

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

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

20

21 **KEYWORDS:** Food chemistry/Biochemistry

22

23 **INTRODUCTION**

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

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

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

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

53 MATERIALS AND METHODS

54 Plant materials

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

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

60 **Transmission electron microscopy**

61 Electron micrographs were prepared in an analogous manner to a previous report (16).
62 Samples of the resulting bran (~ 1 mm thick) were fixed in freshly made 2.5% glutaraldehyde in
63 cacodylate buffer (0.05 M, *pH* 7.4) and washed with freshly made cacodylate buffer. The samples were
64 then post-fixed in 2% osmium tetroxide before being embedded in Spurr resin and polymerized. Thin
65 sections (0.5 μm) were cut and stained with toluidine blue for light microscopy before ultra-thin
66 sections were selected and mounted on copper grids and stained using uranyl acetate and lead citrate
67 for electron microscopy (JEOL 1010 TEM; JOEL Ltd., Herts, UK).

69 **Isolation and washing of oil bodies**

70 Rice bran (100 g) in 500 mL distilled water was mixed on a roller mixer at 50 rpm for 1 h
71 before homogenisation (2 min, Krups blender, maximum speed). The slurry was filtered through three
72 layers of cheese cloth and the filtrate centrifuged (Beckman Coulter, London, UK) at 10,000 *g* for 20
73 min at 5 °C. The upper-most layer (COBs) was collected, resuspended in 9.0 M urea (1:4, *w/v*),
74 vortexed vigorously and centrifuged (10,000 *g*, 20 min, 5 °C). The remaining mass was washed three
75 more times with distilled water in the same manner to furnish OBs (referred to hereafter as purified
76 OBs). Water washed OBs (*Figures 2*) are purified in the same manner, without suspension in any
77 aqueous solution of urea.

79 **Compositional analysis**

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

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

88 **Protein analysis by SDS-PAGE**

89 The proteinaceous material from dried oil body samples from which the water and lipidic
90 material had been separated was denatured using Laemmli's solution (Bio-Rad Laboratories, Herts,
91 UK, 190 $\mu\text{L}/\text{mg}$ protein isolate) and β -mercaptoethanol (Bio-Rad, 10 $\mu\text{L}/\text{mg}$ protein isolate) at 95 °C
92 and chromatographed by SDS-PAGE using 4-20% gradient Protean® Gel Tris-HCl Gel (Bio-Rad).
93 The gels were stained with Imperial Bio-Rad Coomassie blue (R-250). Excess stain was removed using
94 ddH₂O and imaged using a Bio-Rad GX-800 densitometer.

96 **Fatty acid composition**

97 The lipid (phospholipids and triglycerides) fraction separated from the rest of the OB material,
98 vacuum-oven-dried and then dissolved in chloroform (0.01 g/mL), was converted to fatty acid methyl
99 esters using trimethylsulfonium hydroxide (0.25 M in methanol) using an established method (lipid:
100 trimethylsulfonium hydroxide 5:1 ν/ν) (18). A gas chromatograph (Trace GC Ultra, Thermo Scientific,
101 Loughborough, UK) equipped with a mass spectrometer (DSQ II Single Quadrupole GC/MS, Thermo
102 Scientific, Loughborough, UK) was employed, with a flame ionization detector (FID) and auto
103 injection system (CTC Analytics, Essex UK) and operated in the splitless mode. One microlitre of
104 sample was injected into a capillary column (Phenomenex Zebron ZB-FFAP, California, USA) 30 m \times
105 0.25 mm I.D. coated with nitroterephthalic acid modified polyethylene glycol (0.25 μm film thickness).
106 Injection temperature was 200 °C. The oven temperature was initially held at 120 °C for 1 min and
107 increased to 250 °C at a rate of 5 °C/min for 4 min. The carrier gas was helium. Retention times of
108 standards (Supelco 37 Component FAME Mix, Supelco, PA, USA) were used to identify detected fatty
109 acid esters. Identification was also verified by comparing mass spectra with standard library through
110 the Thermo Scientific Xcalibur software programme. Methyl heptadecanoate (250 $\mu\text{g}/\text{mL}$) was used
111 as an internal standard.

113 **Extraction and quantification of tocochromanols and γ -oryzanol**

114 Phytochemicals were extracted from dried oil body samples (~200 mg), using methanol
115 $3 \times 800 \mu\text{L}$ containing 1% butylated hydroxyl toluene through agitation in a mini-Beadbeater-16 for 30
116 sec at 3,450 oscillations/min and then analyzed by using RP-HPLC as described previously (19).
117 Samples (20 μL) were injected with a total run time of 35 min at 25 °C. Identification and quantification
118 were made using calibration curves prepared from standards of α -, β -, γ -, and δ -tocopherols and
119 tocotrienols (2-15 $\mu\text{g}/\text{mL}$) (Sigma, Gillingham, Dorset, UK) and γ -oryzanol (50-300 $\mu\text{g}/\text{mL}$) (Tokyo
120 Chemical Industry UK, Oxford, UK). Waters Millenium^{®32} Chromatography Manager Software
121 (version 3.0) was utilized for data analysis.

