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Relative Changes in Tocopherols, Isoflavones, Total Phenolic Content, and Antioxidative Activity in Soybean Seeds at Different Reproductive Stages

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Immature seeds of soybean are becoming increasingly popular as a snack/vegetable to harness the health benefits of soybean. They are shelled from the immature pods picked from the mother plant at different reproductive stages. Information concerning changes in antioxidant constituents and antioxidant capacity during reproductive phases of soybean seeds is scarce. The aim of the present study was to determine whether immature seeds picked at different reproductive stages differ in tocopherol, isoflavone, total phenolic contents, free radical scavenging activity, and total antioxidative capacity. Seeds shelled from the soybean pods picked at three reproductive stages (R5, R6, and R7) as well as at full maturity were subjected to high-performance liquid chromatography analyses for tocopherol and isoflavone contents. Significantly higher values ($P < 0.05$) were observed for tocopherols and isoflavones in immature seeds picked at late reproductive stages. At the first reproductive stage, that is, R5 stage, δ-tocopherol was the predominant form of tocopherol, whereas in subsequent reproductive stages as well as at complete maturity stage, the γ-isomer contributed maximum proportion to the total tocopherol content. Genistein was, in general, the major form of isoflavone at all reproductive stages. Reduction in free radical scavenging activity, total antioxidant capacity, and total phenolic content in late-picked seeds concomitant with increased concentration of tocopherol and isoflavone isomers was observed. The results show that bioactive constituents other than isoflavones and tocopherols may decline with the advancement of maturity.

KEYWORDS: Soybean; immature seeds; tocopherols; isoflavones; DPPH free radical scavenging activity; FRAP

INTRODUCTION

Soy-based foods have emerged as one of the most economical and easily available "functional foods" worldwide. Tocopherols and isoflavones, the two major biomolecules with nutraceutical value, are in high concentrations in soybean seeds. Tocopherols as free radical scavengers diminish the risk of cancer, cardiovascular diseases, neurodegenerative diseases such as Alzheimer's and Parkinson's, and enhance the immune system (*1, 2*). Soy isoflavones as antioxidants have been shown to give protective effect against cardiovascular diseases and type-2 diabetes (*3, 4*), and their estrogen-like properties have been associated with prevention of hormone-dependent cancers, alleviation of menopausal symptoms, and the enhancement of bone mineral density in women (*5, 6*). Soybean seeds possess all four naturally occurring tocopherols, namely, α-, β-, γ-, and δ, which differ in their ability to quench free radicals due to variation in the number and position of methyl substituents on the chromanol ring as shown in **Figure 1**. As a result, α-tocopherol possesses maximum antioxidative activity, whereas relative activities for the β-, γ-, and δ-isomers are 50, 10, and

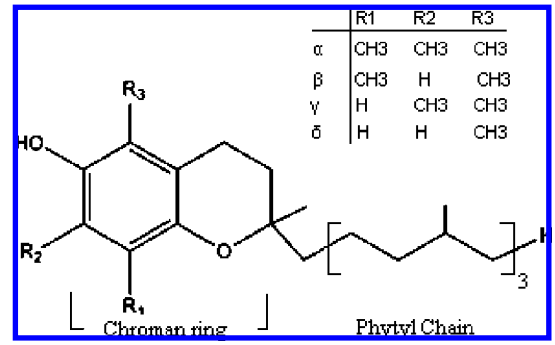

Figure 1. Structure of tocopherol isomers.

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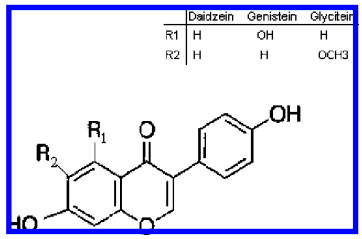

Figure 2. Structure of aglycone forms of isoflavones.

30% of the α-tocopherol activity, respectively (*7*). Isoflavones structurally possess two benzene rings joined by a three-carbon chain and are biologically functional when they are present as aglycones, namely, daidzein, glycitein, and genistein (**Figure 2**). In soybean seeds and soy foods, they are stored as *β*-glucosides and their malonyl and acetyl derivatives; however, they are converted to corresponding aglycones in the human gut before absorption (*8*).

