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DETERMINATION OF VITAMIN E ISOMERS IN PLASMA USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

(Penentuan Isomer-Isomer Vitamin E di dalam Plasma Menggunakan Kromatografi Cecair Berprestasi Lampau)

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Abstract

A rapid method for quantification of vitamin E isomers (α-, β-, γ-, δ- tocopherol and tocotrienols) was developed by using ultra performance liquid chromatography (UPLC) from human plasma. This method involved liquid-liquid extraction of plasma sample to extract the lipophilic portion for injection into UPLC system. The UPLC system was equipped with fluorescent detector (296 nm excitation, 330 nm emissions) and C18 column with 1.7 µm particle size (2.1 x 110 mm column) for particle separation. The mobile phase comprises of 1.0% of H_2O and 99.0% of methanol (HPLC grade) with flow rate adjusted at 0.4 ml min^{-1}. Peak separations were accomplished after 3.50 minutes and 5 µl treated samples were required for injection. Results obtained showed that the UPLC system was able to separate, detect and quantitate six peaks, namely α-, γ-, δ- tocopherol and tocotrienols, respectively. However, it failed to separate and quantificate both, β- tocopherol and tocotrienol due to low amount in nature. The calibration curve was linear for a concentration ranging from 1 µg ml^{-1} – 10 µg ml^{-1} of total vitamin E standard which $r^2 > 0.9996$ for all isomers. The within day and between day coefficients of variation were less than 10.29% (n=4) and 10.73% (n=4) for all isomers. The accuracy of isomers quantification for 1 µg ml^{-1} (n=4) and 10 µg/ml (n=4) vitamin E stocks were 93.52% - 98.13 %. The within day and between day coefficients of variation for vitamin E standard were less than 7.59% (n=4) and 9.74% (n=4) for all isomers while for plasma sample were less than 10.29% (n=4) and 10.73% (n=4) for all isomers. The recovery rates for all isomer were ranging from 98.96 ± 7.22 and 107.50 ±1.19 and the limit of detection was 2.53 ng. The present data suggested that this method required extremely short analysis time i.e. 2.50 min, required less samples for injection and excellent chromatographic reproducibility.

Keywords: UPLC, Tocotrienols, Tocopherol, Plasma, Vitamin E

Abstrak

Satu kaedah yang pantas bagi menentukan kandungan isomer vitamin E (α-, ß-, γ-, δ- tokoferol dan tokotrienol) telah dihasilkan dengan menggunakan Kromatografi Cecair Berprestasi Lampau (UPLC) daripada sampel plasma manusia. Kaedah ini mengunakan teknik pengasingan pada fasa cecair bagi mengekstrak bahagian lipofilik yang terdapat di dalam sampel plasma untuk disuntik ke dalam sistem UPLC. Sistem UPLC dilengkapi dengan pengesan floresen (296 nm eksitasi, 330 nm emisi) dan kolum C18 yang mempunyai saiz partikel 1.7 µm (2.1 x 110 mm kolum) bagi pengasingan kompaun. Fasa gerak terdiri dari 1.0% H_2O dan 99.0% methanol (gred HPLC) dengan kadar aliran 0.4 ml min-1. Pengasingan isomer berjaya dilakukan dalam tempoh 3.50 minit dan hanya sebanyak 5 µl sampel diperlukan untuk disuntik ke dalam sistem. Hasil daripada keputusan yang diperolehi sebanyak enam isomer iaitu (α-,γ-, δ- tokoferol dan tokotrienol yang mampu diasingkan, dikesan dan ditentukan kandungannya oleh sistem UPLC ini. Walaubagaimanapun, ia gagal dalam pengasingan dan penentuan kandungan ß- tokoferol and tokotrienol mungkin disebabkan oleh jumlahnya yang terlalu rendah. Keluk kalibrasi adalah garis lurus, dengan menggunakan kepekatan piawaian vitamin E di antara 1 µg ml-1 – 10 µg ml-1 bagi kesemua isomer dengan nilai $r^2 > 0.9996$. Pekali variasi bagi dalam tempoh sehari dan diantara hari adalah kurang dari 10.29% (n=4) dan 10.73% (n=4) bagi kesemua isomer. Ketepatan kuantifikasi isomer bagi 1 µg ml-1 (n=4) and 10 µg/ml (n=4) stok vitamin E adalah sebanyak 93.52% -

