Soybean (*Glycine max*) Oil Bodies and Their Associated Phytochemicals

Ian D. Fisk and David A. Gray

Abstract: Soybean oil bodies were isolated from 3 cultivars (Ustie, K98, and Elena) and the occurrence of 2 classes of phytochemicals (tocopherol isoforms and isoflavones) and strength of their association with isolated oil bodies was evaluated. Tocopherol is shown to be closely associated with soybean oil bodies; δ-tocopherol demonstrated a significantly greater association with oil bodies over other tocopherol isoforms. Isoflavones do not show a significant physical association with oil bodies, although there is some indication of a passive association of the more hydrophobic aglycones during oil body isolation.

Keywords: genestein, isoflavone, oil body, soybean, vitamin E

Practical Application: Oil bodies are small droplets of oil that are stored as energy reserves in the seeds of oil seeds, and have the potential to be used as future food ingredients. If oil body suspensions are commercialized on a large scale, knowledge of the association of phytochemicals with oil bodies will be valuable in deciding species of preference and predicting shelf life and nutritional value.

Introduction

Soybean typically has a protein content of approximately 34% to 42% (Wolf and others 1982) and a lipid content of approximately 20% (Seal and others 2008) which is primarily located in subcellular oil bodies (Allen and Tao 2007; Iwanaga and others 2008). Oil bodies are storage organelles within seeds and are formed of small droplets of oil surrounded by a half unit membrane of phospholipid and protein, that package the oil for optimum stability during dry storage and mobilization during seed germination (Murphy and others 2001). During conventional solvent extraction of oil from oilseeds, oil bodies are destroyed, allowing the release of crude oil which is then further refined.

In addition to the major components of oil bodies (neutral lipid, protein [oleosin and caleosin], and phospholipids) other minor constituents have also been identified including tocopherol (Fisk and others 2006; White and others 2006), steroleosin (Lin and others 2002), myrosinases (Katavic and others 2006), cruciferins (Katavic and others 2006), phenolic acids (Fisk and others 2006), hydroperoxides (Fisk and others 2008), and flavor volatiles (Hudak and Thompson 1997). The association of these minor components with oil bodies is either due to an intrinsic association with the oil body structure, or is a result of passive association with oil bodies during recovery. Although present at relatively low concentrations these components are likely to affect the quality, functionality, and use of isolated oil bodies.

Oxidation of lipids in food can result in the production of antinutritional factors and undesirable flavor and aroma compounds (Let and others 2005). The process of oxidation may also generate genotoxic and cytotoxic oxygenated aldehydes (Guillen and others 2005). Knowledge of lipid oxidation and the ability to control its development are therefore fundamental to the preservation of the food's nutritional and sensory qualities.

Tocochromanols, which are proposed to be intrinsic components of sunflower oil bodies (Fisk and others 2006), may contribute to an increased oxidative stability through the conversion of lipid and peroxyl radicals to more stable radical or nonradical species. In addition to this fundamental antioxidant mechanism, tocochromanols are amphiphilic and are therefore typically located at membrane interfaces; this will increase oxidative stability due to their spatial location close to the point of initiation of oxidation (the oil/water interface), thereby reducing droplet-to-droplet propagation.

Isoflavones are found in soybeans and are generated via the phenylpropanoid pathway from phenylalanine and are generally recognized to be stored as conjugates in the vacuole (Yu and others 2003) of soybeans, isoflavones are then mobilized as required by the tissue in responses to biological events such as germination or infection. Given the amphiphilic nature of some isoflavones, it is hypothesized that select isoflavone forms might be concentrated in oil bodies extracted from soybeans, either through an intrinsic or a passive association. In food systems isoflavones can exhibit antioxidant properties, but their more significant biological effect is proposed to be through the stimulation of antioxidant protein gene expression (Kameoka and others 1999; Gu and others 2002).

This study is an output of an ongoing research program at the Univ. of Nottingham which has investigated the potential of oil bodies as food ingredients. Previous published works have detailed the physicochemical properties of oil bodies in oat grain (White and others 2006), sunflower seed (Fisk and others 2006; White and others 2009), and soybean (Iwanaga and others 2007; Iwanaga and others 2008). Additionally, research has been carried out to investigate the concentration of oil body-associated phytochemicals and to evaluate the potential use of oil bodies as flavor carriers (Fisk and others 2011), their rheological properties (White and

MS 20110731 Submitted 6/15/2011, Accepted 8/24/2011. *Authors are with Division of Food Sciences, Univ. of Nottingham, Sutton Bonington Campus, Sutton Bonington, Loughborough, Leicestershire LE12 5RD, U.K. Direct inquiries to author Fisk (E-mail:Ian.Fisk@nottingham.ac.uk).*

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others 2008), and their oxidative stability (Fisk and others 2008) in suspension.