Soy products, namely, tofu, miso, and natto, made from mature seeds have been consumed in Southeastern Asian countries since ancient times. In recent times, they have attracted people's attention all over the world as a plethora of reports pertaining to health benefits of soybean have appeared in the literature. Besides, immature seeds shelled from the pods picked at various reproductive stages bear great potential for consumption as a snack/vegetable in many countries such as India and the United States, where the soy products processed from mature seeds could not become as popular as they are in their countries of origin. The immature pods are boiled with a pinch of salt, and the tender seeds contained in them are directly popped into mouth or the green seeds shelled from the pods can be added into stews and soups. Nutritionally, they are rich in vitamin B_1 and minerals (iron, calcium, phosphorus) and low in beany flavor and flatulence-causing factors (*9*, *10*). Antioxidant capacity and the concentrations of some of the antioxidant components, namely, tocopherol isomers and isoflavones, are well documented in mature soybean seeds (*11*−*15*); however, information on these aspects is very limited in the immature seeds (*16*). In the present investigation, soybean pods were picked from plants at three reproductive stages and at full maturity stage. The seeds shelled from them were analyzed for tocopherol and isoflavone content using high-performance liquid chromatography. As total phenolic content is indicative of the total antioxidative activity of any food, we also assessed the soybean seed samples at various reproductive stages for total phenolic content, DPPH free radical scavenging activity, and ferric reducing antioxidant power concomitantly.

MATERIALS AND METHODS

Materials and Reagents. 'JS335' is the most popular soybean cultivar with wide adaptability across different soybean-growing regions of India. Moreover, the pods of the cultivar are glabrous, a desirable trait for pushing the green seeds directly into mouth from the boiled immature pods. Seeds of cultivar 'JS335' were sown in the fields of the National Research Centre for Soybean in the cropping season of 2007, and plants raised under isoclimatic conditions were tagged at the time of flowering. Sufficient numbers of the pods were picked at reproductive stages R5, R6, R7, and full maturity as defined by Fehr et al. (*17*). At R5 stage, soybean seeds grow actively, whereas at R6 stage the pod cavity is completely filled but the seeds are completely green. At R7 stage, soybean seeds begin to attain physiological maturity, and both the pods and the seeds contained in them start turning yellow. At each reproductive stage, fresh weight of 100 seeds was recorded, and moisture percentage of the seed samples was determined using and oven-dry method.

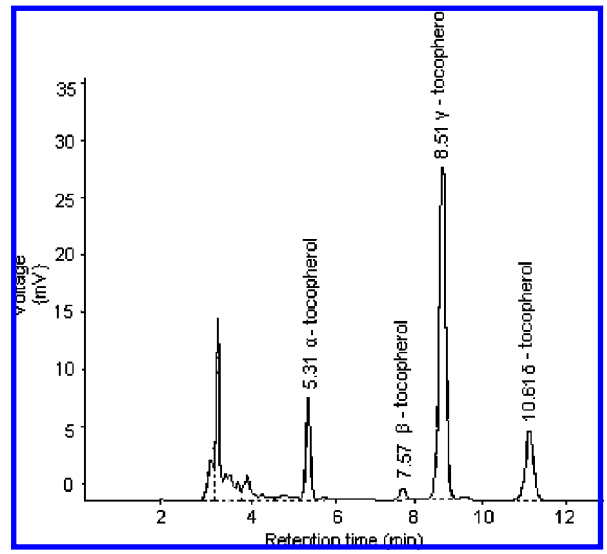

Figure 3. Separation of α-, *β*-, *γ*-, and *δ*-tocopherol isomers using HPLC.

HPLC-grade solvents (acetonitrile, ethyl acetate, *n*-hexane) and individual standards of isoflavones (daidzein, glycitein, genistein) and tocopherols (α, *β*, *γ*, and *δ*) were procured from Sigma Aldrich.

HPLC Determination of Tocopherol Isomers. *Sample Preparation.* Twenty random soybean seeds shelled from pods picked at different reproductive stages were oven-dried, ground using a metallic pestle and mortar into a fine flour, and passed through a 100-mesh sieve. Oil from the finely ground soy flour was extracted using hexane (5 mL) by keeping the flour soaked in hexane for 8 h. The hexane−oil mixture was transferred into vials, and the solvent was evaporated at 40 °C in a vacuum oven until the solvent evaporated completely and only oil was left. A known quantity of the oil taken in an Eppendorf vial was dissolved in HPLC-grade hexane, and the mixture so obtained was passed through a syringe filter (Whatman; 13 mm, 0.5 *μ*m). A 20 *μ*L sample was injected into a Shimadzu HPLC system (LC10AT *vp* series) using a Hamilton syringe.

