finechem, Australia. Acetonitrile, hydrochloric acid and sodium hydroxide were brought from Merck KGaA, Germany. Hexane and diethyl ether were purchased from J.T. Baker, U.S.A.

Determination of total anthocyanins content

Anthocyanin contents of two coloured rice cultivars were extracted according to Jing and Giusti [11] with minor modifications. One gram of each rice sample was ground and 25 ml of 70% aqueous acetone was added. The mixture was stirred for 20 minutes and filtered by Whatman No.1 filter paper. The pellet was re-extracted with 10 ml of 70% acetone until the solution was colourless. The acetone solvent was evaporated by a rotary evaporator at 40°C under vacuum. The monomeric anthocyanin content was measured by the pH differential method and the absorbency of the extract was measured at wavelengths of 510 nm and 700 nm using a spectrophotometer (UV-1601, SHIMADZU) and was calculated as cyanidin 3-glucoside. The total anthocyanin content was expressed in mg/100g dry weight basis.

Determination of free, soluble conjugated and insoluble bound phenolic contents

The free, soluble conjugated and insoluble bound phenolic acids in the coloured rice extracts were isolated according to the procedure described by Krygier *et al.* [12] with slight modifications. All samples were defatted by hexane (1:5, w/v) at ambient temperature and were air-dried for 12 hours. The samples were re-extracted with 20 ml of methanol: acetone: water (7:7:6, v/v/v) six times at room temperature using a homogenizer (IKA®T25 digital ultra-Turrax®, 15 s, 10,000 rpm). The homogenate was centrifuged at 5,000 g for 15 min. and supernatant was collected and evaporated at 30°C under a vacuum to approximately 20 ml. Then supernatants were extracted by using diethyl ether to obtain free phenolics prior to second extraction, the concentrated supernatants were treated with 15 ml of 4 M NaOH for 4 hours at room temperature. The samples were flushed with nitrogen and packed in air-tight glass vials. The resulting supernatants were acidified to pH 2 using hydrochloric acid and centrifuged at 5,000 x g, 15 min, thereafter extracted with diethyl ether. The ether extracts were evaporated until dry at 30°C under vacuum.

The precipitates were treated with 10 ml of 4 M NaOH for 4 hours at room temperature. The samples were flushed with nitrogen and then acidified to pH 2 using hydrochloric acid, followed by centrifugation (5,000x g, 15 min). The supernatants were re-extracted six times with diethyl ether. The ether extracts were evaporated to dryness at 30°C under vacuum to obtain bound phenolics. Dried free, conjugated and bound phenolics were dissolved separately in 1 ml of methanol and stored at -4°C until analysis.

The total phenolics content was determined according to slight modifications to the method described by Singleton and Rossi [13]. A centrifuge tube containing 0.5 ml of methanolic extract was mixed with Folin-Ciocalteu's reagent (0.5 ml) and saturated sodium carbonate solution (1 ml). The volume of the mixture was adjusted to 10 ml using distilled water. The sample was allowed to stand at ambient temperature for 45 min until the blue colour developed and was then centrifuged at 4,000 g for 5 min. An absorbance of the clear supernatant was measured at 725 nm using a spectrophotometer (UV-1601, SHIMADZU). The phenolic content in each extract was expressed as µg ferulic acid equivalent/100g dry weight of the defatted sample (µg FAE /100g DW).

Determination of antioxidant activity

Antioxidant activity was determined by measuring the DPPH radical scavenging activity as described by Choi *et al.* [14] with some modifications. One gram of finely ground sample was mixed with 20 ml of 80% methanol and was shaken for 24 hours at ambient temperature (30°C) before being centrifuged at 8,000 rpm (6,720x g) for 25 minutes. The supernatants were filtered with Whatman No.2 filter paper and then evaporated at 40°C until dry. Dried extracts were re-dissolved in 80% methanol to a concentration of 4 mg/ mL and stored at -4°C until analysis. 0.4 ml crude extracts were mixed with 1.6 ml of 0.2 mM DPPH methanol and vigorously shaken. The reaction mixtures lacking DPPH were used as blanks. All mixtures were placed under subdued light for 10 minutes before measuring the absorbance at 520 nm using a spectrophotometer (UV-1601, SHIMADZU).