There are only a very limited number of studies on the nutritional value of soybean oil bodies; therefore, the objective of this study was to investigate the presence of isoflavones and tocopherol within soybean oil bodies after organelle isolation and to evaluate the strength of association of the phytochemical species with oil bodies after chaotropic washing.

Materials and Methods

All chemicals were of analytical purity (>99%) and were sourced from Sigma-Aldrich UK (Gillingham, U.K.) unless specified. Isoflavone standards were sourced from LC laboratories (Woburn, Mass., U.S.A.). Soybean seeds were supplied by Soya U.K. Ltd. (Southampton, U.K.), and were harvested during 2007 in the Ukraine. Three cultivars were chosen (K98, Elena, and Ustie). The cultivars were previously developed by the Inst. of Agrarian Studies, Kiev, Ukraine specifically for European production, with the main cross-cultivar differences being yield, growth habitat, and earliness of harvest.

Oil body isolation

Oil bodies from soybean were extracted and purified by the method of Tzen and others (1997) with slight modifications as described. Seeds (100 g) in grinding medium (0.5 L, 10 mM sodium phosphate, pH 7.5) were ground in a Kenwood blender (BL315 full power for 60 s; Havant, U.K.). The slurry was filtered through 3 layers of cheesecloth and the filtrate centrifuged in 400 mL batches (8000 RCF for 30 min, 5 °C). The upper layer was isolated using a chilled metal spatula, separated into batches (5 mL), vortexed for 1 min in buffer (25 mL, 10 mM sodium phosphate, pH 7.5), and then centrifuged at 5 °C. A swinging bucket rotor at 2000 RCF for 20 min is used for all centrifugations unless stated otherwise. The upper layer was isolated, mixed with sodium azide (0.02 M), and stored at 5 °C under nitrogen and designated as a water-washed oil body preparation (WWOB). This isolate was then purified by suspending the water washed preparation (5 mL) in washing buffer (25 mL, 9 M urea, 10 mM sodium phosphate buffer, pH 7.5) and centrifuging at ambient temperature. The fat pad was isolated, resuspended (10 mM sodium phosphate buffer, pH 7.5), and centrifuged at room temperature to remove residual urea and extraneous proteins. After centrifugation the centrifuge tube was chilled on ice for 5 min and the upper solidified phase (urea washed oil body preparation) was removed, mixed with sodium azide (0.02 M), and stored at 5 °C under nitrogen, the final preparation being designated as a urea washed oil body preparation (UWOB).

Moisture content

Samples (1.5 g, $n = 3$) were prepared in predried containers, and dried in a Gallenkamp Vacuum Oven (Loughborough, U.K.) at −900 mBar and 40 °C (48 h or until constant weight) to calculate moisture content.

Lipid content

Dried samples (approximately 0.2 g) were vortexed with isooctane (1 mL, 1% [w/v] butylated hydroxytoluene, BHT) and ground (mini-bead beater, Biospec, Bartlesville, Okla., U.S.A.) for 30 s. Samples were centrifuged and the solvent layer was aspirated; this was repeated 3 times and the isolates pooled. Lipid content was measured gravimetrically by the evaporation of the isooctane extracts, and sample residues in mini-bead beater vials were dried (80 °C, 30 min) and dissolved in 1 mL, 20 g/L sodium dodecyl sulfate solution for protein analysis.

Tocochromanol

Tocochromanol composition was analyzed by HPLC as described by Bryngelsson and others (2002) using the isooctane solvent extract. HPLC was performed using a Waters (Elstree, U.K.) 2695 separation module equipped with a Waters 996 photodiode array detector and a Jasco intelligent fluorescent detector P-920 (excitation set at 294 nm and emission at 326 nm, gain 10). Separations were performed using an Inertsil 5 silica ChromSep HPLC column SS 250 × 4.6 mm (Varian BV; Stockport, U.K.) with a ChromSep guard column. Mobile phase was hexane/1,4 dioxane (95:5, v/v) and a flow rate of 1.5 mL/min. Samples (50 μL) were injected with a run time of 20 min at 25 °C. Identification and quantification were made using authentic standards of α-, β-, γ-, δ-tocopherol (Sigma Ltd., Gillingham, U.K.). The coefficient of variation of standards was acceptable at <5%.

