Statistical Analysis and Quantification of Alpha Tocopherol in Edible Seeds and Nuts of Pakistan by Reversed Phase HPLC with UV/Visible Detector

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Summary: In present study, alpha tocopherol was determined in different edible seeds and nuts of Pakistani origin by reversed phase high performance liquid chromatography employing UV/Visible detector. Alpha tocopherol is an important constituent of vitamin E. Extraction of sample oil was carried out by Soxhlet method using n-hexane as a solvent. The maximum concentration of alpha tocopherol was found in wheat germs, i.e. 212.5 mg/100 g and minimum in cashews i.e. 0.15 mg/100 g. Almond, peanut, rice bran, corn, canola seeds, walnut, pine nuts and pistachios contained 37.8, 8.8, 3.6, 1.9, 1.6, 0.6, 0.26 and 0.2 mg of alpha tocopherol/100 g of sample respectively. The results show that the adequate amounts of alpha tocopherol are present in edible seeds and nuts, which emphasizes their recommendation in daily routine diet to remove the deficiency of vitamin E in human body.

Key words**:** Alpha tocopherol, Vitamin E, reversed phase HPLC, nuts and seeds**.**

Introduction

Vitamins are the important constituents of nutrients for human beings. There are basically divided into two sub groups depending upon their solubility i.e. fat soluble and water soluble. Examples of fat soluble vitamins include vitamins A, D, E and K, whereas Vitamin C belongs to water soluble group [1]. Natural vitamin E exists in eight different forms: four tocopherols and four tocotrienols. It contains tocochromanols which are subdivided into α, β, γ, and δ -tocopherol and the respective tocotrienols, which have diverse variations in chemical structure and activity [2-4]. In human beings, alpha tocopherol is the most active form of vitamin E. Its chemical structure is shown in Fig. 1. It is an essential micronutrient and the recommended dietary allowances and reference intakes for vitamin E are currently set at 15 mg/day [5].

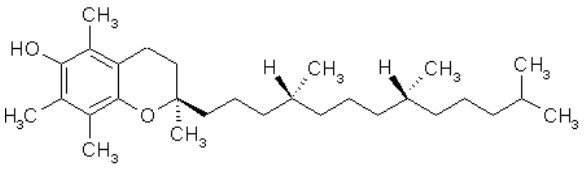

Fig. 1: Chemical structure of alpha tocopherol.

Alpha tocopherol is a lipid-soluble antioxidant, which is usually synthesized only by photosynthetic organisms [6]. It is present as a constituent of unsaponifiable matter in plant tissues and may occur together with phospholipids, carotenoids, chlorophylls and triterpenyl alcohols. The importance of diets rich in plant antioxidants derived from fruits and vegetables are associated with lower risks of cardiovascular problems and cancer. Among the four tocopherols and four tocotrienols (designated as α-, β-, γ-, and δ-) found in food, only alpha tocopherol meets human vitamin E requirements [7, 8].

The deficiency of alpha tocopherol prevents the normal growth of human organisms and results in increased catabolism of unsaturated fatty acids, so that enough quantities are not available for maintenance of cell membranes and other membranous organelles in human body. Alpha tocopherol is highly stable towards heat and acids, and unstable to alkalis, oxygen and UV light. It is degraded on contact with rancid fats, lead and iron. Due to its chelating ability, it is capable of scavenging free radicals via its phenol group. It is a lipophilic compound and considered to play a vital role in reducing the concentration of lipids by oxidation. The cardio-protective effect of alpha tocopherol is due to the capability of inducing inhibition of low density lipoprotein (LDL) oxidation, which is supposed to be a major reason of atherogenic processes [9]. In addition to its cardio-protective effect, alpha tocopherol is assumed to be the most active form in human beings; however gamma tocopherol is the most prevalent form of vitamin E in plant seeds, especially in nuts [10]. Dietary antioxidants provide shield against oxidative attack by various means like reducing oxygen concentration, preventing first-chain initiation by scavenging initial radicals, intercepting singlet oxygen, binding of metal ion catalysts, chain

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breaking to prevent continuous hydrogen removal from substrates and decomposing primary products of oxidation to non-radical compounds [11]. In addition, it has been suggested that it might be a factor in protecting against cancer induced by free radical-generating contaminants, such as ozone and nitrogen dioxide [12].

