Article

Phytosterol Composition of *Arachis hypogaea* Seeds from Different Maturity Classes

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Abstract: The seeds of cultivated peanut, *Arachis hypogaea*, are an agronomically important crop produced for human nutrition, oilseed and feed stock. Peanut seed is the single most expensive variable input cost and thus producers require seed with excellence performance in terms of germination efficiency. During the maturation process, triglycerides are stored in oil bodies as an energy resource during germination and seedling development. The stability of oil body membranes is essential for nutrient mobilization during germination. This study focused on evaluating the phytosterol composition in seed components including the kernel, embryo (heart), and seed coat or skin. Samples of different maturity classes were analyzed for macronutrient and phytosterol content. The three most abundant phytosterols, β-sitosterol, campesterol, and stigmasterol, comprised 82.29%, 86.39%, and 94.25% of seed hearts, kernels, and seed coats, respectively. Stigmasterol concentration was highest in the seed kernel providing an excellent source of this sterol known to have beneficial effects on human health. Peanut hearts contained the highest concentration of sterols by mass potentially providing protection and resources for the developing seedling. The amount of α-tocopherol increases in peanut hearts during the maturation process providing protection from temperature stress and stability required for seedling vigor. These results suggest that phytosterols may play a significant role in the performance of seeds and provides a possible explanation for the poor germination efficiency of immature seeds.

Keywords: phytosterols; mesocarp; oilseed; maturity; pod-blast; α-tocopherol; oil bodies; campesterol; stigmasterol; β-sitosterol

1. Introduction

The seeds of cultivated peanut, *Arachis hypogaea*, store proteins, lipids, and starch required for energy and growth upon germination. The seed can be harvested to serve as human nutrition, stock feed, and biofuels [1,2,3]. Peanuts and other legumes have the ability to fix nitrogen and thus increase the sustainability of agricultural systems [4]. The pressure for peanut seed as plant-based protein and oilseed source, is increasing with a growing world population. Due to its agronomic importance, improvement of peanut seed performance is necessary to meet the demand.

Lipid (oil) is the predominate macro component and generally increases as the peanut seed matures [5]. For mature peanuts, average total oil was reported to average about 50% on a fresh weight basis [6]. Oilseeds, such as peanuts, store most of their lipids in small, intracellular organelles commonly called oil bodies [7,8]. Triglycerides comprise the majority of these oil bodies, and the interior triglycerides are encapsulated by a phospholipid bilayer and embedded oleosin protein. The oil bodies provide a stable energy reserve that can be accessed upon germination [9].

Phytosterols are a special class of structural lipids that provide stability and fluidity in cell membranes including specialized encapsulation of lipids, such as oil bodies [10]. The primary function of phytosterols are membrane reinforcers and precursors for brassinosteroids, an important phytohormone in plants. Unlike animal and fungi counterparts with cholesterol and ergosterol as the dominant sterol, the plant usually synthesizes an array of sterols with different alkyl group substitutions at the sterol side chain. In addition to the primary function, the correct sterol composition is necessary for many aspects to plant biology such as embryonic pattern formation, cell division, cell elongation, cell polarity, cellulose accumulation, and interactions with other signaling pathways [11].

Physiological maturity impacts seed quality through a variety of mechanisms including desiccation tolerance, preparation of storage reserves and establishment of dormancy [12]. Thus, the impact of seed maturity on germination efficiency is of primary importance to peanut producers. To best assess physiological maturation, a method of classification based on color and morphological differences of the mesocarp was described for determining the developmental stages of fresh peanut (Figure 1) [13]. Maturity determination by this method requires removal of a portion of the exocarp to expose the pod mesocarp. The characteristic darkening of the pericarp is part of a progressive change in colors resulting in mesocarp colors from white (immature) to black (mature) [14]. The outer layer of the pod can be removed, and seeds sorted by maturity class (white, yellow, orange, brown or black) in a non-destructive manner allowing for physiological and chemical studies pertaining to the stage of development [12].

