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## Vitamin E-Stabilised UHMWPE for Orthopaedic Implants: Quantitative Determination of vitamin E and Characterisation of its Transformation Products

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### ABSTRACT

The fate of vitamin E and the formation and identification of its transformation products were investigated at different stages of the manufacturing process of commercially produced cross-linked (by  $\gamma$ -irradiation) UHMWPE stabilised with vitamin E (vitamin E infused-post irradiation) used for tibia-components (as articulating surfaces) in total knee arthroplasty (total knee replacement). Vitamin E ( $\alpha$ -tocopherol) and its transformation products were extracted from microtomed Tibia films and the different products were separated, isolated, purified using high performance liquid chromatography (HPLC), and characterised by spectroscopic methods and LC-MS. The amount of vitamin E and that of the products formed in the different Tibia samples and in their extracts were also quantified using FTIR and HPLC analysis and calibration curves. Thorough analysis of the Tibia extracts has shown that a number of vitamin E transformation products were formed at different concentrations at two selected stages of the implant manufacturing process that is before and after sterilisation by  $\gamma$ -irradiation. The identified products were found to correspond mainly to different stereoisomeric forms of a small number of vitamin E transformation products. Most of the observed products were of dimeric and trimeric nature with their identity confirmed through a detailed study of their spectral and chromatographic characteristics. It was found that the products of vitamin E, prior to the sterilisation step but after the crosslinking and doping of vitamin E, were mainly the dihydroxydimers and trimers (Tibia samples at this stage are referred to as "Tibia-VEPE"). After sterilisation and completion of the manufacturing process, additional dimers of vitamin E were also formed (Tibia samples at this stage are referred to as 'Tibia-VEPE-Sterile'), Furthermore, two tocopherol-derived aldehydes (aldehyde 5-formyl- $\gamma$ -tocopherol and aldehyde 7-formyl- $\gamma$ -tocopherol) were also formed but at very low concentrations especially in the Tibia-VEPE-Sterile samples. The question of whether vitamin E becomes chemically reacted (grafted) onto the polymer matrix during the manufacturing process of the Tibia is also addressed.

**Keywords:** vitamin E; Orthopaedic implants; UHMWPE; antioxidant

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## 1. Introduction

Highly crosslinked medical grade UHMWPE has been widely used as acetabular cup and Tibia component in total hip and knee arthroplasty (total joint replacements, TJR) [1], and the crosslinked polymer has replaced the initially used conventional (not crosslinked) UHMWPE due to significant improvements in wear resistance, a critically important property in prosthetic applications involving joints, leading to reduction in osteolysis [2, 3]. Crosslinking of UHMWPE is typically achieved through the use of gamma irradiation [4] resulting in formation of free radicals in the amorphous phase of the polymer and their subsequent recombination, though some of the generated free radicals become trapped mostly in the crystalline phase [5, 6]. The residual free radicals result in oxidative instability of the polymer, thus a number of approaches have been introduced to reduce the adverse effect of these free radicals produced during the  $\gamma$ -irradiation/crosslinking process. Thermal treatment after irradiation (by melting or annealing) has been used to decrease the concentration of the residual free radicals and to minimize or eliminate their adverse effects on the properties of UHMWPE [7-10]. The post-irradiation melting approach releases the trapped free-radicals from the crystalline regions allowing them to recombine forming further cross-links; but this method was shown to also result in reduction in the mechanical properties and fatigue strength of the irradiated UHMWPE due to decrease in the polymer crystallinity [11]. The annealing process (below the polymer peak melting point) was also shown to [12, 13] result in reduction in the free radical concentration with further recombination. Furthermore, the final step of gamma irradiation (at lower dose) which is used for sterilisation of the polymer implant components result in the introduction of additional free radicals in the system [14] contributing further to the oxidative instability of the polymer.

To combat this problem and to promote long-term oxidative stability of UHMWPE and longevity of the implants, vitamin E has been successfully used as an effective biocompatible antioxidant for this purpose. Initially, Vitamin E was introduced by blending into the UHMWPE resin powder before consolidation and radiation crosslinking [15-18], but the presence of this highly efficient free radical scavenger antioxidant during the irradiation process resulted in reduction in the crosslinking efficiency as well as in a partial consumption of vitamin E itself. The amount of vitamin E that could be incorporated into the polymer by this method was found to be limited to approximately 0.3 wt. % [19]. Optimisation of both the added vitamin E concentration and the subsequent radiation dose were therefore examined by different authors [15-19]. Research has led to development of a different method of introduction of vitamin E into the polymer in which the antioxidant vitamin is diffused into the UHMWPE after the radiation crosslinking step [2, 14]. The crosslinking efficiency of UHMWPE was shown not to be adversely affected by this approach due to the absence of vitamin E during the  $\gamma$ -irradiation/crosslinking step. The diffused antioxidant vitamin removes the trapped free radicals due to its well-known high radical-scavenging efficiency, and this method was shown also to give rise to higher concentrations of vitamin E in the polymer of up to about 0.7 wt % [14, 20].

The reactions of vitamin E during melt processing of polymers and particularly polyolefins were extensively investigated in our laboratories since the 1990's and the chemical nature and distribution of oxidative transformation products formed from dl- $\alpha$ -tocopherol (Vitamin E) in processed polyolefins were identified and characterised [21-25]. The extracts of vitamin E stabilised polymers reported from these studies revealed formation of a large number of products but some were attributed to different stereoisomeric forms of the same products. Most of the products formed during melt processing of Vitamin E-stabilised polyethylene and polypropylene were based on dimers and trimers of  $\alpha$ -tocopherol, as well as quinones and aldehydes [21-25].

To our knowledge, there is no published work on the fate of vitamin E, or, the formation and nature of any of its transformation products that may form in UHMWPE orthopaedic implant components (manufactured by Vitamin E antioxidant infused technology) during the various stages of their production; both after the initial high  $\gamma$ -irradiation dose (typically 100 kGy) used for crosslinking (Tibia samples at this stage are referred to here as '**Tibia-VEPE**') and after the second lower dose gamma irradiation (typically 25-40 kGy) used for sterilization (Tibia samples at this stage are referred to as '**Tibia-VEPE-Sterile**'). Some literature findings have made the assumption, based on hexane reflux extraction at 70°C, that vitamin E becomes chemically bonded (grafted) onto the polymer [26, 27], this assumption will be addressed here.

In this study, UHMWPE-Tibia samples produced for Total Knee Arthroplasty were used in order to investigate the fate of vitamin E and the nature of its transformation products (extracted from microtomed Tibia samples) formed at two stages of the implant manufacturing process before and after sterilisation (Tibia-VEPE and Tibia-VEPE-Sterile). The amount of vitamin E was quantified and its transformation products in the different Tibia samples were isolated, quantified and characterised. A fresh Vitamin E solution, vitamin E solution retrieved from the implants doping process and fresh vitamin E samples that were  $\gamma$ -irradiated were also analysed in order to understand the reaction products formed at the different stages of the Tibia manufacturing process.

## 2. Experimental

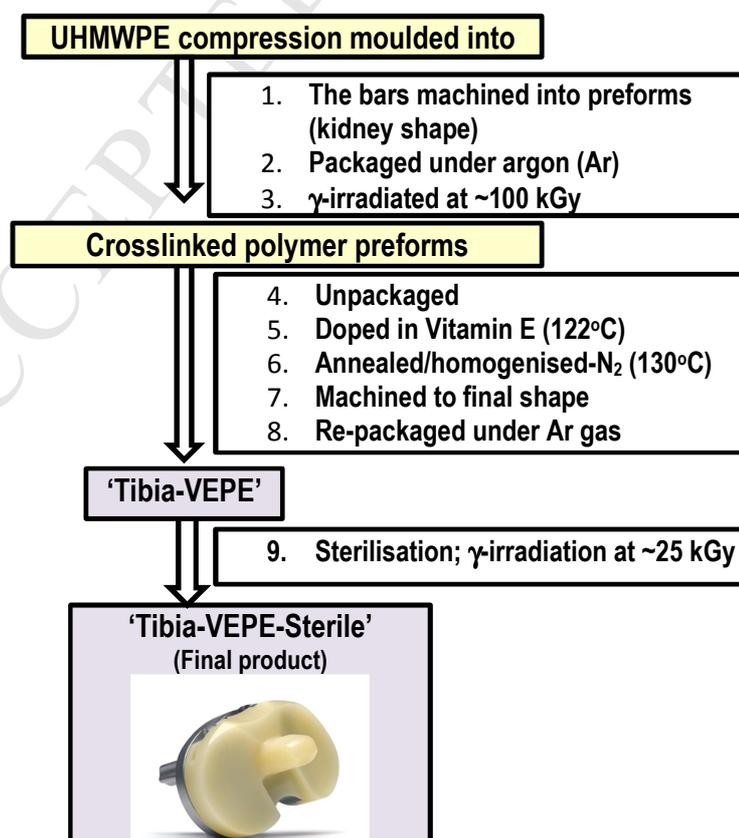
### 2.1. Materials

Tibia component samples, UHMWPE resin and vitamin E solutions were kindly donated by Biomet Inc. (Warsaw, IN). The UHMWPE resin utilized was medical grade GUR1020 (Ticona, Bishop, TX), vitamin E (dl- $\alpha$ -tocopherol) was obtained from Fisher Scientific, Houston, TX. All other solvents used were HPLC (Fisher) and NMR grades (deuterated chloroform, Goss Scientific) and were used without further purification.

### 2.2. Sample Preparations

The manufacturing process of the Tibia samples is described briefly in **Scheme 1**. Isostatically compression moulded UHMWPE (GUR 1020) bar stocks produced at Biomet Inc. were machined into kidney shape preforms. The preforms were packaged under argon gas and the packages were  $\gamma$ -irradiated to crosslink the polymer at a dose of 100 kGy. The irradiated samples were then doped by immersion in a bath containing vitamin E at 122°C under continuous stirring for 5 hours (vitamin E infusion) in order to diffuse through the thickness of the polymer samples and were subsequently annealed at about 130°C under continuous nitrogen flow for at least three days for homogenization. The samples were then machined into Tibia shapes (**Tibia-VEPE**) and packaged under argon gas. The packaged Tibia samples were sterilized (with lower dose of  $\gamma$ -irradiation of 25 k Gy, '**Tibia-VEPE-Sterile**'), see **Scheme 1**, [19, 28-31].

**Scheme 1.** Schematic diagram for the production of vitamin E diffusion-post irradiation process of Tibia samples (the Tibia photograph is courtesy of Biomet Inc)



The Tibia samples were microtomed into thin films (100  $\mu\text{m}$  thicknesses) using a rotary microtome (Leica RM2165). Vitamin E and the various transformation products were extracted from the microtomed films by two methods; reflux in boiling hexane or by accelerated solvent extraction (ASE). For extraction by reflux in hexane, 2g microtomed films were refluxed under nitrogen atmosphere in 100 ml HPLC grade hexane for 24 hrs followed by cooling and evaporation of the solvent in a fume cupboard. Accelerated Solvent Extraction (ASE) method was conducted in a Dionex ASE200 with hexane or with 5% cyclohexane in dichloromethane at different temperatures (ranging from 70°C to 122°C); 2g microtomed films were used and all samples were extracted for 5 cycles, each cycle was 15 min with a pressure of 2000 psi. The extracts were transferred into a beaker and left to dry in a fume cupboard before use.

### 2.3. Evaluation of the Amount of Vitamin E and the Oxidative Induction Time in Microtomed Tibia Films

In order to evaluate the amount of vitamin E in Tibia film samples (microtomed at  $\sim 100 \mu\text{m}$ ), two defined vitamin E indices were determined and were measured as a function of depth by line scanning using a Fourier Transform Infrared microscope (FTIR; Perkin Elmer spectrum GX AutoIMAGE FTIR-Microscope). Spectra were obtained in transmission mode over the range 4000-700  $\text{cm}^{-1}$  using 16 scans and resolution of 4  $\text{cm}^{-1}$  and the line scans were collected from the films across the width of each section (through two edges of the samples spanning two opposite free surfaces) in 100  $\mu\text{m}$  intervals. The concentrations of vitamin E, and vitamin E and transformation products, in the films were quantified by calculating vitamin E indices defined as the ratio of the absorption area under a specific vitamin E peak (at 1263  $\text{cm}^{-1}$  (1275 to 1245  $\text{cm}^{-1}$ ) [6, 28] and at 1210  $\text{cm}^{-1}$  (1225 to 1195  $\text{cm}^{-1}$ ) [32] normalised to that of a polyethylene backbone absorbance peak at 1895  $\text{cm}^{-1}$  (1985 to 1850  $\text{cm}^{-1}$ ).