Major known sources of vitamin E are oils, cereals, seeds, liver, eggs, fish and nuts [13]. For the determination of alpha tocopherol in foods and food products, various methods have been documented, such as colorimetric, spectro-photometric, thin layer chromatography and fluorimetric methods [14]. These methods have certain limitations such as; time consuming, require considerable skill with lots of experience and their precision are low. High performance liquid chromatography (HPLC) is the most common technique for identifying and measuring alpha tocopherol concentrations. A variety of efficient HPLC methods are available for their analysis [14-16]. Reliable and sensitive methods have been developed using reversed-phase and normal-phase HPLC columns, as well as in isocratic as in gradient elution, with fluorescent, electrochemical and UV detection.

The goal of this work was the extraction and quantification of alpha tocopherol in different seeds and nuts using reversed phase HPLC method with C18-column. The C18-column is used because it is compatible with aqueous matrices. It gives fast equilibration in short interval of time when changes of composition in the mobile phase take place.

Results and Discussion

A method based on reversed phase high performance liquid chromatography with UV/Visible detector has been used to determine the concentration of alpha tocopherol in various edible seeds and nuts, which are generally found in Pakistan. Reversed phase HPLC is preferred over normal phase systems because of the advantages like reproducibility of retention times, fast equilibration time and robustness of reversed phase columns over other stationary phases [17]. Experiments were carried out in triplicate fashion for reliability of results in terms of precision and accuracy and results are reported in terms of mean values with standard deviation. Stability of baseline and response linearity of the detector was examined at the beginning of analysis. Detector was able to detect alpha tocopherol at a signal to noise ratio of 3:1. Before analyzing alpha tocopherol in samples, instrument was calibrated using standard solutions. The same operating conditions of the HPLC system were maintained throughout the analysis of all samples of seeds and nuts.

Fig. 2 is showing the calibration curve for standard solutions of alpha tocopherol, whereas Table-1 is showing the concentration of alpha tocopherol in different seeds and nuts. Comparative analysis of concentration of alpha tocopherol in various seeds and nuts is shown in Fig. 3.

Table-1: Concentration of alpha tocopherol in various food items.

Sr. No	Samples	Concentration Mean value ± S.D* (mg/100g)
1	Cashews	0.15 ± 0.003
2	Pistachios	0.2 ± 0.0011
3	Pine nuts	0.26 ± 0.001
4	Walnut	0.6 ± 0.004
5	Canola Seeds	1.6 ± 0.0027
6	Corn	1.9 ± 0.0016
7	Rice bran	3.6 ± 0.0031
8	Peanuts	8.8 ± 0.007
9	Almonds	37.8 ± 0.006
10	Wheat germs	212.5 ± 0.21

S.D*= Standard deviation

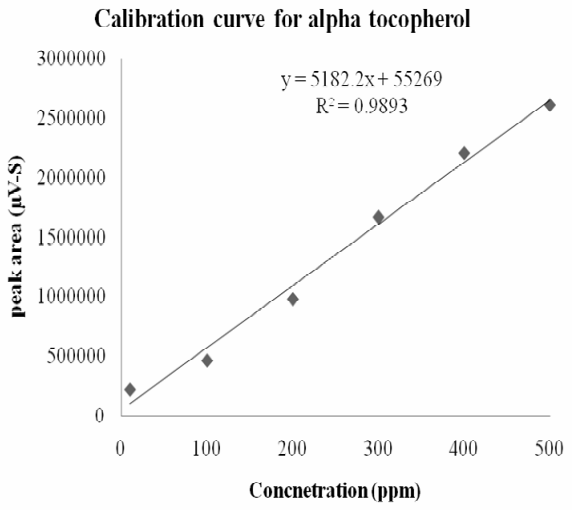

Fig. 2: Calibration curve for standard solutions of alpha tocopherol.

It is observed that wheat germs contain maximum concentration of alpha tocopherol, *i.e.* 212.5 mg/100 g of sample and minimum concentration is found in cashews i.e. 0.15 mg/100 g. Almond, peanut, rice bran, corn, canola seeds, walnut, pine nuts and pistachios contain 37.8, 8.8, 3.6, 1.9, 1.6, 0.6, 0.26 and 0.2 mg/100 g of samples. These values are also comparable to reported values. Little variations are due to genetics, harvest season, origin, environmental conditions, soil composition, maturity level and the methods of cultivation which

highly influence the composition of seeds and nuts [18-22].