98.13%. Pekali variasi bagi dalam tempoh sehari dan diantara hari untuk piawaian vitamin E adalah kurang dari 7.59% (n=4) dan 9.74% (n=4) bagi kesemua isomer manakala sampel plasma pula adalah kurang dari 10.29% (n=4) dan 10.73% (n=4) bagi kesemua isomer. Kadar pemulihan untuk semua isomer adalah di antara 98.96 ± 7.22 dan 107.50 ±1.19 dan tahap pengesan yang dihadkan sebanyak 2.53 ng. Hasil data yang diperolehi menunjukkan bahawa kaedah ini hanya memerlukan masa analisis yang singkat iaitu selama 2.50 min sahaja, serta menggunakan jumlah sampel yang sedikit untuk suntikan dan berkeupayaan menghasilkan kromatogafi yang baik.

Kata kunci: UPLC, Tokotrienol, Tokoferol, Plasma, Vitamin E

Introduction

Vitamin E is an important source of antioxidant from food and consists of eight naturally occurring molecules [four tocopherols (Toc) and four tocotrienols (TCT)]. Molecular structure of vitamin E composed of a polar chromanol ring linked to an isopropanoid chain. The structure of TCT is different from Toc where TCT possess three double bond in its isopropanoid side chain, while Toc has a saturated hydrocarbon phytyl tail. The isomer of both Toc and TCT (α-, β-, γ- and δ-) are different with regards to the number and position of methyl groups on chromanol ring. Tocopherol and TCT are known as lipid soluble compound, being well represented in vegetable, fruits, seeds, dairy foods, nuts and oils. Biological activities of Toc and TCT are generally due to their antioxidant action, i.e. inhibiting lipid peroxidation in biological membranes by breaking free radical driven chain reactions. Tocopherols and TCT were found to possess a cholesterol-lowering property [1, 2] by down-regulating 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, which is the rate limiting enzyme in the mevalonate pathway that contributes to the synthesis of cholesterol [3, 4]. Tocopherols and TCT have been identified to show other effects such as anticancer, anticholesterolemic, antihypertensive, antioxidant, immunomodulatory and neuroprotective [5].

Several chromatographic methods were available in the literature for the determination of Toc and TCT isomers using both reversed and normal phase columns [6]. Ultra performance liquid chromatography (UPLC) is a tremendous method for quantification and characterization of vitamin E. The Acquity UPLC system consist of high-pressure fluidic modules (binary pump), efficient auto samplers characterized by fast injection cycles, low injection volumes, temperature control and high-speed detectors. Thus, the system gives UPLC a rapid, sensitive and high-resolution for separation of vitamin E. The UPLC system is equipped with special column contain bridged ethylsiloxane – silica hybrid (BEH) adsorbent, as 1.7 µm particles, which ensure a wide pH operating range [7]. The particles were created with a special design which is capable to resist high back pressure (< 15000 psi) as compared to conventional column for high performance liquid chromatography (HPLC).

Hence, the main objective for this study was to develop and validate an UPLC method for quantification of all vitamin E isomers in human plasma.

Experimental

Chemical

Methanol (grade for liquid chromatography), *n*-hexane and absolute ethanol were purchased from Merck Sdn Bhd. Ascorbic acid were obtained from Sigma-Aldrich. Complete natural vitamin E (E-8; α-, β-, γ-, δ- Toc and TCT) were purchased from Hovid which consist of 181.82 mg, 4.30 mg, 91.39 mg, 32.26 mg, 5.56 mg, 0.56 mg, 11.11 mg and 2.78 mg of α-, β-, γ-, δ- Toc and TCT, respectively.