A Mettler Toledo Differential Scanning Calorimeter (DSC model 832e) interfaced with a PC was used to measure the Oxidative Induction Times (OIT) of Tibia films (100  $\mu\text{m}$ ) before and after extraction according to ASTM-D3895-03. 2-5 mg microtomed film samples (done in triplicates) were placed in open DSC pans and heated from 40°C to 190°C at a rate of 25°C/min under nitrogen flow. The samples were held at 190°C for 5 min and then the nitrogen was switched over to oxygen keeping the same gas flow rate (40 ml/min). The oxidation induction time was obtained as the time between the onset of the oxygen gas flow and the intersection of the isothermal base line with the tangent at 1 mW deviation from the isothermal baseline.

### 2.4. Quantification of Vitamin E and its Transformation Products from Tibia Extracts

Vitamin E and its transformation products were extracted from different Tibia components and quantified using an analytical HPLC method developed for this purpose. The HPLC analysis was performed on a Thermo Scientific UltiMate 3000 HPLC system equipped with vacuum degasser, quaternary pump, an autosampler and a UV/Vis diode array detector. The chromatographic separation of extracts of microtomed Tibia films was carried out in an isocratic mode using a Zorbax-RX-SIL (5 $\mu\text{m}$ , 4.6 x 250 mm) analytical column at operating temperature of 30°C, constant flow rate of 1 ml/min, UV detection wavelength of 297 nm and with a 25  $\mu\text{l}$  injection volume. The mobile phase was pre-mixed 98.0 hexane / 2.0% dioxane. The concentration of the products was determined from their HPLC peak area using the method described below.

A method was developed based on analytical HPLC analysis and calibration curves constructed for vitamin E, and for each of the isolated and purified transformation products. The calibration curves were produced from HPLC peak areas (analysed using the isocratic conditions described above) plotted against the compound's

known concentrations (expressed in mg/ml hexane) obtained from stock solutions of 1 mg/ml hexane of each of the isolated pure products. The Tibia extracts from 2 g microtomed films (extracted on ASE using cyclohexane/DCM from 85-122°C or by reflux with hexane) were dried and then dissolved in 10 ml hexane and analysed using the analytical HPLC method described above. The peak area for each product and its calibration curves were used to calculate the amount of each product (in mg) in the extracts, which was used to calculate the concentration of the products in the Tibia based on the amount extracted. Therefore, the amount of transformation products formed, and that of vitamin E, were quantified and presented as weight percent of the total amount extracted (concentrations were calculated as part per million (ppm) based on weight of the polymer used and the amounts of the quantified products). The different vitamin E solutions were analysed by dissolving a 100 mg in 100 ml hexane (using the same method), the calibration curves were then used to calculate the amount of each product and vitamin E in the solutions. Repeat analysis of at least three samples taken from different locations of the implants were analysed and the resulting concentration averages of each compound are recorded.

In spite of this, it is important to point out that caution need to be exercised regarding the absolute accuracy of the calculated concentrations, particularly, for some of the products for a number of reasons. If the amount of an isolated transformation product was very small then their purity may not have been absolute (although each was purified at least twice through the HPLC process). Further, the detection wavelength used in the HPLC analysis was fixed at 297 nm rather than using the optimum wavelength for each compound. This, in addition to the fact that some of the products, e.g. Dimer-Ox, were not highly stable (though analysis of these compounds was done immediately after their collection and with maximum care to ensure minimum error).

## 2.5. Separation of Vitamin E Transformation Products

The final sterilised **Tibia** samples (**VEPE-Sterile**) were microtomed into 100 µm films, 20 g of the microtomed films were placed in 2L round bottom flask with 1L HPLC grade hexane, stirred and refluxed under nitrogen atmosphere for 24 hrs followed by drying (in a fume cupboard) and re-dissolving in hexane (HPLC grade) for HPLC fractionation. A Thermo Scientific UltiMate 3000 HPLC system was used for semi-preparative and preparative work for separation and collection of the different product fractions (the system which was described in section 2.3, also comprised of a fraction collector). The normal phase separation was achieved using Zorbax RX-SIL columns (7µm, 21.2 x 250 mm for preparative and 5µm, 9.4x250 mm for semi-preparative HPLC) with a mobile phase (used in both Prep- and Semi-Prep-HPLC systems) of varying ratios of 1,4-dioxane: hexane (from 0.5 to 2.0% dioxane in hexane) and UV detection at four different wavelengths in order to ascertain the presence of the different products.

## 2.6. Spectroscopic Characterisation of the Separated Transformation Products

The purified transformation products of vitamin E isolated from Tibia samples were characterised by different spectroscopic methods; NMR, FTIR and UV-Vis as well as by LC-MS spectroscopy. Proton and carbon nuclear magnetic resonance (<sup>1</sup>H and <sup>13</sup>C NMR) spectra were recorded on a Bruker Avance-300 spectrometer at ambient temperature using tetramethylsilane (TMS) as internal standard. 1D and 2D NMR spectra were recorded using a 5 mm normal dual detection probe and operating at controlled temperatures. The NMR samples were prepared by dissolving 20 mg of the purified transformation products in 0.7 ml deuterated chloroform (CDCl<sub>3</sub>) solvent which contained 0.05% TMS. For **<sup>1</sup>H NMR**, the spectra were recorded at 300.13 MHz using a high-resolution dual (<sup>1</sup>H <sup>13</sup>C) gradients probe. Spectra were recorded using the zg30 pulse program with 32 scans and referenced to an internal TMS standard at 0.0 ppm. **PENDANT** <sup>13</sup>C NMR spectra were obtained at 75 MHz for carbon. The pendant pulse program was used with waltz16 decoupling during acquisition with 2048 scans and phased for CH<sub>3</sub>/CH positive and quaternary carbons and CH<sub>2</sub> negative with internal reference TMS at 0.0 ppm. 2-

Dimensional  $^1\text{H}$ - $^{13}\text{C}$  Heteronuclear Multiple Bond Coherence (**HMBC**) spectra, which correlate  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts through multiple-bond heteronuclear scalar coupling ( $^nJ_{\text{CH}}$ ,  $n = 2$  or  $3$ ) were also recorded. The cross peaks in the  $^1\text{H}$ - $^{13}\text{C}$  HMBC spectra shows the chemical shifts of  $^1\text{H}$  on one axis (horizontal) which correlated to  $^{13}\text{C}$  (on the vertical axis) that belongs to H and C atoms which are separated by two or three chemical bonds. The 2-Dimensional  $^1\text{H}$ - $^{13}\text{C}$  Heteronuclear Single Quantum Coherence (**HSQC**) spectra, which correlate  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts through single-bond heteronuclear scalar coupling ( $^1J_{\text{CH}}$ ) were also recorded. The cross peaks in the  $^1\text{H}$ - $^{13}\text{C}$  HMBC spectra shows the chemical shifts of  $^1\text{H}$  on one axis (horizontal) which correlated to  $^{13}\text{C}$  (on the vertical axis) that belongs to H and C atoms which are chemically bonded.

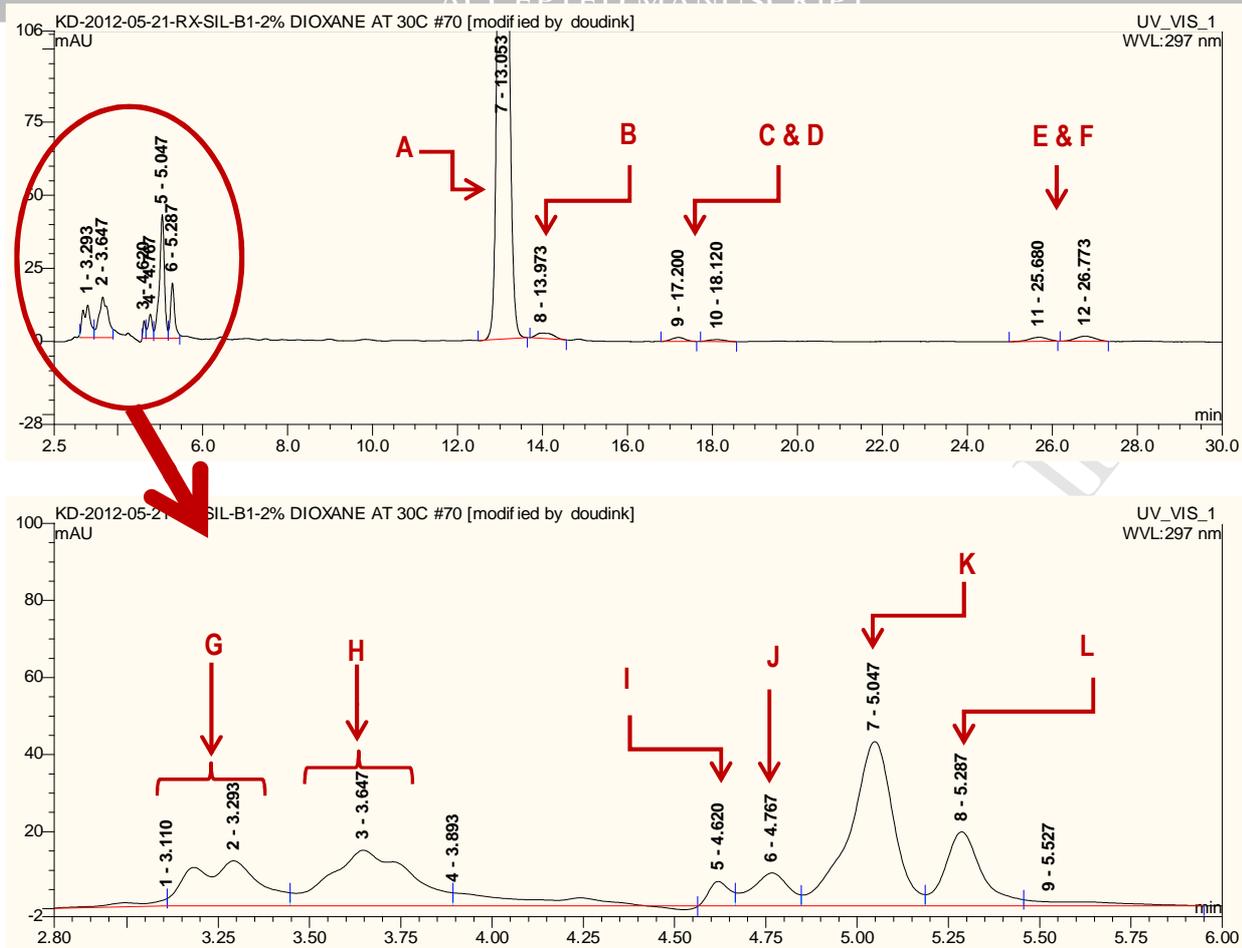
Fourier Transform Infrared (FTIR) measurements were performed on a Perkin Elmer Spectrum One, over the range of  $4000\text{--}400\text{ cm}^{-1}$ ; spectral collection was taken over 16 scans with resolution of  $4\text{ cm}^{-1}$ . Ultraviolet-visible (UV-Vis) spectra of solutions and thin Tibia polymer films were recorded at room temperature on Cary 5000i (Agilent) UV-Vis spectrophotometer at a scanning rate of  $600\text{ nm/min}$ . The Liquid chromatography-Mass Spectroscopy (LC-MS) detection analysis was performed using Thermo MSQ plus instrument coupled to the analytical HPLC system (described above) used in negative mode soft ionisation at Atmospheric Pressure Chemical Ionization (APCI) mode with cone voltage of  $100\text{ V}$ , probe Temperature  $500^\circ\text{C}$  and mass detection range of  $100\text{--}2000\text{ m/z}$ .

### 3. Results and Discussion

#### 3.1. Characterisation of Vitamin E and its transformation products from Tibia components

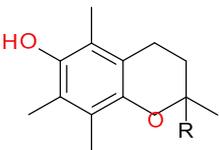
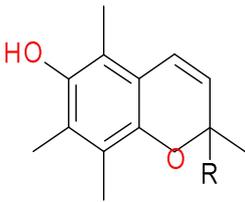
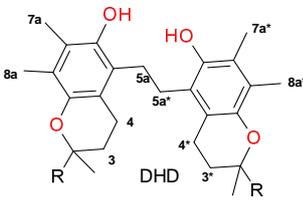
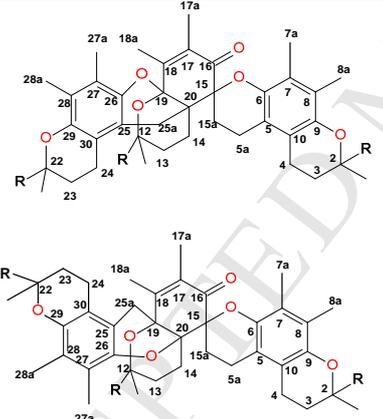
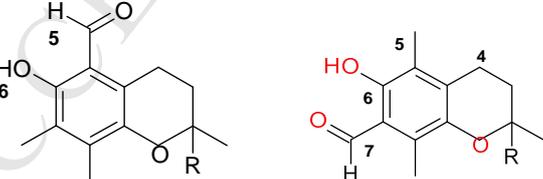
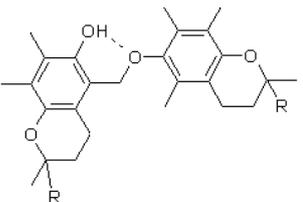
The nature of vitamin E transformation products that may have formed in the final Tibia product (Tibia-PEVE-Sterile), see **Scheme 1**, was investigated through their characterisation and identification using complementary spectroscopic methods followed by quantification of the products. The different transformation products were extracted from the Tibia samples and were then separated.