Experimental design and statistical analysis

A two factor factorial experiment was conducted and tested with a Randomized Complete Block Design (RCBD) with five replications. The factors were the cultivars (black and red rice) and the oxygen concentrations (0, 5 and 10%). The controlled atmosphere system was set up to obtain desired oxygen concentrations. The samples were kept in plastic containers filled with 0% O_2 (100% N_2), 5% O_2 (95% N_2) or 10% O_2 (90% N_2). Oxygen concentration in the containers was regularly checked with OXYBABY® V (WITT- GASETECHIK GmbH & Co HG, Germany). The data were collected at monthly intervals from each treatment.

Microsoft Excel was used to perform the descriptive statistics, including mean, standard deviation and regression analysis. All the measured parameters were expressed as means ± standard deviations from five replications. All statistical analyses were conducted using the SAS system for Windows v 6.12. The least significant difference (LSD) test was used to compare treatment means at $p<0.05$ level.

Results and Discussion

Total anthocyanins content

Recently, anthocyanins have been recognized for their health benefits such as antioxidant capacity and antimutagenic and chemopreventive activities. These activities contribute to the reduction of incidence of chronic diseases [11], neuronal and cardiovascular illness, cancer and diabetes, among others [15]. The total anthocyanins content of the black rice before storage was significantly greater than the content in red rice (Table 1) and it might be due to cultivar difference. The total anthocyanins content of black rice has been shown to vary according to cultivars or species [16, 17].

Intensity and colour stability of anthocyanins are influenced by several factors such as pH, structure, concentration, co-pigmentation, metal complexing, temperature, light, oxygen, acetaldehyde, ascorbic acids, sugars and their degradation products including sulphur dioxide, amino acids, catechin and some others [18]. Oxygen may cause degradation of anthocyanins by a direct oxidation mechanism and or by indirect oxidation whereby oxidized constituents of the medium react with the oxygen [11]. The total anthocyanin contents of both rice cultivars were not significantly altered by the different O_2 concentrations over the four-month storage period (Table 2) and were relatively stable during storage (Table 1). Oxygen and hydrogen peroxide easily oxidize anthocyanins [19]. A two-fold increase in the half-life values of hordeumin (a protein-tannin-anthocyanin complex) at pH 5.0, 6.0 and 7.0 was observed when the samples were kept under nitrogen flow [20]. Bordignon-Luiz, *et al.* [21] reported that the half life values of Isables grape samples kept in the presence of oxygen were lower when compared to the values of the samples kept under nitrogen flow. The result of this research was in agreement with this previous work. The stability of total anthocyanins content in this study might be due to the fact that lack of oxygen or low oxygen concentration in the package may cease or delay the physiological functions of the grain during storage. Besides, moisture content of the rice sample during storage was reduced to 7%, so it may cause the inactivation of enzymes which hasten anthocyanins degradation. It can be concluded that lack of oxygen or low oxygen concentrations can preserve the total anthocyanins during storage, although the effect of low O_2 concentrations in this study was not significant.

Table 1. Changes in total anthocyanins content of the black rice and red rice during storage.

Storage time (months)	Total anthocyanin content (mg/100g DW)	
	Black rice	**Red rice**
0	28.91±1.09	0.53±0.05
1	29.43±1.63	0.53±0.04
2	30.93±1.07	0.51±0.06
3	32.18±1.69	0.48±0.05
4	31.06±1.40	0.50±0.05
Cultivar	**	
Storage time	ns	
Cultivar×Storage time	ns	
$LSD_{0.05}$	2.73	
C.V. (%)	24.12	

Data were presented as means ± standard errors. Data represents 15 samples of each treatment. Ns=not significant.

Table 2. Changes in total anthocyanins contents of black and red rice stored under different O_2 concentrations.

O_2 concentration (%)	Total anthocyanins content (mg/100g DW)	
	Black rice	**Red rice**
0	30.48±1.18	0.57±0.03
5	30.26±1.13	0.51±0.04
10	30.77±0.97	0.42±0.05
Cultivar	**	
O_2 concentration	ns	
Cultivar×O_2 concentration	ns	
LSD $_{0.05}$	2.13	
C.V. (%)	24.12	

Data were presented as means ± standard errors. Data represents 25 samples of each treatment. Ns=not significant.