Standard Solution Preparation

Vitamin E standard solution was prepared from vitamin E (E-8; Hovid) where 1 mg of the stock was accurately weighted and dissolved in methanol as a stock solution. Standard curve consist of a blank, together with a series of stock solution diluted with different concentrations i.e. 1 µg ml^{-1}, 2 µg ml^{-1}, 4 µg ml^{-1}, 6 µg ml^{-1}, 8 µg ml^{-1} and 10 µg ml^{-1}). The standard solution were prepared daily before being analyzed using UPLC.

Sample Preparation

Blood samples were collected from healthy volunteers (man and women) and transferred into EDTA tube. The samples were centrifuged at 3000 rpm for 15 minutes. Plasma was pooled and transferred into polypropylene tube, stored at -20°C until analysis [8]. Sample analysis was done by transferring 200 µl of plasma into new polypropylene tubes containing 50 µl of 10% ascorbic acid (1 mg in 10 ml of absolute ethanol) and vortexed for

10s. The ascorbic acid was added to minimize the oxidation of target analytes. One ml of absolute ethanol was added to precipitate protein, followed by vortexing for 10 s and centrifuged at 3000 rpm for 15 min at 4°C. After that, 3 ml of *n*-hexane was added (for extraction of vitamins) and mixed using Shaker Orbit P4 at 500 rpm for 10 min. Then, the samples were centrifuged again at 3000 rpm for 15 min at 4°C. Supernatants (2.5 ml) were transferred into polypropylene tubes and evaporated using Vacum Concentrator (Thermo ISS110-230). Then, the residues were dissolved with 50 µl of methanol, filtered through 0.45 µm filter and diluted 30 times before 5 µl of samples were injected onto the UPLC system.

Validation of Method

The method was validated by measuring the accuracy, precision, recovery and limits of detection. Accuracy of this method was determined by measuring the ability of the system to quantitate the known concentration of vitamin E standard at lower and higher concentrations. The precision of method was evaluated by determination of the value of intra-assay (within run) and inter-assay (between run) of vitamin E in standard solution and plasma sample. The reproducibility of extraction method was measured by determining the recovery of spike from plasma sample. The limit of detection or sensitivity of the florescent detector were determined by preparing vitamin E stock of different concentration over the range 0.1 µg ml^{-1} – 0.9 µg ml^{-1} and the amplitude of the peak were measured.

Chromatography Condition

The chromatographic system consists of a Water Acquity ultra performance liquid chromatography (Water, Massachusetts, U.S.A) equipped with a fluorescent detector at the wavelength of 296 nm and 330 nm for excitation and emission. Particle separation were done using reversed phase C_{18} column (2.1 x 110 mm, 1.7 µm) and the temperature of column was set at 30°C. The mobile phase was eluting isocratically with 1.0% of H_2O and 99.0% of methanol with flow rate adjusted at 0.4 ml min^{-1}. Peak separations were accomplished after 3.50 minutes and 5 µl treated samples were required for injection. The data was collected and processed using Empower chromatographic software (Water, Massachusetts, U.S.A). All preparations were conducted in low light environment in order to minimize the photodegradation of the solution.

Results and Discussion

Peak Separation

Figure 1 presented a good six peaks separation of Toc and TCT from E-8 standard in reversed-phase UPLC on a C_{18} column and peak separations were accomplished after 3.50 minutes. The order of elution for chromatogram is α-TCT, γ-TCT, δ-TCT, δ-Toc, γ-Toc and α-Toc. The result was comparable to that reported by Xu [9]. Retention times were 1.19, 1.32, 1.44, 1.71, 1.94 and 2.12 min for α-TCT, γ-TCT, δ-TCT, δ-Toc, γ-Toc, and α-Toc, respectively. However, the system failed to detect β-TCT and β-Toc.

