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ORIGINAL ARTICLE

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 most important edible oil crop in worldwide and its kernels show the highest levels of Vitamin E among edible oil seeds. Among tocochromonals, tocopherols alone plays a substantial role in immune responses and several other metabolisms. Additionally it also tends to be a healthy diet for human consumption, but also in protecting lipids against oxidation and, thus, lengthening the shelf-life of edible oils. Aims: The aim of the present study is to analyse the four tocopherol homologous (α-, γ-, δ-, β) in peanut oil from the common cultivars of *Arachis hypogaea* L. Materials: The seeds tested for different tocopherol isomers were ALR 1, 2, TMV 2 and VRI 5 for. Normal Phase-HPLC (High performance Liquid Chromatography) equipped with Photo Diode Array detector based method has been adopted to determine the tocopheryl isomers (α, γ, δ and β–Tocols) in a single step manner to ease the tocopherol quantification limits. Results: The tocopherol distribution variability found for ALR 1, and TMV 2 and VRI 5 for different α–, γ–tocopherol isomers is at the highest of 41.9 $\mu g.g^{-1}$ and 171.6 $\mu g.g^{-1}$ of segregated peanut oil. Also, oil quantified for Vitamin E in Tocopheryl acetate derivation is highest about 91.37 $mg.g^{-1}$. The ALR-1,2 varieties, TMV-2 cv' and VRI-5 cv' peanut oils were considered as an excellent source for raw consumptions with some exemptions like half roasted and with peel at low heat treatment extracted oils but not after long frying in food processes. Conclusion: Consumption of ALR 1, 2 and TMV 2 peanut varieties will provide a more balanced tocopherol ratio of alpha and gamma, as well as antioxidant advantages, than VRI 2 cultivars.

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

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INTRODUCTION

Vitamin-E is an essential nutrient of human diet, commonly termed as tocochromonols, so far it is characterised with eight structural isoforms and that further differentiated into four isomers each namely, tocopherols and tocotrienols [1]. Dietary tocopherols are profound from the plants during the photosynthesis and it is stored and found to be rich in seed crops. Peanut legumes are the top sources of Vitamin-E enriched edible oils apart from its high proteins, minerals and also involved in immune functions and regulation of certain metabolic processes [2]. Also, Vitamin-E or tocopherols present in peanut oils naturally tends to provide additional nutritional benefits such as strong antioxidants, and protect the high amounts of PUFA in dietary oils. Recent report from WHO reveals that the 90% of populations are not meeting with the daily recommended intake of Vitamin through the diet. Many researches demonstrate that there is more vitamin E in peanuts that was accomplished. An ounce of peanuts provides 20% of our daily needs and is considered as an excellent natural dietary source too. That implies that two servings provide almost half of our daily needs [3]. But

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in the recent days the fortified Vitamin oils also had gained much attention in the commercial commodity to meet the consumer demands. Because mostly the peanut oil has been used for deep frying and hence it is not focused on basic daily needs in consumption of oils. The present study centre is to conquer the difficulties with better knowledge around every particular variety at its oxidative fortitude will answer the diet aspects and its need for local breeders to meet its consumer demands [4].

Peanut oil with rich vitamin E which is commonly known as an antioxidant agent, the commitment which is utilized mainly as a cooking oil with a high smoke point (450 °F) that aides in oxidative strength during the process of deep frying. [5]. It is, therefore, significant that the vitamin E tocopherol distribution levels in the peanut oil be expressed as an isomer-tocopherol equivalent as well as its contribution to the fatty acid components into diet benefits. Although the concentrations of tocopherols and its crucial interaction of agronomic trait attributes (e.g., seed number and weight) it's essential in many crop improvement programmes, data available on this specific point remains scarce and is restricted with few reports using different environments and field preliminaries [6]. The right cultivar and the most appropriate growing conditions may be selected to obtain crops with effective storage of Vitamin E in kernels and for the most benecial consumption of human nutrition and health [7].

To date, studies are expending for the prevalent quality oil varieties with rich antioxidants and healthy diet compositions are yet inaccessible. Consequently, the neutraceutical potent Vitamin E in oilseed plants has also attracted industrial and researchers in the most recent decade [8]. Recognizing peanut genotypes with high tocopherol concentrations could lead to the possibility of diminishing other aspects of nutritional composition and additionally agronomic execution, for example, seed yield (since they can be intently related to unfavourable genes). Subsequently, investigating and understanding the relationship between tocopherol concentrations and other agronomical qualities will also ensure that the choice for high tocopherol concentrations won't be made to the detriment of other significant attributes [9].

