Phytochemical composition of *Oryza Sativa* (Rice) Bran Oil Bodies in crude and purified isolates

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ABSTRACT: In this paper, we describe a procedure for isolating and purifying oil bodies (OBs) from *Oryza sativa* bran, and present evidence that strongly suggests a physical association between the OB organelles and several anti-oxidant phytochemicals (γ-oryzanol and several tocochromanols). This in turn provides a valuable comparison with similar analyses of tocochromanols in other plant species, as well as indicating that rice bran, normally a waste product from the rice industry, may provide a valuable source of anti-oxidants as well as protein and unsaturated fat, for mammalian consumption.

KEYWORDS: Food chemistry/Biochemistry

INTRODUCTION

Oryza sativa, Asian rice, in common with many other food crops harvested for their seed, contains a significant quantity of fatty-acid-based energy storage molecules. Typically, the fatty acid residues contained within these triglycerides are *mono*- or *poly*-unsaturated and are thus sensitive to oxidative degradation (become rancid). The rate of this oxidation is a function both of exposure to oxygen in the air and to the number of unsaturated (olefin) bonds present in the fatty acid residues.

The facile oxidation of *poly*-unsaturated fatty acid residues that gives rise to rancid fats is therefore at odds with the observation that seeds containing such *poly*-unsaturated fatty acids are able to exist in a stable, unoxidised state for some time before germination. This begs the question of what mechanism is employed by plants to protect their triglycerides from oxidation. An obvious chemical candidate is the group of lipophilic compounds related to tocopherol (vitamin E), called tocochromanols, and one steroid-based anti-oxidant, γ-oryzanol. These are known phytochemicals that have been isolated from plant species (1,2) and are amongst other phenolic compounds in the seeds of plants (3-5).

Rice bran, a by-product from rice milling, is 15-20 % fatty acids, depending on the variety of *Oryza* and type of milling (6). They are known to contain the anti-oxidants noted above although levels recorded are influenced by the origin of the material (7), bran processing (8) and the method of extraction and purification (9, 10). As well as anti-oxidant properties, they also help to maintain the stability of cellular membranes, and prevent intracellular lipid oxidation (11).

The fatty acids produced by plants are stored as triglycerides in organelles called oil bodies (OBs) (12, 13). Such OBs serve as an energy source for germination and radicle growth. OBs are composed of a triglyceride (fat) core surrounded by a *mono*-layer of complex lipids embedded with proteins called oleosins, caleosins and steroleosins (14). Transmission electron microscopy has shown that the highest concentrations of OBs are located within the aleurone, sub-aleurone and germ, and not the starchy endosperm of oat and rice grain (15, 16). However, OBs have not been isolated from rice bran, nor has any attempt been made to establish their phytochemical composition. Thus we report two methods for isolating rice bran OBs. This has allowed us to determine their phytochemical content and provides evidence for a physical association between tocochromanols and γ-oryzanol in OBs of rice bran.

MATERIALS AND METHODS

Plant materials

Oryza sativa (Basmati brown rice) from India, 2007 was purchased from East End Food plc. (Birmingham, UK). The grains were milled using NW1000 Turbo rice mill (Natrawee technology,

Bangkok, Thailand). The bran was sieved through a 600 μm screen immediately and before further processing.

Transmission electron microscopy

Electron micrographs were prepared in an analogous manner to a previous report (16). Samples of the resulting bran (~1 mm thick) were fixed in freshly made 2·5% glutaraldehyde in cacodylate buffer (0·05 M, *p*H 7·4) and washed with freshly made cacodylate buffer. The samples were then post-fixed in 2% osmium tetroxide before being embedded in Spurr resin and polymerized. Thin sections (0·5 μm) were cut and stained with toluidine blue for light microscopy before ultra-thin sections were selected and mounted on copper grids and stained using uranyl acetate and lead citrate for electron microscopy (JEOL 1010 TEM; JOEL Ltd., Herts, UK).

Isolation and washing of oil bodies

Rice bran (100 g) in 500 mL distilled water was mixed on a roller mixer at 50 rpm for 1 h before homogenisation (2 min, Krups blender, maximum speed). The slurry was filtered through three layers of cheese cloth and the filtrate centrifuged (Beckman Coulter, London, UK) at 10,000 *g* for 20 min at 5 ºC. The upper-most layer (COBs) was collected, resuspended in 9·0 M urea (1:4, *w/v*), vortexed vigorously and centrifuged (10,000 *g*, 20 min, 5 ºC). The remaining mass was washed three more times with distilled water in the same manner to furnish OBs (referred to hereafter as purified OBs). Water washed OBs (*Figures 2*) are purified in the same manner, without suspension in any aqueous solution of urea.