Twelve well separated peaks of vitamin E and its transformation products are observed from the analytical HPLC chromatogram, see **Figure 1** peaks **A** to **L**. These were subsequently isolated and a detailed characterisation of each of the isolated products was carried out using different spectroscopic methods as described below (see structures in **Table 1**).



**Figure 1:** HPLC chromatogram of Tibia (Tibia-VEPE-Sterile) extracts using analytical HPLC method

**Table 1:** Structures of vitamin E and its transformation products isolated from Tibia samples along with selected spectroscopic characteristics

COMPOUND (code)	STRUCTURE	FTIR main peaks cm <sup>-1</sup>	UV λ max (nm)	MS, molecular ion peaks
Vitamin E		C-O-C: 1262 C-O-H: 1212 O-H: 3470	297, 289	429
Tocopherol-3-ene (Toc-ene)		C=C: 1600/1550 C-O-C: 1261	335, 281, 272	427
Dihydroxydimers (DHD)		C-O-C; 1261 C-O-H; 1211	298, 291, 281	858
Trimers (TRI)		C=O; 1739 and 1693 C-O-C; 1260 C-O-H; 1208	295	1284 - 1285
Aldehydes (Ald-5 & Ald-7)		C=O: 1635 C-O-C: 1262 O-H 3467	387, 281, 272	443
Dimer-Ox		C-O-C: 1265 O-H : 3420 (973 )	292	858

**Fraction A:**

The main peak A, eluted at 13 min, was characterised as the parent compound vitamin E. The vitamin (dl  $\alpha$ -Tocopherol) was fully characterised (using 1D NMR;  $^1\text{H}$  and  $^{13}\text{C}$  Pendant, 2D NMR; HSQC and HMBC, FTIR, UV and LC-MS) in order to use it as a reference compound to aid with the characterisation of the different transformation products.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of vitamin E are shown in **Figure 2**. The main signals that are used here for the purpose of analysis and characterisations of the transformation products are based on the groups attached to the phenol and the chroman rings. In the  $^1\text{H}$  NMR spectra (**Figure 2A**) there are three  $\text{CH}_3$  groups on the phenol ring ;  $5_a$  and  $7_a$  at 2.14 ppm and  $8_a$  at 2.19 ppm, and on the chroman ring there are two methylenes ( $\text{CH}_2$ ); 3 at 1.80ppm and 4 at 2.63ppm in addition to the  $\text{CH}_3$  group ( $2_a$ ) at 1.26ppm. The  $^{13}\text{C}$  Pendant NMR signals are assigned in **Figure 2B** where the carbons of the rings are labelled as 2 -10 and the carbon for the  $\text{CH}_3$  groups on the phenol ring are labelled  $5_a$ ,  $7_a$  and  $8_a$ , in addition to the  $\text{CH}_3$  group ( $2_a$ ) on the chroman ring.

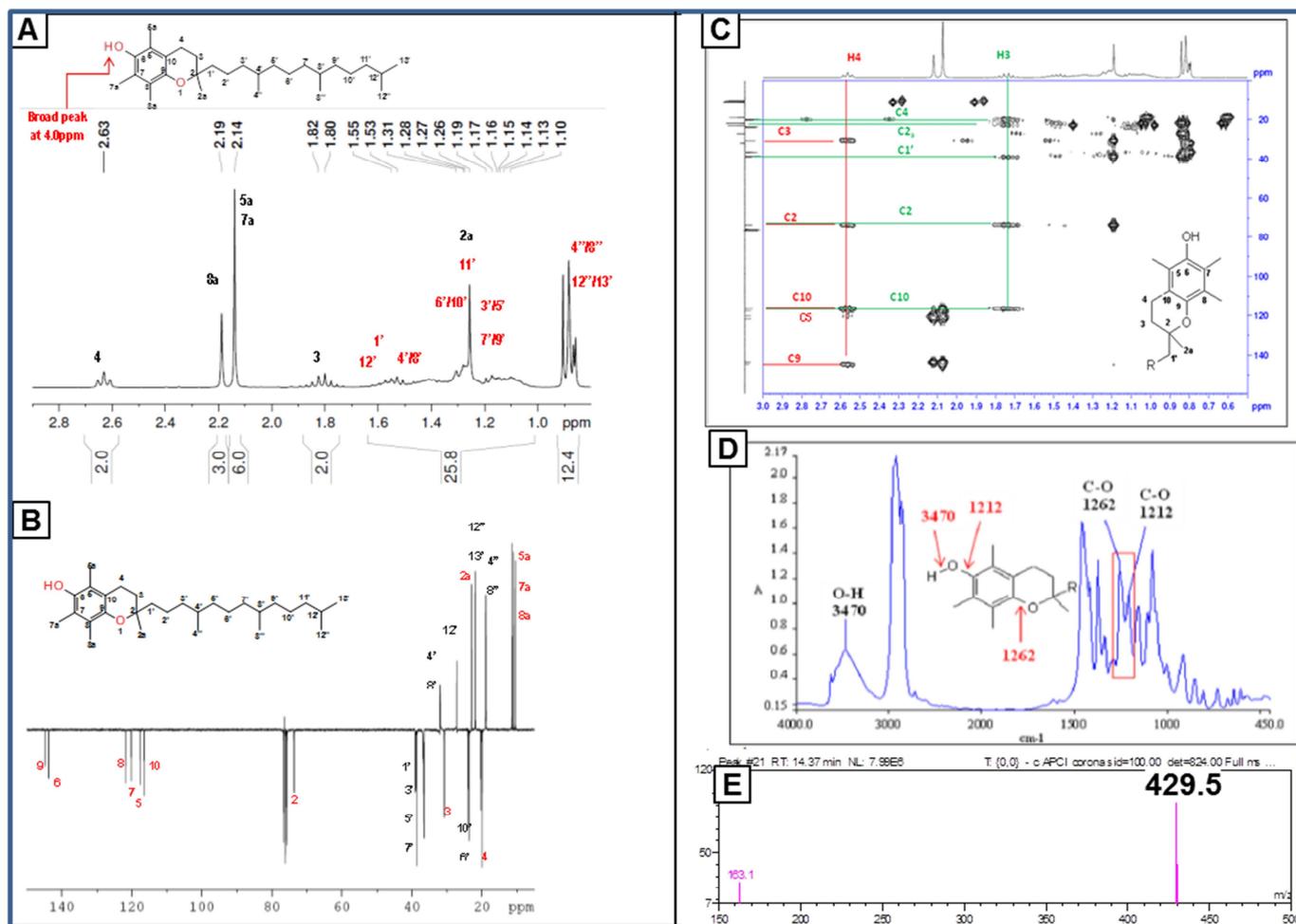
**Figure 2C** shows the 2D HMBC NMR spectrum of fresh vitamin E, of a special interest here is the correlation between the protons on the two methylene groups ( $\text{CH}_2$ : H3 and H4) in the chroman ring and various carbons as this is the part of the vitamin molecule where the changes would occur in most of its transformation products. The correlation between protons on carbon number 3 (H3) and carbons C4, C2<sub>a</sub>, C1', C2 and C10 which are two or three bonds away from protons H3 (the green lines) is clearly illustrated, as well as the correlation between the protons on carbon number four (H4) and carbons C3, C2, C10, C5 and C9.

The FTIR spectrum of Vitamin E, see **Figure 2D** and **Table 1**, shows the presence of two important absorptions at  $1262\text{ cm}^{-1}$  (ether C-O stretching of the chroman ring) and at  $1212\text{ cm}^{-1}$  (C-O stretching of the phenol hydroxyl group) with the former being normally used to quantify the amount of vitamin E in the polymer [32]. In this work, both peaks were used to estimate the amounts of vitamin E and the transformation products in the polymer samples before extraction [2, 28, 32]. The UV-spectrum of vitamin E shows absorptions at 291 and 297 nm, and its MS spectrum (from LC-MS) has given a molecular ion peak with a mass of 429 (**Figure 2E**) confirming that this HPLC peak A is that of vitamin E.

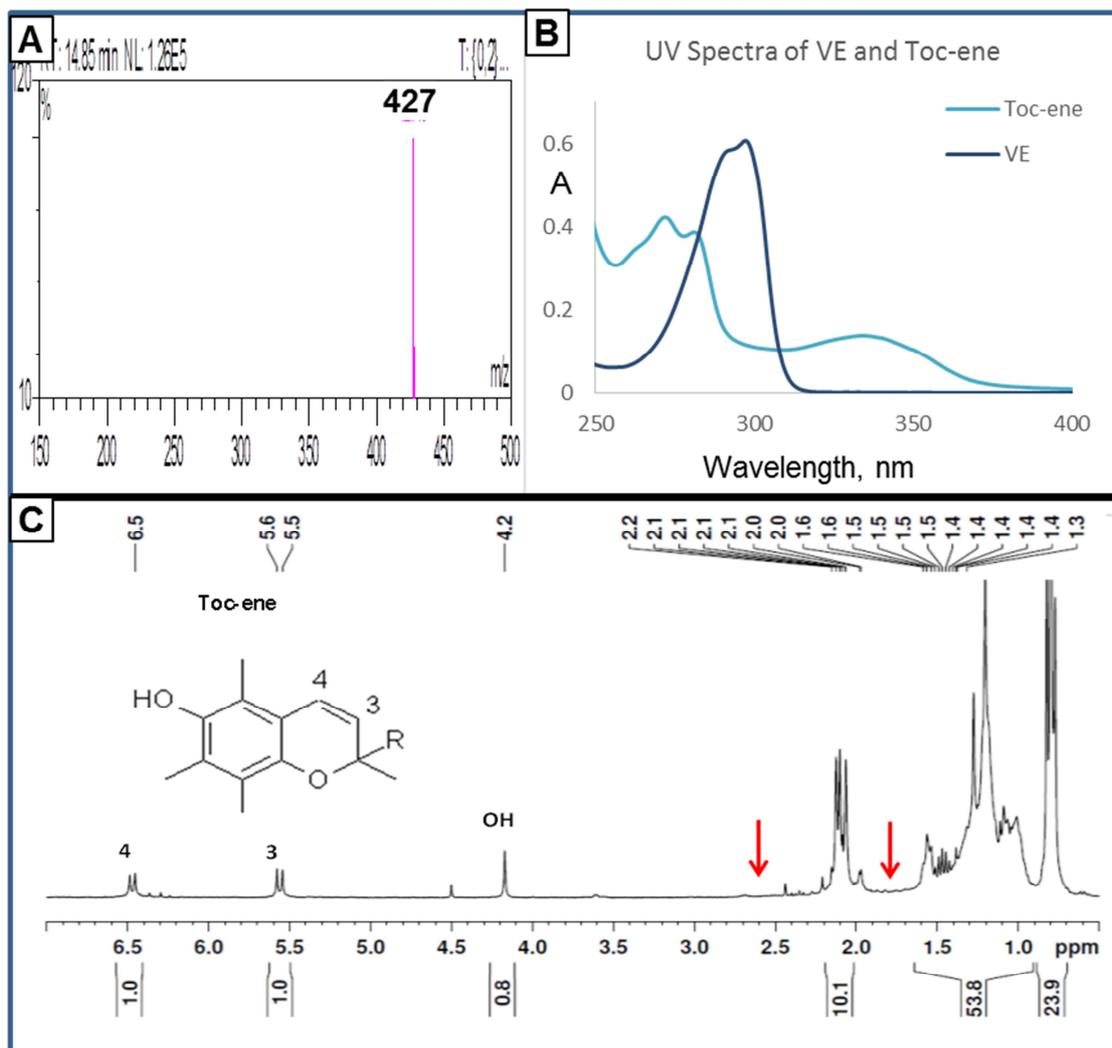
**Fraction B:**

The second HPLC peak B that appeared in the chromatogram after vitamin E at a retention time of 14.0 min (see **Figure 1**) corresponds to a monomer product based on one vitamin E molecule having a molecular ion in its MS spectrum with a mass of 427 (**Figure 3A**). The UV spectrum of this product (**Figure 3B**) showed three  $\lambda_{\text{max}}$  at 272, 281 and a longer wavelength absorption at 335 nm compared to that of vitamin E which gave absorptions at the low wavelengths of 291 and 297 nm only.

The  $^1\text{H}$  NMR spectrum of this isolated product (see **Figure 3C**) is very similar to that of vitamin E except for protons H3 and H4 appearing at 5.55 and 6.45 ppm with each peak being a doublet suggesting that they are two single neighbouring protons (with coupling constants of 10Hz for cis protons) positioned next to a double bond. Protons H3 and H4 of vitamin E which appeared at 1.80 and 2.63 ppm (**Figure 2A**) are completely absent in compound B (see **Figure 3C**). These characteristics suggest that this product is **Tocopherol-3-ene** (see **Table 1** for **Toc-ene** structure), which we have not seen in our previous studies on melt processing of polyethylene with vitamin E [23].



