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Analysis of Vitamin E (Tocopherols) using Normal Phase HPLC from common cultivars of Peanut (*Arachis hypogaea* L.)

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Abstract

Background: Peanut is the world's most important edible oil crop, and its kernel contains the highest levels of vitamin E among edible oil seeds. Among tocochromonals, tocopherols alone play a substantial role in immune responses and several other metabolisms. Additionally, it tends to be a healthy diet for human consumption, while also protecting lipids against oxidation which in turn helps to extend the shelf-life of edible oils.

Aims: The aim of the present study is to analyze the tocopherol homologous (α-, γ-, δ-) in peanut oil from the common cultivars of *Arachis hypogaea* L.

Materials: The tested ALR 1, 2, TMV 2, and VRI 5 peanut cultivars, cultivated under an adaptive strategy, for different tocopherol isomers. Papain-enzymatic extraction of oils was done. To ease the tocopherol quantification levels, normal phase HPLC equipped with a photo diode array detector-based method has been used to determine the tocopheryl isomers (α, γ, and δ −Tocols) in a single-step quantification.

ISSN PRINT 2319 1775 Online 2320 7876
 Journal Volume 10, Iss 04, 2021

Results: The Normal phase NP- HPLC used in this experiment showed a lot of variation in the amount of α- and γ-tocopherol in ALR 1, 2, TMV 2, and VRI 5 peanuts. The peanut oil showed the highest differences found for α- and γ-tocopherol were 78.42 $\mu g.g^{-1}$ and 158.70 $\mu g.g^{-1}$, respectively. Additionally, the oil with the highest vitamin E quantification in tocopheryl acetate was about 58.42 $\mu g.g^{-1}$. The DPPH assay, which ranged from 78.61 to 88.56 $mg.L^{-1}$ ascorbic acid equivalent, and the FRAP assay, which ranged from 85.04 to 91.98 mmol/L vit C g^{-1}dw, revealed excellent sources for antioxidants. These findings also suggest that the cold-assisted papain extraction utilized in the quantification of tocopherol in peanut cultivars yielded the maximum amount of oil.

Conclusion: Among the cultivars, consumption of ALR 1, 2, TMV 2, and VRI 5 peanut varieties will provide a more balanced gamma and alpha tocopherol ratio, as well as antioxidant advantages.

Keywords Vitamin E. Peanut oil. Tocopherol. homologs. NP-HPLC.

Abbreviations

Normal Phase-HPLC - High performance Liquid Chromatography

Introduction

In the human diet, Vitamin-E, also known as tocochromonols, is an essential nutrient. It is characterized by eight structural isoforms, which further differentiate into four isomers, namely, tocopherols and tocotrienols.[1] Plants produce dietary tocopherols during photosynthesis, which they store and find to be rich in seed crops. Peanut legumes are the top sources of vitamin-E-enriched edible oils, apart from their high protein and mineral content, and they are also involved in immune functions and the regulation of certain metabolic processes. [2] Also, vitamin E, or tocopherols, present in peanut oils naturally tend to provide additional nutritional benefits, such as strong antioxidants, and protect the high amounts of PUFA in dietary oils. A recent report from the WHO reveals that 90% of populations are not meeting the daily recommended intake of vitamin E through their diet. An ounce of peanuts provides 20% of our daily needs and is also considered an excellent natural dietary source. That implies that two servings provide almost half of our daily needs. [3] But in recent days, fortified

vitamin oils have also gained much attention as a commercial commodity to meet consumer demands. [4]

Peanut oil, with its high smoke point of 450 °F, was primarily utilized as a cooking oil. This oil is abundant in vitamin E, which is well-known for its antioxidant properties and helps maintain the stability of the oil during deep-frying. [5] It is critical that the levels of vitamin E tocopherol in peanut oil, as an isomer-tocopherol equivalent, and its role in fatty acid components confer health benefits. While many crop improvement programs rely on the concentrations of tocopherols and their crucial interaction with agronomic trait attributes (e.g., seed number and weight), the available data on this specific point remains scarce and restricted, with few reports using different environments and field preliminaries. [6]