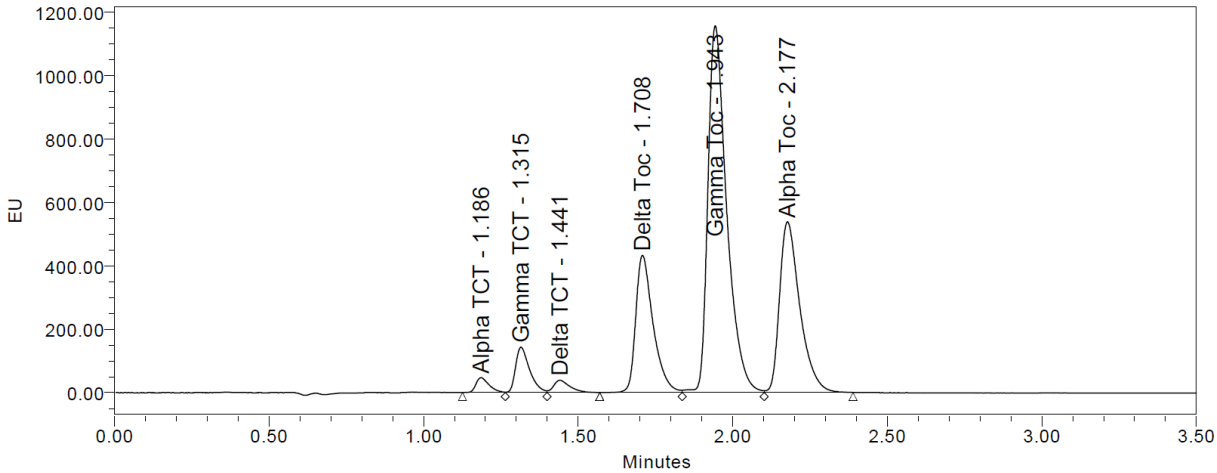


Figure 1: Chromatogram of a standard vitamin E (E-8, Hovid) retention time: α-TCT, 1.19 min; γ TCT, 1.32 min; δ-TCT, 1.44 min; δ-Toc, 1.71 min; γ-Toc, 1.94 min; and α-Toc, 2.18 min

Calibration Curve

Figure 2 showed the overlay chromatograms consisting of blank and seven different concentrations of standard solution and the retention time for each isomer appear within 5%. The calibration curve was analyzed using Empower chromatographic software and showed in Table 1. The mean linearity of the calibration curve for each isomer was 0.9996 ± 0.00019. The r^2 value (0.9996) showed a good linearity of the analytical method.