Polyphenol content

Phenolic compounds are excellent antioxidants by virtue of the electron donating activity of the 'acidic' phenolic hydroxyl group, which can stabilize and delocalize unpaired electrons within its aromatic ring [22]. Fardet *et al.* [23] suggested that these phenolic acids are believed to act mainly as free radical scavengers and/or chelators of transition metals (minerals or trace elements). Phenolic compounds are ubiquitous in cereals [24] and a wide range of phenolic acids, belonging mainly to the benzoic acid and cinnamic acid classes, contained in cereals [25]. Among the phenolic acids found in *Gramineae*, and in rice particularly, are ferulic acid and p-coumaric acid, the main molecules found in the cell walls [26, 27]. In whole grain cereals, ferulic acid can be found in the free, soluble conjugate or esterified and insoluble-bound forms [28]. The free, soluble conjugated and insoluble bound phenolic contents of the black rice were significantly higher than those of the red rice (Table 3). Shahidi and Naczk, [24] revealed that the phenolics content in plants depends on cultivation techniques, cultivar, growing conditions and ripening process. In addition, processing and storage conditions also influence the level of phenolics in plant sources. Storage time significantly influenced the changes in all phenolic forms of both rice samples (Table 3). Free, insoluble bound and total phenolic contents decreased as the storage time increased while soluble conjugated phenolics declined after the first two months of storage and gradually increased after that. These significant changes are consistent with the findings of Tsugita *et al.* [29] and Zhou *et al.* [27], who observe the oxidation of ferulate esters of hemicelluloses that causes a reduction in bound phenolics. The total phenolics decreased during storage, which agrees with the results of the free and insoluble bound phenolics because of the greater contribution of bound phenolics to the total phenolic content.

Fig. 1 shows the effect of oxygen concentration on free, soluble conjugated, insoluble bound and total phenolic contents of the black and red rice. The rice stored at 0% O_2 showed the highest free and soluble conjugated phenolic, whereas insoluble bound phenolic content was the highest in rice stored at 5% O_2. Total phenolic contents were not significantly different among different oxygen concentrations during storage.

The oxygen concentration did not significantly affect free, insoluble bound and total phenolic contents of the black rice, whilst significant effect was observed in soluble conjugated phenolic content of the black rice. The black rice stored at 0% O_2 maintained the highest soluble conjugated phenolics and it contributed to the highest total phenolics among all treatments. Contradictions were observed in the red rice samples. The highest free phenolic, and lowest bound phenolic contents were found in the red rice stored at 0% O_2, leading to the lowest total phenolic content compare to the others. The polyphenols were no different between both rice cultivars stored at 5 and 10% O_2 concentrations. Unfortunately, there is no available information on the effects of low oxygen storage on polyphenol contents in either coloured rice or cereals. There were no significant differences in total free phenolic of the mangosteen fruit when stored under low O_2 (0.25%) at 6°C (84% RH), or at room temperature (30°C, 71.5% RH) compared with fruit in normal air conditions [30]. Zheng *et al.* [31] studied the changes of strawberry phenolics in response to high oxygen treatments.

This study showed that total phenolic content in high O_2 treated and control fruit increased during the first 7 and 10 days respectively of the storage, thereafter they decreased gradually during the storage of 14 days. No significant differences in total phenolic content were found among all high O_2 treated and air control fruit throughout the experimental period. The data obtained in this work pointed out that rice samples stored at 0% oxygen concentration showed the minimum losses of the free and soluble phenolic contents during storage.

Table 3. Effect of storage time on polyphenol contents of the black and red coloured rice.

Cultivar	Storage time (month)	Polyphenol (µg FAE/100g DW)			
		Free	Soluble conjugated	Insoluble bound	Total
Black rice	0	12.61±0.59 a	23.96±1.12 bc	38.12±0.70 a	74.69±1.58 a
	1	10.77±0.32 b	21.08±0.65 d	33.95±0.59 b	65.81±0.52 b
	2	7.09±0.41 b	22.83±0.81 cd	31.029±0.79 c	60.95±0.90 d
	3	11.42±0.40ab	25.48±0.95 b	27.41±0.74 d	64.31±0.74 cd
	4	10.30±0.75 b	28.20±2.76 a	23.85±0.65 e	62.34±1.72 cd
Red rice	0	10.74±0.27 dc	15.44±0.80 e	24.42±1.12 e	50.60±1.21 e
	1	7.39±0.17 d	11.80±0.56 f	23.40±0.90 e	42.59±1.11 f
	2	11.09±0.73 dc	15.68±0.53 e	22.99±0.97 e	49.76±0.74 e
	3	8.37±0.50 dc	15.14±0.37 e	17.67±0.91 f	41.18±0.68 f
	4	6.55±0.46 d	14.94±0.62 e	12.59±0.81 g	34.08±1.48 g
Cultivar		**	**	**	ns
Storage time		**	**	**	ns
Cultivar× Storage time		**	**	**	**
$LSD_{0.05}$		1.36	2.53	2.35	3.13
C.V.(%)		16.84	16.84	8.94	7.33

Data were presented as means ± standard errors. Data represents 15 samples of each treatment. **$p \leq 0.0$, ns = not significant. Figures in the same small letter in the same column were not statistically different.