Protein content

Dried samples (approximately 0.2 g) were vortexed with methanol (1 mL, 1% [w/v] butylated hydroxytoluene [BHT]) and ground (mini-bead beater, Biospec) for 30 s. Samples were centrifuged and the solvent layer was aspirated; this was repeated 3 times and the isolates pooled. The protein content of defatted dried seed tissue was determined using the BCA (bicinchoninic acid) assay following solubilization of proteins in 20 g/L sodium dodecyl sulfate solution. Samples were then vortexed (1 min) and centrifuged (2000 RCF for 1 min). The supernatant was then aspirated, diluted (to within the range of the standard curve), and assayed for protein content as per the BCA assay (Smith 1985).

Total phenolic content

Total phenolic content (TPC) was analyzed by the Folin-Ciocalteau method with some modifications (Fisk and others 2006). Folin-Ciocalteau reagent (0.25 mL) was allowed to react with a methanol extract (50 μL) for 1 min, then 20% w/v sodium carbonate solution (0.75 mL) was added, the sample vortexed and left for 1 min then resuspended in distilled water (3.95 mL), vortexed and left for 2 h. The sample was passed though a syringe filter (0.45 μm nylon filter) and absorbance measured using a cuvette at 760 nm using gallic acid as a standard. The coefficient of variation of standard curve was acceptable at >0.1%.

Isoflavone

Isoflavone analysis was conducted as described by Kim and others (2007) with slight modifications. A Waters 2695 separation module equipped with a Waters 996 photodiode array detector and YMC ODS AM-303, 5 μm pore (250 × 4.6 mm I.D.) column were utilized for chromatographic separation and assessment of the absorption. Solvents were vacuum degassed and consisted of A, 0.1% (v/v) acetic acid in distilled water and B, 0.1% (v/v) acetic acid in acetonitrile. Solvent ramp was as follows: (A [85%], B [15%]) ramped over 60 min to (A [40%], B [60%]), held for 5 min, then returned to (A [85%], B [15%]) over 5 min; solvent flow rate was consistently maintained at 1.5 mL/min and injection volume was 20 μL (methanol isolate, as described previously). Eluent was then passed to a mass spectrometer for mass determination and additional identification (Micromass, Waters, U.K.) with an ESP + interface. Settings on the mass spectrometer were m/z range 200 to 600; cone voltage 18 V; capillary voltage 4.8 kV; source temperature 100 °C, and desolvation temperature 350 °C. Gas flow was set at 750 mL/h. All identified peaks were confirmed

with authentic standards. The coefficient of variation of standards was acceptable at <5%.

Experimental design and data analysis

All experiments were conducted on a fully balanced randomized experimental design with 3 true replicates for each analysis. Data were analyzed as appropriate by XL-STAT 7.5.2 (Addinsoft, New York, U.S.A.) by ANOVA ($P < 0.05$) with Fishers LSD post hoc, or linear regression analysis.

Results and Discussion

Soybean oil bodies

Oil bodies were prepared from soybeans of 3 cultivars, K98, Elena, and Ustie, and their lipid and protein content was measured (Figure 1) at each washing stage. In all cases there was an increase in the concentration of lipid and a decrease in the concentration of associated proteins in oil bodies during washing compared with seed; this was further enhanced after rigorously washing the oil body preparations with urea. Urea is a potent chaotropic agent that is capable of removing any extraneous proteins carried over during the recovery of oil bodies (Millichip and others 1996). Proteins that are intrinsic to oil bodies (such as oleosin) are not easily removed with chaotropic agents.

Tocopherol concentration of soybean and isolated oil bodies

All tocopherol isoforms were identified in all samples (α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol) and are detailed in Figure 2. Tocotrienols were identified at lower concentrations but were close to the limit of detection of the HPLC-fluorescence detector and therefore were not further analyzed. The total concentration of tocopherol isoforms in the Elena seed was 341 ± 102 mg/kg dwb, K98 had a tocopherol concentration of 335 ± 31 mg/kg dwb, and Ustie 271 ± 74 mg/kg dwb. There was no difference in total tocopherol concentration between the seed samples ($P < 0.05$).