123 **Total phenolic content (TPC)**

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

132 **RESULTS AND DISCUSSION**

133 In order to determine the size of rice bran OBs *in vivo*, images of this system were taken using
134 Transmission Electron Microscopy (TEM). The OBs appear as light grey spherical droplets in brown
135 rice (*Figure 1A*) and rice bran (*Figure 1B*), and were all 0.5-1.0 μm in diameter. In general, OBs were
136 observed in the aleurone and sub-aleurone layers (*Figure 1*). The distribution of OBs observed is in
137 agreement with previous studies on OBs from *Oryza* species (15,20).

138 It should be noted that the bran was richer in both protein ($15.0 \pm 0.65\%$), and
139 lipids/triglycerides ($16.3 \pm 0.52\%$) than brown rice ($8.9 \pm 0.63\%$ and $2.5 \pm 0.15\%$ respectively) and
140 white rice ($8.6 \pm 0.75\%$ and $0.6 \pm 0.03\%$, respectively). Yields of OBs from whole-grain basmati

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

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

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

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

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

171 The retention of tocopherols, tocotrienols and oryzanols in purified OBs compared with the
172 starting bran material is shown in *Table 1*. Notably, concentrations of tocochromanols and oryzanols
173 with respect to total lipid content both decline between that in bran and that in crude oil bodies, and
174 from crude oil bodies to purified oil bodies. However, there is a higher retention of tocotrienols (41%)
175 compared with the tocopherols (17%) between purified OBs and the *Oryza sativa* bran they were
176 isolated from. This also suggests that, *in vivo*, there is an enrichment of tocotrienols in the OB
177 compared with tocopherols. Certainly, there is reduction of >90% in total phenolic compounds, with
178 >80% of the total phenolic compounds being removed during the urea-washing step alone. This
179 demonstrates that there is a significant pool of phenolic compounds that are removed during washing
180 steps along with extraneous proteins. Additionally, these data demonstrate that the step in which the
181 OBs are washed in urea results in some loss in tocopherols and tocotrienols (27% and 23%
182 respectively), and oryzanol (9%). This in turn suggests that a quarter of the tocochromanols and about
183 a tenth of the oryzanols that are present in the bran are either not as strongly linked to the OBs as the
184 phytochemical fraction that remains after purification, or are not associated with OBs at all. Further,
185 the reduction in total phenolic compounds (*Table 1*) is relatively large with respect to the reduction in
186 tocochromanols. Before purification (*i.e.* in the bran), tocochromanols represent <7% of the total
187 phenolic fraction. After purification, this group of compounds represents around a third of the
188 phenolic compounds that remain.