The normal phase HPLC-DAD method was accustomed for analysing the dietary tocopherol ratios in view to peanut oil comprehension of diet composition and disease counteractive action parametric of peanuts with which super abound in. Concentrates revealed the deficient ingestion of Vitamin E Tocopherol and Alpha Tocopherol [10]. Studies reported the inadequate ingestion of Vitamin E Tocopherols and alpha tocopherol. Considering this, it is imperative to substantiate the distribution of Tocopherols in relation to alpha tocopherol levels in the oils analysed. As the Dietary Reference Intakes (DRI), the daily recommendation of vitamin E for healthy individuals for adults is of 15 mg of alpha-tocopherol. This suggestion depends on investigation of vitamin deficiency incited in humans and in preliminaries of hemolysis *in-vitro* caused by oxidatitive stress [11]. Regardless of gamma-tocopherol prevalence in the detriment of alpha-tocopherol in contrast to arachis oil, this is the primary contributor for the ingestion of vitamin E for the population accompanying high oxidative balance [12]. Considering the findings of this paper, the utilization of oils with higher concentrations of alpha-tocopherol in peanut needs to be stimulated in order to improve the ingestion of this vitamin.

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. Oil Quantitate nuts were washed 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 of 1%, 1.25% and 1.5% (v/w, based on raw peanut weight) which was then optimized with 1.25% and proceeded further for the extraction and incubated for 60 minutes at 45°C. After sufficient enzymatic hydrolysis, the mixture was transferred to a boiling water bath 40°C for 5 min to inactivate the papain enzyme activity. The mixture was centrifuged at 14000 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 in case of long-term storage. Peanut oil bodies of 1g were accurately weighed and a mixture of 0.8 ml isopropanol and n-hexane (1:1 v/v) was mixed to extract 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 500 µl and filtered using syringe filters (0.22

ìm, PTFE syringe filters) [Himedia, Mumbai, India]. Followed by 100 to 500 ppm standard solutions of Vitamin E tocopherol preparation and 20 ìl 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)

$$\%Oil\ yield = \left[\frac{W_2 - W_3}{W_1}\right] \times 100$$

Where:

W_1 = Original weight of the sample

W_2 = Weight of pre-extraction + oil

W_3 = Weight of dried sample + post-extraction.

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 (1500 X *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:

$$\text{Concentration CX of Vitamin E } (\mu M) = \frac{A_C - A_T}{A_C} \times 1000 \quad ...(2)$$

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.

$$\frac{Peak\ area\ of\ the\ sample}{Peak\ area\ of\ the\ standard} \times 1000 \quad ...(3)$$

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 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 μm; Phenomenex, USA) with dimensions of 250 × 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 50 μl 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 37°C. The percentage of incubation was calculated from the following equation:

$$\%\ \text{of inhibition} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad ...(4)$$

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 (500mg mL^{-1}). The absorbance at 593 nm was recorded after 30 min of incubation at 37°C. 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.

RESULTS

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 and improved pod setting properties (yield, seed and maturity) over the other peanut cultivars. 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 of a field cultivated seeds.

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 [17, 18]. The present study were also validated the relavance among the seed size , sensory quality and testae colour with tocopherol accumulation were also listed in Table 1 and Figure 2. In order to investigate tocopherol accumulation we have evaluated the use of papain enzymatic (crude) extraction method from kernels. This resulted in an average of 52.56g to 56.05 percentage, with seed mass ranging from 46.38 to 49.45 100 seed weight. In all four cultivars, g-1 was found. Similarly, the vitamin E tocopheryl acetate concentration measured ranged from 73.14 to 91.37 $\mu g.g^{-1}$.

Determination of Vitamin E Alpha Tocopherol Analysis Using NP-HPLC

The samples were compared to the standard absorption spectra of α-T, β-T, 5-T, δ-Tocopherols with retention times of 3.8, 4.8, 5.2, and 6.2, respectively. Normal phase HPLC analysis carried out on the oil separated from the dry peanut seeds varieties demonstrated that the peak area calculated for 5-, α-Tocopherol levels were high in Alr1 and Alr2 (Figure 2 and Graph 1). Vitamin E δ- and β-Tocopherol were also detected below quantification limit upto 4.86 ($\mu g.g^{-1}$) DW of oil. Highest contents of α-T, β-T, γ-T, δ-Tocopherol detected were 69.31, 9.11, 27.99, 4.36 ($\mu g.g^{-1}$) DW individually in all varieties. The α-tocopherol concentration in *Arachis* oil is in range of 31.29 ($\mu g.g^{-1}$) DW, for β-tocopherol in oil it ranges upto 2.71 ($\mu g.g^{-1}$) DW and for δ-tocopherol is in range upto 4.36 ($\mu g.g^{-1}$) DW, whereas in γ-tocopherol, it contains about 13.25 to 28 ($\mu g.g^{-1}$) 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 presence of vitamin E- tocopherol acetate was identified in all the varieties, ranging from 73.14 to 91.37 $\mu g.g^{-1}$ to the subjected cultivar varieties. Relatively the antioxidant activity using DPPH and FRAP values are also concordant to the Vitamin E tocopheryl acetate, which was identied among dierent cultivar genotypes