The profile of tocopherol isoforms was extremely sensitive to the process of oil body isolation and urea washing (Figure 2); α-tocopherol was the most sensitive and δ-tocopherol was the most resistant to removal with chaotropic washing, and β-tocopherol and γ-tocopherol were equally resistant to the process of urea washing ($P < 0.05$).

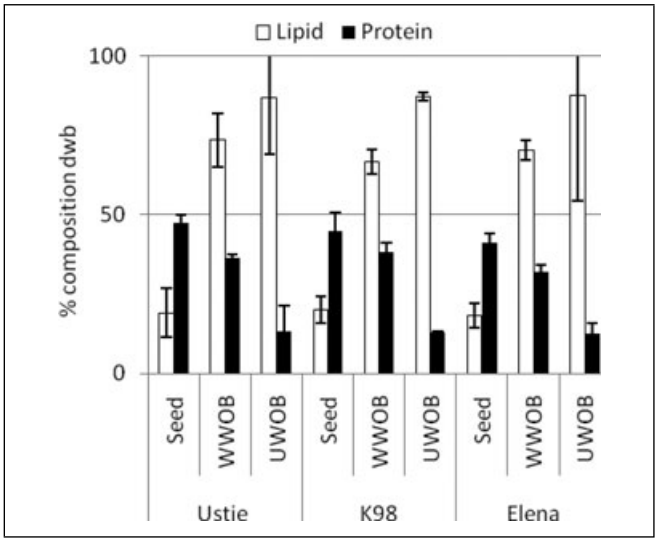

Figure 1–Composition (% protein and lipid dwb ± 1 SD) of soybean and isolated oil bodies for Ustie, K98, and Elena.

The relatively tight association of specific tocopherol isoforms with WWOB is shown in Figure 2; δ-tocopherol was selectively enriched during the isolation process in K98 and Ustie ($P < 0.05$). The selective association of δ-tocopherol with the oil body structure has never been previously reported although interestingly a 1976 paper (Yamauchi and Matsushita 1976) on soybeans eluded to the enrichment of γ-tocopherol in the spherosome fraction (oil body) that floated on centrifugation, but did not provide any substantiating data.

The previous literature has suggested a specific cellular distribution of tocochromanols in cereals, with tocotrienols being concentrated in the outer bran layer (White and others 2006), α-tocopherol being concentrated within the chloroplasts, and the other isoforms being distributed and associated with bulk lipid and membranous regions. The data presented support this hypothesis and forward the argument that δ-tocopherol may be more closely associated with soybean oil bodies than the other tocopherol isoforms and may therefore have a defined spatial distribution across the seed. Falk and others (2004) suggest a similar argument with barley grains, and have identified a defined distribution of tocochromanols across the grain, tocotrienols were concentrated within the germ, and tocopherol was more equally distributed across the grain.

The association of tocopherol with oil bodies contributes significantly to the oxidative stability of the lipidic component of the oil bodies both *in vivo* and *ex vivo*. A previous study has shown the enhancement of oxidative stability of sunflower oil bodies over laboratory manufactured emulsions with the same tocopherol profile (Fisk and others 2008), which indicated a combination of tocopherol concentration; location and matrix effects may drive the enhanced storage stability of lipid within oil body structures.

Total phenolic content of soybean and isolated oil bodies

The TPC of each soybean cultivar and their respective oil body preparations was measured. The TPC of the seed was variable, which may be due to batch variation. The TPC of the isolated oil bodies was less than or equal to that of the seed ($P < 0.05$), and with urea washing the TPC was further reduced for all samples (Figure 3) ($P < 0.05$); average TPC values for WWOB for all cultivars were 10.2 ± 1.9 mg GAE/g dwb and 4.8 ± 1.4 mg GAE/g dwb for the UWOB material.

The loss of phenolic compounds during oil body extraction and subsequent further loss with urea washing is comparable to the previous data for oilseeds (Fisk and others 2006) (sunflower) and cereals (White and others 2006) (oat). Most phenolic compounds found in the seed are phenolic acids (conjugated or free) (Lee and others 2008); any free phenolic acids present will be hydrophilic and are removed with the wash water, and conjugated phenolic acids are removed at the filtration stage or the first centrifugation stage due to their association with non-oil body associated seed material.