**Figure 2:** Spectra of vitamin E: (A)  $^1\text{H}$  and (B)  $^{13}\text{C}$  Pendant NMR; (C) HMBC NMR; (D) FTIR spectrum and (E) mass spectra, from LC-MS (from HPLC fraction A)



**Figure 3:** Spectra of **Toc-ene** obtained from Tibia extracts (HPLC fraction B): (A) Mass spectrum (from LC-MS); (B) UV spectrum (compared with that of vitamin E) obtained from Tibia extracts (HPLC fraction B); (C) <sup>1</sup>H NMR spectrum

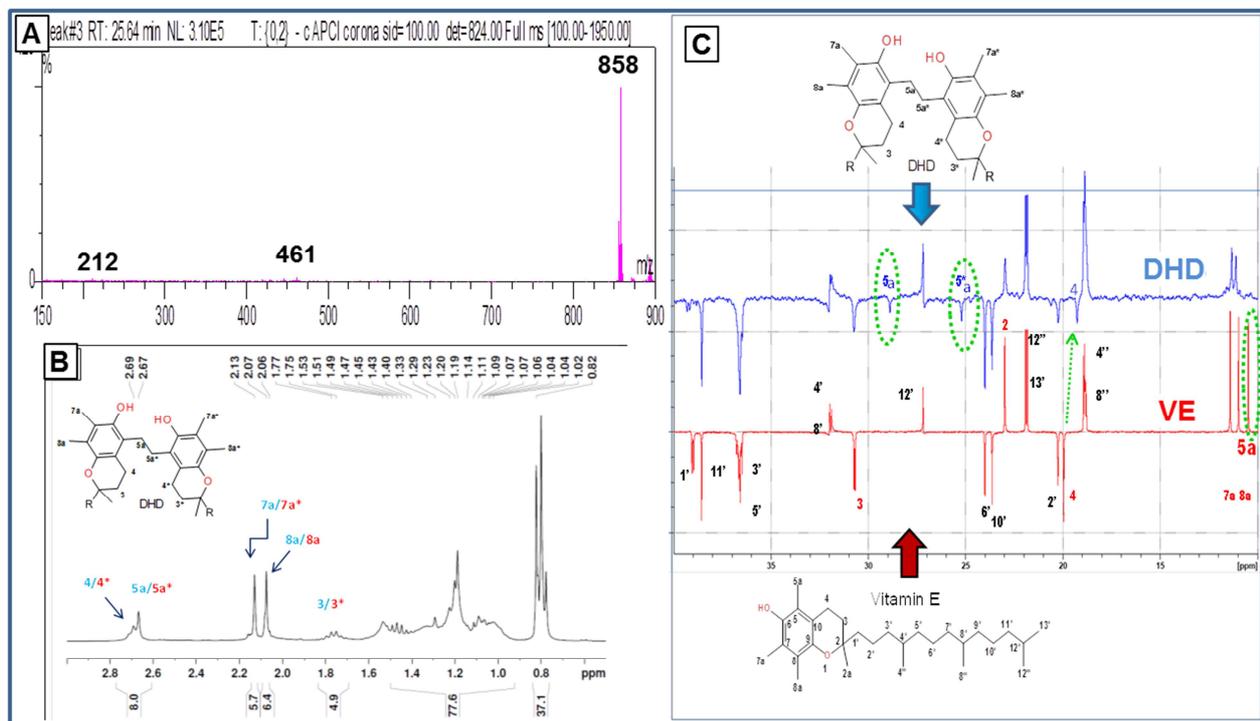
### Fractions C and D;

The amount of these products was very small for full isolation and characterisation, but their chromatographic retention and UV and MS analyses (not shown here) fit well with the characteristics of spirodimers of vitamin E (**SPD**) with their full characterisation reported in our previous work [21-25].

### Fractions E and F:

Peaks with retention times of 25.7 and 26.8 min (see **Figure 1**) were found to have very similar NMR, UV and MS characteristics with their molecular ion peak appearing at m/s of 858, **Figure 4A and B**, suggesting a dimeric structure. The <sup>1</sup>H NMR spectra (**Figure 4B**) of these compounds show that there are only two types of CH<sub>3</sub> groups on the phenol rings (7a/7a\* and 8a/8a\*) at 2.13 and 2.07 ppm. The 5a/5a\* methylene protons are shifted here downfield and appeared at 2.67ppm, and are shown to have a very close chemical shift to that of the CH<sub>2</sub> groups in the chroman rings 4/4\* at 2.69 ppm. The <sup>13</sup>C pendant NMR (**Figure 4C**) confirms the observations in the <sup>1</sup>H NMR spectrum and shows that one of the CH<sub>3</sub> groups in the vitamin E phenol ring (proton 5a, +ve at 10.5

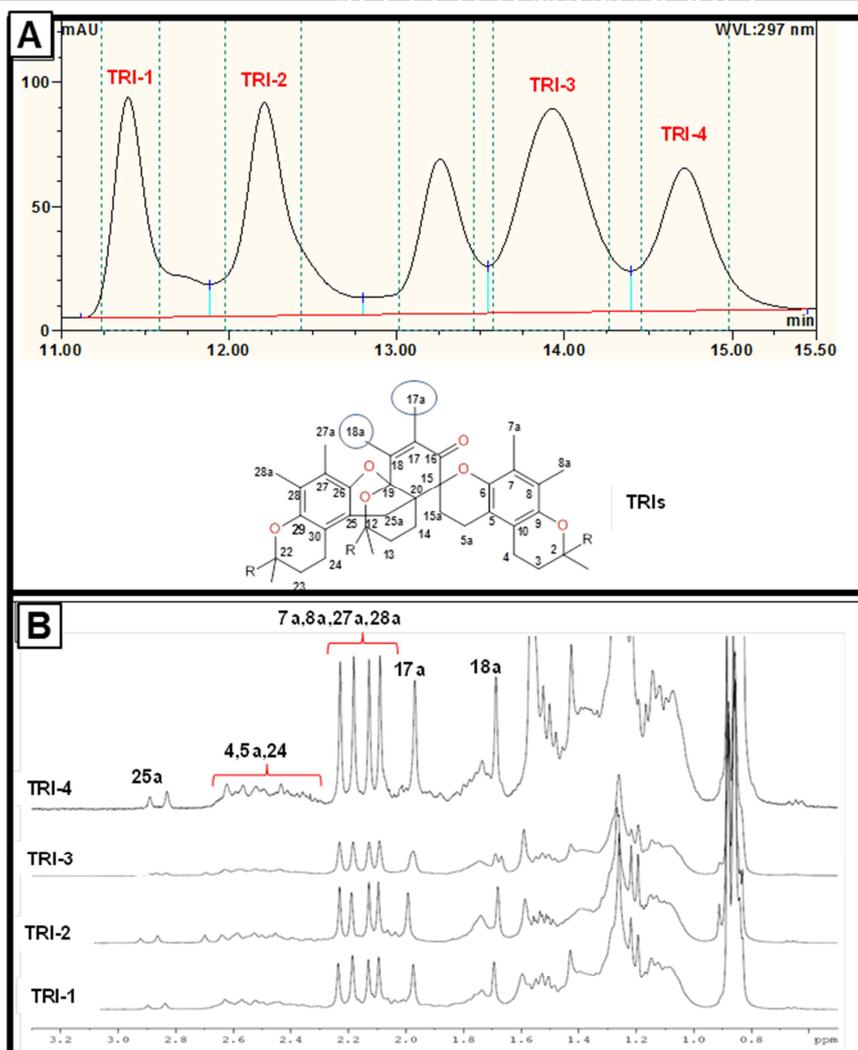
ppm, **Figure 4C-VE**) has been converted to CH<sub>2</sub> groups in the product (protons 5a, 5a\*, -ve at 25.2 and 28.9 ppm, **Figure 4C-DHD**). The <sup>13</sup>C spectrum also shows the shift of C4 from 20 ppm in vitamin E to 19.2 ppm in compounds E and F which suggest that the change in the molecular structure has taken place in a position near to C4 but not on this carbon. The UV analysis of this product showed similar results to vitamin E with an absorption band at λ<sub>max</sub> of 298 nm. These characterisations, and also based on our earlier literature [23, 25], confirm that these two products are stereoisomers of dihydroxydimer (DHD), see **Table 1** for structure.



**Figure 4:** spectra of Dihydroxydimers (HPLC fractions E and F) obtained from Tibia extracts: (A) MS (from LC-MS); (B) <sup>1</sup>H NMR; (C) <sup>13</sup>C pendant NMR spectra compared with that of vitamin E

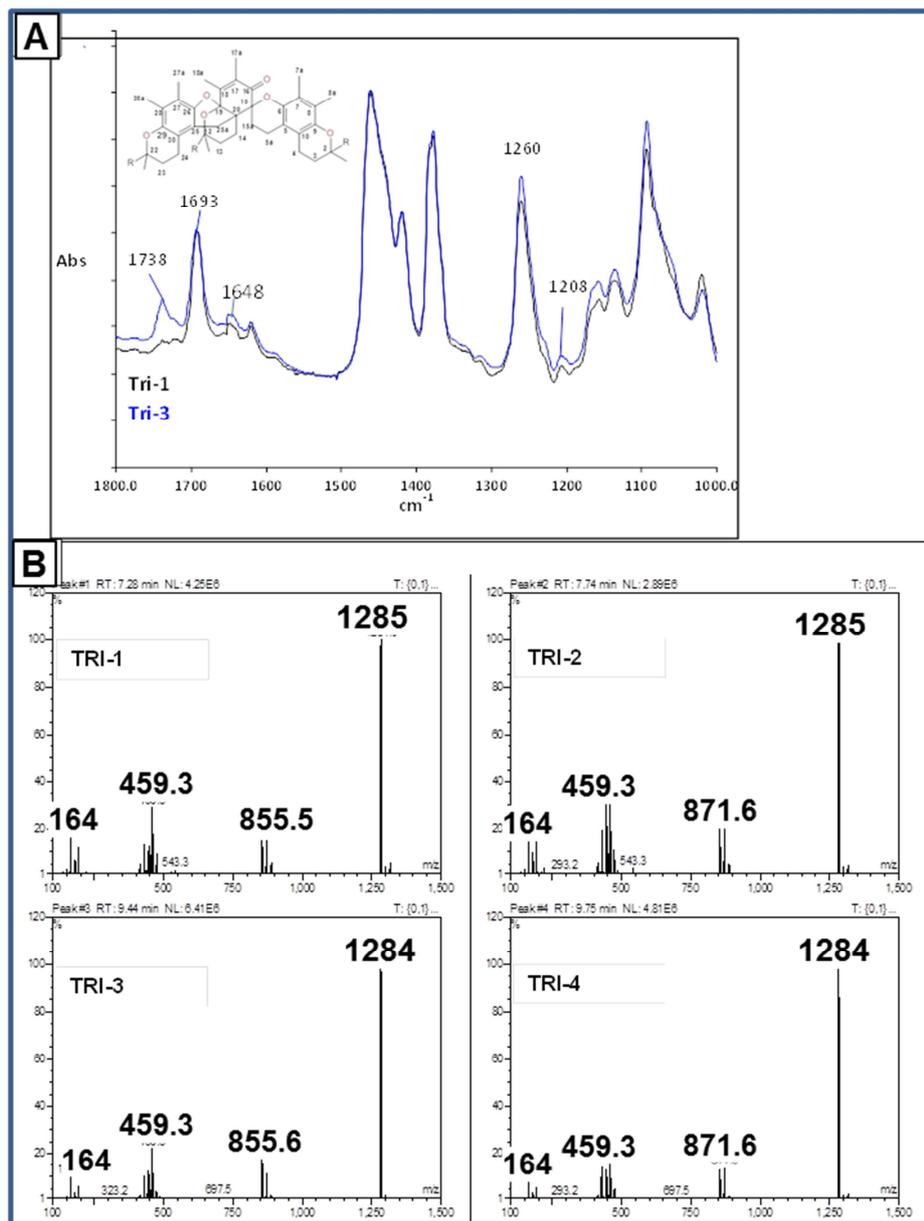
### Fractions G and H:

The first few product peaks that eluted in the analytical HPLC analysis between 3.2-5.2 min (**Figure 1**) were collected and re-run on a preparative HPLC to give five separated peaks (one was a mixture of two of the isomeric products), see **Figure 5A**, which were then isolated and fully characterised. The spectral characteristics (NMR, UV, MS, FTIR) of each of the four main pure isolated fractions were found to be very similar to those of stereoisomers of the same trimeric product of vitamin E that we have reported earlier [23, 24]. For example, the <sup>1</sup>H NMR spectra of the four compounds isolated from the Tibia-VEPE-Sterile extracts shown in **Figure 5B** clearly show similar characteristics except for some minor chemical shift differences for the signals of protons 17a and 18a. Similarly, their UV spectra were also very similar having one main absorption at λ<sub>max</sub> of 296 nm. The identity of these compounds as trimers is further evidenced from their FTIR spectra (**Figure 6A**) showing main absorptions at 1693 and 1648 corresponding to >C=O and >C=C<, respectively (see TRI structure), and their MS (from LC-MS) spectra giving a molecular ion peak corresponding to the Trimers at 1285 and 1284 (3 times molar mass of Toc) – 6 H], **Figure 6B**.



