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Determination and Characterization of Tocopherols in Vitamin E Extracted from Goat Milk

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ABSTRACT

Vitamin E was extracted from milk samples using saponification methods and the separation was achieved using high performance liquid chromatography (HPLC). Results from HPLC revealed that extracted vitamin E from milk existed in forms of α , β and δ tocopherols with α -tocopherol being the dominant form. The resulting vitamin E was characterized using UV-Visible and FTIR spectroscopy. A series of standard solution were prepared from the stock solution of 500 ppm vitamin E. From the Uv-visible, a maximum wavelength, λ_{\max} obtained was 292.0 nm and the molar absorptivity calculated from graph 1 was $4220.8 \text{ L mol}^{-1} \text{ cm}^{-1}$. The FTIR spectra for both fresh vitamin E and oxidized vitamin E were compared and analysed. The spectrum for oxidized vitamin E showed similar absorption bands as fresh vitamin E except for C=O stretching which indicated that the oxidation process has occurred.

Keywords: Vitamin E, tocopherol, HPLC, UV-Visible, FTIR spectroscopy

1.0 INTRODUCTION

Vitamin E is a fat-soluble vitamin commonly found in many foods, fats, and oils. It is also an antioxidant, a substance that may help prevent damage to the body's cells. Antioxidants may provide protection against serious diseases including heart disease and cancer (Burton, and Traber 1990). Vitamin E is necessary for structural and functional maintenance of skeletal, cardiac, and smooth muscle (Yusuf et al. 2000). It also assists in the formation of red blood cells and helps to maintain stores of vitamins A and K, iron, and selenium (Food and Nutrition Board, 2000). It may have a positive effect on immune health, protect against the oxidative damage that can lead to

heart disease, have preventive effects against cancer, help relieve symptoms of Alzheimer disease, and may help prevent some diabetes-related damage, particularly to the eyes (Behl, and Moosman 2002). It is an essential micronutrient for humans and achieving an optimal status is assumed to produce beneficial health outcomes (Galli et al. 2017). This vitamin is extremely important in the early stages of life, from conception to postnatal development. During pregnancy, placental transfer of vitamin E to the fetus is limited, making breast milk the only source of this nutrient for infants that are exclusively breastfed. This intake represents an important way to supply the new born with an essential antioxidant protection and stimulate immune

system development (Debieer 2007). Vitamin E dietary intake is established in many countries around the globe and refers to its important role as a chain breaking antioxidant in preserving the integrity of the cell membrane. The current intake recommendations for vitamin E vary between 3 and 15 mg/day in different countries and depending on the age of the person (Wu 2014). For example, in the US, the Recommended Dietary Allowance (RDA) for vitamin E is 15 mg α -tocopherol in adults for both men and women with a minimum age of 14 years (IOM Vitamin E 2000).

Vitamin E is divided into two groups which are tocopherols and tocotrienols. They are identified by prefixes alpha- (α -), beta- (β -), gamma- (γ -), delta- (δ -), and epsilon (ϵ -). Tocopherols are found in virtually all photosynthetic plants, notably in vegetable oil and nuts, whereas tocotrienols are mainly encountered in monocot seeds (Mène-Saffrané and Pellaud, 2017). The most famous of the vitamin E group is α tocopherol (Mene-Saffrane 2010). It has been one of the most intensely studied nutrients. This is associated with its function as a free radical damage protector. Vitamin E acts as an antioxidant. Previous studies revealed that α -tocopherol is the most important of all vitamins E and has a lot of biological functions and serving as antioxidants (Cohn, 1997, Liu et al. 2002). Tocopherols are widely distributed throughout the plant kingdom and inside the body of some animals (Terentis et al. 2002). α -Tocopherol, the most common form of vitamin E in human tissues, has long been considered as a cytoprotective factor with suggested roles in preventing inflammatory and degenerative processes in the liver during the exposure to a range of xenobiotics, environmental pollutants and dietary factors (Aboul-Soudy et al. 2011). Vitamin E acts as a peroxy radical which