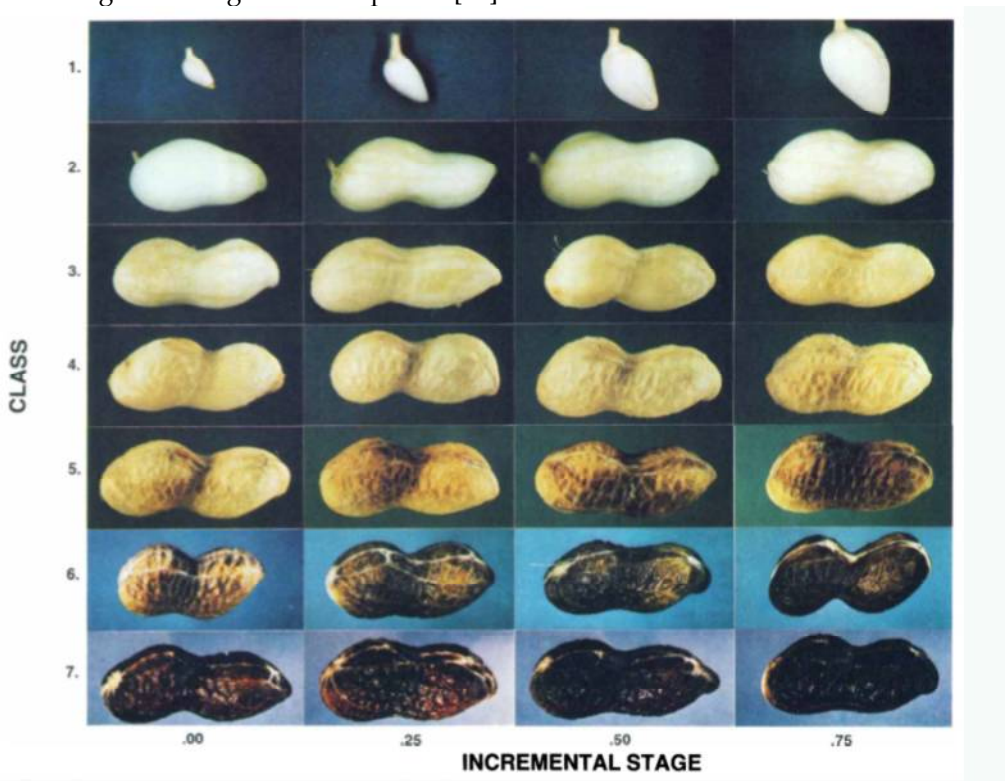

Figure 1.
Florunner mesocarps (exocarp removed) of seven maturity classes subdivided into incremental stages.

Seed maturity proceeds with the thickening of cell walls and addition of oil bodies [15]. For mature peanuts of the highest classification (black mesocarp), the cytoplasm of the parenchyma cells is essentially full of oil bodies. The specific aim of the project seeks to clarify the role of phytosterols in the formation of oil bodies as the seed matures. To accomplish this aim, seeds were organized into different maturity classes and the phytosterols extracted and identified. It is hypothesized that establishment of a critical mass of phytosterols provides the membrane stability for proliferation of oil bodies during maturation.

2. Results

2.1 Stage of peanut maturity and macronutrient composition in different maturity classes

Peanut seed development can be classified into 7 classes with 4 incremental stages in each class (Figure 1) [13]. Based on the color of the mesocarp, the last three classes are described and named as "orange", "brown", or "black", respectively. Pooled samples from each maturity classes were analyzed to determine macronutrient percentages and results summarized in Table 1. Generally, fat % increased from 50.95% to 51.96% when immature samples (orange mesocarp class) were compared to mature seeds (black mesocarp class). Protein % decreased and sugar % increased when comparing immature and mature seed samples. These results are consistent with observed trends in peanut crop analysis conducted by the Biochemistry Research Laboratory at Lubbock Christian University during the 2011-2017 CYs. The USDA National Nutrient Database for Standard Reference (Release 27, Basic Report 16087) reports macronutrient composition as 49.2% fat, 25.8% protein, and 4.7% sugars. The reported results are significantly different as compared to the standard values with respect to total fat and protein percentages but could be attributed to a specific variety versus average trends across all market types of cultivated peanut.

Table 1. Macronutrient Composition of *Arachis hypogaea* Seeds of Different Maturation Classes.

Pod Color	Fat (%)	Protein (%)	Sugar (%)
Orange	50.95	20.90	3.43
Brown	51.49	19.90	3.63
Black	51.96	18.00	4.42

Method error ±0.33% fat, 0.648-0.798% protein, and -0.33-0.52% sugar.

2.2 Isoprenoids and phytosterol composition in different tissues

Using GC/MS, we analyzed three classes of peanut samples for their isoprenoid and sterol composition. We have positively identified 11 compounds (Figure 2) based on the mass spectra and retention time relative to the authentic standards.