To date, studies are expanding for the prevalent quality of oil varieties with rich antioxidants and healthy diet compositions, which are yet to be accessible. As a result, the potent neutraceutical vitamin E in oilseed plants has received increased industrial and research attention in the most recent decade. [7, 8] Identification peanut genotypes with high tocopherol concentrations may potentially reduce nutritional composition and agronomic performance, such as seed yield, due to their close association with unfavorable genes. Investigating and understanding the relationship between tocopherol concentrations and other agronomical qualities will ensure that choosing high tocopherol concentrations doesn't compromise other significant attributes. [9]

The normal phase HPLC-DAD method was used to analyze the dietary tocopherol ratios, taking into account the dietary composition of peanut oil and the disease-preventive action parameters of peanuts, which are abundant. [10] Studies reported inadequate ingestion of vitamin E tocopherols. This idea is supported by the findings of the inquiry into vitamin E insufficiency in humans as well as the preliminary hemolysis *in vitro* that was caused by oxidative stress. [11] Given this, it is critical to substantiate the distribution of tocopherols in relation to gamma and alpha-tocopherol levels in the analyzed oils. Despite the prevalence of gamma-tocopherol over alpha-tocopherol in comparison to arachis oil, this is the primary source of vitamin E ingestion by the population, accompanied by a high oxidative balance. To obtain crops with effective vitamin E storage in kernels and for the most beneficial consumption of human nutrition and health, one can select the right cultivar and the most

appropriate growing conditions. According to the findings of this article, the use of oils with higher concentrations of gamma and alpha-tocopherol in peanuts can improve vitamin E consumption in the diet.

Materials and methods

Experiment

The tocopherols standards (> 99 % purity) for the study were commercially availed from Sigma Aldrich (Cat # 258024, T1782, T2028, T3634), Supelco (46401-U), Bangalore,India. The solvents such as n-hexane, ethyl acetate, and molecular grade water were procured from Merck Bengaluru, India. All the chemicals were of HPLC analytical grade.

Collection of seed

The peanuts (*Arachis hypogaea*), was kindly provided by the Department of Centre for Oil crop research, Tamil Nadu Agricultural University of Coimbatore, Tamilnadu. Peanuts were germinated and grown under adaptive cultivation condition. After maturity seeds shelled out and stored at -80°C in air tight bags separately for the analysis.

Cold temperature assisted papain enzymatic extraction of *Arachis* oil:

Peanut oil was extracted by papain extraction method as described below with required modifications. [13] Briefly, the seeds were freeze dried for about 12 hours and crushed using liquid nitrogen in triplicates. This was mixed intermittently and papain (latex taken from unripe papaya) was added to the mixture to a final concentration in the range of 1 %, 1.25 % and 1.5 % (v/w, based on raw peanut weight) which was then optimized to 1.25% and proceeded further for the extraction and incubated for 20 minutes at 60-65°C. Peanut oil bodies of 1g were accurately weighed and a mixture of 2-propanol and n-hexane (0.2:1 v/v) was used to separate the fractions at 4ºC for 6 to 8 hrs. After sufficient enzymatic hydrolysis, the mixture was centrifuged at 12000 rpm for 15 minutes at 4°C for the collection of creamy oil bodies,

ampicillin (0.01% w/v) was added to avoid microbial spoilage for long-term storage. The supernatant was further evaporated, 200 µl of oil from each sample were taken (weight was determined) and re-dissolved in HPLC grade methanol to a final volume of 500µl and filtered using syringe filters (0.22 µm, PTFE syringe filters) [Himedia, Mumbai, India]. Followed by 100 to 500 ppm standard solutions of Vitamin E tocopherol preparation and 20 µl of each sample was injected with the run time of 10 minutes at 30°C for the analysis.

Oil content in seeds

The percentage oil (w/w) content in peanut seeds was evaluated from dried extracts of seeds. For each sample, the triplicate value of the average was determined using AOAC (1995) method. [14]

Oil content was calculated using Eq. (1)

$$\% \text{ Oil yield } = \left[\frac{W2 - W3}{W1}\right] x100$$

Where:

W_1=Original weight of the sample

W_2=Weight of pre-extraction + oil

W_3=Weight of dried sample + post-extraction.