HPLC Conditions. Separation of four tocopherols was carried out using a silica amino column (Phenomenex, 5 *μ*m with dimension of 250 × 4.6 mm) fitted in a Shimadzu HPLC system. The samples were eluted isocratically by employing mobile phase (*n*-hexane/ethyl acetate, 3:1 v/v) at a flow rate of 1.0 mL/min using an LC10AT pump. For the best resolution of all the isomers of tocopherols, the temperature of the column oven was maintained at 35 °C. The tocopherols were detected with a UV detector (SPD 10 AT *vp*) at 495 nm. The resolution of the tocopherol isomers is shown in **Figure 3**, and the peak area of these isomers was compared with the respective standard curve generated from various concentrations of external standards to calculate the relative amounts of four isomers of tocopherol using software CSW version 1.7. The concentrations of tocopherols were determined in the oil fraction of the oven-dried seeds; therefore, to express the concentration per gram of seed, oil content (percent) at each reproductive stage was estimated gravimetrically using a semiautomated Soxhlet system (Pelican Equipment, Chennai, India) and employed to convert the estimated concentrations per gram of oil into per gram of soy flour.

HPLC Determination of Isoflavones. *Sample Preparation.* Seeds from different reproductive stages were finely ground after complete drying in an oven at 75 °C and passed through a 100-mesh sieve. Finely ground soy flour (125 mg) was extracted with 80% ethanol (5 mL) and concentrated HCl (1 mL) for 2 h in a boiling water bath using a standard method (*18*), which relies on acid hydrolysis of 12 endogenous isoflavone isomers to their respective aglycone forms, that is, daidzein, glycitein, and genistein. The suspension resulting after the extraction was centrifuged at 10000 rpm for 10 min.

HPLC Conditions. The supernatant obtained after centrifugation was passed through a syringe filter (Whatman 0.5 *μ*m, 13 mm diameter) before loading into the HPLC system. Twenty microliters of the syringe-filtered sample was injected into a Shimadzu chromatograph (LC-10AT VP), equipped with a UV detector (SPD 10AT VP) and oven (CTO-

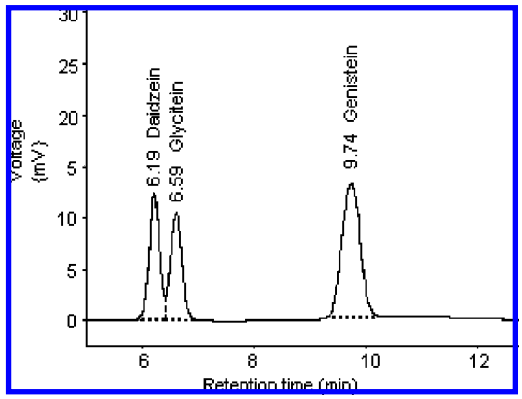

Figure 4. Separation of isoflavones (daidzein, glycitein, and genistein) using HPLC.

10) housing a C-18 silica column (Phenomenex; 5 μm with dimension of 250 × 4.6 mm), preceded by a guard column (Phenomenex, 4.0 × 3.0 mm). The column oven was maintained at 40 °C. The separation and elution of isoflavones was accomplished by employing a binary gradient mode with solvent A (10% ACN) and solvent B (38% ACN) at a flow rate of 0.8 mL/min for 25 min. The solvent system was run as follows (% solvent A/solvent B): 0 min (0/100), 5 min (10/90), 20 min (0/100), and 25 min (0/100). The resolution of isoflavones as detected at 260 nm is shown in **Figure 4**. A standard curve for daidzein, glycitein, and genistein was generated by injecting various concentrations of the standards of these isoflavones procured from Sigma-Aldrich. The relative concentration of individual isoflavones in the sample was calculated by software CSW version 1.7 after superimposing the chromatogram of the sample on the standard curve. Individual isoflavone concentration was expressed as micrograms per gram on a dry weight basis. Concentrations of aglycones were summed to compute total isoflavone concentration.

Extraction of Antioxidants from Seeds. Oven-dried seeds of different reproductive stages were finely ground into flour and made to pass through a 100-mesh sieve. Soy flour (1.0 g) was extracted with 15 mL of 70% aqueous acetone at 25 °C in the dark overnight. The mixture was centrifuged at 3000 rpm for 10 min. The residues were re-extracted with 5 mL of the 70% acetone. Both extracts were combined and stored at 4 °C in the dark for further analyses of total phenolic content, DPPH free radical scavenging activity, and ferric reducing antioxidant power assay. The extraction and analysis were performed in triplicate.

DPPH Free Radical Scavenging Activity. DPPH free radical scavenging capacity of the extract was evaluated using an absolute ethanolic solution of DPPH following the procedure of Mellore and Tappel (*19*). The absorbance of sample (A_{sample}) was measured using a spectrophotometer (UV160A, Shimadzu) at 517 nm against ethanolic blank. A negative control was run after the addition of DPPH solution to 0.1 mL of the extraction solvent (70% acetone). Decrease in absorbance at 517 nm showed reduction of DPPH radical. The percent inhibition of the DPPH radical by the soybean antioxidant extract was calculated using the formula

$$\%\ \text{DPPH free radical scavenging activity} = [1 - (A_{sample}/A_{control})] \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample after incubation for 10 min at room temperature.