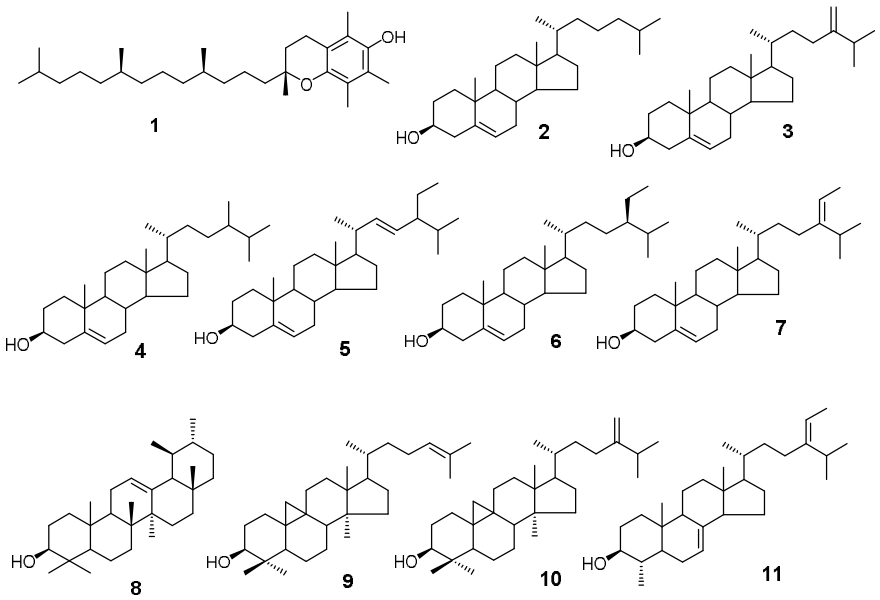

Figure 2. Structures of compounds identified by GC/MS.

We found that of the three seed component classes analyzed, the kernel and seed coat generally contained the same percentage of α-tocopherol (0.63% and 0.64%, respectively), decreasing slight with maturity in brown versus black mesocarp classes, 0.69% to 0.63% kernel composition and 0.71% to 0.64% seed coat composition (Table 2). In contrast, the hearts contained significantly less

tocopherol by percentage (Table 2) as compared to the kernel and seed coat. Also, the amount of α-tocopherol increased from 0.09% in the orange mesocarp class, to 0.19% in the brown mesocarp class, and 0.24% in the black mesocarp class. Vitamin E is comprised of eight structurally related molecules including four forms of tocopherols. Peanuts, like many other oilseeds, contain tocopherols [16]. Within plants, these molecules are found in cell membranes and possess antioxidant activity which protects organelles from reactive oxygen species (ROS) [17]. Like phytosterols, tocopherols contribute to maintaining membrane fluidity [17].

Table 2. Phytosterol and Isoprenoid Composition of *Arachis hypogaea* Seed Components of Different Classes.

		Heart			Kernel			Seed Coat		
	Sterols and Isoprenoids[1]	**Black**	**Brown**	**Orange**	**Black**	**Brown**	**Orange**	**Black**	**Brown**	**Orange**
1	Alpha-Tocopherol	0.24	0.19	0.09	0.63	0.69	0.57	0.64	0.71	0.66
2	Cholesterol	0.19	0.17	0.19	0.21	0.22	0.18	0.54	0.57	0.46
3	24-Methylenecholesterol	0.85	0.86	0.92	0.27	0.86	0.22	0.13	0.12	0.12
4	Campesterol	17.42	16.65	16.78	12.14	11.12	11.25	14.71	14.71	15.11
5	Stigmasterol	2.91	2.87	2.63	11.63	11.61	10.70	3.76	3.95	4.57
6	Sitosterol	61.99	62.03	62.33	62.61	58.70	63.92	75.78	76.45	75.19
7	Isofucosterol	9.75	10.20	11.01	10.70	14.32	11.99	2.67	2.09	2.18
8	alpha-Amyrin	0.38	0.38	0.36	0.61	0.65	0.38	0.99	1.05	0.89
9	Cycloartenol	2.35	2.45	1.95	0.70	0.44	0.37	0.73	0.24	0.74
10	24-Methylenecycloartanol	1.95	2.10	1.65	0.33	1.18	0.21	0.01	0.04	0.00
11	Citrostadienol	1.97	2.12	2.10	0.17	0.21	0.21	0.06	0.08	0.06
	Total (μg/mg)	**8.68**	**8.47**	**10.25**	**1.04**	**0.93**	**0.91**	**5.40**	**5.51**	**5.71**

[1] Sterols and Isoprenoids content is AVE %.