Compositional analysis

The moisture content was determined by drying the sample (~200 mg) to constant mass in a vacuum oven at 40 ºC (48 h). The lipid (phospholipid and triglyceride) content of the dried oil body sample was determined gravimetrically by repeated extraction with *iso*-octane (3 × 500 μL) using a Mini-Beadbeater-16 (Biospec, OK, USA) for 30 sec at the shaking speed of 3,450 oscillations/min at room temperature. The lipid extracts were evaporated under nitrogen stream to dryness. Protein

content of the remaining material was measured using a bicinchoninic acid (BCA) assay (17) calibrated against bovine serum albumin (BSA).

Protein analysis by SDS-PAGE

The proteinaceous material from dried oil body samples from which the water and lipidic material had been separated was denatured using Laemmli's solution (Bio-Rad Laboratories, Herts, UK, 190 μL/mg protein isolate) and β-mercaptoethanol (Bio-Rad, 10 μL/mg protein isolate) at 95 °C and chromatographed by SDS-PAGE using 4-20% gradient Protean® Gel Tris-HCl Gel (Bio-Rad). The gels were stained with Imperial Bio-Rad Coomassie blue (R-250). Excess stain was removed using ddH_2O and imaged using a Bio-Rad GX-800 densitometer.

Fatty acid composition

The lipid (phospholipids and triglycerides) fraction separated from the rest of the OB material, vacuum-oven-dried and then dissolved in chloroform (0·01 g/mL), was converted to fatty acid methyl esters using trimethylsulfonium hydroxide (0·25 M in methanol) using an established method (lipid: trimethylsulfonium hydroxide 5:1 *v*/*v*) (18). A gas chromatograph (Trace GC Ultra, Thermo Scientific, Loughborough, UK) equipped with a mass spectrometer (DSQ II Single Quadrupole GC/MS, Thermo Scientific, Loughborough, UK) was employed, with a flame ionization detector (FID) and auto injection system (CTC Analytics, Essex UK) and operated in the splitless mode. One microlitre of sample was injected into a capillary column (Phenomenex Zebron ZB-FFAP, California, USA) 30 m × 0·25 mm I.D. coated with nitroterephthalic acid modified polyethylene glycol (0·25 μm film thickness). Injection temperature was 200 °C. The oven temperature was initially held at 120 °C for 1 min and increased to 250 °C at a rate of 5 °C/min for 4 min. The carrier gas was helium. Retention times of standards (Supelco 37 Component FAME Mix, Supelco, PA, USA) were used to identify detected fatty acid esters. Identification was also verified by comparing mass spectra with standard library through the Thermo Scientific Xcalibur software programme. Methyl heptadecanoate (250 μg/mL) was used as an internal standard.

Extraction and quantification of tocochromanols and γ-oryzanol

Phytochemicals were extracted from dried oil body samples (~200 mg), using methanol 3 × 800 μL containing 1% butylated hydroxyl toluene through agitation in a mini-Beadbeater-16 for 30 sec at 3,450 oscillations/min and then analyzed by using RP-HPLC as described previously (19). Samples (20 μL) were injected with a total run time of 35 min at 25 ºC. Identification and quantification were made using calibration curves prepared from standards of α-, β-, γ-, and δ-tocopherols and tocotrienols (2-15 μg/mL) (Sigma, Gillingham, Dorset, UK) and γ-oryzanol (50-300 μg/mL) (Tokyo Chemical Industry UK, Oxford, UK). Waters Millenium®[32] Chromatography Manager Software (version 3·0) was utilized for data analysis.

Total phenolic content (TPC)

The TPC of a methanolic extract of dried oil bodies was determined using a modified procedure based on Folin-Ciocalteau method (19). Samples were left at room temperature for 30 minutes before measuring absorbance at 750 nm with gallic acid as a standard. Results are expressed as gallic acid equivalents per g of lipid (mg GAE/gm lipid). Error values are based on the standard error of measurements (n = 3) for n = 3 samples. Error values quoted are propagated from the standard error of each n.

