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ORIGINAL PAPER

# Variations in Bran Carotenoid Levels within and between Rice Subgroups

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**Abstract** Rice (*Oryza sativa* L.) is a major grain in the human diet and carotenoids are valuable antioxidants. However, little is known about varietal differences in the carotenoid contents of the rice bran. The objective of this study is to determine the relative differences in bran carotenoid levels among all the five subgroups of rice. Measurements were made by a recently described, rapid non-destructive fluorescence quenching method. Confirmation by high performance liquid chromatography (HPLC) after solvent extraction of the bran indicated that the major carotenoid was lutein. Our data showed that carotenoid levels were stable over 10 years of storage. Tropical japonica rice, the most consumed subgroup in the United States, tended to have the lowest levels of carotenoids in the bran while temperate japonicas had the highest. These differences in carotenoid content may open up new opportunities for identifying or breeding rice varieties with higher nutritional value.

**Keywords** Rice (*Oryza sativa* L.) · Bran · Carotenoids · Lutein · Rice subgroups · Fluorescence spectrometry

## Introduction

For humans, carotenoids are essential nutritional components for healthy skin and eyesight and their antioxidant characteristics have been demonstrated to improve human health [for reviews, see 1, 2]. Deficiencies in dietary carotenoids particularly vitamin A precursors in humans can lead to xerophthalmia, blindness, and even premature death. Carotenoids other than vitamin A precursors have been found to serve as lipophilic antioxidants and have important roles in the prevention of age-related macular degeneration, atherosclerosis, and some cancers. These carotenoids include lycopene, lutein, and zeaxanthin, and their oxidative metabolites.

Removal of the hull from a rice kernel leaves brown rice which is comprised of the bran, embryo, and endosperm. The bran is about 5–7% (*w*/*w*) of the brown rice [3]. Carotenoids are known to be a constituent of rice bran [4–10].

Historically, rice has been classified into subgroups according to geographical location, cropping systems, and weather patterns to which the rice is adapted. The five subgroups are indica, tropical japonica (TRJ), temperate japonica (TEJ), aus, and aromatic. The indica and japonica subgroups can be differentiated by chlorate resistance, phenol reaction, apiculus hair length [11], cold-tolerance, grain length, and disease resistance [12]. These separations are largely borne out by molecular studies. Garris et al. [13] used simple sequence repeats (SSR) to divide rice from across its geographic range into the five subgroups. Another SSR study, of U.S. rice cultivars [12], largely confirmed the long-standing subgroup classifications of the cultivars.

Understanding the source and structure of genetic diversity provides a means for breeders to understand and utilize the genetic variation that is available [12]. The breeders should current survey is information rice breeders should find of interest in their efforts to improve the nutritional value of rice by increasing its antioxidant capacity. We found that the level of carotenoids varies among and within rice subgroups. The development of a carotenoid measurement technique

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that can be used on small samples provided a means of surveying a large number of rice cultivars for bran carotenoid levels. The technique utilized to generate the data for this survey could be useful to breeders developing nutritionally enhanced rice as it is sensitive enough to monitor the carotenoid content of a single rice seed and, by being non-destructive, allows any high content seeds to be sprouted and grown into mature plants for breeding purposes.

Using this method, the rice subgroups and cultivars can be compared for their further use as breeding material to improve the nutritional value of brown rice.

## Materials and Methods

### Seeds for Extraction of Bran for High Performance Liquid Chromatography (HPLC) Analysis

The carotenoid levels were measured by fluorescence spectrometery of dehulled Ponta Rubra, Spring, Bengal, and Teqing rice seeds ($n$=8) to ensure that a range of levels were present. These seeds had been produced in different years and stored as paddy rice for 1, 1, 3, and 8 years, respectively. The rice bran was removed from the endosperm using a Pearlest milling device (Kett, Tokyo, Japan). Four grams of bran were ground with a mortar and pestle in 5 ml of tetrahydrofuran (THF) in which carotenoids are highly soluble [14]. The liquid was decanted into a glass centrifuge tube with the solid bran retained in the mortar and the grinding repeated with a second addition of THF. The liquid and solid material were combined in the glass centrifuge tube and placed in a sonication bath for 1 min, then stored overnight at 4 °C. The sample was then centrifuged (1,500 rpm for 15 min) and the supernatant decanted into a clean glass conical tube. THF was added to rinse the bran and decanted into the conical tube. Five ml THF was again added to the bran, vortexed, and both tubes were stored overnight at 4 °C. The next morning, the tube with the bran was centrifuged at 1,500 rpm for 15 min. The supernatant was decanted into the conical tube containing the previously collected extractions. The extraction was concentrated by vacufuge and the extraction was brought to a final volume of less than 400 μl for measurement by HPLC.