Figure 1: Peanut Cold Assisted Enzymatic Oil Segregation of Tocopherols

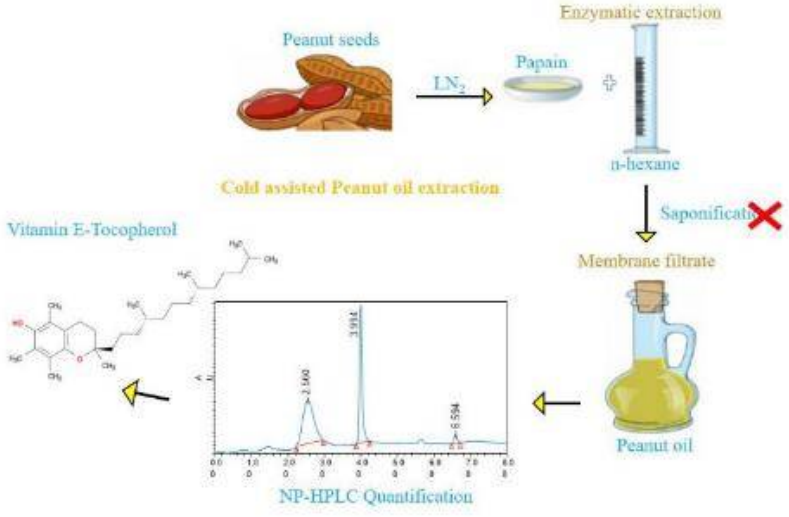


Table 1: Determination of Peanut Seed Characteristics of Cultivars ALR 1, 2, TMV 2 and VRI 5 Varieties. The data's are calculated from three replicated analysis of each sample ± SD

Peanut Varieties	Oil yield using Papain (%)	Vitamin E Toc-acetate (µg.g^{-1})	DPPH inhibition (equivalent as mg.L^{-1} Ascorbic acid)	FRAP assay (mmol/L Vit C.$^{g-1}$DW)	Seed size	Sensory Quality parametric	Colour of testae	Seed mass of 100 seed.g^{-1}
ALR 1	49.56±0.45	80.81±0.57	86.78±1.91	90.32±0.82	Medium large	Strong nutty	Red	49.45±0.12
ALR 2	55.88±0.59	91.37±0.54	88.56±2.76	91.98±0.3	Small	Sweet nutty	Light pink	48.4±0.17
TMV 2	53.26±1.12	73.14±0.56	78.61±2.02	85.04±0.64	Medium large	Mild nutty	Pink	49.32±0.44
VRI 5	51.05±1.06	84.31±0.33	87.41±1.71	85.34±2.85	Small	Nutty	Red	46.38±0.2