protects human from potentially damaging free radicals and lowering the risk of developing blood clots. It is a powerful lipid antioxidant that protects cell membranes from the damaging effects induced by free radicals (Yokota et al. 2002, Teresawa et al. 2000). In addition, several tocopherol isoforms show beneficial roles in delaying brain aging reducing the risk of developing Alzheimer's disease [7]. Vitamin E also functions as an enzymatic activity regulator which involve in smooth muscle growth (Bulger and Maier 2003). Besides, Vitamin E can protect lipid, prevents oxidation of polyunsaturated fatty acids and plays a role in neurological and inhibition of platelet aggregation (Kasim et al. 2010). Despite low incidence of overt vitamin E deficiency, research shows that many American adults have suboptimal α -tocopherol status (Maras et al. 2004) (Ford et al. 2006) and around 90% of the population in the US has vitamin E intakes below the EAR (Dwyer et al. 2013).

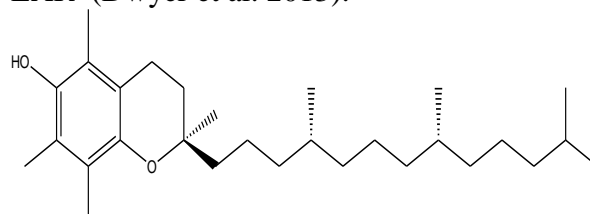


Fig.1: Alpha- (α -) tocopherol

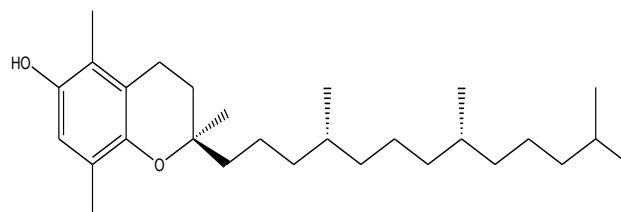


Fig. 2: Beta- (β -) tocopherol

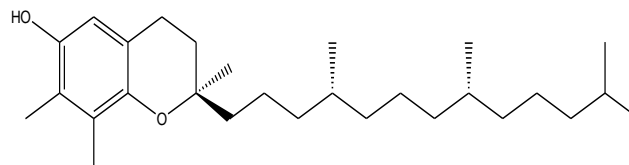
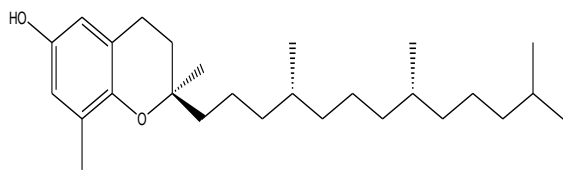


Fig.3: Gamma- (γ -) tocopherol

Fig. 4: Delta- (δ -) tocopherol

Vitamin E can be found in different food materials such as milk, soya bean, palm oil etc. The production of vitamin E, which has the balance content of tocopherols and tocotrienols, increased considerably which is accompanied by the number of the industries making the production increasing (Traber, and Stevens 2011). Extraction of Vitamin E can be achieved by several methods (Mottier et al. 2002, Prieto, 2017). Among the methods, Solvent-based extraction is widely applied to extract the natural product (Gupta, et al. 2017). It usually uses organic solvents such as hexane, chloroform and short chain alcohols. Ethanol and isopropanol are preferred to be used than hexane and chloroform, due to the health hazard issues. Also, ethanol and isopropanol tend to extract more non-glyceride material than hexane due to their greater polarity.

The aim of this work is therefore to extract vitamin E from Milk and characterize it using UV-Visible, FTIR spectroscopy

2. MATERIALS AND METHODS

All organic solvents used in this research work were of analytical grade. KOH, HCl and pyrogallol were purchased from Fluka Chemica, Switzerland. α and γ -tocopherol, tocopherol acetate, methanol, ethanol and hexane were obtained from Sigma Aldrich, USA. Ultra-pure water was used throughout the study.