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

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

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

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

217 **ACKNOWLEDGMENTS**

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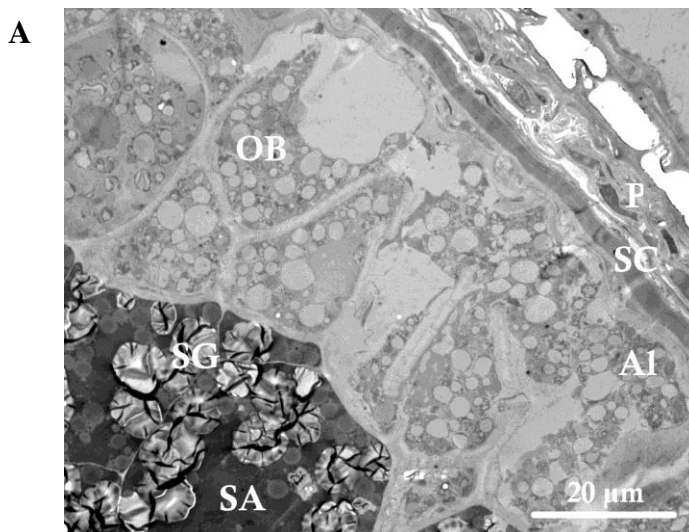
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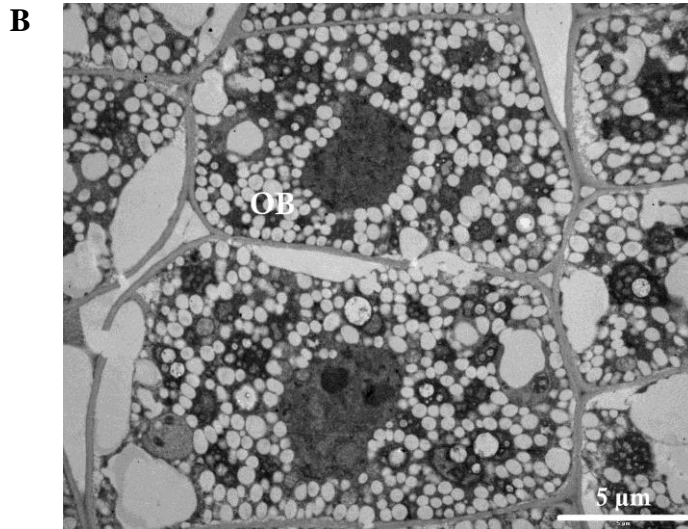
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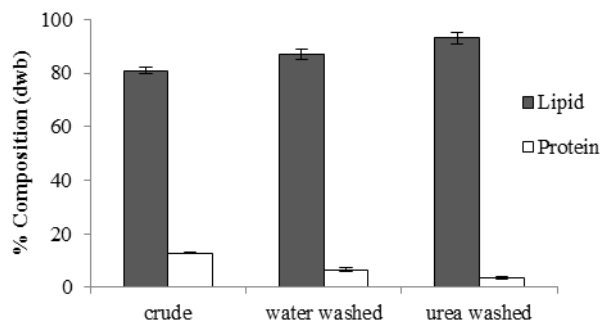
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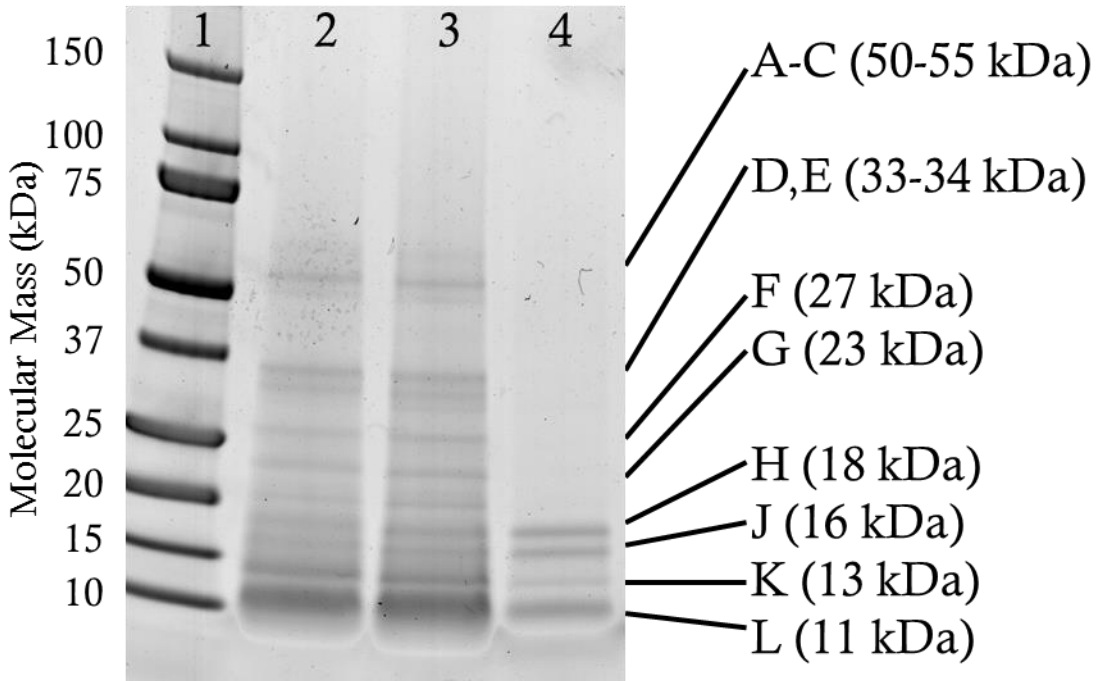


314 *Figure 1. Electron micrograph of oil bodies in (A) brown rice surface and (B) aleurone layer of*
 315 *rice bran. OB: Oil body P: Pericarp, SC: Seed coat, Al: Aleurone, SA: Sub-aleurone, SG: Starch*
 316 *granule*



318

319 *Figure 2. Lipid and protein levels (% dry weight basis) in crude rice bran oil bodies and in OBs*
 320 *after washing with water and 9.0 M urea.*



323

324

Figure 3. SDS-PAGE profiles in oil body preparations from rice bran. OBs washed in urea