Isoflavone concentration of soybean and isolated oil bodies

Isoflavone concentration was measured in all samples and is fully detailed in Table 1, with a breakdown by isoflavone class shown in Figure 4. In the seed the most concentrated isoforms were genistin, malonyl daidzin, daidzin, and acetyl daidzein although a wide range of other isoforms were identified; the total isoflavone concentration was 2300 ± 23 mg/kg dwb (averaged across all cultivars).

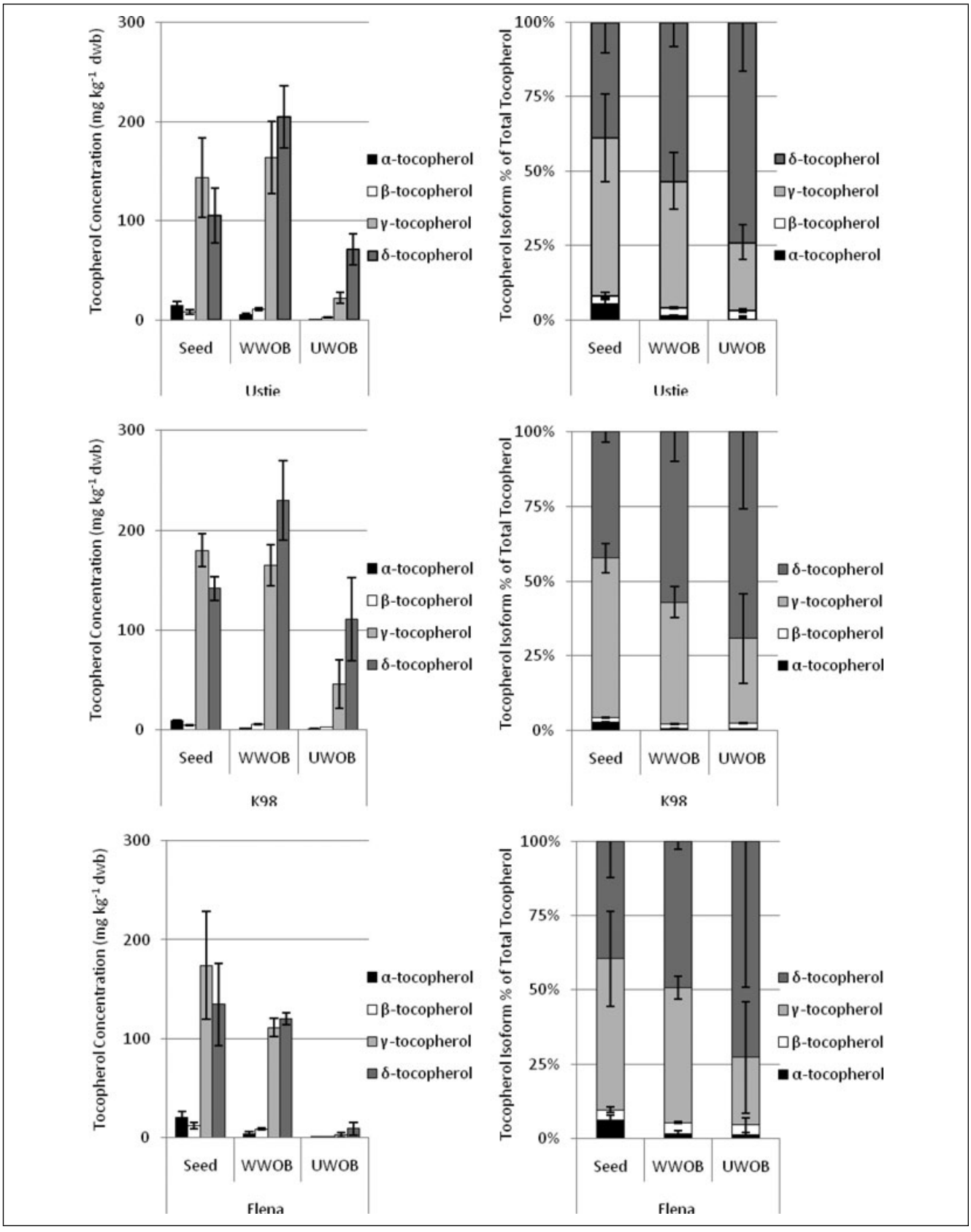

Figure 2–Tocopherol concentration (mg/kg dwb ±1 SD) in soybean and isolated oil bodies for Ustie, K98, and Elena.