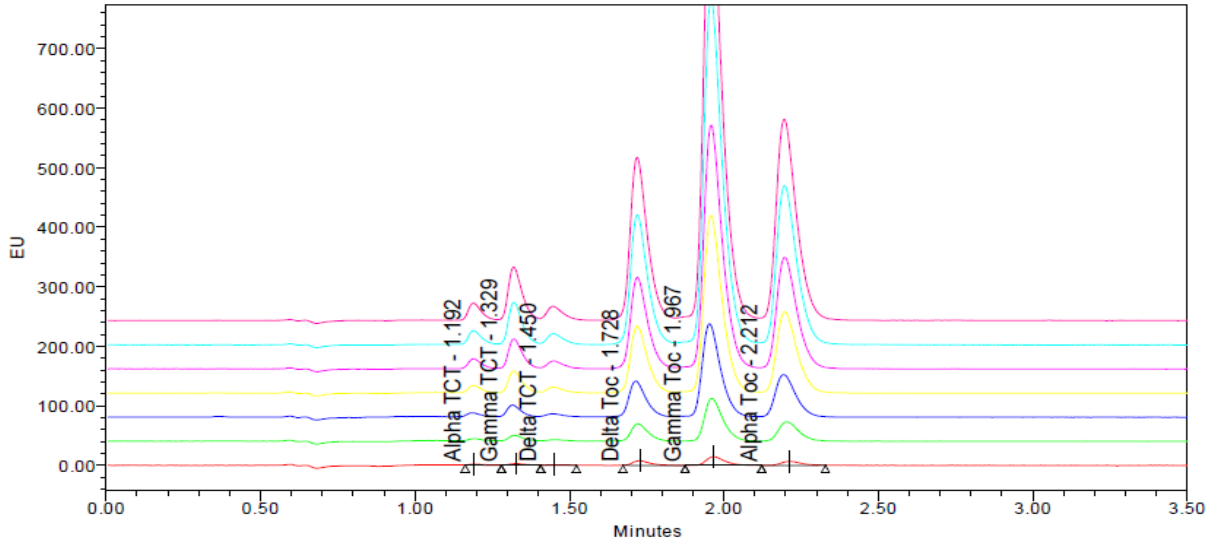
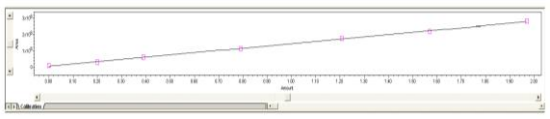
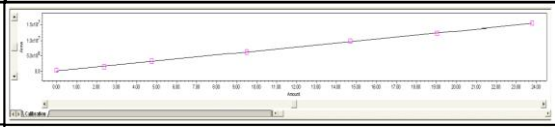
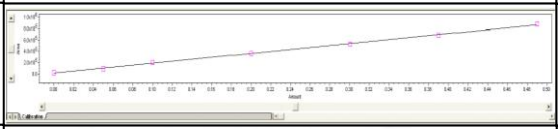
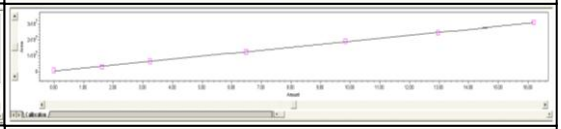
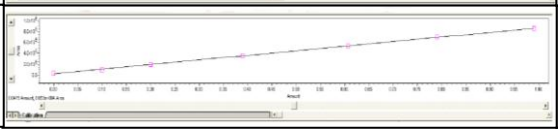
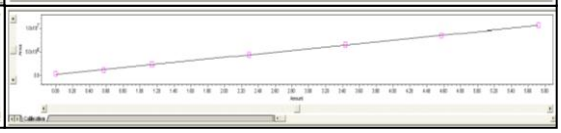


Figure 2: Overlay chromatogram of different concentrations of vitamin E isomers

Table 1: Calibration curve of standard solution: α-TCT; 0.9995, γ-TCT; 0.9997, δ-TCT; 0.9993, δ-Toc; 0.9997, γ-Toc; 0.9997, α-Toc; 0.9997

	Tocotrienol	Tocopherol
α		
γ		
δ		

Determination of Vitamin E Isomers in Human Plasma

Figure 3 showed the chromatogram of vitamin E isomers from pooled human plasma. This system was able to detect 6 isomers, except β-TCT and β-Toc, where it is similar to results found in the determination of the isomers in vitamin E standard. The mean values of each isomer were shown in Table 2.