**Figure 5: (A)** Prep HPLC chromatogram of the **trimeric products** isolated from Tibia extracts; **(B)** their  $^1\text{H}$  NMR spectra (fractions G and H)

However, it is interesting to note that these different stereoisomeric trimers have some slight differences in the FTIR of their carbonyl region. For example, the trimer that eluted at 3.6 min (TRI 3) showed a stronger absorption in the  $1738\text{ cm}^{-1}$  region compared to that of the trimer that had eluted at 3.3 min (TRI 1) suggesting a slightly different environment around the carbonyl group of these two trimers which may be due to two main isomeric structures of the trimers, TRI-A and TRI-B, which have been identified in the literature previously [21-25] [33-35]. The structure of **Trimer-A** was identified in our earlier work [24], it has its carbon number 26 bridging through the hydroxyl oxygen to carbon number 19 of the second part of the molecule and the carbon of the methyl group (that has become methylene group, C 25a) bridges to carbon number 20 of the second molecule. Structure **Trimer-B** was reported in earlier literature [21-25] [33-35], in which carbon number 26 bridges through the hydroxyl oxygen to carbon number 20, whereas the methyl group carbon (carbon 25a) bridges to carbon number 19 of the second part of the molecule, see structures in **Table 1**. Each of these two different structures can form a number of stereoisomers, hence the presence of several peaks in the HPLC chromatogram that have very similar characteristics but all belonging to the trimeric structure.

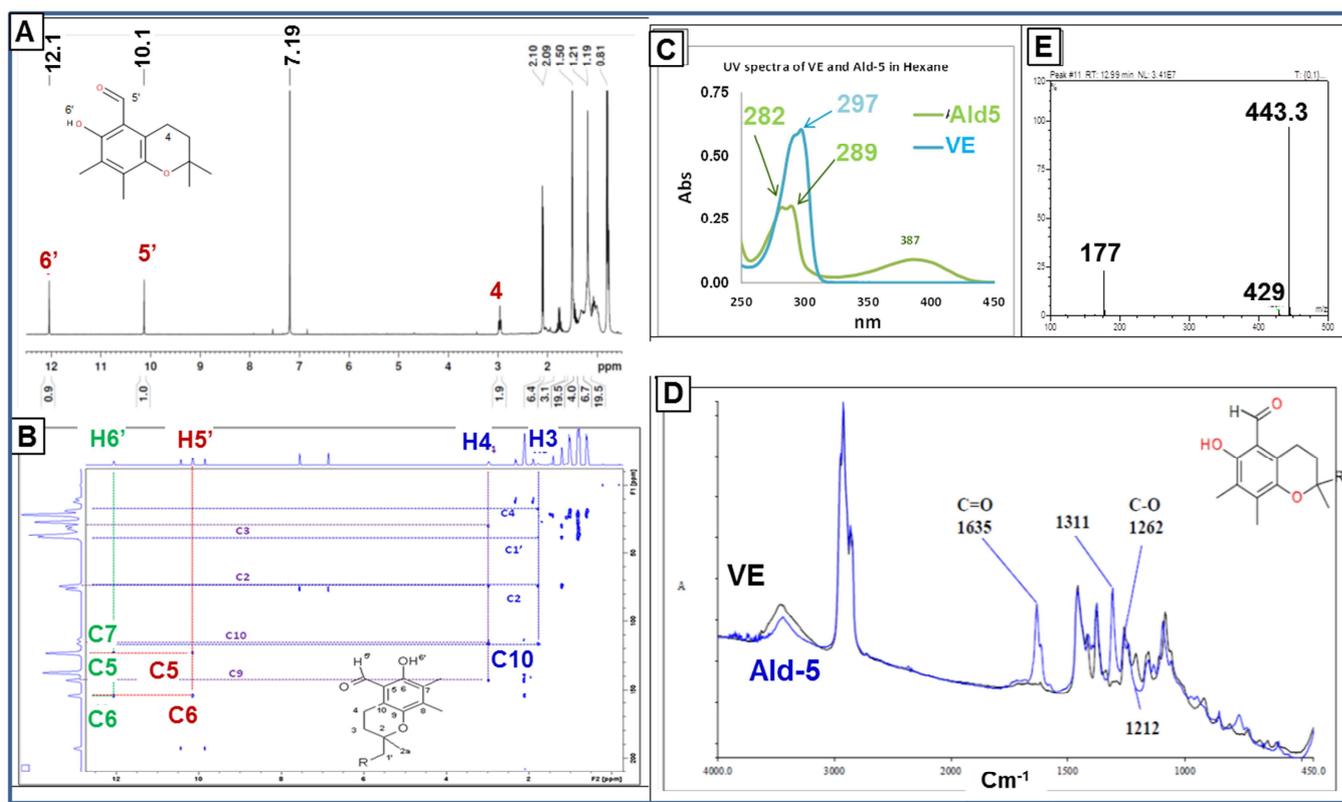


**Figure 6:** Spectra of Trimeric products isolated from *Tibia* extracts (HPLC fractions G and H): (A) their FTIR spectra and (B) their MS spectra

### Fractions I and L:

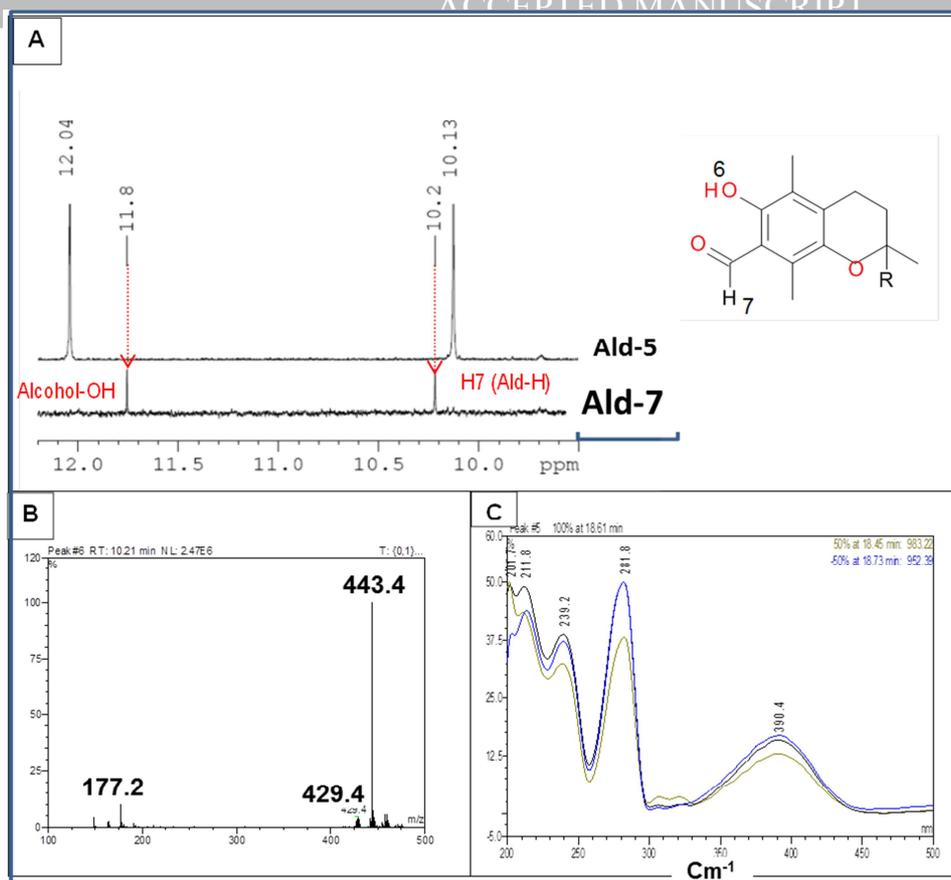
These two peaks which had retention times of 4.6 and 5.3 min (see **Figure 1**) were found to each contain an extra oxygen atom. The  $^1\text{H}$  NMR spectrum, **Figure 7A**, of the compound corresponding to peak L (elution at 5.3 min) shows clearly the presence of an aldehydic proton ( $\text{H}5'$ ) at 10.1 ppm as well as a phenol proton ( $\text{H}6'$ ) at 12.0 ppm. The 2D  $^1\text{H}$ - $^{13}\text{C}$  HMBC NMR spectra of compound L, **Figure 7B**, shows that the two protons are correlated to the carbon of the phenol ring (C5 and C6) and also the aldehydic proton ( $\text{H}5'$ ) showed a  $J^1$  correlation to the aldehyde carbons **C5'**; two contour plots correlation to the carbon at 193 ppm, confirming the structure as that of **Tocopherol-5-aldehyde (ALD-5)**. Further support of this aldehyde structure is clear from its UV spectrum showing a long wavelength absorption maximum at 387 nm, see **Figure 7C** attributed to the presence of an aryl aldehyde group. The FTIR spectrum of compound L (**Figure 7D and Table 1**) shows a distinct carbonyl absorption ( $-\text{C}=\text{O}$ ) at 1635  $\text{cm}^{-1}$  and a ( $-\text{C}-\text{O}$ ) stretch absorption at 1311  $\text{cm}^{-1}$  typical of aldehydic carbonyl. The

above characterisation, along with the MS results showing a molecular ion peak with  $m/s$  of 443, **Figure 7C**, and similarity to literature characteristics [23-25] further confirms the identity of compound L as **Tocopherol-5-aldehyde (ALD-5)**, see **Table 1**.



**Figure 7:** Spectra of **Ald-5** isolated from Tibia extract (HPLC fraction L): (A)  $^1\text{H}$  NMR; (B) HMBC-NMR; (C) mass spectrum; (D) FTIR spectrum (compared with that of vitamin E)

The NMR of the compound corresponding to peak I (elution time of 4.6 min) also showed both aldehydic and phenolic protons that appeared at 10.2 and 11.8 ppm, respectively, very slightly shifted compared to those seen in Ald-5, **Figure 8A**, and a corresponding aldehydic carbon signal in its  $^{13}\text{C}$  NMR at 195 ppm (not shown here). The UV spectrum of this compound I, **Figure 8C** showed a  $\lambda_{\text{max}}$  at 390 nm quite similar to the 387 nm absorption observed in the other aldehyde, Ald-5, these characteristics and the observed molecular ion at  $m/s$  of 443, **Figure 8B**, confirm the identity of this compound as **Tocopherol-7-aldehyde (ALD-7)**, see **Table 1**.



**Figure 8:** Spectra of **Tocopherol-7-aldehyde (Ald-7)** isolated from Tibia extracts (HPLC fraction I): (A)  $^1\text{H}$  NMR spectra (compared with that of Ald-5); (B) MS spectrum; (C) UV-Vis spectra

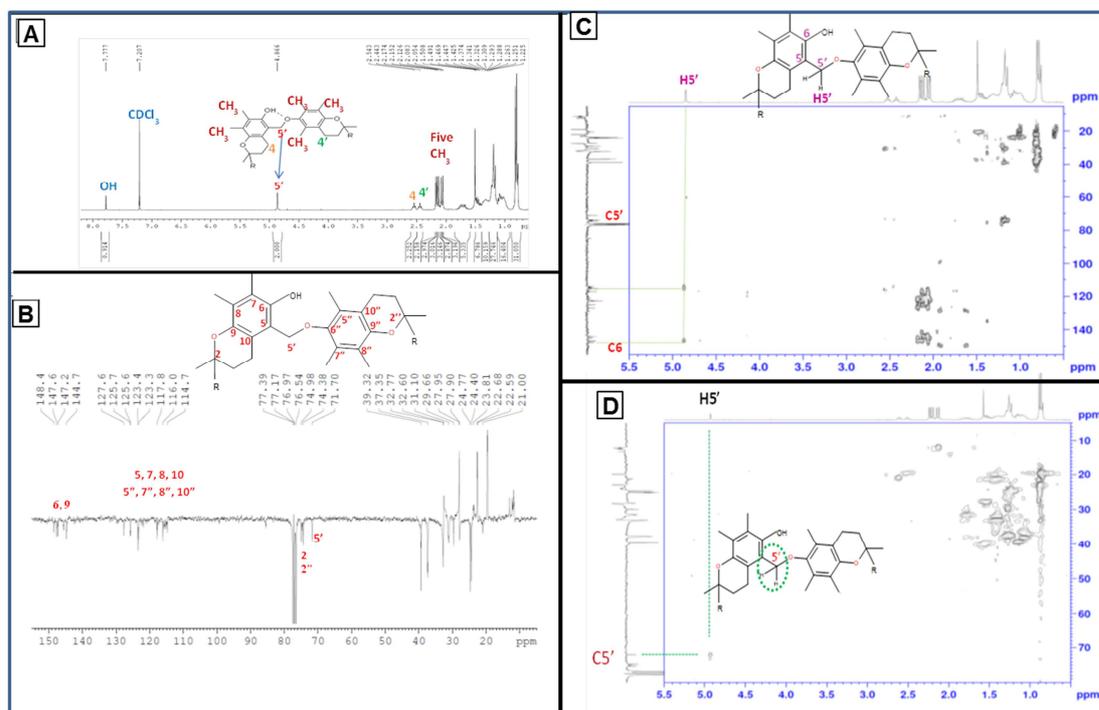
### Fractions J and K:

The compound corresponding to peak **K** which had a retention time of 5.0 min (see **Figure 1**) was isolated, purified and characterised by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. **Figure 9A** gives the  $^1\text{H}$  NMR spectra for this fraction (analysed immediately after being collected) and shows methylene ( $\text{CH}_2$ ) group protons at a chemical shift of 4.87 ppm ( $\text{H}5'$ ) and a corresponding carbon ( $\text{C}5'$ ) having  $^{13}\text{C}$  NMR shift at 71.7 ppm (**Figure 9B**). The  $^1\text{H}$  NMR spectrum also shows the presence of five  $\text{CH}_3$  groups located on aromatic rings (chemical shift between 2.0 and 2.2 ppm which are different from each other). It is important to note that this compound has only one  $-\text{OH}$  group having a chemical shift of 7.8 ppm compared to the phenolic  $-\text{OH}$  of vitamin E which appears at 4 ppm. The sharpness of the signal for this proton and its chemical shift suggests that this proton is associated with a close-by atom through a uniform hydrogen bonding.