During the preparation of WWOB there was a loss of most identified isoflavones (Table 1). Generally, the more hydrophilic compounds (malonyl-glycosides and acetyl-glycosides) were lost to the greatest extent, whereas the more hydrophobic aglycone compounds (genistein, glycitein, daidzein) were lost to a lesser extent (β-glycosides have an intermediate loss). The differential loss is highlighted in the change in the relative distribution of isoforms by class in Figure 4. After urea washing, the absolute concentration of all isoflavone classes was very significantly reduced and the elevated presence of aglycones (relative to total isoform concentration) shown in WWOB was lost; Figure 4 illustrates the return of the relative distribution of isoform classes to a similar profile as found in the native seeds.

In addition to the known isoflavone isoforms, 2 unknowns were identified. The first unknown (Unk) was tentatively identified as a diazin variant, due to its UV–VIS absorption maxima of 248.5 nm and m/z of 242. The second unknown (Unk2) is less defined and had a UV–VIS absorption maxima of 261 nm and m/z of 393. Both unknown compounds were undefined by the mass spectrum library (NIST/EPA/NIH mass spectrum library). Both unknown compounds were highly associated with the oil bodies and Unk2 was significantly more concentrated in the oil body preparation than the seed. Unk2 was highly hydrophobic log $P >$ 2.41 (identified due to order of elution as the log P of genistein was estimated as 2.41 from the literature [Leo 1995] and Unk2 eluted later in the chromatogram).

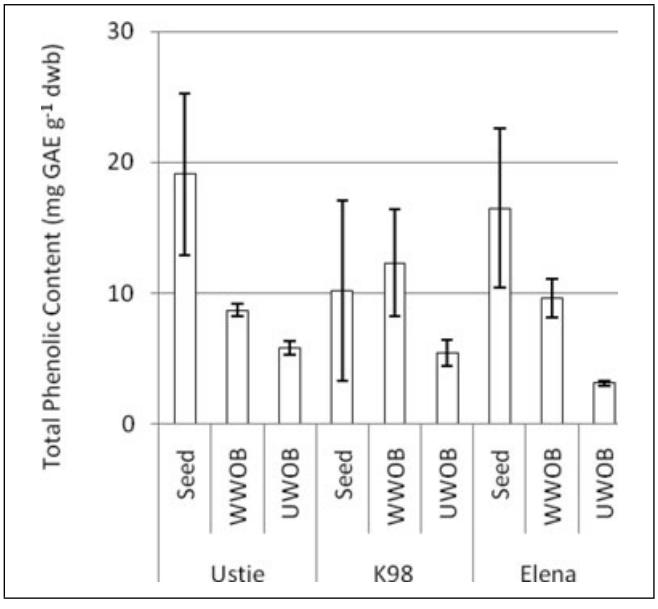

Figure 3–Total phenolic content (gallic acid equivalents mg/g dwb ± 1 SD) in soybean and isolated oil bodies for Ustie, K98, and Elena.

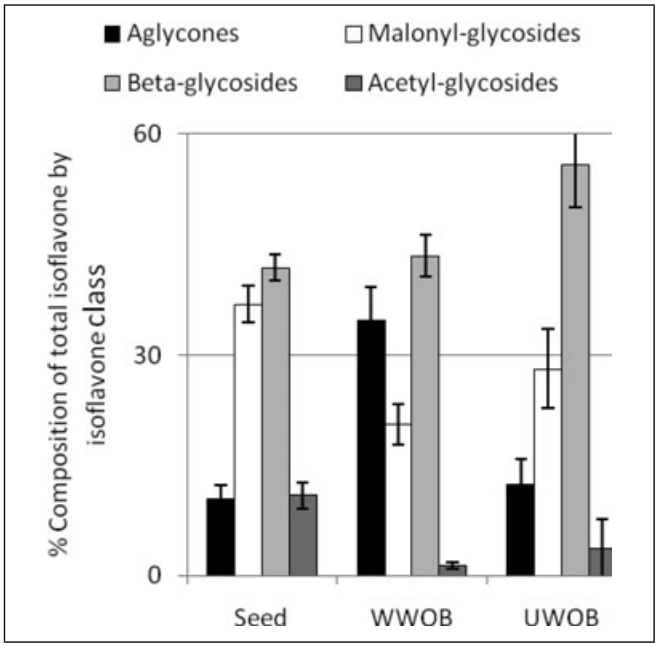

Figure 4–Variation in % distribution of isoflavone class (averaged across all seeds) in soybean and isolated oil bodies.