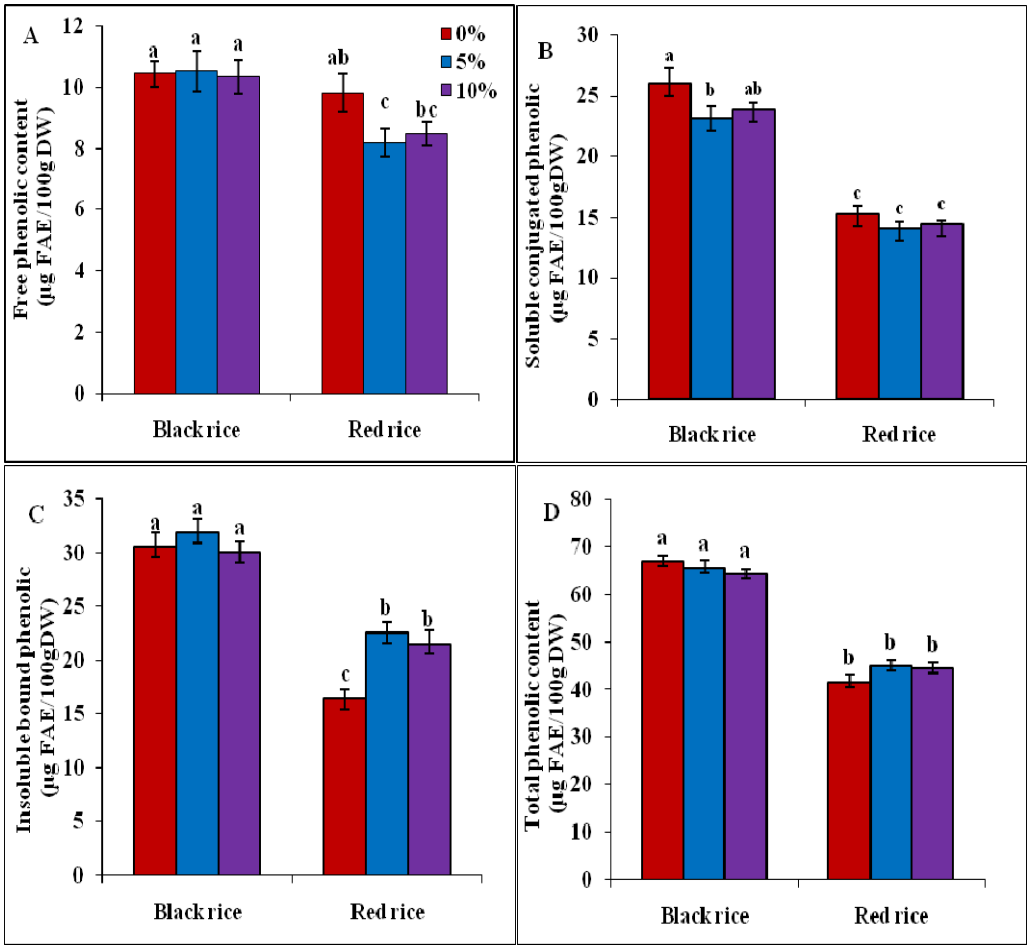

Figure 1. Effect of O_2 concentration on polyphenol contents of the black and red rice. Data represents 25 samples of each treatment. The error bar indicates the standard error of the treatment.

Radical scavenging activity

The antioxidant activities of the methanolic extract from the black and red rice were significantly different. The red rice had greater radical scavenging activity than the black rice (Fig. 2). The radical scavenging activity was significantly affected by storage time in both rice cultivars (Fig.2), but not by oxygen concentration (Table 4). Therefore, the data from all oxygen concentrations were combined and analyzed for the effects of storage time. A significant interaction between storage time and variety was observed in this study, but not the interaction between oxygen concentration and storage time. The antioxidant activities slightly declined during the stages of initial storage and then significantly increased after two months of storage. The highest antioxidant activities were observed in samples stored at 0% O_2 for black rice (87.05±0.70%) and for red rice (91.51±0.45%) after four months of storage. The radical scavenging activity of both cultivars increased during storage and increasing rates were significantly different between varieties. After four months storage, radical scavenging activity of the black rice increased by 10% of the initial value, while only a 6% increase was observed in the red rice.