### HPLC Analysis

For HPLC analysis, dried samples were brought up to 400 μl with THF. These solutions were placed into an ultrasonic bath for 5 min and then centrifuged at 14,000 rpm for 5 min. Then 200 μl of the samples were filtered through a 0.45 μm nylon filter (13 mm) and 20 μl were injected into the HPLC. Separation was performed on a Chromquest system (Thermoquest, San Jose, CA) using a Prevail (Alltech, Deerfield, IL) C18 column (250 mm× 4.6 mm, 5 μm). The mobile phase consisted of a gradient of 90% MeOH:10% THF (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml per min. Solvent A was initially at 10% for the first 3 min, ramped to 90% from 3 to 12 min and remained so for a further 2 min. The following calibration standards were used: astaxanthin, canthaxanthin, lycopene, lutein, zeaxanthin (Roche, Parsippany, NJ), and β-carotene (Sigma, St. Louis, MO). The detector utilized was a model UV6000LP Photo Diode Array (Thermo Separation Products, San Jose, CA) and chromatograms were monitored at 450 nm. Since lutein was the predominant carotenoid present, levels of of carotenoids were evaluated by integration of the lutein peak.

### Seeds for Screening for Relative Carotenoid Levels

The measurement of bran carotenoids by subgroup used rice grown in the field in 2008 in Stuttgart, AR. Rice was harvested, dried, and stored at room temperature until measurement. Rice was dehulled manually or by using a TR200 dehuller (Kett, Tokyo) prior to carotenoid measurements of the bran.

### Fluorescence Spectroscopy

A non-destructive synchronous fluorescence (or luminescence) technique [8], was utilized to determine the presence of carotenoids in rice bran. Plant cells are highly autofluorescent while carotenoids have a high absorptivity, which allows them to quench the natural fluorescence of the rice cell. Fluorescence levels were measured from 400/410 nm to 600/610 nm using a wavelength difference of 10 nm on single kernels of rice with their bran left intact (i.e., unmilled or brown rice). The spectrum was integrated from 505 nm to 565 nm to obtain a net integrated fluorescence intensity (NIFI) value that reflected the relative amount of carotenoids present.

### Stability of Bran Carotenoids

A test was carried out to determine the stability of carotenoids in the bran of rice stored as intact kernels. Rice from Kaybonnet, for which intact kernels of different ages stored at 4 °C was available, was tested. Kernels were dehulled just prior to carotenoid measurement by fluorescence spectrometry. Measurements were made of 8 kernels from each time point.

### Screening of Rice by Subgroup

Numerous methods have been used to categorize rice by subgroup. Only rice cultivars that could be verified as to subgroup in the literature were utilized (Table 1).

**Table 1** Rice subgroup and reference for cultivars used. Subgroups are temperate japonica (TEJ), aromatic, indica, aus, and tropical japonica (TRJ)

| Subgroup | Cultivar | Reference |
|---|---|---|
| TEJ | Aichi Asahi | [13] |
| TEJ | Mansaku | [13, 37] |
| TEJ | M401 | [12, 38, 39] |
| TEJ | Colusa | [12, 39] |
| TEJ | Jouiku 393 | [13] |
| TEJ | NPE 835 | [13] |
| TEJ | Agostano | [13] |
| Aromatic | Kitrana 508 | [13] |
| Aromatic | Tchampa | [13] |
| Aromatic | Bico Branco | [13] |
| Aromatic | Cuba 65 | [13] |
| Indica | Pin Kaeo | [13, 37] |
| Indica | GP-2 | [40] |
| Indica | R312 | [40] |
| Indica | Taducan | [12, 13, 37] |
| Indica | Xiangzaoxian No. 1 | [41] |
| Indica | Zanuo #1 | [40] |
| Indica | IR-8 | [12, 13] |
| Aus | Phudugey | [13] |
| Aus | DV 85 | [13, 37] |
| Aus | Dhala Shaitta | [13, 37] |
| Aus | Kalamkati | [13] |
| Aus | BJ1 | [13, 37] |
| Aus | Arc 10352 | [13] |
| Aus | Black Gora | [13] |
| TRJ | Wells | [13, 40, 42] |
| TRJ | Newbonnet | [38–40, 42] |
| TRJ | Rexoro | [13] |
| TRJ | LaGrue | [40, 42] |
| TRJ | Drew | [40, 42] |
| TRJ | Kaybonnet | [40] |
| TRJ | Fortuna | [13] |
| TRJ | Lemont | [13, 38–40] |

### Statistical Analysis

Data was analyzed statistically using one-way ANOVA. Significance was accepted at the 5% level. A Fisher's protected LSD was performed to determine the significant difference between the results. Significance was accepted at the 5% level.