Recovery of Peanut oil (%) = [(Oil content (in 100 g))/mass of papain enzymatic oil extract (76 %)] x100

Here, the expected mass of peanut oil using papain enzymatic extraction is 76 % wt: wt.

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Vitamin E Analysis

Total Vitamin E acetate content assay:

Arachis oil sample of 0.5ml was taken into 5ml amber vial, about 5% methanol and 0.005% Triton X 100 was added and shaken vigorously and kept for incubation at 4°C for 1 minute. About 3ml of xylene was added, the test tube plugged and vigorously shaken for another 1 minute. The tube was centrifuged to separate the extract (1500x*g*, 5 to 7 minutes); simultaneously, 0.05% of 0.25ml solution of Ferrozine was used as standard. About 1.5 ml of the extract (Upper layer) was collected and transferred, into the test-tube and mixed and was analysed at 517 nm against the blank.

The concentration of cx of Vitamin E (α-Tocopheryl acetate) was calculated using the following equation:

Concentration CX of Vitamin E (µM) = $(A_C – A_T) / A_C \times 1000$ –EQ 1

Where A_c is the absorbance of the control (Blank without extract) and A_T is the absorbance of the sample.

Preparation of Standard Solution

Stock solution of alpha tocopherol was prepared in methanol. 0.1g of alpha tocopherol was dissolved in 100 mL of methanol. Dilutions of 100-500 ppm alpha tocopherol standard solutions were prepared from stock solutions.

$$\text{Vitamin E Tocopherol (mg /g}^{-1}) = \frac{\text{Peak area of the sample}}{\text{Peak area of the standard}} \times 1000 \quad (1)$$

Normal phase HPLC Analysis:

For the determination of the eluted Tocopherol, an analytical gradient HPLC waters system (Waters HPLC, Vienna, Austria) equipped with a PDAD method was employed. The normal phase Symmetry® (NH_2) column (4.6x 250 mm I.D; 5.0 um) were used and the eluent was n-hexane and ethyl acetate (both HPLC quality) of (70:30 v/v) [Himedia, Mumbai, India]. The measurement is performed at 30°C. Twenty microliters of syringe – filtered samples were

ISSN PRINT 2319 1775 Online 2320 7876
 Journal Volume 10, Iss 04, 2021

injected into the column and eluted isocratically with HPLC grade methanol (100% v/v) [Merck, Mumbai, India]. The Vitamin E - tocopherols were detected using PDAD 2998; the 292nm absorption band is typical for the determination of alpha-tocopherol and correlates with the concentration in the added external standard read. The tocopherol content were also determined using a Luna NH_2 (amino) column (5 um; Phenomenex, USA) with dimensions of 250 x 4.6 mm. Twenty microliters of a syringe – filtered sample were injected into the column and eluted isocratically with HPLC – grade n-hexane and ethyl acetate (70:30 v/v) [Himedia, Mumbai, India] at a flow rate of 1.0 ml/min. Data represent the average of three replicates ± SE. The chromatographic system was controlled and the data was collected and prepared by the PC integrator (Empower software version 2.0).

DPPH antioxidant activity:

The ability of the test solutions to scavenge DPPH radical was assessed spectrometrically.[15] Briefly 50µl of ascorbic acid test solution (at 500µg mL^{-1}) was mixed with 450 µL Tris-HCL buffer (at 50mmol L^{-1}, pH 7.0) and 2.5 ml DPPH (0.1mmol L^{-1} in methanol), the resultant absorbance was recorded at 517nm after 30 mins at 37ºC. The percentage of incubation was calculated from the following equation:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] \text{ X } 100$$

Where, A_0 was absorbance of the control (blank, without test solution) and A_1 was absorbance in the presence of the test solution.

Ferric reducing antioxidant power (FRAP) assay

The FRAP, a method for measuring total reducing power assay, was assessed with modification. Briefly, 6mL of working FRAP reagent (0.1M acetate buffer: 0.02M $FeCl_3$: 0.01M TPTZ=10:1:1) freshly prepared, was mixed with 20 µL of sodium acetate (500µg mL^{-1}). The absorbance at 593 nm was recorded after 30 min of incubation at 37ºC. Ascorbic acid, was used as the standard at 0.2mg mL^{-1}. [16]

Statistical analysis

Standard curves were formulated against concentration versus peak area by linear regression analysis and mean ± standard deviation was chosen to determine the significant difference at the $p < 0.05$ level among the samples using the program IBM Sigma stat 21. Samples were analysed in triplicate ($n = 3$) and data were reported as mean ± SD using the IBM Sigma stat 21 program.