RESULTS AND DISCUSSION

In order to determine the size of rice bran OBs *in vivo*, images of this system were taken using Transmission Electron Microscopy (TEM). The OBs appear as light grey spherical droplets in brown rice (*Figure 1A*) and rice bran (*Figure 1B*), and were all 0·5-1·0 μm in diameter. In general, OBs were observed in the aleurone and sub-aleurone layers (*Figure 1*). The distribution of OBs observed is in agreement with previous studies on OBs from *Oryza* species (15,20).

It should be noted that the bran was richer in both protein (15·0 ± 0·65%), and lipids/triglycerides (16·3 ± 0·52%) than brown rice (8·9 ± 0·63% and 2·5 ± 0·15% respectively) *and* white rice (8·6 ± 0·75% and 0·6 ± 0·03%, respectively). Yields of OBs from whole-grain basmati

brown rice and white rice proved low and thus these sources were judged to be too inefficient for commercial application. The focus of this investigation was therefore on OBs isolated from the bran only.

In order to determine both the presence of tocochromanols and γ-oryzanol and any physical association with OBs quantitatively, several steps were required. First, the OBs were separated from the bran and purified. The success of this step was monitored by determining the protein, lipid and triglyceride composition of the isolates (compositional analysis). The purification process was successful in removing proteinaceous material not associated with OBs, leading to a relative increase in lipid content (*Figure 2*). This analysis was supported by determining the protein composition (using gel electrophoresis, *Figure 3*) and the fatty acid profile of the isolate (*Figure 4*). Finally, RP-HPLC was used to determine the presence of tocochromanols and γ-oryzanol in given material, measured against an independent standard based on commercially available samples of these anti-oxidants.

Although high concentrations of urea are known to have a denaturing effect on proteins (22), it appears that proteins associated with OBs are perhaps not as sensitive to this effect as others, since the OBs remain intact despite repeated treatment with concentrated urea solution. Gel electrophoresis of the purified protein fraction arising from the purified OBs (*Figure 3*) showed several bands, including ones relating to masses of the known isoforms of oleosin (16-18 kDa, bands H and J). Other masses include the larger ones observed (50+ kDa, bands A-C), the medium-sized ones (33-34 kDa, band E) and smaller ones (10 and 13 kDa, bands K and L). Whether or not the unknown bands provide evidence for aggregations (dimers, trimers *et cetera*) of known proteins, unknown OB proteins or fragments of known proteins respectively is not clear. However the fact that several other bands from the purified OB material are consistent with the known composition of OBs (23, 24) (*Figure 3*) suggests that the OBs have been purified correctly.

Like the protein composition, the fatty acid composition of purified OBs is similar to the fatty acid composition of the crude OBs and thus washing the crude oil body material has no effect on it (*Figure 4*). The lipid fraction was extracted from crude OBs using *iso*-octane, hydrolysed and methylated before purification to afford fatty acid methyl esters. The data is shown in *Figure 4* and indicates that around 75% of fatty acids are unsaturated, with no evidence for oxidation of unsaturated

fatty acids (as measured by GC-MS). This indicates that the OBs that were isolated remained intact during purification, as no oxidation or loss of fatty-acid-derived material was observed.

The retention of tocopherols, tocotrienols and oryzanols in purified OBs compared with the starting bran material is shown in *Table 1*. Notably, concentrations of tocochromanols and oryzanols with respect to total lipid content both decline between that in bran and that in crude oil bodies, and from crude oil bodies to purified oil bodies. However, there is a higher retention of tocotrienols (41%) compared with the tocopherols (17%) between purified OBs and the *Oryza sativa* bran they were isolated from. This also suggests that, *in vivo*, there is an enrichment of tocotrienols in the OB compared with tocopherols. Certainly, there is reduction of >90% in total phenolic compounds, with >80% of the total phenolic compounds being removed during the urea-washing step alone. This demonstrates that there is a significant pool of phenolic compounds that are removed during washing steps along with extraneous proteins. Additionally, these data demonstrate that the step in which the OBs are washed in urea results in some loss in tocopherols and tocotrienols (27% and 23% respectively), and oryzanol (9%). This in turn suggests that a quarter of the tocochromanols and about a tenth of the oryzanols that are present in the bran are either not as strongly linked to the OBs as the phytochemical fraction that remains after purification, or are not associated with OBs at all. Further, the reduction in total phenolic compounds (*Table 1*) is relatively large with respect to the reduction in tocochromanols. Before purification (*i.e.* in the bran), tocochromanols represent <7% of the total phenolic fraction. After purification, this group of compounds represents around a third of the phenolic compounds that remain.