Ferric Reducing Antioxidant Power (FRAP) Assay. Total antioxidant capacity of the soybean extract was determined using the FRAP assay as described by Benzie and Strain (*20*). FRAP reagent [3 mL containing 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1] was mixed with 0.1 mL of soybean extract and incubated for 15 min at 37 °C; absorbance was recorded at 593 nm. FRAP value was expressed as millimoles per 1000 g on a dry weight basis using the calibration curve of Fe^{2+}. The linearity range of the calibration curve was 0.015−0.075 μM.

Determination of TPC. The TPC of the soybean extracts was determined by Folin−Ciocalteu reagent following the method of Singleton and Rossi (*21*). An aliquot (0.05 mL) of 70% acetone soy flour extract was mixed with 0.5 mL of Folin−Ciocalteu reagent and 0.5 mL of 20% sodium carbonate, and the final volume was made to 5 mL with distilled water. The absorbance was measured at 700 nm against distilled water as blank after incubation for 30 min at room temperature. Total phenolic content was expressed as gallic acid equivalents (milligrams of GAE per gram on a dry weight basis) through standard calibration curve of freshly prepared propyl gallate.

Statistical Analyses. Samples were prepared in triplicate for carrying out the above biochemical analyses. All of the statistical analyses were carried out using the M-STATC program.

RESULTS AND DISCUSSION

Physical Parameters at Different Reproductive Stages. **Table 1** exhibits the physical characters of the immature seeds shelled from the pods at different reproductive stages. Weight of the 100 seeds (fresh weight) was maximum at R6 stage, that is, second picking. Moisture content declined slowly between stages R5 and R6 and, thereafter, it declined rapidly until complete maturity. The rate of increase of dry matter was maximum between stages R5 and R6, with maximum dry mass accumulated by R7 stage (third picking). After R7 stage, there was little increase in the dry mass of the seeds.

Tocopherol Profile at Different Reproductive Stages. Tocopherol isomers and total tocopherol content varied significantly ($P < 0.05$) between various reproductive stages (**Table 2**). In the tender seeds obtained from the first picking, that is, at R5 stage, all four isomers of tocopherols (α, β, γ, and δ) were observed,; however, δ-tocopherol was the predominant form, comprising about 70% of the total tocopherol concentration. In the seeds from the second picking (R6 stage), δ-tocopherol was 52.6% of the total tocopherol content. γ-Tocopherol increased about 3-fold, whereas the α- and β-isomers remained unchanged compared to the R5 stage. At R7 stage, that is, seeds obtained from the third picking, the concentration of the γ-isomer increased further and was slightly higher than that of the δ-isomer. During this period, though, the concentration of the α-isomer increased significantly ($P < 0.05$); however, the β-isomer remained unchanged. At complete maturity, the concentration of δ-tocopherol, which remained static between stages R5 and R7, increased slightly, whereas the α-, β-, and γ-isomers exhibited 7.0-, 5.0-, and 3.5-fold increases over their respective concentration in the previous picking stage (R7 stage). Consequently, total tocopherol content increased to the magnitude of 3-fold between R7 stage and complete maturity. γ-Tocopherol contributed the maximum (57.6%) and the β-isomer the minimum (4.1%) proportion to total tocopherol content in mature seeds. Furthermore, as evident from **Figure 5**, maximum accumulation of total tocopherol content occurred when the rate of increase in oil content was the lowest.

Individual Forms of Isoflavones and Total Isoflavones at Different Reproductive Stages. Individual forms of isoflavones and total isoflavone concentration changed significantly between seeds picked at various reproductive stages (**Table 3**). Total isoflavones increased in each picking starting from R5 to complete maturity stage. An increase of 5.1-fold (from 333.1 to 1696.9 $\mu g\ g^{-1}$ on a dry weight basis) was observed for total isoflavone concentration. For accumulation of isoflavones, the growth period between stages R5 and R6 was the most active phase, exhibiting a 117% increase, whereas the period between R7 and complete maturity was the most sluggish phase with only a 40% increase. Compared to the R5 stage, daidzein,

Table 1. Physical Parameters of the Seeds at Different Reproductive Stages of Soybean in Cultivar 'JS335'[a]

growth stage	seed coat color	100-seed fresh wt (g)	moisture (%)	100-seed dry wt (g)	% dry matter accumulation
R5	green	11.10 ± 0.41 c	74.8 ± 0.84 a	2.80 ± 0.05 d	31.1 d
R6	green	22.01 ± 0.60 a	68.7 ± 0.61 b	6.88 ± 0.06 c	76.4 c
R7	yellow green	18.48 ± 0.73 b	47.8 ± 1.23 c	9.64 ± 0.17 b	94.5 b
complete maturity	creamy yellow	11.41 ± 0.52 c	10.5 ± 0.40 d	10.20 ± 0.14 a	100 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($P < 0.05$) for each physical parameter.