Result

A total of four peanut cultivars was obtained from Department of Pulses and Oil Seed researches, Tamilnadu Agricultural University Coimbatore, India's peanut breeding field trials. These lines were chosen for this study because of their outstanding agronomic characteristics for adaptive ability under controlled condition with improved pod setting properties (yield, seed and maturity) over the other peanut cultivars that are taken for studies. The HPLC-Normal phase analysis using photo diode array detector-based analysis methods of peanut oil separated all the Tocopherols in four different varieties with same culture conditions.

Determination of papain enzymatic extraction of *Arachis* oil, seed characteristics and vitamin E tocopherol acetate assay:

The concentration of distinct tocopherol homologues in several oilseed crops is affected by meteorological conditions throughout the year and genetic variability. There was also a lot of variation in seed size, sensory quality, and testae colour as listed in figure 2 and table 1. To investigate tocopherols in oil, the present study evaluated the use of papain enzymatic separation to assess differences in variation among genotype and agronomic characters of peanut seeds. This resulted oil yield ranged from in an average of 49.56 to 55.88 percentage, with seed mass ranging from 46.32 to 49.45 100 seed weight. Similarly, the vitamin E tocopheryl acetate concentration measured ranged from 28.22 to 58.42 $\mu g.g^{-1}$.

Determination of Vitamin E alpha tocopherol analysis using NP-HPLC

The samples were compared to the standard absorption spectra of α-T, γ-T,δ-Tocopherols with retention times of 3.9,5.38, and 6.5, respectively. Normal phase HPLC analysis carried out on the oil separated from the dry peanut seeds varieties demonstrated that the peak area calculated

for γ-Tocopherol level were high in Vri 5 and ALR2 (Fig.3 and Graph 1). Vitamin E δ-Tocopherol was also detected at the quantification limit of 10.05 (µg.g^{-1}) DW of oil. Highest contents of α-T, γ-T, δ-Tocopherol detected were 78.42, 158.70 and 12.36 (µg.g^{-1}) DW individually in all varieties. The α-tocopherol concentration in *Arachis* oil it ranged from 30.23 to 78.42 (µg.g^{-1}) DW, for δ-tocopherol it ranged from 10.05 to 12.36 (µg.g^{-1}) DW, whereas in γ-tocopherol, it contains about 99.8 to 158.70 (µg.g^{-1}) DW .

Determination of DPPH and FRAP assay

In vitamin E –Tocopherol acetate, antioxidant activity of extracted tocopherols was estimated using DPPH and FRAP assays and the results are given in table 1. The antioxidant activity using DPPH and FRAP values are also concordant to the Vitamin E tocopheryl acetate, which was identified among different cultivar genotypes from seeds collected (Table 1). The DPPH, FRAP values were found about 78.61 to 88.56 equivalent as mg.L^{-1} Ascorbic acid and 85.04 to 91.98 mmol/L Vit C.$^{g-1}$DW respectively in all the four varieties.

Discussion

Vitamin E Natural antioxidants; peanut varieties rich in alpha tocopherol and gamma tocopherol, in particularly, provide a higher antioxidant potential to the oil. [17] Regarding to the ratios of gamma tocopherols to alpha tocopherols, it was found that the average gamma tocopherols/tocopherols ratios of peanuts were high: (Graph 1), distinguishing between peanut oil with gamma tocopherols/tocopherols ratios [18-20]. Therefore, present reports can help consumers and researchers detect peanut oil in all qualitative and quantitative detections. The oil content had effective range of tocopherol variation than the seed biomass and this was also the reason to their antioxidant properties. [21] Tocopherol concentration in edible oil crops mostly depends on various factors that are responsible for oil biosynthesis and its oxidative stability.