The possible physical association of tocochromanols and especially γ-oryzanol with OBs *ex vivo* suggested by these results agrees with a similar study of *Avena sativa* that posited an intrinsic association between tocochromanols and OBs in the seeds in that species (16). The retention of the major tocochromanol isomer in *Avena* and *Oryza* species during OB isolation is at odds with the retention-during-purification data for some other seeds for which data has been published to date, *viz.* *Echium plantagineum* and *Helianthus annuus* (*Table 2*, 26, 27). Taken together, these data (*Table 2*) suggest that cereals (*Avena* and *Oryza*) are enriched in tocotrienols, whereas the oilseeds (*Echium* and *Helianthus*) are enriched in tocopherols. Overall the profile of tocochromanol isomers in the plant

material (seed/grain/bran) is reflected in the oil bodies whether they are crude or washed, although, as mentioned above, the rice bran oil bodies show a measureable increase (73% - 86%) in γ-tocotrienol. An estimate of total tocochromanol present in the purified oil bodies can be made on comparing the concentration of these phytochemicals per unit mass of lipid in the starting material with that in the purified OBs. This calculation strongly suggests that the majority of oil seed tocochromanols are strongly associated with oil bodies, which appears not to be the case for the cereals for which data is available.

The placement of these anti-oxidants in OBs *in vivo* may help to explain the oxidative stability of the OBs *ex vivo* (26,28-30). However, unlike dormant oil seeds, it is notable that *Oryza* bran material is prone to oxidation through the release of fatty acids due to the action of lipase enzymes. What is not clear is the intracellular origin of these fatty acids and whether any are derived from the OBs themselves.

Rice bran is currently a waste product from the rice industry, thus OBs recovered from rice bran *via* a simple wet milling process provides a rich source of these anti-oxidants as well as unsaturated fat and protein. This material is also in a format (OBs) that has potential as a functional food ingredient as it is easy to handle, and is in a natural form that allows emulsification simply by dispersing the oil bodies in an aqueous medium. This reduces the number of ingredients required to produce food products from it and offers a novel food ingredient that will offer a reduced carbon footprint with enhanced label credentials.

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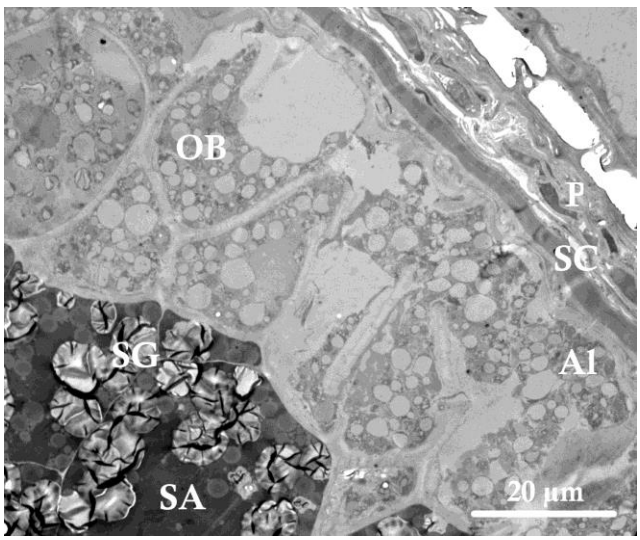


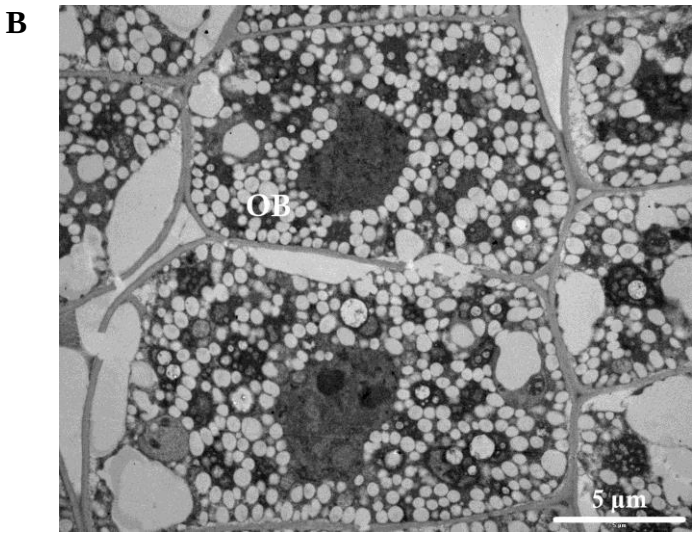


Figure 1. Electron micrograph of oil bodies in (A) brown rice surface and (B) aleurone layer of rice bran. OB: Oil body P: Pericarp, SC: Seed coat, Al: Aleurone, SA: Sub-aleurone, SG: Starch granule