Table 2. Concentrations (Micrograms per Gram on a Dry Weight Basis) of Tocopherol Isomers at Different Reproductive Stages in Cultivar 'JS335'[a]

reproductive stage	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol
R5	3.42 ± 0.23 c (6.0)	1.10 ± 0.09 b (1.9)	12.06 ± 0.94 d (21.4)	39.60 ± 1.31 b (70.7)
R6	3.70 ± 0.09 c (4.3)	1.43 ± 0.16 b (1.6)	35.33 ± 2.71 c (41.4)	44.83 ± 1.62 b (52.6)
R7	7.31 ± 0.14 b (7.7)	2.12 ± 0.17 b (2.2)	46.01 ± 2.14 b (47.7)	41.01 ± 1.80 b (42.5)
complete maturity	53.34 ± 1.85 a (19.6)	11.20 ± 0.96 a (4.1)	156.80 ± 4.83 a (57.6)	51.20 ± 2.07 a (18.7)

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($P < 0.05$) for each isomer of tocopherol. Values given in parentheses indicate percentage of the total tocopherol concentration.

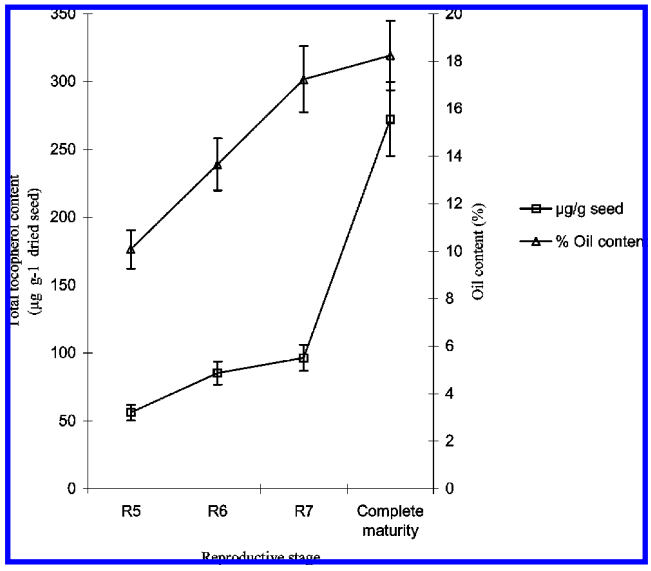

Figure 5. Total tocopherol concentration and oil content during different reproductive stages of soybean in cultivar 'JS335'. Numbers represent the average of three independent samples ± SE.

Table 3. Concentrations (Micrograms per Gram on a Dry Weight Basis) of Isoflavones at Different Reproductive Stages of Soybean in Cultivar 'JS335'[a]

reproductive stage	daidzein	glycitein	genistein	total isoflavones
R5	76.38 ± 2.61 d	172.80 ± 3.60 c	83.92 ± 0.93 d	333.10 ± 3.64 d
R6	166.16 ± 5.40 c	183.28 ± 5.40 c	375.57 ± 2.72 c	725.00 ± 5.86 c
R7	301.53 ± 4.92 b	326.04 ± 6.42 b	583.57 ± 5.40 b	1211.14 ± 2.87 b
complete maturity	349.59 ± 6.51 a	539.54 ± 6.35 a	807.75 ± 6.71 a	1696.88 ± 7.69 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($P < 0.05$) for individual forms of isoflavones and total isoflavones content.

glycitein, genistein, and total isoflavone contents increased 4.6-, 3.12-, 9.6-, and 5-fold at complete maturity.

For both daidzein and genistein, maximum percent increases were between stages R5 and R6 with magnitudes of 117 and 347.7%, respectively, whereas the minimum increases of these forms of isoflavones were between R7 and complete maturity with magnitudes of 15.9 and 38.4%, respectively. For glycitein content, it is the growth phase between stages R6 and R7 that exhibited maximum increase (77.8%) for its accumulation.

Table 4. DPPH Free Radical Scavenging Activity, FRAP, and TPC at Different Reproductive Stages of Soybean in Cultivar 'JS335'[a]

reproductive stage	DPPH (% reduction)	FRAP (mmol/1000 g on dry wt basis)	TPC (gallic acid equiv g^{-1})
R5	58.54 ± 1.31 a	55.43 ± 1.24 a	3.13 ± 0.05 a
R6	53.02 ± 1.24 b	32.01 ± 0.73 b	1.94 ± 0.02 b
R7	44.95 ± 0.89 c	30.92 ± 0.42 b	1.50 ± 0.04 c
complete maturity	43.87 ± 0.76 c	20.75 ± 0.35 c	1.34 ± 0.01 d

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($P < 0.05$) for DPPH free radical scavenging activity, FRAP value, and TPC.