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

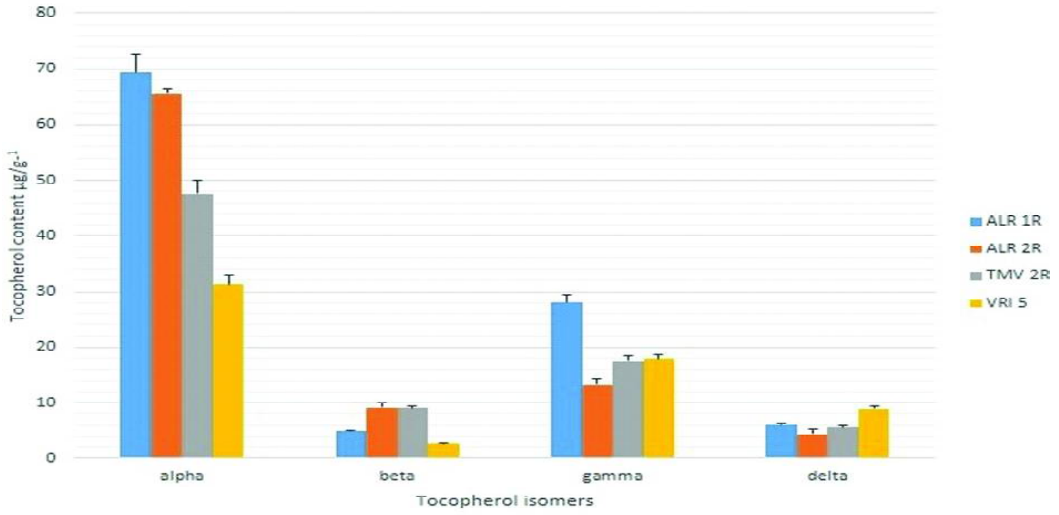
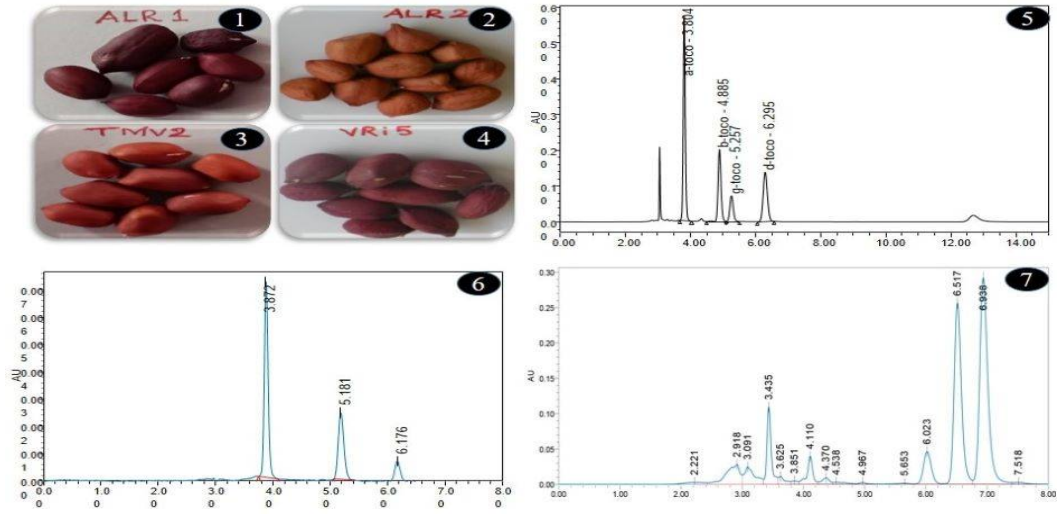


Figure 2: Peanut Seed Varieties. (1) to (4) are Peanut Seeds and (5) Represent NP-HPLC Standards Chromatogram of Tocopherols and Seed Chromatogram of (6) ALR2, (7) VRI5 Peanut Varieties



from seeds collected from field developments (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; varieties rich in alpha tocopherol and gamma tocopherol, in particularly, provide a higher antioxidant potential to the oil . In terms of alpha tocopherol/tocopherol ratios, it was discovered that the average alpha tocopherol/tocopherol ratios of peanuts were nearly identical, especially in cold assisted extraction: distinguishing tocopherols in decreasing order respectively to their isomer parts. [19, 20]. Therefore, present reports can help consumers and researchers detect peanut oil in all assortments individually for other qualitative and quantitative detections. The oil content had effective range of variation than the seed biomass and this was also the reason to their antioxidant properties [20]. Tocopherol concentration in edible oil crops mostly depends on genotype and season it has grown .Antioxidant stability of edible oil is important and to be maintained because the products are usually exposed to highlight and other unfavourable conditions, when and until it reaches the consumers [21]. Similarly, the concentration of each vitamin E isomer in peanut seed oil samples revealed that the colour of the testae peel contributed to tocopherol differences among the cultivars studied. [22]. Though NP-HPLC study of the composition of four tocopherols done for the Tamilnadu cultivar varieties has revealed that they contain γ -T, α -T, β-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 β–Tocopherols as well as 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, present reports may help the consumers and researchers for other qualitative and quantitative detection of *A. hypogaea* oil individually in all assortments.

CONCLUSION

In summary, the results confirmed that the distribution of tocopherol vary in the above four portions and their antioxidant potentials accordingly to their varietal origin and environment concerns. Secondly, there is a demand to analyse the cultivated commercial *A. hypogaea* oil sold in localities to check for vitamin E bioavailability in peanut is scarce. The present scenario of climatic change and the shift of peanut growing in delta regions [India] have a positive effect on the tocopherol content accumulations in peanut kernels due to the effect of cultivation conditions increasing this content were revealed. The objective of selecting for high tocopherol content from a phenotype is easily attainable because of its high heritability, whenever the adequate parents are chosen. The separation and quantification of Tocopherol distribution in *A. hypogaea* oil, according to this study, would lead to a better understanding of the occurrence of vitamin E in relation to the genotypic contribution of screened *A. hypogaea* types. Additionally, peanut oil compositions along with tocopheryl vitamer hold the stand to supplement the LDL levels in least portions to moderate proportions in daily diet concert were relatively disclosed.