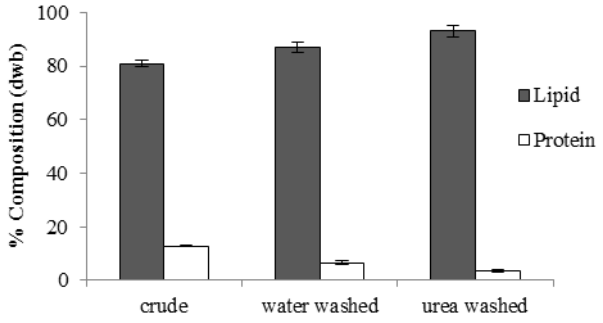


Figure 2. Lipid and protein levels (% dry weight basis) in crude rice bran oil bodies and in OBs after washing with water and 9·0 M urea.

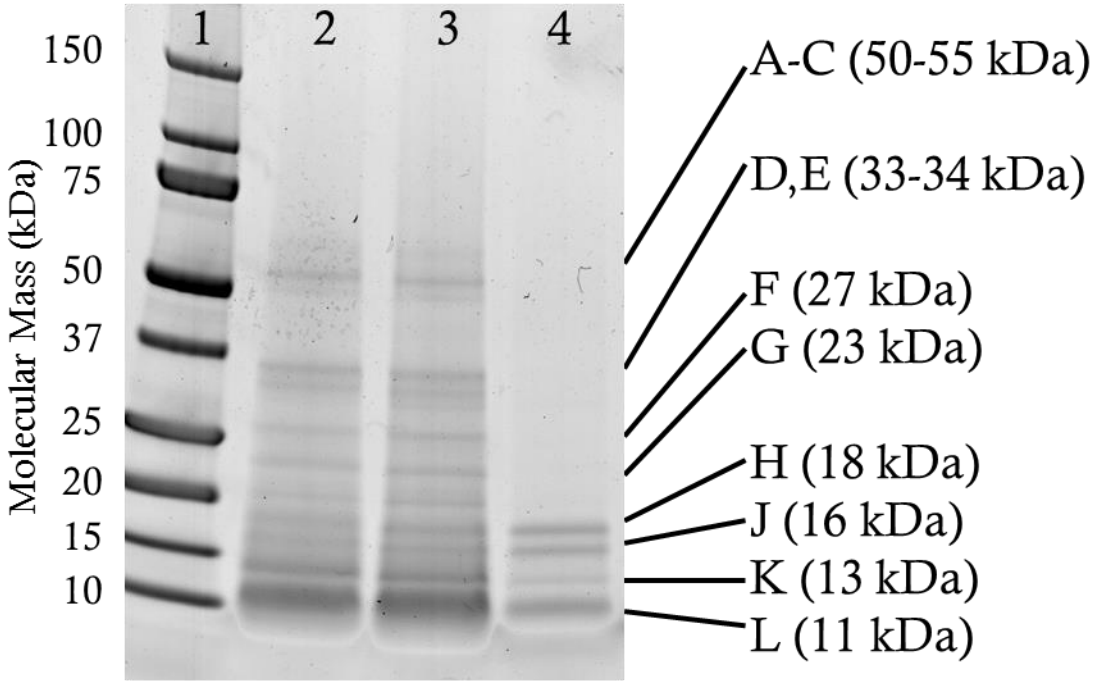
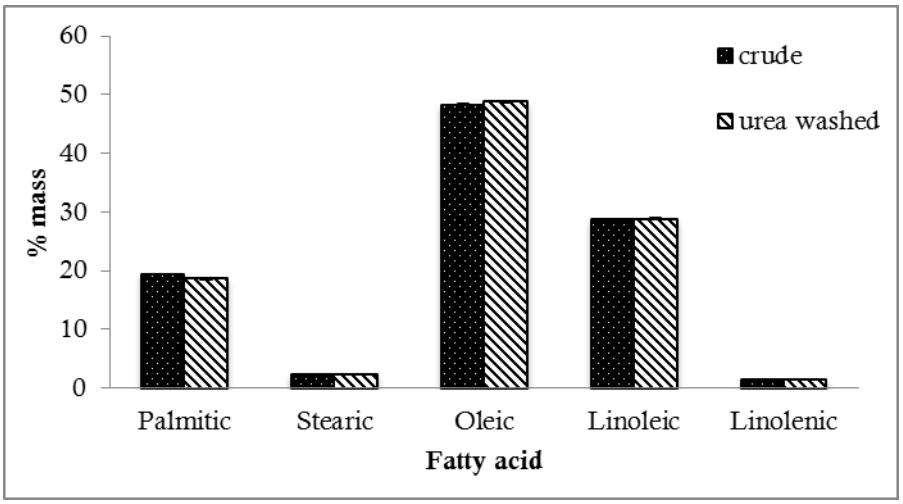