Table 1–Isoflavone concentration (mg/kg dwb ± 1SD) in whole soybeans and isolated oil bodies. Values are quoted relative to the dry mass of seed or oil body preparation. Unk1 and Unk2 are unknown compounds and are further discussed in the text.

Isoflavone class	Isoflavone	Retention time	Ustie (mg/kg)			K98 (mg/kg)			Elena (mg/kg)		
			Seed	WWOB	UWOB	Seed	WWOB	UWOB	Seed	WWOB	UWOB
Aglycones	Daidzein	32	165 ± 18	85 ± 6.3	9 ± 1.8	123 ± 22	100 ± 3.5	12 ± 12	118 ± 73	70 ± 9.8	7 ± 1.1
	Glycitein	36	25 ± 3	5 ± 0.4	0 ± 0	23 ± 6.2	7 ± 0.46	0 ± 0	31 ± 11	7 ± 1.5	0 ± 0
	Genistein	47	95 ± 10	54 ± 7.3	4 ± 0.2	76 ± 13	56 ± 6.25	5 ± 3.6	59 ± 11	39 ± 5	4 ± 0.4
Malonyl-glycosides	Malonyl diadzein	18	89 ± 18	7 ± 3	0 ± 0	89 ± 8.2	6 ± 1.5	0 ± 0	86 ± 17	6 ± 1.3	0 ± 0
	Malonyl daidzin	20	555 ± 157	35 ± 31	8 ± 2	562 ± 78	28 ± 8.4	3 ± 4.2	694 ± 140	43 ± 14	5 ± 2.3
	Malonyl genistein	26	110 ± 19	13 ± 4.2	0 ± 0	99 ± 14.1	10 ± 1.3	0 ± 0	103 ± 19	10 ± 1.5	0 ± 0
	Malonyl glycitein	38	44 ± 56	29 ± 8.6	22 ± 25	33 ± 21	30 ± 5.6	29 ± 29	13 ± 2.7	30 ± 18	27 ± 5.4
β-glycosides	Diadzin	11	402 ± 68	70 ± 17	8 ± 0.2	395 ± 29	92 ± 30	12 ± 13	353 ± 33	91 ± 7.3	9 ± 0.78
	Glycitin	13	69 ± 19	4 ± 1.2	1 ± 0.33	66 ± 2.1	10 ± 4.8	1 ± 1.1	76 ± 12	7 ± 2.8	1 ± 0.17
	Genistin	18	471 ± 61	82 ± 23	18 ± 8.5	421 ± 42	66 ± 9.2	17 ± 20	378 ± 34	64 ± 6.8	21 ± 0.74
	Unk 1	28	43 ± 23	8 ± 9.3	56 ± 55	76 ± 36	14 ± 9.7	20 ± 17	107 ± 36	20 ± 0.93	32 ± 12
Acetyl-glycosides	Acetyl daidzein	21	202 ± 46	6 ± 2.98	11 ± 14	276 ± 52	5 ± 0.93	3 ± 4.2	264 ± 46	2 ± 0.32	0 ± 0
	Acetyl genistin	39	1 ± 0.24	1 ± 0.43	0 ± 0	1 ± 0.61	1 ± 0.26	0 ± 0	3 ± 0.27	1 ± 0.41	0 ± 0
Unknown	Unk2	55	6 ± 2.2	25 ± 9	22 ± 2.3	10 ± 5.1	22 ± 14	15 ± 0.77	10 ± 1.8	16 ± 3.7	23 ± 7.5
	Total		2277 ± 670	424 ± 170	159 ± 82	2250 ± 650	447 ± 120	117 ± 111	2395 ± 510	406 ± 104	129 ± 28

Conclusion

Tocopherol is closely associated with soybean oil bodies, and δ-tocopherol demonstrates a significantly greater association with oil bodies over other tocopherol isoforms. Isoflavones do not show a significant physical association with oil bodies, although there is some indication of a passive association of the more hydrophobic aglycone species during oil body isolation.

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