## Results and Discussion

### Stability

In order to have enough seed to extract the bran for HPLC, we used individual cultivars that had been harvested in a different year and stored since then. To ensure that source materials from different storage times could be compared, the stability of carotenoids in the bran of dehulled kernels was measured. The carotenoid levels of seed that had been stored for various lengths of time were stable over 10 years of storage as paddy rice at 4 °C (Fig. 1).

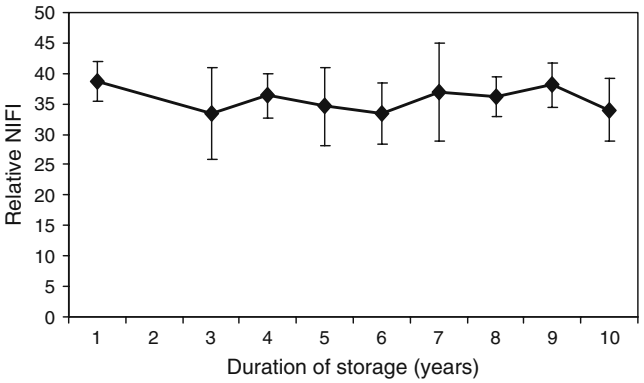


**Fig. 1** Carotenoid levels in rice bran of Kaybonnet over 10 years of storage. Bars indicate standard deviations. Means were not significantly different at the 5% level

The degradation of carotenoids dissolved in solvents by oxidation and light is rapid, being measured in hours, and usually results in the complete breakdown of the carotenoids in the absence of protective antioxidants [15, 16]. However, the stability of carotenoids contained in plant material is much higher. The decline in carotenoid contents of wheat seeds [17, 18] and flour [19] is slow, being measured in weeks and months, and then the carotenoids are not completely degraded. Wheat seeds still retained 40% of their carotenoids at two weeks under accelerated aging (high temperature and humidity) conditions [18]. Cereal grains (wheat, barley, rye, corn, and oats) retained from 27 to 64% of their carotenoids over four months of storage after being milled [17]. Menkir et al. [20] and Quackenbush et al. [21] found that carotenoid levels were similar among yellow corn samples harvested at different

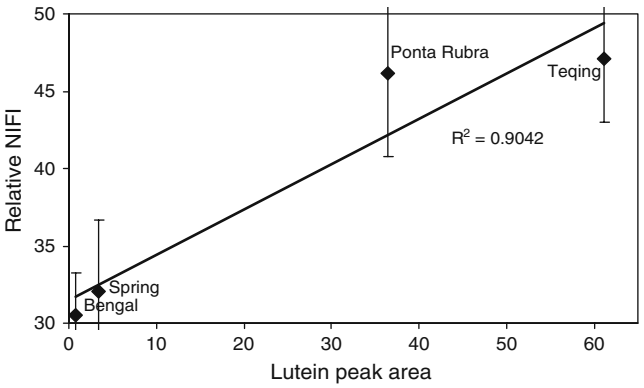


**Fig. 2** Comparison of carotenoid measurements by high performance liquid chromatography (HPLC) and by fluorescence quenching. Bars indicate standard deviations