The  $^1\text{H}$  NMR spectrum also shows the presence of two types of  $\text{CH}_2$  groups (see **Figure 9, 4 and 4'**). The 2D HMBC NMR analysis, **Figure 9C**, of this compound showed correlations between the protons of the  $\text{CH}_2$  at 4.87 ppm ( $\text{H}5'$ ) and the aromatic ring carbons C5, C6 and C10. Similarly, the 2D HSQC NMR spectra (**Figure 9D**) show the correlation between the same  $\text{CH}_2$  group (at 4.87 ppm,  $\text{H}5'$ ) and the carbon at 71.8 ppm ( $\text{C}5'$ ) confirming that these protons are directly bonded.

The UV-vis analysis of this product showed a very similar spectrum to that of vitamin E with a  $\lambda_{\text{max}}$  at 292 nm compared to 297 nm for vitamin E. The LC-MS analysis of this product gave a mass of 429 which is the same as that of vitamin E. This suggests that the product is not very stable and fragments easily under heating during the

MS-analysis into two molecules of vitamin E. Based on all the above characterisation a dimeric structure dimerised via a  $-C-O-C$  link is suggested for compound **K** with the structure of a **Dimer-Ox** shown in **Table 1**. The compound corresponding to peak **J** was an unstable product which has rearranged rapidly to the DHD during its characterisation.



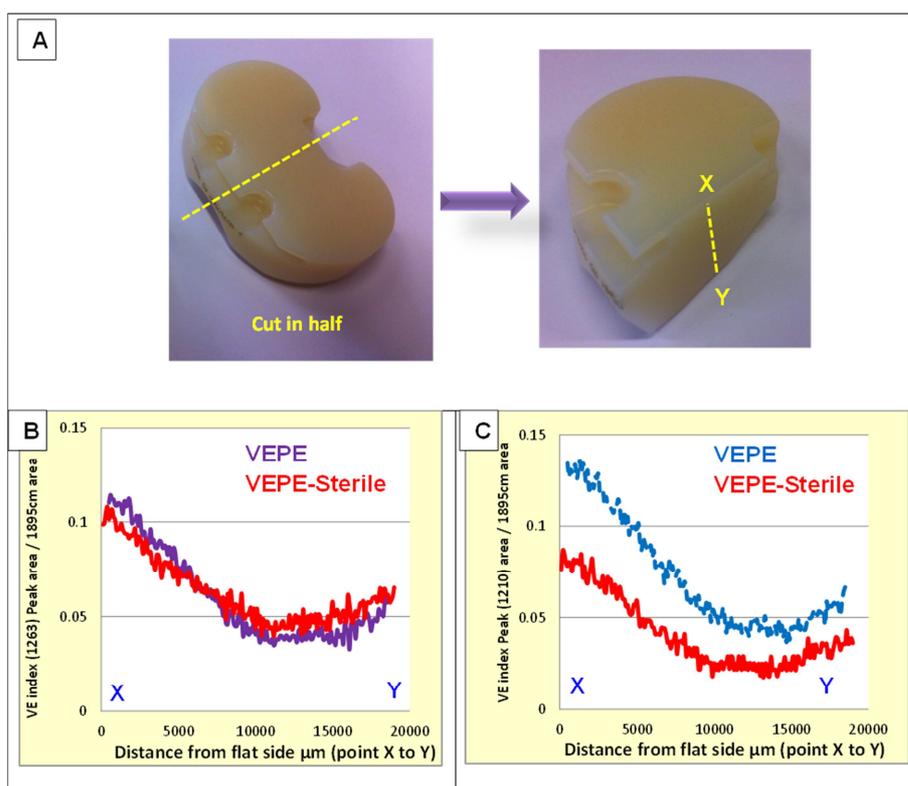
**Figure 9:** Spectra of **dimer-Ox** (HPLC fraction K) extracted from Tibia: (A)  $^1\text{H}$  NMR; (B)  $^{13}\text{C}$  NMR; (C) 2D HMBC-NMR; (D) HSQC NMR (all NMR analysis was carried out immediately after dissolving the sample in the NMR solvent)

### 3.2. The Concentration Profile of Vitamin E and Transformation Products as a Function of Depth of the Tibia Samples

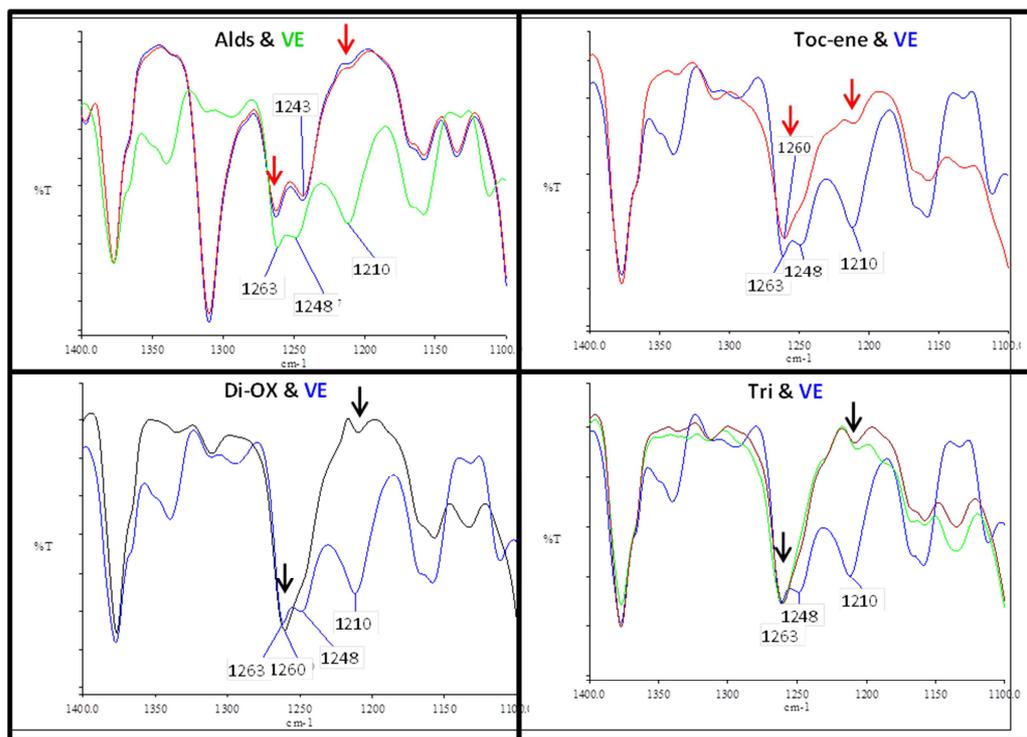
The concentration profiles of vitamin E and transformation products were determined across the depth of the Tibia polymer samples (both before and after the sterilisation stage) using vitamin E indices determined by micro-FTIR line-scanning. Each Tibia sample was first sliced in half and FTIR spectra were collected across the width of microtomed thin sections (through the freshly cut face from the centre of the flat face to the top of the sample along points X-Y in **Figure 10A**) in 100  $\mu\text{m}$  intervals. Vitamin E index based on the  $1263\text{ cm}^{-1}$  peak [6, 12] which corresponds to the aryl-O-alkyl ether group (in the chroman ring) is less accurate as a measure of the vitamin E concentration than the  $1210\text{ cm}^{-1}$  peak index [32] which corresponds to the C-O stretch of the phenol ring. This is mainly due to the fact that all the transformation products discussed above have at least one chroman ring, thus the  $1263\text{ cm}^{-1}$  index would give rise to a measure of the combined amount of vitamin E and all its products in the samples as can be seen from **Figure 11**. However, although measurements based on the  $1210\text{ cm}^{-1}$  peak index (C-O of the phenol ring) can be considered to be a more accurate assessment of vitamin E concentration in the polymer of the Tibia samples, attention should be drawn here to the fact that even for this index, a mild interference from some of the products that have a similar phenol C-O absorption (e.g. DHD, Toc-ene, Aids) could be expected. However, **Figure 11** shows that the IR-absorption peaks of these transformation products at  $2010\text{ cm}^{-1}$  are very small compared to that of the vitamin peak at  $1210\text{ cm}^{-1}$  and also compared to the products

absorption peak at  $1263\text{ cm}^{-1}$ , therefore, the  $1210\text{ cm}^{-1}$  peak index can still be considered a more accurate measure of vitamin E concentration in the Tibia polymer.

Based on the  $1263\text{ cm}^{-1}$  peak index, **Figure 10B** shows that both Tibia-VEPE and Tibia-VEPE-Sterile samples give a similar total concentration profile of vitamin E and its products across the depth of both set of samples. However, the  $1210\text{ cm}^{-1}$  index shows clearly that the **Tibia-VEPE-Sterile** samples have lower amount of vitamin E than is the case for **Tibia-VEPE** samples, see **Figure 10C**, suggesting that a higher amount of transformation products must have formed in the Tibia-VEPE-Sterile samples. These results are in good agreement with the quantified amounts of the products collected from the Tibia-VEPE-Sterile extracts, see next section.



**Figure 10:** Vitamin E depth profiles (based on FTIR indices for peaks  $1263\text{ cm}^{-1}$  and  $1210\text{ cm}^{-1}$ ) for **Tibia-VEPE** and **Tibia-VEPE-Sterile** samples



**Figure 11:** FTIR spectra of vitamin E and transformation products showing the two peaks at  $1263\text{ cm}^{-1}$  and  $1210\text{ cm}^{-1}$  in the spectrum of vitamin E and the appearance of mainly the  $1263\text{ cm}^{-1}$  in the transformation products.

### 3.3. Quantitative Analysis of Vitamin E and its Transformation Products in Tibia Extracts and Mechanism of their Formation

In order to have a better understanding of the amount and distribution of the different transformation products formed in the Tibia samples at the two selected stages of their manufacture (before and after the sterilisation process), the different transformation products were extracted from Tibia-VEPE and Tibia-VEPE-Sterile samples and their concentrations quantified using the analytical HPLC method described earlier. Similarly, transformation products formed in a vitamin E solution that was retrieved from the doping chamber, and from one that was  $\gamma$ -irradiated, were also analysed and quantified.