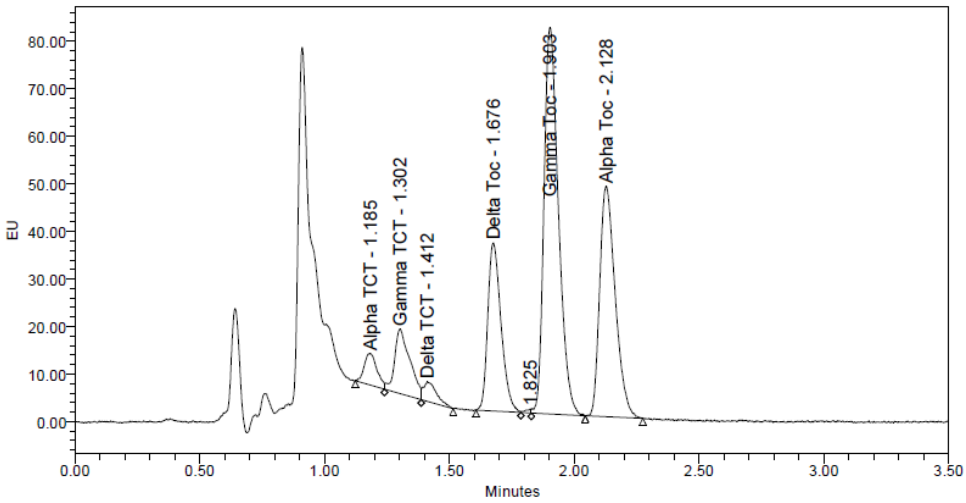


Figure 3: Separation of a vitamin E isomers (α-, γ-, δ- Toc and TCT) in human plasma on reversed phase column (C18 column with 1.7 µm particle)

Table 2: The concentration of isomers vitamin E in pooled plasma

Isomers	Mean ± SD			n
α-TCT	0.122	±	0.008	4
γ- TCT	0.250	±	0.018	4
δ-TCT	0.051	±	0.004	4
δ-Toc	0.338	±	0.025	4
γ-Toc	1.015	±	0.079	4
α-Toc	3.317	±	0.232	4

Validation of Method

Table 3 showed the accuracy of this method in order to measure standard solution at 1 ug ml^{-1} (lower concentration) and 10 ug ml^{-1} (higher concentration). The accuracy to detect isomers at 1 ug ml^{-1} was between 93.52 ± 7.96 and 98.13 ± 4.36 while 94.78 ± 0.77 and 96.60 ± 0.53 for 10 ug ml^{-1}. Meanwhile, for precision of the method, the coefficient variation for intra-assay and inter-assay of the isomers for standard solution were less than 7.59% (n=4) and 9.74% (n=4) for all isomers (Table 4). The coefficient value in plasma was below 10.29% and 10.54% (Table 5). The recovery for each isomer in spike pooled plasma was shown in Table 6 where it is ranging from 98.96 ± 7.22% and 107.50 ±1.19%. The limit of detections showed that all detected TCT and Toc isomers can be measured at 0.1 µg ml^{-1} except for the δ-TCT, where it can only be detected at 0.3 µg ml^{-1}. The lowest detection of UPLC was calculated by times with the percentage of δ-TCT in the solution, thus showed that the limit of detection in this study was 2.52 ng.

Table 3: Accuracy of 1 ug ml^{-1} and 10 ug ml^{-1} of isomers vitamin E determination in standard solution

Isomers	1 µg/ml				10 µg/ml			
	Mean ± SD			n	Mean ± SD			N
α-TCT	93.52	±	7.96	4	96.11	±	0.87	4
γ- TCT	95.59	±	4.90	4	95.39	±	1.14	4
δ-TCT	96.05	±	1.09	4	95.24	±	1.81	4
δ-Toc	98.06	±	4.39	4	96.60	±	0.53	4
γ-Toc	98.13	±	4.36	4	95.92	±	1.28	4
α-Toc	95.99	±	3.96	4	94.78	±	0.77	4

Table 4: Coefficient variation of intra-assay (within run) and inter-assay (between run) of isomers vitamin E determination in standard solution

Isomers	Intra-assay					Inter-assay				
	Mean ± SD			n	C.V	Mean ± SD			N	C.V
α-TCT	1.31	±	0.07	4	5.14	1.33	±	0.12	4	8.83
γ- TCT	2.93	±	0.22	4	7.50	3.17	±	0.19	4	6.03
δ-TCT	0.73	±	0.05	4	7.26	0.72	±	0.77	4	9.74
δ-Toc	10.00	±	0.74	4	7.43	10.00	±	0.62	4	6.24
γ-Toc	19.78	±	1.23	4	6.22	20.85	±	1.26	4	6.06
α-Toc	25.55	±	1.94	4	7.59	28.15	±	2.40	4	8.54

Table 5: Coefficient variation of intra-assay (within run) and inter-assay (between run) of isomers vitamin E determination in plasma sample