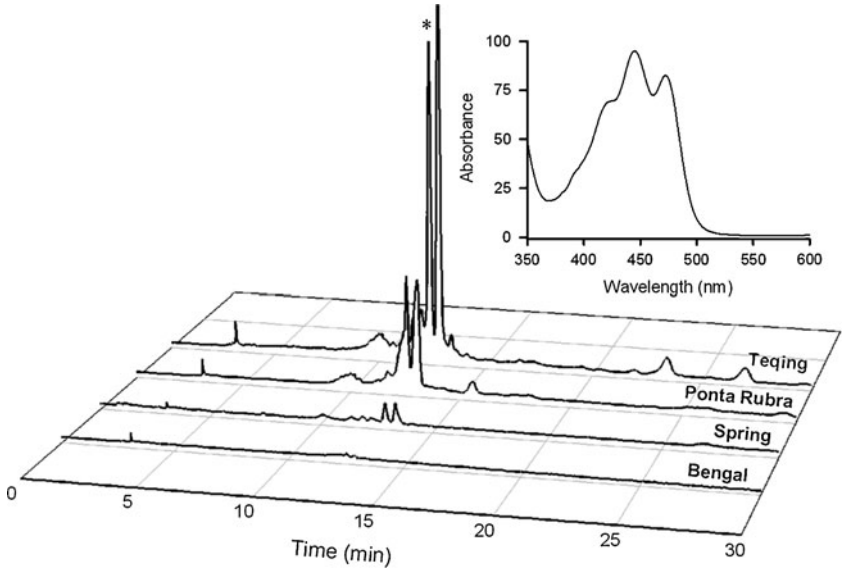


**Fig. 3** High performance liquid chromatography (HPLC) chromatograms of carotenoids from four different rice cultivars. The starred peak is lutein, shown in inset

locations and years. On average, over 90% of the lutein was retained in corn through three years of storage at 7 °C [22]. Even when stored as whole or white flour for eight months, lutein or total carotenoids in wheat flour was reduced a maximum of 50% at room temperature, and less at cooler temperatures [23]. Our results indicate that bran carotenoid levels are stable for years when the rice is stored at cool temperatures with the covering hull intact (Fig. 1).

## Identification of Carotenoids

Previous calculations [8] showed that the amount of carotenoid measured by fluorescence quenching was comparable to that measured by extraction, although bran extraction measures carotenoids in the entire bran while fluorescent quenching only measures the carotenoids present on the surface of a small area of the bran. Measurements of bran in cultivars previously determined to have higher levels of carotenoids by fluorescence quenching had correspondingly higher levels when extracted and measured by HPLC. The brans of four cultivars representing a range of carotenoid contents were extracted and analyzed by HPLC and the results were compared to the relative amounts found by fluorescence spectrometry (Fig. 2). The values corresponded well, especially at low levels of carotenoid.

In each of the four cultivars, the primary carotenoid in the bran was identified as lutein (Fig. 3, starred peak). Although efforts were made to ensure that kernels were mature and not still green, the second large peak was

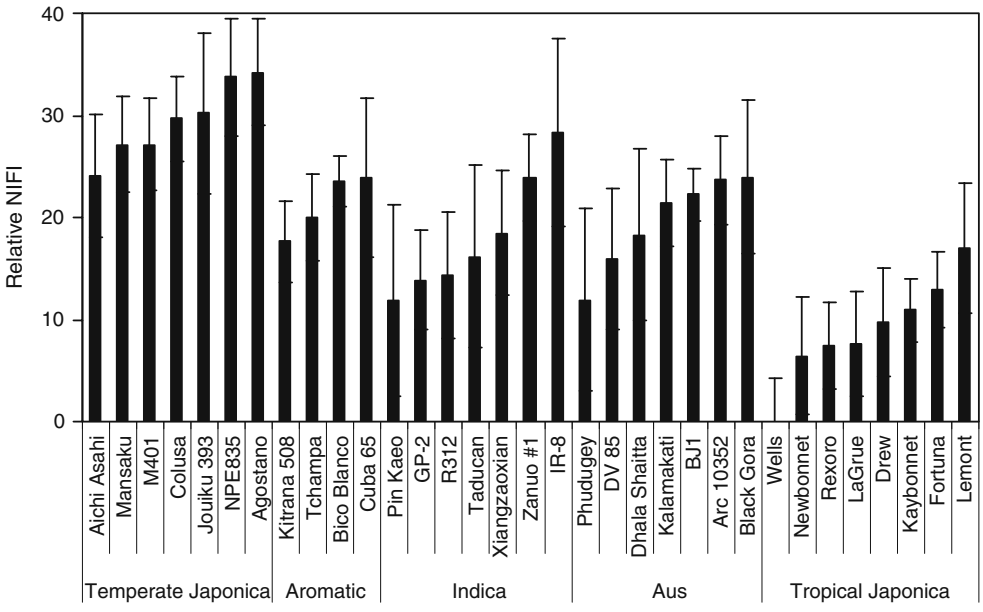


**Fig. 4** Relative carotenoid levels in bran of cultivars in the five subgroups of rice. Bars indicate standard deviations. Fisher's protected LSD=1.80 ($p<0.05$)

identified as chlorophyll b. Depending on the cultivar, a variable number of smaller peaks had absorption spectra indicative of carotenoids and eluted in the region for carotenoids. Though the peaks did not exactly correspond to the retention time of any of our standards, these minor peaks had characteristics of β-carotene or zeaxanthin.