325

demonstrate the same protein composition as those washed only in water. Lane 1, molecular

326

mass marker; lane 2, crude oil bodies; lane 3, water-washed oil bodies; lane 4, urea-washed oil

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bodies. Bands are identified in accordance with reported work on OB proteins (12,21,23,25)

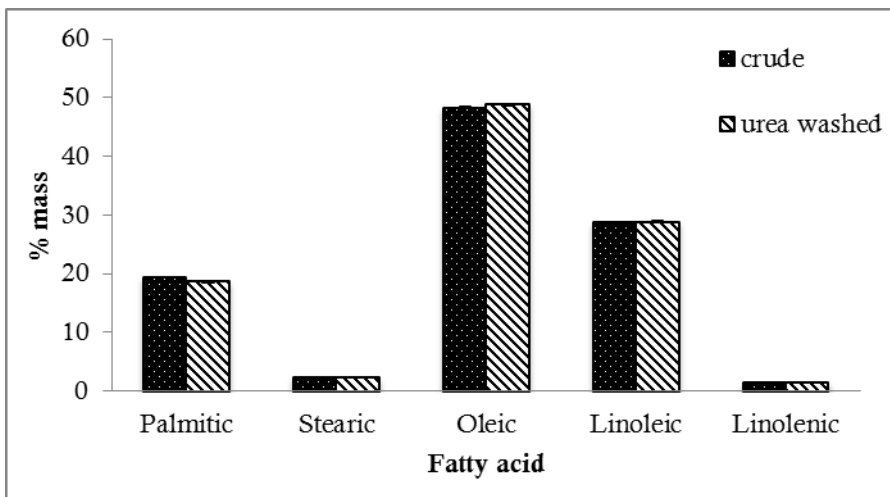
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thus: A-G (lanes 1 and 2 only) are unknown, H and J are identified as oleosin isoforms, K and

329

L unknown. All samples were applied at a total protein loading of 10 µg.

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331

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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 \pm 3	277 \pm 15	58 \pm 4	41 \pm 1	15 \pm 1
γ -tocopherol	17 \pm 1	104 \pm 5	30 \pm 1	22 \pm 1	22 \pm 1
δ -tocopherol	1.5 \pm 0.1	9.4 \pm 0.6	4.8 \pm 0.2	4.3 \pm 1.4	46 \pm 18
Total tocopherols	64 \pm 3	390 \pm 16	93 \pm 10	68 \pm 7	18 \pm 3
α -tocotrienol	19 \pm 2	116 \pm 11	48 \pm 5	33 \pm 1	36 \pm 4
γ -tocotrienol	126 \pm 3	771 \pm 19	425 \pm 9	316 \pm 7	39 \pm 4
δ -tocotrienol	25 \pm 1	153 \pm 5	90 \pm 2	82 \pm 2	54 \pm 3
Total tocotrienols	170 \pm 4	1,040 \pm 22	563 \pm 4	432 \pm 2	42 \pm 1
Tocochromanols (All)	233 \pm 4	1,430 \pm 27	655 \pm 11	500 \pm 7	35 \pm 1
Total Phenolic Content	3,470 \pm 181	21,200 \pm 1,102	7,820 \pm 157	1,530 \pm 147	8 \pm 1
γ -Oryzanol	2,310 \pm 40	14,200 \pm 250	9,690 \pm 130	8,830 \pm 97	62 \pm 2

334

335

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

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Plant material	Major tocopherol isomer	Seed/Grain/Bran	COB	WWOB	UWOB	Total seed/grain/bran tocopherols associated with purified oil bodies ^a
Whole <i>Avena</i> Grain	α -tocotrienol	66.3 \pm 2.2	77.2 \pm 18.2	60.8 \pm 13.7	69.3 \pm 8.2	20
<i>Oryza</i> Bran	γ -tocotrienol	74.2 \pm 3.3	75.5 \pm 2.1	ND	73.2 \pm 1.9	35
<i>Echium</i> Seed	γ -tocopherol	92.9 \pm 5.5	93.7 \pm 7.9	93.4 \pm 3.3	93.7 \pm 0.6	86
<i>Helianthus</i> Seed	α -tocopherol	94.4 \pm 8.3	ND	93.5 \pm 10.7	93.9 \pm 12.8	80

339

340 *Table 2. The retention of the major tocopherol isomer during purification of OBs from*
341 *several species, Avena (16), Oryza (present study), Echium (26), Helianthus (27). ND, not*
342 *determined. ^aThis estimate is calculated based in the total tocopherol concentrations per unit*
343 *mass lipid. There is an assumption that most of the lipid in these tissues is present in oil bodies.*
344