2.1 Collection of Milk Samples

The Goat Milk sample was collected from the Al-Qalam university animal house and was stored in sterile plastic containers. After collection milk was immediately kept in the freezer at -10°C until analysis.

Analyses were performed within 2 days of collection.

2.2 Preparation of standards

Stock solutions of α -tocopherol standards (10 ppm) was prepared by dissolving 0.0043g of α -tocopherol in 1L of ethanol and kept for one week at -10°C . Spiking solutions of α -tocopherol standards were prepared on the day of analysis by taking certain amount of the prepared stock solution and diluted to the required volume. Other tocopherol standards were prepared in the same way. α -Tocopherol was used as internal standard because its chemical structure was similar to those of the other forms of tocopherol assayed (i.e. β , δ and γ -tocopherol and tocopherol acetate).

2.3 Extraction of Vitamin E from the Goat Milk

The milk samples stored in a sterile plastic container was collected and warmed to 35°C and briefly sonicated to ensure sample homogeneity. 5 mL of the milk sample was spiked with 20 μL α -tocopherol standard (7–10 μg). To disrupt milk fat globules, Methanol (1 mL) containing pyrogallol (3%, w/v) then 1 mL aqueous KOH (10%, w/v) were added. Samples were vortex-mixed and placed in a water bath. Saponification was performed at 70°C for 30 min. After the first 15 min of saponification the tubes were briefly vortex-mixed. After cooling of the tubes on ice, samples were acidified to pH 2 with 1 mL HCl. Then 4 mL hexane was added. Tubes were vigorously vortex-mixed for 20 s, three times, and kept on ice between mixing. To separate the emulsion the tubes were centrifuged at room temperature at 1300g for 10 min. The top, organic, layer was carefully transferred to a clean Pyrex container and evaporated, under a gentle stream of nitrogen, on a warm plate at 40°C . The fatty residue was reconstituted in 0.5 mL methanol–propan-2-ol (1:1 v/v) with warming to 30°C (Ramos-Lledó et al. 2001).

2.4 Chromatographic separation

High performance Liquid Chromatography (HPLC) was performed with a Waters (USA) system incorporating an Alliance 2690 separations module and a 996 photodiode-array detector and operated by Millennium32 software. Tocopherols were separated on a Waters Symmetry C18 column (3.9 mm × 150 mm) with a Waters Symmetry C18 (3.9 mm × 10 mm) guard column. The mobile phase was initially a linear gradient of acetonitrile in water (from 95 to 100% in 10 min) then 100% of acetonitrile for 10 min; the flow rate was 1 mL min⁻¹. The column temperature was 45°C and the wavelength-range scanned was 275– 350 nm. Samples were kept in an auto sampler at 30°C and the injection volume was 25 µL. Peak areas of tocopherols were integrated at 295 nm. Tocopherols were identified by co-elution after spiking the extracted samples with solutions of the tocopherols in ethanol. To ensure reproducible retention on the column, after each batch of 30–40 milk extracts the column was washed with propan-2-ol (1 mL min⁻¹ for 60 min at 45°C) (Korchazhkina et al. 2006).

2.5 Characterization of the Vitamin E

The wavelength max (λ_{max}) was determined by using 150 ppm of vitamin E solution prepared with the aid of a UV-Vis spectrometer ((Varian CARY 50 probe), with blank ethanol used as reference. After determining the λ_{max} , each of the standard solutions prepared were measured.

The surface functional groups of the extracted vitamin E were determined using Fourier Transform Infrared (FT-IR) spectroscopy (Thermo Scientific FTIR spectrometer, San Jose CA, USA). The spectrum was recorded and compared with the spectrum of fresh vitamin E.