Other cereal grains have been found to contain lutein: yellow corn [20, 24], black barley [25, 26], bread and durum wheat [25, 27], triticale [27], oat, and spelt [25]. The levels of lutein can vary greatly among varieties of wheat [27–29] and corn [20, 24, 30]. Wild rice (*Zizania aquatica* L.), however, contains violaxanthin and β-carotene but does not contain lutein [31].

The amount of total carotenoid measured and the types of carotenoids identified in rice bran varies among cultivars. Lamberts and Delcour [32] found primarily lutein and smaller amounts of zeaxanthin and β-carotene in the bran of five cultivars. Trace amounts of lycopene were also detected in some of the samples. Seven of the eleven cultivars analyzed for carotenoids by Tan et al. [33] contained lutein and β-carotene. The other four cultivars either had no carotenoids, unidentified carotenoids, and/or β-carotene or lutein alone. The bran of several high yielding rice cultivars contained carotenoids of which β-carotene was a small percentage in a subset of the cultivars [34]. Aruna et al. [35] did not find lutein in commercial rice bran oil. Abdul-Hamid et al. [10] found lycopene and β-carotene in the bran from bulk rice obtained from a commercial mill and Frei and Becker [7] measured β-carotene in colored and non-colored Asian landraces. Abdul-Hamid et al. [10] identified β-carotene and lycopene in rice bran obtained from a local mill; Nakornriab et al. [36] identified β-carotene in the brans of two Thai black rices.

## Diversity in Levels of Bran Carotenoids

Cultivars were selected and grown in the field in 2008 for each of the five subgroups of *Oryza sativa* that are cultivated and consumed: TRJ, TEJ, indica, aus, and aromatic (Table 1). The tabulated data were organized into categories of rice by subgroup with cultivars ranked from low to high relative bran carotenoid content. As shown in Fig. 4, each rice subgroup has a range of low to high carotenoid containing lines. The presence of carotenoids in the bran is not limited to a rice subgroup; each subgroup has lines available for breeding carotenoids into bran in order to increase antioxidant levels in the brown rice.

The subgroups have been shown to be genetically, physiologically, and morphologically separable. Most cultivated rice is divided between indica or japonica. Indica are typically lowland rices from the tropics that are nonsticky when cooked [13]. Japonicas are grown in the U.S. and are divided into TRJ and TEJ for the regions from which they were developed and are primarily what is grown in the United States. Aus and aromatics are genetically distinct from indica and japonicas. The aus are drought-tolerant, early maturing rice cultivars while the aromatics have a popcorn-like scent [13]. The tightness of the range of carotenoid levels in these two subgroups may be representative of the recency of the population bottleneck that resulted in these subgroups, while the diversity of carotenoid levels within all the subgroups, may be accounted for by the lack of selective pressure for rice bran to contain or not to contain carotenoids [13].

As described here, the interplay of the strong fluorescence of the rice kernel and the strong absorptivity or fluorescence quenching properties of the carotenoids provided a highly sensitive and non-destructive method to qualitatively detect the presence of carotenoids in rice bran. Although HPLC allows identification of specific carotenoids, it requires relatively large amounts of rice to obtain enough bran for extraction. The fluorescence method alleviates the need for large amounts of rice so that estimates of carotenoid levels can easily be obtained on a large number of small samples. The fluorescence method also reduces the concern for degradation of carotenoids during extraction and clean up and is far quicker to obtain than the more labor-intensive HPLC procedures.

A major obstacle to the utilization of rice bran as a food or food additives is its tendency to degrade and become rancid [6]. Enzymes, both of plant and microbial origins, are the major cause of bran deterioration. Lipases and oxidases are localized in the testa-cross layer region. Once this region is disrupted during hulling and milling, enzymatic hydrolysis is possible and microorganisms present on the hull then also have access to the bran. As long as the rice is stored as whole seeds, carotenoids in the bran are stable over several years of storage.

It appears that particular subgroups tend to have certain relative bran carotenoid levels. The temperate japonicas measured had the highest bran carotenoid levels while the tropical japonicas had the lowest measurements. Nonetheless, the levels measured here indicate enough variation that promising candidates for breeding to increase antioxidants in brown rice are available in each subgroup.

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