Antioxidant stability of edible oil is important to be maintained because the products are usually exposed to high heat and other unfavourable conditions, when and until it reaches the consumers. [22] Tocopherol concentration in edible oil crops mostly depends on various factors that are responsible for oil biosynthesis and its oxidative stability. [23] Though NP-HPLC study of the composition of four tocopherols done for the Tamilnadu cultivar varieties has revealed

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 Journal Volume 10, Iss 04, 2021

that they contain γ -T, α -T and δ-Tocopherol in the decreasing order; whereas seeds consist of a good quantity of γ-Tocopherol, followed by a small portion of α–Tocopherol, while δ-Tocopherols are also detectable to their quantification limits. Additionally, research is needed to not only validate these findings, but also to understand the oil biosynthesis basis and /or the physiological processes which are responsible for the tocopherol accumulation and its hierarchical distribution in edible oil seeds. Hence, the present reports may help consumers and researchers in the preliminary screening of vitamin E tocopherols in edible oils.

Conclusion:

In summary, the results confirmed that the distribution of tocopherol vary in the above isomer portions and their antioxidant potentials accordingly to their cultivation condition. Secondly, there is a demand to analyse the cultivated commercial *Arachis* oil sold in localities to check for vitamin E tocopherol bioavailability in peanut is scarce. The objective of selecting for high γ -tocopherol content from a phenotype is easily attainable because of its adaptability. The separation method and quantification of Tocopherol distribution in *Arachis* oil, according to this study, would lead to a better understanding of the occurrence of vitamin E in relation to the cultivar contribution of screened *Arachis hypogaea* L.

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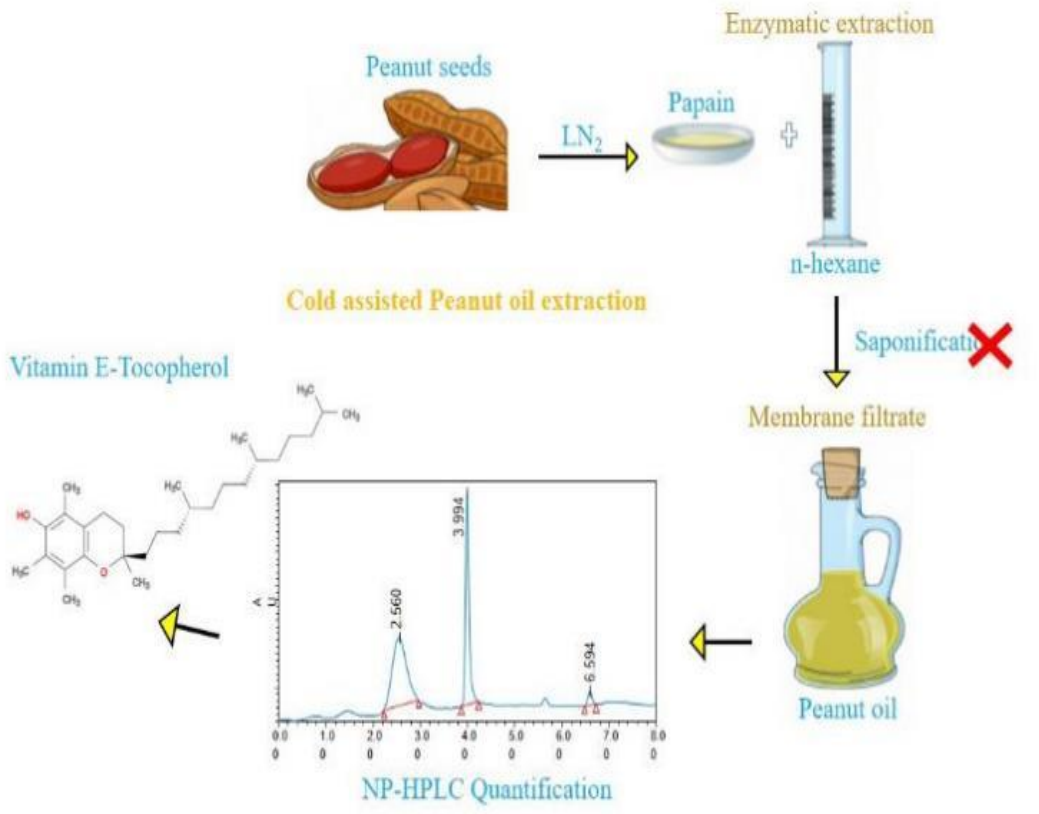