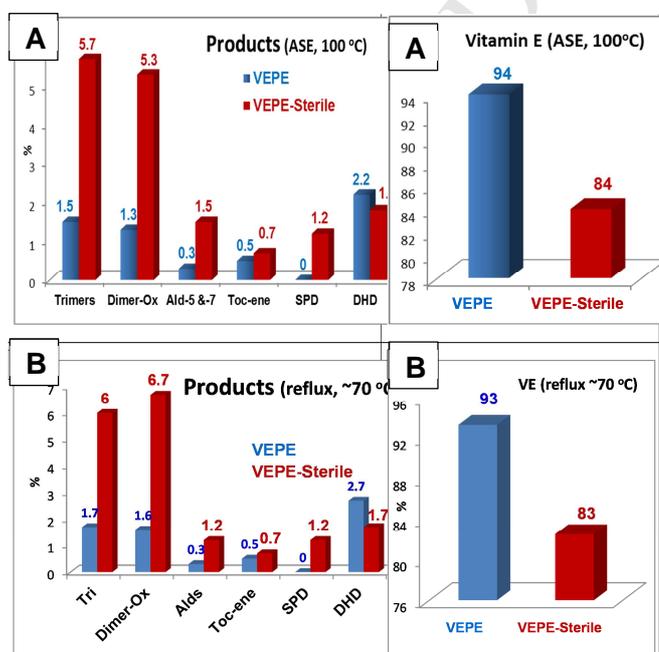
HPLC analysis of the liquid fresh untreated vitamin E sample showed it to be  $\sim 99.3\%$  pure and contained only about  $0.7\%$  of other  $\alpha$ -tocopherol isomers, whereas a vitamin E sample that had been  $\gamma$ -irradiated with a dose of  $300\text{ kGy}$  had  $\sim 98.7\%$  vitamin E in addition to low concentration of Toc-ene ( $0.5\%$ ) along with the formation of a small concentration of DHD (fresh vitamin E does not contain any DHD). Further, analysis of a vitamin E sample that was retrieved from the doping chamber of the manufacturing process, showed it to have an even lower amount of the vitamin E ( $\sim 97\%$ ) with some concentration of Toc-ene ( $0.6\%$ ) and a higher amount of DHD ( $2.3\%$ ), **Table 2**.

For the manufactured Tibia samples (Tibia-PEVE and Tibia-PEVE-Sterile), the amount of Vitamin E and that of the formed transformation products were analysed, using different extraction methods, quantified and compared. An overview of the outcome of the distribution of transformation products and their relative concentrations in each

type of the Tibia samples is summarised in **Figure 12** and **Table 2**. **Figure 12** shows a comparison of the amounts (averages of at least three repeats, from different parts of different batches of any one type of Tibia sample, normalised as percent of the total) of vitamin E and each of its main products after extraction by reflux with hexane and by ASE with cyclohexane/DCM at 100°C, while **Table 2** shows the concentrations as averages from ASE extractions at a range of temperatures of 85-122°C (a number of repeats per sample at each temperature, results are expressed both as percent of the total extract and by weight in part per million, ppm).

It is clear from **Figure 12** and **Table 2**, that based on the ASE at 100°C extraction method, the **Tibia-VEPE** samples (before sterilisation) contained an average amount of vitamin E of about 94% with the main products formed being the dihydroxydimers (DHD~ 2.2 %), an amount similar to that quantified in the retrieved vitamin E solution from the doping chamber, trimers (TRI~ 1.5 w%), the Dimer-Ox (~1.3 w%) along with small amounts of Toc-ene and Ald-5 (Ald-7 was not detected). On the other hand, the sterilisation process involved in the final stage of the **Tibia-VEPE-Sterile** production has given rise to a reduction in both the amount of vitamin E (down to ~84%) and that of the DHD (reduced by about 20% compared to the Tibia-VEPE samples) accompanied by an almost 4 fold increase in the concentrations of the TRIs, Dimer-Ox, aldehydes, and formation of a small concentration of the spirodimer (not present/ not detectible in Tibia-VEPE samples).

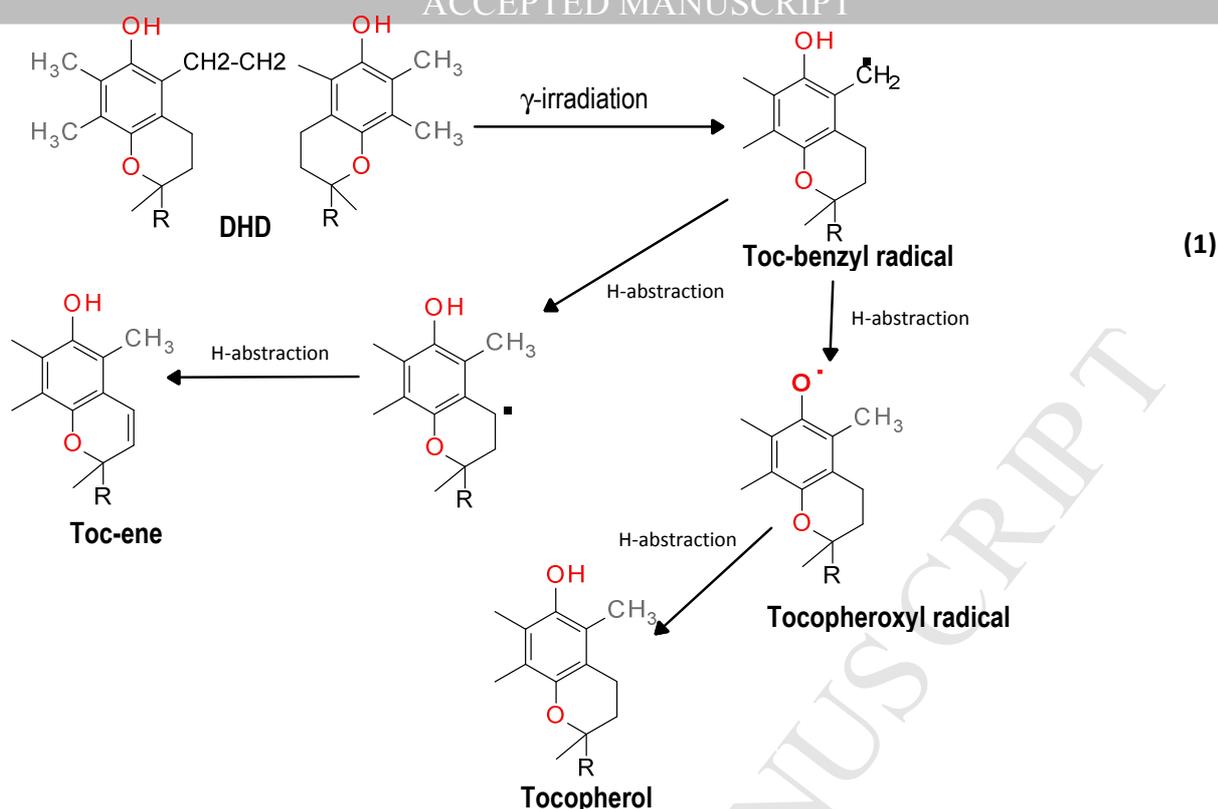
The consistently lower concentration of DHD (under all extraction conditions and repeat analysis) and the higher concentration of Toc-ene formed in the **Tibia-VEPE-Sterile** samples compared to **Tibia-VEPE**, see **Figure 12**, suggests that the second low dose of  $\gamma$ -irradiation contributes to breaking-down some of the DHD giving rise to the generation of Toc-ene and more tocopherol, see **Reaction 1**. However, the amount of tocopherol (VE) found in the Tibia-VEPE-Sterile samples was shown to be lower than that in the Tibia-VEPE, but this can be explained by the fact that the  $\gamma$ -irradiation conditions used for sterilisation would almost certainly result in further transformations from the tocopherol leading to its reduction and the observed, almost 4-fold, increase in the other products formation.



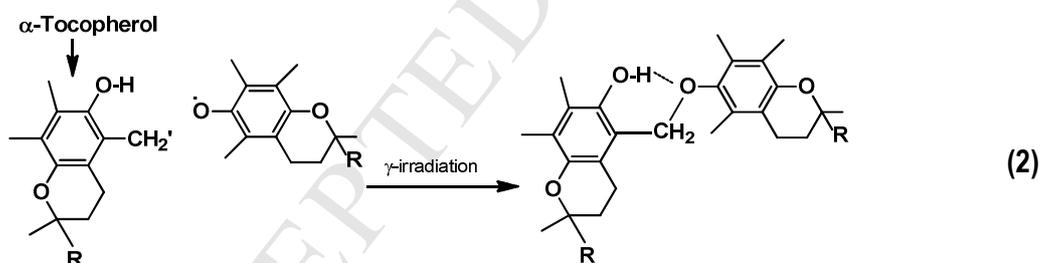
**Figure 12.** Average amount (normalised as % of total) of VE and its transformation products separated from **Tibia-VEPE** (blue) and **Tibia-VEPE-Sterile** (red) samples using ASE extraction at 100°C, **A**, (each result is an average of TWO samples tested) and extraction by reflux in hexane, **B**, (~70°C) for Tibia-VEPE, results are average of FOUR samples and for Tibia-VEPE-Sterile average of THREE samples)

**Table 2:** Quantification of Vitamin E and transformation products in extracts (ASE, cyclohexane/DCM, 100°C) of Tibia-VEPE and Tibia- VEPE-Sterile Samples, and in vitamin E solutions  $\gamma$ -irradiated, and a retrieved sample from the doping chamber of the manufacturing process. The results are the **total average** of 7-8 samples analysed taken from at least 3 or 4 extractions at temperatures ranging from 85 °C to 122 °C at different locations of the Tibia samples.

Compounds extracted from Tibia Samples	Tibia-VEPE (pre-sterilisation) Average (w%)	Tibia-VEPE (pre-sterilisation) Average (ppm)	Tibia-VEPE-Sterile (post-sterilisation) Average (w%)	Tibia-VEPE-Sterile (post-sterilisation) Average (ppm)
Vitamin E (VE)	94.2	3869	83.8	3130
Trimers	1.5	60	5.7	212
Ald-7	~0	2	0.2	7
Dimer-Ox	1.3	54	5.3	197
Ald-5	0.3	13	1.3	49
Toc-ene	0.5	21	0.7	26
SPD	0.0	0	1.2	44
DHD	2.2	89	1.8	70
TOTAL	100%	4108	100%	3735
	$\alpha$ -tocopherol %	DHD w%	Toc-ene w%	Unstable Dimers w%
Fresh VE	(99.3 + 0.7) = 100	0	0	0
VE-300 kGy	98.7	0.5	0.5	0.3
VE-doping	97.1	2.3	0.6	0



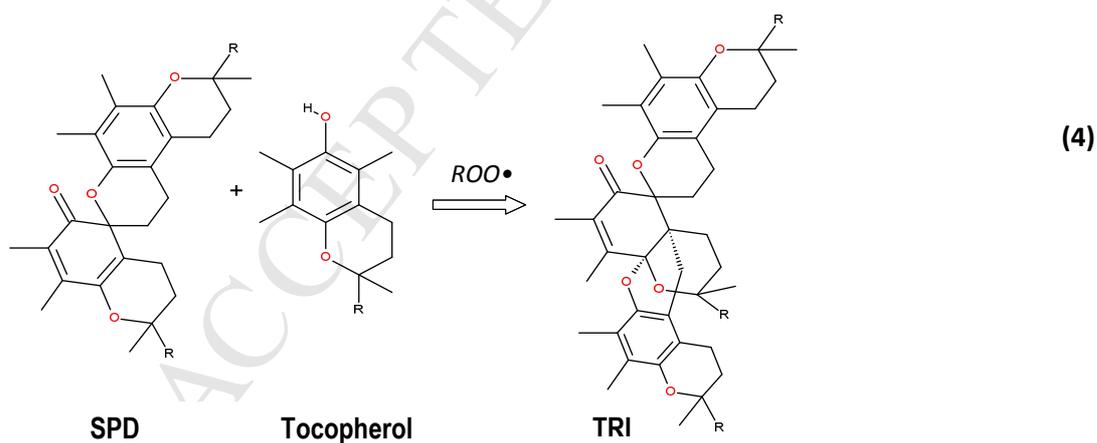
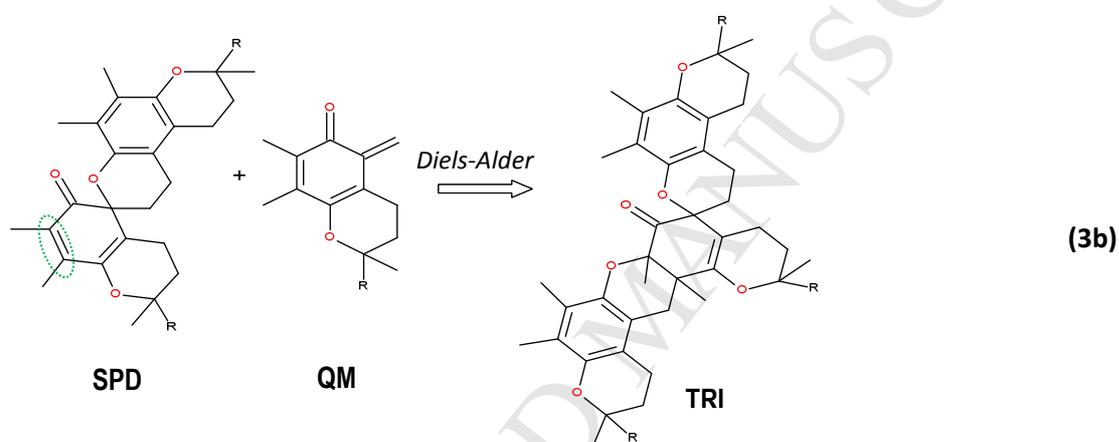
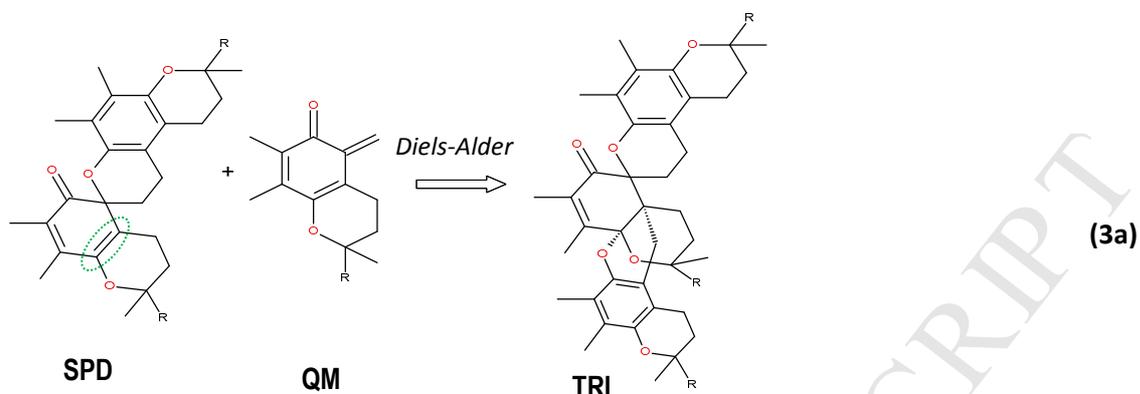
The much higher amount of Dimer-ox found in the **Tibia-VEPE-Sterile** samples is almost certainly formed directly from tocopherol via coupling of the tocopheroxy radical and tocobenzyl radical, **reaction 2**.