Many phytosterols are present in nature [18]. In peanuts, the three predominant phytosterols are β-sitosterol, campesterol and stigmasterol. In 2004, it was reported that these three sterols comprise approximately 95% of total peanut sterols [18]. Our analysis confirmed that these sterols are the major components of total peanut sterols in each component, 82.29% of hearts, 86.39% of kernels, and 94.25% seed coats (Table 2) but at lower percentages as compared to the 2004 reported values. Other minor component phytosterols were detected (Table 2) including those not previously identified most likely due to advances in technology. Some variation may also be due to varietal differences as these samples were isolated from Runner type peanuts as compared to Spanish, Valencia, and Virginia market types.

When comparing sterol composition in the different seed components, the relative percentage of stigmasterol should be noted (Table 2), heart 2.80%, kernel 11.31%, and seed coat 4.09%. This result differs from the reported value of stigmasterol in Runner peanuts as 11.0% [18]. However, the kernel is the largest component of the seed by mass and as a result, would more closely reflect data on the entire seed.

2.3 Isoprenoid and phytosterol composition in different maturity classes

It is critical for farmers to harvest peanut at the optimized maturity to maximize the crop value and a suitable biomarker could help farmers to harvest at the right time. We compared the profiles of the sterols and isoprenoids from the three stages and there are no statistically significant differences among the major sterols. However, we found that α-tocopherol in the peanut hearts changed dramatically crossing the maturation stages. The absolute amount of this important metabolite was increased correspondingly from 0.92 μg/mg in orange to 1.61 μg/mg in brown and peaked at 2.08 μg/mg in black. Given the fact that Vitamin E is a very important metabolite to plant biology and a valuable compound to human health, we think that the content of Vitamin E could be used as a

biomarker for peanut harvesting. We plan to develop a user-friendly and portable method to determine Vitamin E content, which may have important practical value to peanut farmers.

3. Discussion

Peanuts are a nutrient rich plant-based protein source that contain vitamins, minerals, antioxidants, and bioactive phytochemicals leading to the perception that peanuts are a "super food" [19]. Peanut phytosterols have been shown to help lower LDL cholesterol by competing in the digestive tract with cholesterol and preventing absorption [20]. One of the major phytosterols, stigmasterol, has been investigated for its pharmacological importance as an antihypercholesterolemic, anti-inflammatory, antioxidant, hypoglycemic and antitumor effector [21,22]. In this study, it is reported that the kernel contains a higher percentage of stigmasterol as compared to the heart and seed coat. Different manufacturing processes may remove the seed coat or heart, so it is beneficial to the health of the consumer that the kernel possess relatively high concentrations of stigmasterol.

In addition to potential health benefits, stigmasterol is thought to play a role in temperature stress tolerance in plants [23]. Drought and extreme heat in the growing season can increase the sensitivity of the plant to opportunistic organisms [24]. Accumulation of critical phytosterols, such as stigmasterol, during pod development may set the foundation for physiological maturation processes and resistance to stress.

Tocopherol content can vary with environmental stress and growing location in addition to other factors [25]. In this study, hearts contained the lowest percentage of α-tocopherol as compared to the other seed components, but the amount increased during maturation. During germination, the stability of the peanut heart, or embryo, is critically important to the development of the seedling and stand establishment under adverse environmental conditions [17]. Alpha-tocopherol deactivates ROS generated during photosynthesis and is upregulated during stressful events [17]. Immature peanut seed is less resistant to stress and as a results, is more likely to be adversely affected during germination. The results suggest that the synthesis and accumulation of α-tocopherol in developing peanut hearts may be vitally important to seedling vigor upon germination.

4. Materials and Methods

4.1 Materials and reagents

Epicoprostanol 5β-Cholestan-3α-ol, heptane (99%), anhydride pyridine (99.9%), and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade n-Hexane, HPLC grade methanol, potassium hydroxide (85%) (KOH), acetone, and HPLC grade dichloromethane, were purchased from Thermo Fisher (Waltham, MA, USA).

4.2 Pod blasting

Freshly harvested pods, 2017 Crop Year (CY), from one specific genotype of Runner peanuts grown under conventional cultural practices were obtained. Pods were removed from the plant material, and 'pod blasted' to reveal the mesocarp. Pod blasting is a process by which in-shell peanut pods are placed in a wire basket and a residential style pressure washer is used to spray the shell exterior with high pressure water, removing the outer portion of the peanut hull and exposing the colored mesocarp layer underneath. The blasted pods were separated by color into three different maturity classes, orange, brown, and black. After separation, the remainder of the pod outer layer was removed, and the seeds segregated for additional chemical analyses.