3. RESULTS AND DISCUSSIONS

The results of chromatograms recorded at $\lambda = 300$ nm showing the elution profile of δ , γ , and α -tocopherol in spiked and unspiked milk specimens are shown in Fig. 1. It shows that δ -tocopherol was either absent from milk or native trace amounts were usually below the limits of UV quantification. The major peak appeared in the chromatogram is that α -tocopherol which is the most important vitamin E present in the milk. Also smaller peaks of γ -tocopherols and tocopherol acetate have appeared which shows that they were in minor quantity in some milk samples.

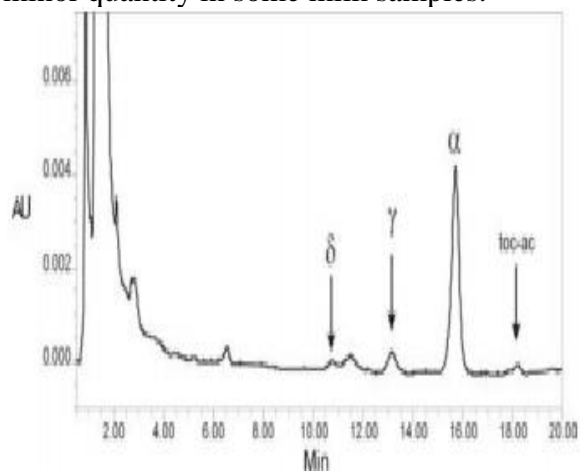


Fig. 5: Chromatogram of an unspiked milk sample, showing the peaks of different forms of vitamin E

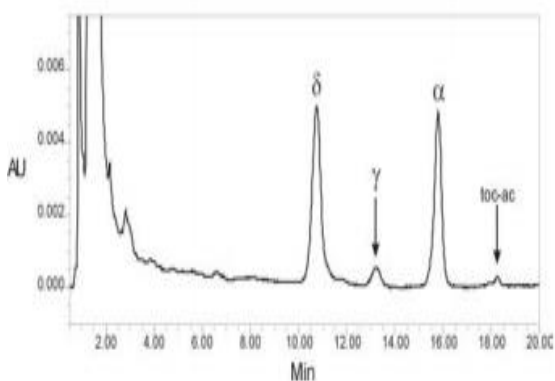


Fig. 6: Chromatogram of a spiked milk sample, showing peaks of different forms of vitamin E

The maximum wavelength, λ_{\max} of Vitamin E obtained is 292.0 nm. From the figure 7, the molar absorptivity obtained is $4220.8 \text{ L mol}^{-1} \text{ cm}^{-1}$. From the UV-Vis spectrometry results, it showed that vitamin E absorbed in low intensity absorptions (molar absorptivity calculated in the range of 10^3 - 10^4) indicating the presence of aromatic compound which is a type of chromophore.

In the conjugated chromophore, the electrons jump between energy levels that are extended pi orbitals, created by a series of alternating single and double bonds, often in aromatic systems. This conjugation gives a stability of p electron and result in a decrease in transition energy from p to p* orbital. Thus, bathochromic shift occurred. In the experiment, the concentration of the unknown is 42.29 ppm and the volume of standard solution taken to make unknown is 2.11 mL. Both value are determined by using equation $A = \epsilon bc$, (where A is the absorbance, ϵ is the molar absorptivity ($\text{Lmol}^{-1}\text{cm}^{-1}$), b is the pathlength (cm) and c is the concentration (molL^{-1})), and equation $C_1V_1 = C_2V_2$ (where C_1 and C_2 are the initial and final concentrations and V_1 and V_2 are the initial and final volume respectively). The detection limit of vitamin E at 1ppm showed an absorbance at 0.010.. It is a function of both signal strength (sensitivity) and signal stability. Therefore, this sensitivity make UV-Vis spectroscopy is a good technique to be used to determine the detection limit of vitamin E (Figure 7).