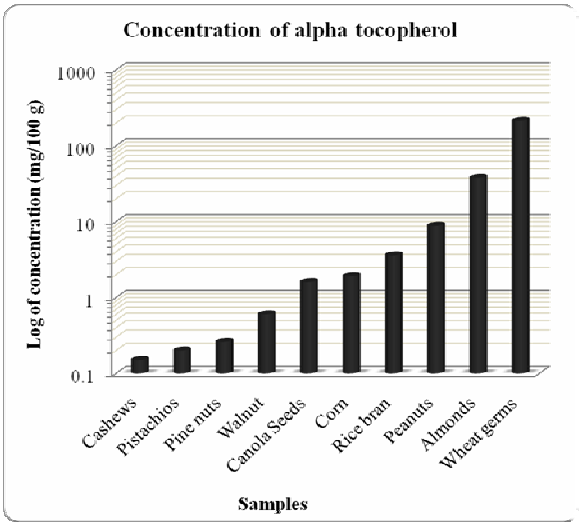

Fig. 3: Comparative concnetration of alpha tocopherol in various seedss and nuts.

The results revealed that these nuts and edible seeds are good dietary sources of alpha tocopherol and can contribute to a balanced intake of vitamin E. The recommended average intake of nuts and edible seeds is 30 g/day, in order to overcome the deficiency of vitamin E [13].

Experimental

Chemicals and Reagents

Methanol, n-hexane, dl-alpha tocopherols, potassium hydroxide and anhydrous sodium sulphate were purchased from Fluka. All the chemicals were of HPLC analytical grade.

Seed Collection

The almonds (*Prunus amygdalus var. dulcus*), walnuts (*Juglans regia*), canola (*Brassica napus*) seeds, peanuts (*Arachishypogaea)*, rice (*Oryza sativa*) bran, wheat (*Triticum aesativum*) germ, corn (*Zea mays*), pine nuts (*Pinus girardiana*), cashews (*Anacardium occidentale*) and pistachios (*Pistacia vera*) were purchased from local markets of Lahore, Pakistan. They were washed, crushed and stored in air tight glass jars separately.

HPLC Specifications

The HPLC system used was a Perkin Elmer 200 Series with reciprocating pump, vacuum solvent delivery degasser, chromatography interface link 600 series, UV/Visible detector, 20 µL loop injector with Rheodyne valve (Model 7125). The chromatographic system was controlled and the data collected and processed by the computer integrator (Total chrome software).

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. Then 10, 100, 200, 300, 400 and 500 ppm standard solutions of alpha tocopherol were prepared by proper dilution of the stock solution.

Sample Preparation

Oil extraction from different seeds and nuts was accomplished with a Soxhlet extractor using n-hexane as a solvent. 15.0 g of the dried, homogenized sample of various seeds and nuts were placed in thimbles separately and extracted for 6 hours at 80 ^{0}C. Then extra solvent form sample oils were removed by rotary vacuum evaporator. For the separation of alpha tocopherol, these oil samples were saponified in the following way; 5.0 g of oil was mixed with 10 mL of 4.0 M KOH in methanol at 80 $^\circ$C for 30 min. This solution is subsequently transferred to a separating funnel along with 50 and 120 mL distilled water successively in order to get rid of the aqueous phase. After that, the organic phase was washed three to four times with 50 mL distilled water and filtered on anhydrous Na_2SO_4 for the removal of moisture. The resulting organic solution is evaporated on a rotary evaporator to obtain the residue. The residue was dissolved in 50 µL methanol [12, 13]. All the samples were prepared in the similar way and stored in air tight glass vials.

Quantification of Alpha Tocopherol

Alpha tocopherol contents in edible seeds and nuts were measured by reversed phase HPLC and UV-detection at 295 nm based on the reported with minor modifications [12, 13]. 20 µL of sample was injected into a reversed-phase C18 column (ODS C18, 25cm × 4.6 mm, 5 mm i.d.). Pure methanol was used as mobile phase during analysis with a flow rate of 1- 2 mL min^{-1}. Same procedure is followed with standard solutions. The column was purged with methanol for 10 min after each sample analysis to remove the traces of impurities and residues. Chromatographic peaks of alpha tocopherol were observed at 7.0-7.44 min. The chromatograms were registered according to their retention time for determining the peak area of all standard and sample solution of alpha tocopherol as mention in Table-2.

Table-2: Peak area of chromatograms of standard solutions of alpha tocopherol

Concentration (ppm)	Peak area (µV-S)
10	223745
100	464209
200	980000
300	1670000
400	2208558
500	2610157

Conclusion

A simple, robust and reliable reversed phase high performance liquid chromatography method has been employed to find out the concentration of alpha tocopherol in various edible seeds and nuts, which are generally available in Pakistan. The concentration of alpha tocopherol in these samples ranges between 0.15 - 212.5 mg/ 100 g of sample. It is observed that for the extraction of tocopherol saponification is needed for samples high in fat in order to remove the fatty acids in excess before their HPLC analysis.

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