Despite a decrease of polyphenols in rice samples, radical scavenging activity significantly increased during storage. It should be considered that, by using methanol as the extraction solvent, antioxidants other than phenolics, such as tocopherols, tocotrienols and γ –oryzanol are also extracted [32]. These antioxidants also exhibit radical scavenging activity against DPPH and leading higher radical scavenging activity [32]. In this study, slight but not significant increased amounts of total anthocyaninin in rice samples may also have contributed to increased radical scavenging activity during storage.

There were the linear relationships between total anthocyanins, phenolics (free, soluble conjugated and insoluble bound) and radical scavenging activity of the colored rice cultivars. The radical scavenging activity was significantly related with the insoluble bound phenolic (R^2=0.690, p<0.05), and total phenolic (R^2= 0.531, p<0.05) and. Only a weak correlation was observed with the free phenolic (R^2= 0.170, p>0.05), soluble conjugated phenolic (R^2= 0.120, p>0.05) and total anthocyanin content (R^2= 0.356, p>0.05).

Linear relationships between radical scavenging activity and insoluble bound, total phenolic content confirm that insoluble bound, total phenolic contents may be major responsible compounds for the radical scavenging activity against DPPH. On the other hand, weak correlation of free and soluble conjugated phenolics and total anthocyanin and radical scavenging activity indicated that small portions of soluble fractions contributed less to the antioxidant activity in coloured rice. Significant amounts of bound phenolics rather than free and soluble conjugated phenolics mainly contributed to the radical scavenging activity of coloured rice cultivars. Thus, this research finding demonstrated the importance of bound phenolicin in the radical scavenging activity of coloured rice samples.

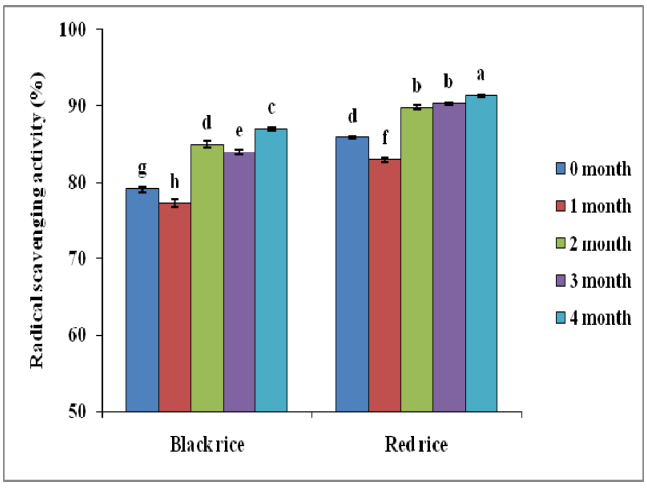

Figure 2. Changes in radical scavenging activities of the black and red rice cultivars stored for four months.

Data represents 15 samples of each treatment. The error bar indicates the standard deviation of the treatment. Point not shown the error bar is hidden by the symbol.

Table 4. Changes in radical scavenging activities of black and red rice stored under different O_2 concentrations.

O_2 concentration (%)	Radical scavenging activity (%)	
	Black rice	**Red rice**
0	82.98±3.63	88.07±3.13
5	82.38±4.12	87.99±3.35
10	82.06±4.27	88.12±3.58
Cultivar	ns	
O_2 concentration	ns	
F-test	ns	
LSD $_{0.05}$	2.09	
C.V. (%)	1.43	

Data were presented as means ± standard errors. Data represents 25 samples of each treatment. ns = not significant

Conclusions

The present study determined the changes in radical scavenging activity and bioavailable compounds of coloured rice under low oxygen storage. The research data point out the importance of oxygen concentration in the packaging on retaining the radical scavenging activity and bioactive compounds of coloured rice cultivars during storage. The results show that storage time and low O_2 concentration had strong influences on radical scavenging activity, the total anthocyanins and polyphenol contents of coloured rice cultivars. Rice stored at 0% O_2 can preserve antioxidant activity resulting in maintaining the minimum losses of bioactive compounds during storage. The research findings will help develop guidelines for new packaging design to preserve the antioxidant properties of rice for the consumer, rice traders and growers. Further research is needed to examine the effects of aging and oxygen concentration on non-phenolic antioxidant compounds, such as vitamin E, carotenoids, minerals and trace elements. Moreover, this study also points towards further studies to understand the changes of individual anthocyanin and phenolic acid profile during storage under controlled atmosphere.

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