Barring the R5 stage when glycitein content represented maximum proportion of the total isoflavones' content, at all other picking stages, genistein was predominantly high.

TPC, DPPH Free Radical Scavenging Activity, and FRAP at Different Reproductive Stages. Table 4 exhibits the concentrations of total phenolic content, free radical scavenging activity, and FRAP of the tender seeds picked at various immature stages and at complete maturity. Total phenolic content was maximum (3.13 GAE g^{-1} on a dry weight basis) in immature seeds obtained from the first picking; however, it declined thereafter continuously until maturity with maximum decline observed between stages R5 and R6. DPPH free radical scavenging activity was highest at R5 stage and declined in the subsequent two pickings, that is, at R6 and R7 stages with maximum percent decline between stages R6 and R7. It did not change significantly between R7 stage and complete maturity. Similar to free radical scavenging activity, FRAP was maximum at R5 stage. It declined sharply at R6 stage, remained the same between stages R6 and R7, and again declined between R7 and complete maturity stage.

In the first two pickings, R5 and R6 stages, although all four isomers were present, it is δ-tocopherol that predominates. As evident from the proposed pathway for the biosynthesis of tocopherol isomers as given in **Figure 6** (*2*), two precursors for tocopherol synthesis, that is, homogentisic acid and phytyl diphosphate, are obtained from the shikimate and isoprenoid pathways, respectively. The concentration of different tocopherols is dependent upon the activities and substrate of E1 (homogentisic acid prenyltransferase), E2 (2 methyl-6-phytylbenzoquinol, that is, MPBQ tocopherol cyclase), E3 (MPBQ specific methyl transferase), E4 (2,3-dimethyl-5-phytybenzoquinol specific translocase), and E5 (γ -tocopherol methyl transferase). We speculate that high levels of δ-tocopherol at

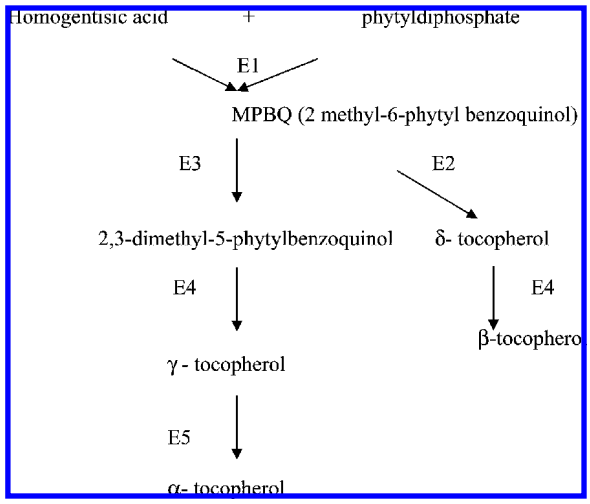

Figure 6. Final steps of tocopherol biosynthetic pathway as given by Bramley et al. (*2*): E1, homogentisic acid prenyltransferase; E2, 2-methyl-6-phytylbenzoquinol (MPBQ) specific tocopherol cyclase; E3, MPBQ specific methyl transferase; E4, 2,3-dimethyl-5-phytylbenzoquinol specific translocase; E5, γ-tocopherol methyl transferase.

the early reproductive stages may be because of higher activities of MPBQ tocopherol cyclase (E2) and/or low activities of one or more enzymes in the chain involving MPBQ specific methyl transferase (E3), 2,3-dimethyl-5-phytybenzoquinol specific translocase (E4), and γ-tocopherol methyl transferase (E5). It would be interesting to study the differential activities of the enzymes involved in the proposed pathway at different reproductive stages of soybean seed.