4.3 *Isolation of peanut seed components*

For each maturity class, 10-50g of redskin kernels were weighed and dried in a forced air oven at 130°C for 45 minutes. After cooling to ambient temperature, the kernels were manually separated by removing the skin, breaking open the kernel, and removing the heart to subdivide the samples into three subsections consisting of seed coats, hearts, and kernels. The subsections were scaled at a specific mass for 5 replicates. Each replicate of the heart and kernel contained 50mg while the seed coat replicates contained 20mg. Each scaled replicate was placed in a 2mL microcentrifuge tube with locking lid.

4.4 *Fat, protein and sugar analysis*

To analyze the seed sample for total fat by organic solvent extraction, the exact mass of 10g ±0.1g sample is recorded and the sample pre-dried in a forced air oven at 130°C for 45 minutes to remove moisture. After cooling to ambient temperature, the dried sample is quantitatively transferred to an explosion-proof blender jar. 60mL Dichloromethane (DCM) is added and the mixture blended at high speed for 1 minute. After allowing the blender jar to cool for 30 seconds before opening, remove the blender lid and wash down the sides of the blender with DCM in a wash bottle. Replace lid and blend at high speed for an additional 1 minute. Allow blender jar to cool for 30 seconds before opening and wash down sides of blender with DCM a second time to remove all residue. Carefully pour blender contents into a Büchner funnel vacuum filter apparatus with a Toxicity Characteristics Leaching Procedure (TCLP) glass fiber filter, rinsing the blender jar residue with DCM into the funnel until all residue is removed. Filter the mixture, and transfer the filtrate from the vacuum flask to a tared stainless beaker, rinsing the vacuum flask with DCM into the stainless beaker to ensure all residue is transferred. Evaporate the solvent in the stainless flask over a steam bath until all solvent has been removed. Monitor evaporation and weigh beaker/remaining oil as needed by removing the beaker from the steam bath and allowing it to come to ambient temperature. Evaporation is complete when the mass of the oil remaining in the stainless beaker stabilizes (≤0.03g change in mass over a 30-minute span on the steam bath). Record the final weight of the beaker and oil and calculate the percent oil using the following formula:

$$\% \text{ Oil} = \frac{\text{Weight of Beaker with Oil} - \text{Empty Beaker Weight}}{\text{Sample Weight}} \times 100$$

The protein analysis (reference methods AOAC 992.15; AACC 46-30) was conducted by Medallion Labs (Minneapolis, MN, USA) and the sugar analysis (Sugar by HPLC) was conducted by North Carolina Extension/North Carolina State University (Raleigh, NC, USA).

4.5 *Preparation of nonsaponifiable fraction (NSF)*

Prepare 10% KOH/methanolic solution by dissolving 50g KOH in 50mL deionized water and bringing to volume of 500mL with methanol. Prepare internal standard by mixing the epicoprostanol 5β-Cholestan-3α-ol with heptane to a final concentration of 1mg/mL. After adding 25µL of internal standard solution and 1mL 6N methanolic KOH to each tube, the samples were saponified at 80°C for 2 hours using a Thermomixer (Eppendorf, Hamburg, Germany) with constant shaking at 500rpm. Once cooled to ambient temperature, the nonsaponifiable fraction (NSF) containing free sterols was extracted with 1mL of n-hexane. The hexane was pooled in a 1.5mL microcentrifuge tube and the hexane was removed by evaporation in a fume hood overnight. To the residual, 20µL of acetone was added to dissolve the sterols.

4.6 *GC-MS analysis*

2µL of acetone solution was injected into an Agilent GC-MS (Agilent 6890 BC coupled with 5973 mass selective detector (MSD)) (Agilent, Santa Clara, CA, USA). The GC was equipped with an Agilent DB-5Ms+DG narrow bore capillary column (30m x 0.25mm x 0.25µm with 10m Duraguard). The injection mode was splitless, with helium carrier gas at a constant flow of 1.2mL/minute. The GC

oven was initialed at 170°C, held for 1 minute, the temperature was ramped to 280°C at 40°C/minute and held at 280°C for 25 minutes. The MSD was in electron ionization (EI) mode, scan range was from 50-550amu, temperature of the ion source was 230°C, the quadrupole temperature was 150°C, and the interface was 280°C.

The GC-MS data was processed with ChemStation software (Agilent, Santa Clara, CA, USA) and Automated Mass Spectral Deconvolution & Identification System (AMDIS) (National Institute of Standards and Technology, United States Department of Commerce, Washington, DC, USA). The sterol peaks were deconvoluted using AMDIS after baseline correction and identified by their relative retention time to cholesterol and comparison to the mass spectra from commercial mass database (NIST08 mass spectral library). The GC peak representing the sterol amount generated from total ion current (TIC) was integrated using the software default parameters.

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Conflicts of Interest: The authors declare no conflict of interest.

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