Figure 3. SDS-PAGE profiles in oil body preparations from rice bran. OBs washed in urea demonstrate the same protein composition as those washed only in water. Lane 1, molecular mass marker; lane 2, crude oil bodies; lane 3, water-washed oil bodies; lane 4, urea-washed oil bodies. Bands are identified in accordance with reported work on OB proteins (12,21,23,25) thus: A-G (lanes 1 and 2 only) are unknown, H and J are identified as oleosin isoforms, K and L unknown. All samples were applied at a total protein loading of 10 μg.



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332 *Figure 4. Fatty acid composition in crude oil bodies and oil bodies after washing with 9·0 M*

333 *urea.*

Phytochemical	Bran		Crude OBs (mg/Kg lipid)	Urea washed OBs (mg/Kg lipid)	Phytochemical retention between Urea washed OBs and Bran (%)
	mg/Kg mass	mg/Kg lipid			
α-tocopherol	45 ± 3	277 ± 15	58 ± 4	41 ± 1	15 ± 1
γ-tocopherol	17 ± 1	104 ± 5	30 ± 1	22 ± 1	22 ± 1
δ-tocopherol	1·5 ± 0·1	9·4 ± 0·6	4·8 ± 0·2	4·3 ± 1·4	46 ± 18
Total tocopherols	**64 ± 3**	**390 ± 16**	**93 ± 10**	**68 ±7**	**18 ± 3**
α-tocotrienol	19 ± 2	116 ± 11	48 ± 5	33 ± 1	36 ± 4
γ-tocotrienol	126 ± 3	771 ± 19	425 ± 9	316 ± 7	39 ± 4
δ-tocotrienol	25 ± 1	153 ± 5	90 ± 2	82 ± 2	54 ± 3
Total tocotrienols	**170 ± 4**	**1,040 ± 22**	**563 ± 4**	**432 ± 2**	**42 ± 1**
Tocochromanols (All)	**233 ± 4**	**1,430 ± 27**	**655 ± 11**	**500 ± 7**	**35 ± 1**
Total Phenolic Content	**3,470 ± 181**	**21,200 ± 1,102**	**7,820 ± 157**	**1,530 ± 147**	**8 ± 1**
γ-Oryzanol	2,310 ± 40	14,200 ± 250	9,690 ± 130	8,830 ± 97	62 ± 2

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335 *Table 1. The concentrations of phytochemicals at various stages of the process.*

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Plant material	**Major toco-chromanol isomer**	**Seed/Grain/Bran**	**COB**	**WWOB**	**UWOB**	**Total seed/grain/bran tocochromanols associated with purified oil bodies[a]**
Whole *Avena* Grain	α-tocotrienol	66·3 ± 2·2	77·2 ± 18·2	60·8 ± 13·7	69·3 ± 8·2	20
Oryza Bran	γ-tocotrienol	74·2 ± 3·3	75·5 ± 2·1	ND	73·2 ± 1·9	35
Echium Seed	γ-tocopherol	92·9 ± 5·5	93·7 ± 7·9	93·4 ± 3·3	93·7 ± 0·6	86
Helianthus Seed	α-tocopherol	94·4 ± 8·3	ND	93·5 ± 10·7	93·9 ± 12·8	80

Table 2. The retention of the major tocochromanol isomer during purification of OBs from several species, Avena *(16),* Oryza *(present study),* Echium *(26),* Helianthus *(27). ND, not determined. [a]This estimate is calculated based in the total tocochromanol concentrations per unit mass lipid. There is an assumption that most of the lipid in these tissues is present in oil bodies.*