Figure1: Peanut cold assisted enzymatic oil segregation of Tocopherols

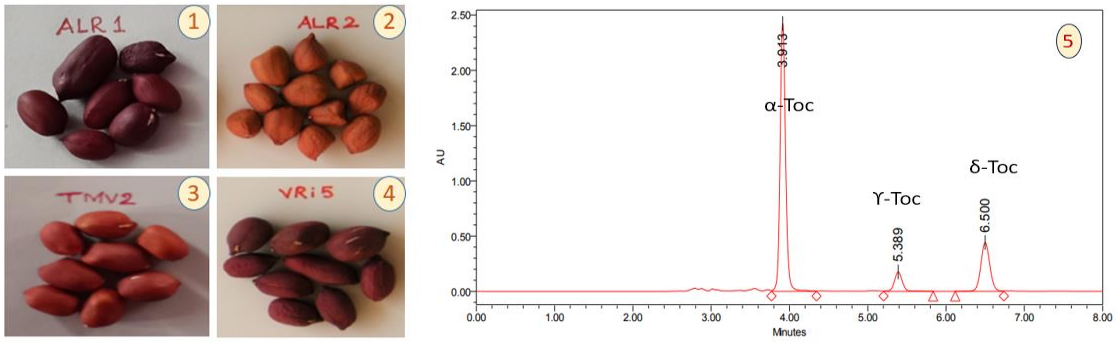

Figure 2: Seed morphology of cultivated seed varieties. (1) to (4) are peanut seeds and (5) represent NP-HPLC standards chromatogram of Tocopherols.

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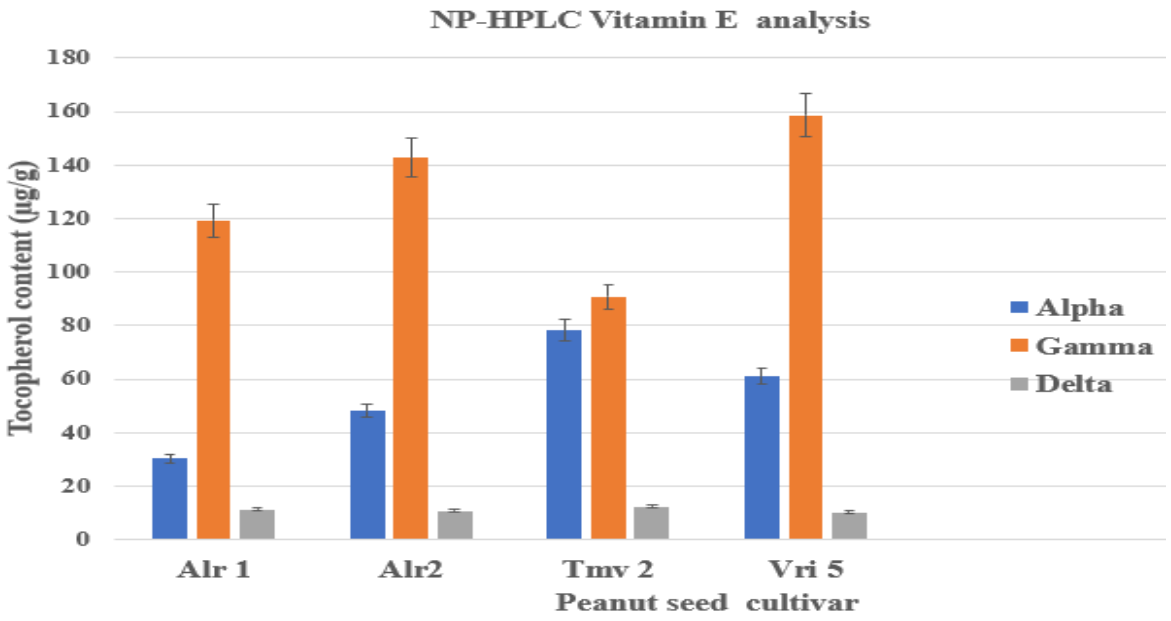
ISSN PRINT 2319 1775 Online 2320 7876
 Journal Volume 10, Iss 04, 2021