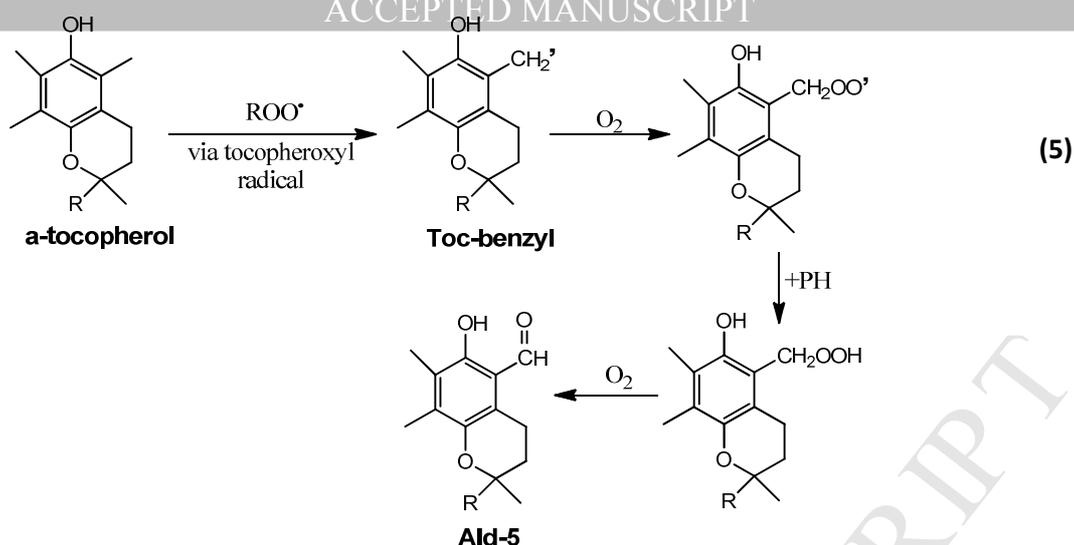


Further, the small amount of **SPD** formed in the **Tibia-VEPE-Sterile** samples (but not in Tibia-VEPE) accounts for the much higher concentration of the TRIs formed in the Tibia-VEPE-Sterile, since the main path to the formation of the TRIs is via the SPD, **reactions 3 and 4**, it is also known that the SPDs are generally less stable under more severe conditions [22, 25]. The fact that SPDs are not detected in **Tibia-VEPE** samples does not necessarily mean that they are not formed, almost certainly they are formed but transformed immediately to the TRIs and their small concentration (not detected) would be responsible for the formation of the observed much lower concentration of the TRIs in the Tibia-VEPE samples (compared to four times higher level in Tibia-VEPE-Sterile samples).

We have also shown in our earlier literature [22] that Toc-Alds are generally formed at higher amounts under more severe and oxidative conditions. In this case, it is the influence of the second dose of  $\gamma$ -irradiation, though

under a controlled environment with limited amount of oxygen (vacuum packed under Ar), would be responsible for the formation of the small concentrations of Ald-5, and even lower amount of Ald-7, observed in the **Tibia-VEPE-Sterile** samples. The aldehydes are formed as a consequence of oxidation of the Toc-benzyl radical followed by hydrogen abstractions, see **reaction 5** for **Ald-5** [22].





### 3.4. The Question of Graftability and Extraction of Vitamin E from Tibia Samples

Some earlier literature has claimed that small amount of vitamin E grafts on the UHMWPE during the manufacturing process of the Tibia, this conclusion was based on results of extraction of Tibia samples in hexane at 70°C [36]. To address the question of whether vitamin E does actually get chemically linked to the Tibia polymer during their manufacturing process, we have subjected Tibia samples to different extraction methodologies conducted at different temperatures followed by examination of their Oxidative Induction Times (OIT) as an indicative measure of the polymer stability due to any remaining unextractable (chemically linked) vitamin E.

**Table 3** shows that the OIT of the **virgin** (unstabilised) **UHMWPE** polymer (a Bar stock) is, as expected, very low (less than a minute), whereas the **Tibia-VEPE-Sterile** gave ~ 85 min. Extraction in hexane at 70°C and by ASE at 80°C reduced the OIT down to a quarter of its initial value (to ~20 min) suggesting that some of the stabilisers were not completely extracted under these conditions. Therefore, harsher conditions were used in order to check if vitamin E could possibly have become grafted during the manufacturing process. Extraction using ASE with cyclohexane (5%) in dichloromethane at 110°C gave rise to complete loss of the OIT in Tibia-VEPE-Sterile samples down to less than a minute which is the same as that for a completely unstabilised UHMWPE. These findings show clearly that vitamin E and its transformation products are fully extractable and the vitamin does not graft onto UHMWPE which is used for making orthopaedic implants by the vitamin E infused manufacturing technology.

**Table 3:** OIT for the Tibia films before and after extraction in ASE with hexane and with a solvent mixture (5% cyclohexane in DCM) at different temperatures (5 cycles 15min each at 2000 psi), each sample was re-extracted for a second time under the same conditions; standard deviation is shown in brackets

Sample/Extraction	OIT of Tibia-VEPE-Sterile, min.
virgin UHMWPE	0.5 ( $\pm 0.2$ )
Before extraction	87 ( $\pm 7$ )
Reflux in hexane (70°C)	22 ( $\pm 1$ )
ASE in hexane at 80°C	21 ( $\pm 1$ )
ASE in cyclohexane/DCM at 100°C	11 ( $\pm 1$ )
ASE in cyclohexane/DCM at 105°C	3 ( $\pm 1$ )
ASE in cyclohexane/DCM at 107°C	2 ( $\pm 1$ )
ASE in cyclohexane/DCM at 110°C	0.5 ( $\pm 0.5$ )

#### 4. Conclusion

Different vitamin E transformation products formed during the manufacturing process of crosslinked UHMWPE Tibia samples produced by the 'the vitamin E antioxidant infused technology' before (Tibia-VEPE) and after (Tibia-VEPE-Sterile) the sterilisation stages were separated, isolated, purified, identified and quantified. The crosslinked ( $\gamma$ -irradiation dose of  $\sim 100$  kGy) and vitamin E-doped polymer in the Tibia-VEPE samples were found to retain about 94% of the vitamin with the formation of mainly different stereoisomers of dihydroxydimers (DHD), trimers (TRI), dimer-Ox, and a small amount of Toc-ene and aldehyde-5. The final Tibia product, the Tibia-VEPE-Sterile samples (after sterilisation with about 25 kGy), on the other hand, was shown to have a lower amount of vitamin E of  $\sim 84\%$  and a reduced amount of the DHD, but this was accompanied by an almost a four-fold increase in the concentrations of the TRI, dimer-ox, aldehydes and a marginal increase in Toc-ene as well as the formation of spirodimer that was not detected in the Tibia-VEPE sample. The latter, is suggested to be, at least in part, responsible for the increased amount of the trimers formation, whereas the DHD reduction is also suggested to be responsible for the formation of more Toc-ene and regeneration of vitamin E which upon the second dose of  $\gamma$ -irradiation for sterilisation is further consumed to generate more transformation products, thus the higher overall concentration of products in the Tibia-VEPE-Sterile samples, the mechanism of the formation of these products was outlined. It was also shown categorically that vitamin E does not chemically graft to UHMWPE during the manufacturing process in contrast to some previous literature suggestions.

#### Acknowledgments

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**Caption of Figures**

- Figure 1:** HPLC chromatogram of Tibia (Tibia-VEPE-Sterile) extracts using analytical HPLC method
- Figure 2:** Spectra of **vitamin E**: (A)  $^1\text{H}$  and (B)  $^{13}\text{C}$  Pendant NMR; (C) HMBC NMR; (D) FTIR spectrum and (E) mass spectra, from LC-MS (from HPLC fraction A)
- Figure 3:** Spectra of **Toc-ene** obtained from Tibia extracts (HPLC fraction B): (A) Mass spectrum (from LC-MS); (B) UV spectrum (compared with that of vitamin E) obtained from Tibia extracts (HPLC fraction B); (C)  $^1\text{H}$  NMR spectrum
- Figure 4:** Spectra of **Dihydroxydimers** (HPLC fractions E and F) obtained from Tibia extracts: (A) MS (from LC-MS); (B)  $^1\text{H}$  NMR; (C)  $^{13}\text{C}$  pendant NMR spectra compared with that of vitamin E
- Figure 5:** Preparative HPLC chromatogram of the **trimeric products** isolated from Tibia extracts (A); their  $^1\text{H}$  NMR spectra, fractions G and H (B)
- Figure 6:** Spectra of **Trimeric products** isolated from Tibia extracts (HPLC fractions G and H): (A) their FTIR spectra and (B) their MS spectra
- Figure 7:** Spectra of **Ald-5** isolated from Tibia extract (HPLC fraction L): (A)  $^1\text{H}$  NMR; (B) HMBC-NMR; (C) mass spectrum; (D) FTIR spectrum (compared with that of vitamin E)
- Figure 8:** Spectra of **Tocopherol-7-aldehyde (Ald-7)** isolated from Tibia extracts (HPLC fraction I): (A)  $^1\text{H}$  NMR spectra (compared with that of Ald-5); (B) MS spectrum; (C) UV-Vis spectra
- Figure 9:** Spectra of **dimenr-Ox** (HPLC fraction K) extracted from Tibia: (A)  $^1\text{H}$  NMR; (B)  $^{13}\text{C}$  NMR; (C) 2D HMBC-NMR; (D) HSQC NMR (all NMR analysis was carried out immediately after dissolving the sample in the NMR solvent)
- Figure 10:** Vitamin E depth profiles (based on FTIR indices for peaks  $1263\text{ cm}^{-1}$  and  $1210\text{ cm}^{-1}$ ) for Tibia-VEPE and Tibia-VEPE-Sterile samples
- Figure 11:** FTIR spectra of vitamin E and transformation products showing the two peaks at  $1263\text{ cm}^{-1}$  and  $1210\text{ cm}^{-1}$  in the spectrum of vitamin E and the appearance of mainly the  $1263\text{ cm}^{-1}$  in the transformation products.
- Figure 12:** Average amount (normalised as % of total) of VE and its transformation products separated from **Tibia-VEPE (blue)** and **Tibia-VEPE-Sterile (red)** samples using ASE extraction at  $100^\circ\text{C}$ , **A**, (each result is an average of TWO samples tested) and extraction by reflux in hexane, **B**, ( $\sim 70^\circ\text{C}$ ) for Tibia-VEPE, results are average of FOUR samples and for Tibia-VEPE-Sterile average of THREE samples)

**Caption of Tables**

- Table 1:** Structures of vitamin E and its transformation products isolated from Tibia samples along with selected spectroscopic characteristics
- Table 2:** Quantification of Vitamin E and transformation products in extracts (ASE, cyclohexane/DCM, 100 °C) of Tibia-VEPE and Tibia- VEPE-Sterile Samples, and in vitamin E solutions  $\gamma$ -irradiated, and a retrieved sample from the doping chamber of the manufacturing process. The results are the **total average** of 7-8 samples analysed taken from at least 3 or 4 extractions at temperatures ranging from 85°C to 122°C at different locations of the Tibia samples.
- Table 3:** OIT for the Tibia films before and after extraction in ASE with hexane and with a solvent mixture (5% cyclohexane in DCM) at different temperatures (5 cycles 15min each at 2000 psi), each sample was re-extracted for a second time under the same conditions; standard deviation is shown in brackets.