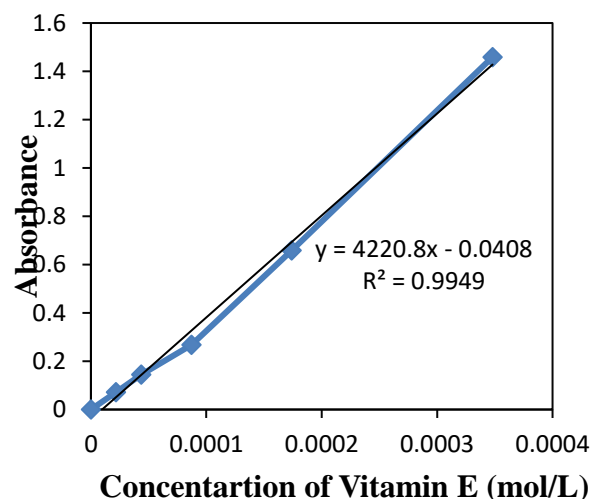


Fig 7: Absorbance versus Concentration of Vitamin E in mol/L

The IR result for fresh vitamin E showed O-H stretching (3456.5 cm^{-1}), Csp³-H stretching ($2928, 2858.2 \text{ cm}^{-1}$) and C=C stretching vibration (1456.2 cm^{-1}). The regions at 1371.8 cm^{-1} , 1254.3 cm^{-1} and 1085.5 cm^{-1} indicate for C-H (sp³) of -CH₂-bending vibration, C-H (sp³) of -CH₃ (methyl group) bending vibration and symmetric C-O stretching vibration of C-O-C respectively. The IR result for oxidized vitamin E showed similar absorption bands as fresh vitamin E. The appearance of C=O stretching at 1713.1 cm^{-1} indicate the oxidation process is occurred (tocopherol oxidized to tocopherone). The oxidation process was not complete since the O-H stretching still exists on the spectrum. This error may due to incomplete heating of vitamin E at $110 \text{ }^\circ\text{C}$ and present of water in air which presence in oxidized vitamin E.

Table 1: The absorption bands for fresh vitamin E:

Wave number (cm ⁻¹)	Remarks
3456.5	O-H stretching vibration
2928, 2858.2	C(sp ³)-H stretching (asymmetric and symmetric stretching vibrations)
1456.2	C=C stretching vibration
1371.8	C-H (sp ³) of -CH ₂ - bending vibration
1254.3	C-H (sp ³) of -CH ₃ (methyl group) bending vibration
1085.5	symmetric C-O stretching vibration of C-O-C
916.69	trans ,CH ₂ stretching
733.18	C-C of -(CH ₂) ₃ - skeleton vibration (rocking), long chain band

Table 2: The absorption bands for oxidized vitamin E:

Wave number (cm ⁻¹)	Remarks
3456.5	O-H stretching vibration
2928, 2858.2	C(sp ³)-H stretching vibration (asymmetric and symmetric stretching vibrations)
1713.1	C=O (carbonyl group) stretching vibrations
1632.4	C=C (conjugated with C=O) stretching vibration
1456.2	C=C stretching vibration
1371.8	C-H (sp ³) of -CH ₂ - bending vibration
1254.3	C-H (sp ³) of -CH ₃ (methyl group) bending vibration
1085.5	symmetric C-O stretching vibration of C-O-C
916.69	trans ,CH ₂ stretching
733.18	C-C of -(CH ₂) ₃ -skeleton vibration (rocking), long chain band

4.0 Conclusion

Based on the experiments conducted, the predominant form of vitamin E extracted from the milk sample is α -tocopherol. Results from characterization revealed that the wavelength of maximum absorption, λ_{\max} and molar absorptivity which were determined by using UV-Visible absorption spectrophotometer obtained are 292.0nm and 4220.8 L mol⁻¹ cm⁻¹ respectively. The detection limit (absorbance) of vitamin E at 1 ppm is was found to be 0.010. While for FTIR, the result obtained for fresh vitamin E and oxidized vitamin E both showed the C=O stretching which confirmed that the oxidation is occurred.

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