Interestingly, concentrations of three tocopherol isomers, that is, the α-, β-, and γ-isomers, increased dramatically between R7 stage and maturity. Britz and Kremer (*22*) suggested that biosynthesis of the tocopherols as free radical scavengers is triggered as a response to stress conditions. Yadav et al. (*23*) studied the electronic spin resonance (ESR) spectra of oxy radicals during seed development and observed a sudden burst of free radicals after R7 stage when there was rapid drying of the seeds as indicated by a sharp decline in moisture content in our study (**Table 1**). A sudden rise in the tocopherols between R7 stage and maturity may be a response to quench the intensity of free radicals generated during the period. The presence of all four naturally occurring isomers in considerable concentration at all the reproductive stages is in contrast to other tocopherol-containing crops. Tocopherols in pea (*Pisum sativum*) exist predominantly as γ-tocopherol with negligible concentrations of α- and δ-isomers (*24*), whereas in peanuts total tocopherol content is composed mainly of the α- and γ-isomers of tocopherol (*25*). Individual forms of isoflavones as well as total isoflavone content increased throughout the seed development, which was in consonance with the results obtained by Kim et al. (*16*) in two Korean cultivars, 'Sojinkong' and 'Daepungkomg'. However, unlike tocopherol isomers, individual forms of isoflavones did not increase dramatically between R7 stage and complete maturity. The most active phase of accumulation of isoflavones, that is, between stages R5 and R6, coincided with the active phase of dry matter accumulation.

Tocopherols and isoflavones increased in the late reproductive stages. The increasing trend of total tocopherol concentration towards seed maturity in *Brassica napus* L. as observed by Goffman et al. (*26*) was in conformity with our results. Similarly, increase in total isoflavone content toward maturity was in consonance with the early study carried out during soybean seed development (*16*). However, free radical scavenging, total antioxidative activity, and total phenolic content in our study declined as the developing soybean seeds approached complete maturity. Soybean is known to possess phenolic acids, namely, syringic acid, ferulic acid, sinapic acid, coumaric acid, gentisic acid, chlorogenic acid, caffeic acid, and hydroxybenzoic acid, in mature seeds (*27*). Among these, caffeic and chlorogenic acid are strong scavengers. They are more potent DPPH scavengers than isoflavones. In rye, Weidner et al. (*28*) observed a decline in the phenolic acids, namely, coumaric acid, caffeic acid, sinapic acid, and ferulic acid, as well as the total phenolic content as the grains approached maturity. Therefore, the decline in free radical scavenging activity and total antioxidative capacity despite the increase in tocopherols and isoflavones may be due to a decline in phenolic acids with stronger antioxidant activity than these two biological components. A steep decline in total phenolic content as the seed approached maturity strengthens this suggestion.

Conclusively, our results show that immature seeds picked at early reproductive stages possess low concentrations of tocopherols and isoflavones, but they have high free radical scavenging and total antioxidative power. As a decline in total phenolic content was observed in late picking stages, we speculate that a decline in bioactive compounds other than tocopherols and isoflavones may be responsible for the low antioxidative value of picked seeds from late reproductive stages. In this context, it would be of interest to investigate the concentrations of various phenolic acids, proanthocyanidins, and saponins, the biomolecules present in the soybean seeds and known to have antioxidative activity, at different reproductive stages.

ABBREVIATIONS USED

DPPH, 2,2-diphenyl-2-picrylhydrazyl radical; FRAP, ferric chloride reducing antioxidant power, HPLC, high-performance liquid chromatography; TPC, total phenolic content; GAE, gallic acid equivalents.

LITERATURE CITED

(1) Kamal-Eldin, A.; Appleqvist, L. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **1996**, *31*, 671–667.

(2) Bramley, P. M.; Elmadfa, I.; Kafatos, A.; Kelly, F. J.; Manios, Y.; Roxborough, H. E.; Schuch, W.; Sheehy, P. J. A.; Wagner, K. H. Vitamin E. *J. Sci. Food Agric.* **2000**, *80*, 913–938.

(3) Damasceno, N. R. T.; Apolinario, E.; Flauzino, F. D.; Fernandes, I.; Abdall, D. S. P. Soy isoflavones reduce electronegative low-density lipoprotein (LDL) and anti-LDL auto-antibodies in experimental atherosclerosis. *Eur. J. Nutr.* **2007**, *46*, 125–132.

(4) Nordentoft, I.; Jeppesen, P. B.; Hong, J.; Abdula, R.; Hermansen, K. Increased insulin sensitivity and changes in the expression profile of key insulin regulatory genes and beta cell transcription factors in diabetic kkAy-mice afetr feeding with a soybean protein rich diet high inisoflavones content. *J. Agric. Food Chem.* **2008**, *56*, 4377–4385.

(5) Sarkar, F. H.; Li, Y. W. The role of isoflavones in cancer chemoprevention. *Frontiers Biosci.* **2004**, *9*, 2714–2724.

(6) Song, Y.; Paik, H. Y.; Joung, H. Soybean and soy isoflavone intake indicate a positive change in bone mineral density for 2 years in young Korean women. *Nutr. Res. (N.Y.)* **2008**, *28*, 25–30.

(7) Schneider, C. Chemistry and biology of vitamin E. *Mol. Nutr. Food Res.* **2005**, *49*, 7–30.