Peanut Varieties	Oil yield using Papain (%)	Vit. E Toc- acetate (UV-Spec method) (µg.g-^{1})	DPPH inhibition (equivalent as mg.L^{-1} Ascorbic acid)	FRAP assay (mmol/L Vit C.g-^{1}DW)	Seed size	Colour of testae	Seed mass of 100 seed.g-^{1}
ALR 1	49.56±0.45^{d}	45.14±0.24^{b}	86.78±1.91^{c}	90.32±0.82^{b}	Medium large	Red	49.45±0.12^{a}
ALR2	55.88±0.59^{a}	58.42±0.17^{a}	88.56±2.76^{a}	91.98±0.3^{a}	Small	Light pink	48.4±0.17^{b}
TMV2	53.26±1.12^{b}	42.31±0.38^{c}	78.61±2.02^{d}	85.04±0.64^{d}	Medium large	Pink	47.32±0.44^{c}
VRI 5	51.05±1.06^{c}	28.22±0.03^{d}	87.41±1.71^{b}	87.34±2.85^{c}	Small	Red	46.32±0.2^{d}

Table 1: Determination of cultivated peanut seed characteristics of ALR 1, 2, TMV 2 and VRI 5 varieties. The data's are calculated from three replicated analysis of each sample ± SD.



Graph 1: Determination of Tocopherol Vitamin E content using NP- HPLC. The data's are calculated from three replicated analysis of each sample ± SD.

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ISSN PRINT 2319 1775 Online 2320 7876
 Journal Volume 10, Iss 04, 2021

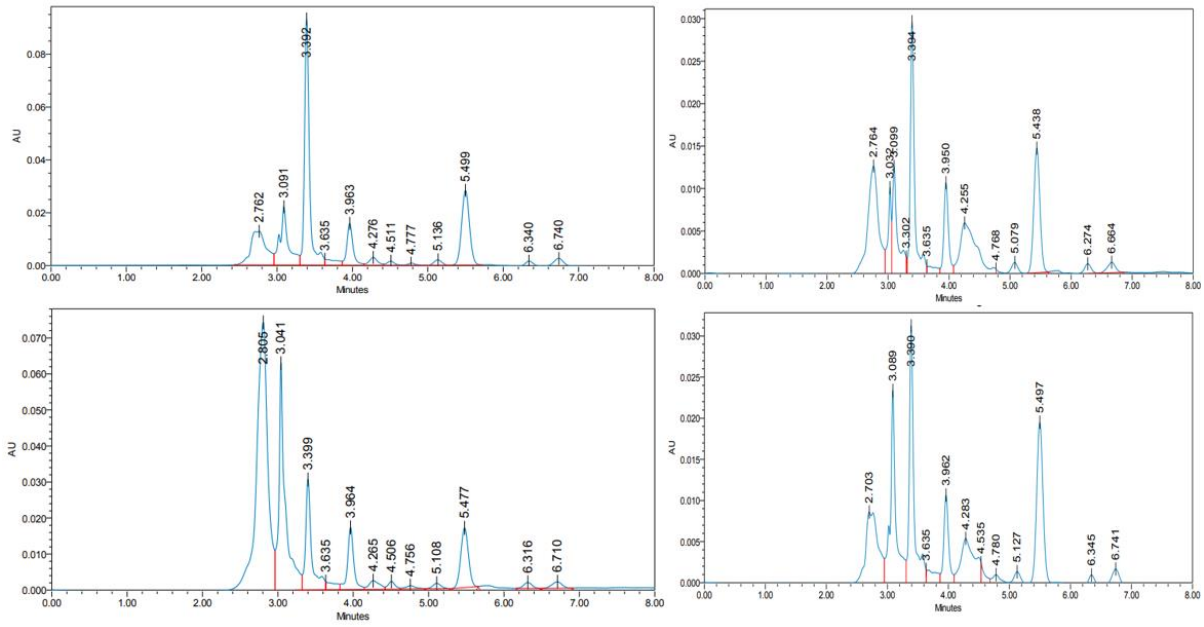

Figure 3: Determination of Tocopherol Vitamin E using NP- HPLC chromatogram. (1) ALR 1, (2) ALR 2, (3) TMV 2 and (4) VRI 5 field cultivar peanut varieties.

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