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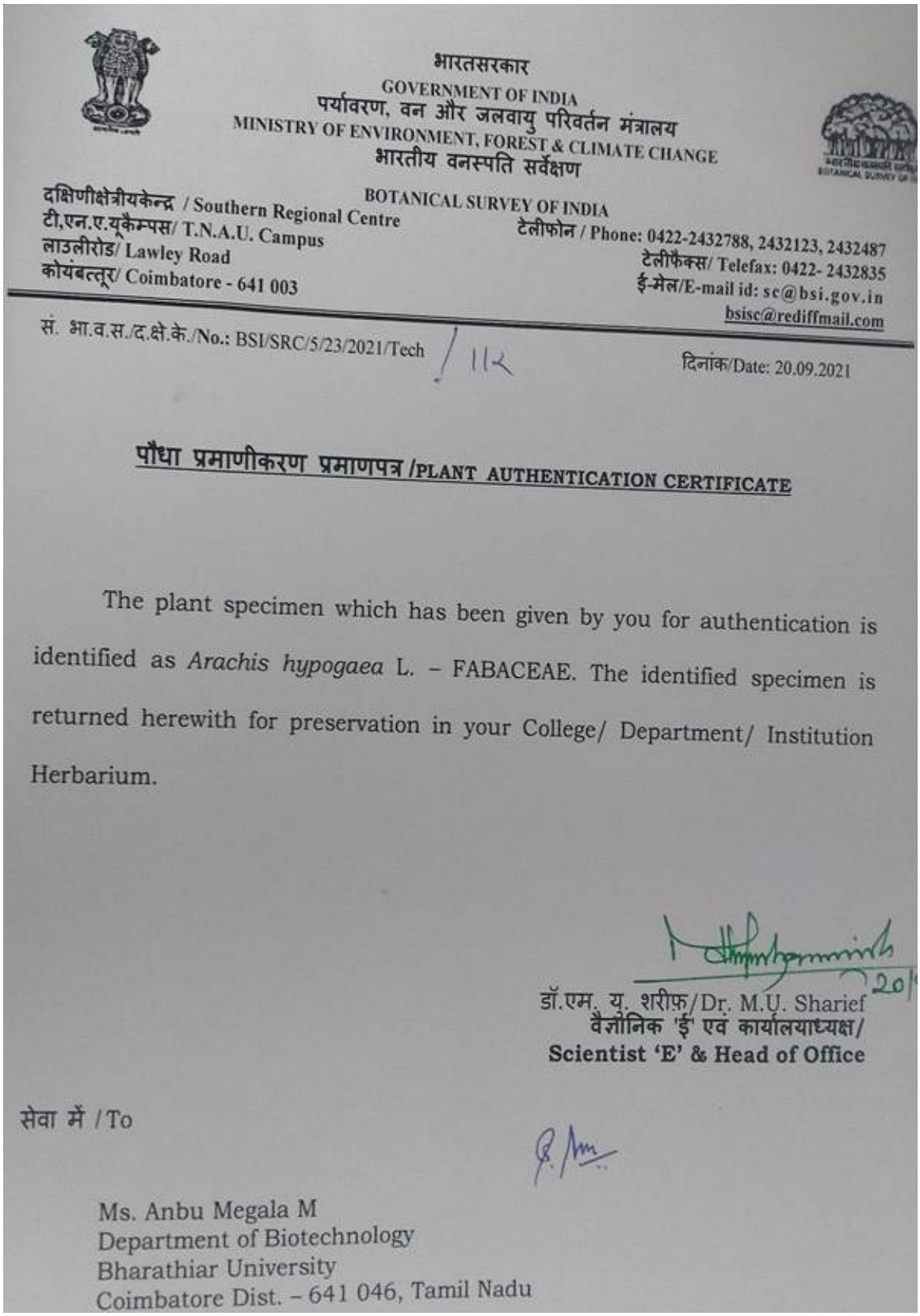
ANNEXURE



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दिनांक/Date: 20.09.2021

पौधा प्रमाणीकरण प्रमाणपत्र /PLANT AUTHENTICATION CERTIFICATE

The plant specimen which has been given by you for authentication is identified as *Arachis hypogaea* L. – FABACEAE. The identified specimen is returned herewith for preservation in your College/ Department/ Institution Herbarium.

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डॉ.एम. यू. शरीफ़/Dr. M.U. Sharief
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