(8) Setchell, K. D.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *129*, 758S–767S.

(9) Shanmughamsundaram, S.; Marr, M. R. Global expansion of high value vegetable soybean. *Proc. VII World Soybean Res. Conf.* **2004**, 921–928.

(10) Kumar, V.; Rani, A.; Chauhan, G. S. A comparative study of oligosachharides in immature and mature seeds of soybean genotypes. *J. Food Sci. Technol*. **2007**, *44* (1), 49–51.
(11) McCord, K. L.; Fehr, W. R.; Wang, T.; Welke, G. A.; Cianzio, S. R.; Schbebly, S. R. Tocopherol content of soybean lines with reduced linolenate in the seed oil. *Crop Sci*. **2004**, *44*, 772–776.
(12) Riedel, K. M.; Lee, J. H.; Renita, M.; Martin, K. S.; Schwartz, S. J.; Vodovotz, Y. Isoflavones profiles, phenol content, and antioxidant activity of soybean seeds as influenced by cultivar and growing location in Ohio. *J. Sci. Food Agric*. **2007**, *87*, 1197–1206.
(13) Lee, S. J.; Kim, J. J.; Moon, H. I.; Ahn, J. K.; Chun, S. C.; Jung, W. S.; Lee, O. K.; Chung, I. M. Analysis of isoflavones and phenolic compounds in Korean soybean seeds of different seed weights. *J. Agric. Food Chem*. **2008**, *56*, 2751–2758.
(14) Xu, B. J.; Yuan, S. H.; Chang, S. K. C. Comparative analyses of phenolic composition, antioxidant capacity and color of cool season legumes and other selected food legumes. *J. Food Sci*. **2008**, *72* (2), S167−S176.
(15) Rani, A.; Kumar, V.; Verma, S.; Shakya, A.; Chauhan, G. S. Tocopherol content and profile of soybean: genotypic variability and correlation studies. *J. Am. Oil Chem. Soc*. **2007**, *84*, 377–383.
(16) Kim, J. A.; Chung, I. M. Changes in isoflavones concentration of soybean (*Glycine max*) seeds at different growth stages. *J. Sci. Food Agric*. **2007**, *87*, 496–503.
(17) Fehr, W. R.; Caviness, C. E.; Burmood, D. T.; Penninton, J. S. stage of development descriptions for soybeans, *Glycine max* (L.) Merril. *Crop Sci*. **1971**, *11*, 929–931.
(18) Vyn, T. J.; Yin, X.; Bruulsema, T. W.; Jackson, J. C.; Rajcan, I.; Bruder, S. M. Potassium fertilization effects on isoflavones concentrations in soybean. *J. Agric. Food Chem*. **2002**, *50*, 3501–3506.
(19) Mellors, A.; Tappel, A. L. The inhibition of mitochondrial peroxidation by ubiquinon and ubiquinol. *J. Biol. Chem*. **1996**, *241*, 4353–4356.
(20) Benzie, I. F.; Strain, J. J. The ferric reducing ability of plasma as a measure of 'antioxidant power': the FRAP assay. *Anal. Biochem*. **1996**, *239*, 70–76.
(21) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdate and phosphotungstic acid reagent. *Am. J. Enol. Vitic*. **1965**, *16*, 144–158.
(22) Britz, S. J.; Kremer, D. F. Warm temperatures or drought during seed maturation increase α-tocopherol. *J. Agric. Food Chem*. **2002**, *50*, 6058–6063.
(23) Yadav, S.; Bhatia, V. S.; Guru Prasad, K. N. Oxyradical accumulation and rapid deterioration of soybean seeds due to field weathering. *Indian J. Biochem. Biophys*. **2006**, *43*, 41–47.
(24) Yoshida, H.; Tomiyama, Y.; Saiki, M.; Mizushina, Y. Tocopherol content and fatty acid distribution of pea (*Pisum sativum* L.). *J. Am. Oil Chem. Soc*. **2007**, *84*, 1031–1038.
(25) Wyatt, C. J.; Carballido, S. P.; Mendez, R. O. α- and γ-tocopherols content of selected foods in the Mexican diet. *J. Agric. Food Chem*. **1998**, *46*, 4657–4661.
(26) Goffman, F. D.; Velasco, L.; Becker, H. C. Tocopherols accumulation in developing seeds and pods of rapeseed. *J. Agric. Food Chem*. **1999**, *49*, 4990–4994.
(27) Stroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol*. **2003**, *41*, 753–758.
(28) Weidner, S.; Frqczek, E.; Amarowicz, R.; Abe, S. Alterations in phenolic acids content in developing rye grains in normal environment and during enforced dehydration. *Acta Physiol. Planta*. **2000**, *23* (4), 475–482.

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