Isomers	Intra-assay					Inter-assay				
	Mean ± SD			n	C.V	Mean ± SD			N	C.V
α-TCT	0.23	±	0.01	4	5.74	0.22	±	0.02	4	8.14
γ- TCT	0.30	±	0.03	4	10.29	0.29	±	0.02	4	5.54
δ-TCT	0.08	±	0.00	4	5.43	0.06	±	0.01	4	10.54
δ-Toc	0.53	±	0.04	4	7.62	0.54	±	0.05	4	9.55
γ-Toc	1.20	±	0.11	4	9.38	1.12	±	0.10	4	8.99
α-Toc	4.04	±	0.25	4	6.26	3.80	±	0.33	4	8.80

Table 6: Mean recovery of all isomers vitamin E in human plasma

Sample	Recovery (% mean ± SD)	
Isomer	**TCT**	**Toc**
α	107.50 ±1.19	100.79 ± 3.31
γ	99.21 ± 5.04	98.96 ± 7.22
δ	103.00 ± 7.02	101.63 ± 6.79

Ultra performance liquid chromatography (UPLC) system that was used in this study for sample separation using column packed with 1.7 µm provide a superior resolution, increase in speed of analysis, improve in sensitivity of detection and resist to the higher back pressure (<15000 psi). The UPLC also uses very low mobile-phase flow rates

(0.4 ml min^{-1}), which is more economically important in processing large samples. Methanol was used in mobile phase and all the stationary phases because it was found to improve the retention and separation of the compounds [10].

The β-TCT and β-Toc were not able to be detected in this study using E-8 as a standard, since a reversed-phased column that was used cannot separate β-TCT with γ-Toc [11]. Other studies also speculated that the two isomers were commonly reported together as the sum of β- and γ- Toc [12, 13]. In addition, the separation process using reversed-phase column is difficult to separate β-, γ- and δ- Toc due to the position of the methyl group on the aromatic ring [8]. Another probability on failure to separate and quantitate β-TCT and β-Toc is due to its very low amount in vitamin E.

In addition, in normal-phase HPLC on a silica column, seven peaks of Toc and TCT was eluted and there are α-TCT, β- TCT, γ-TCT, δ-Toc, α-Toc, γ-Toc and δ-Toc [9]. The normal-phase diol column gave reproducible and consistent separations of all the Toc and TCT (α-, β-, γ-, δ- Toc and TCT) as compared to reversed-phase column which is lack of separation [14]. Tocotrienol was eluted first compared to the Toc because of the chemical structure, i.e. the tocols with unsaturated side chain have shorter retention time than those with saturated side chain. In addition, the substituents on the chromanol ring also affect the retention times of tocotrienol and tocopherol, where this effect only seen in reversed-phase column rather than normal-phase column [9].

The precision of the assay was evaluated by determining the intra- and inter-assay coefficient of variation (CV) of the vitamin E concentration in the standard solution and the plasma sample. The standard solution and the sample plasma were analyzed four times daily (intra-assay) for five days (inter-assay). Results from the intra-assay and inter-assay analysis shown, that this method are precise and reliable, except for the inter-assay analysis of δ-TCT. This result might be due to very low concentration of δ-TCT in plasma sample. The results obtained from these analytical analyses were presented in Table 4 and 5. The result was considered acceptable with the CV values obtained were not exceeding 10%.

The accuracy study was done by preparing two different concentrations of standard solution (l μg ml^{-1} and 10 μg ml^{-1}) and was evaluated for 4 samples which were prepared individually. The accuracy was calculated as mean and associated standard deviation which was presented in Table 3. The mean accuracy obtained, expressed as percentage, ranges between 93.52 - 98.13 % for all isomers.

The results of recovery were presented in Table 6, based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out four times. The mean recoveries were in the range between 99 – 108% with a trend of full recovery and of good reproducibility. This range is within the acceptable recovery limits (90 -110%).

The limit detection of this analysis was assessed for all the vitamin E isomers in the range of concentration of 0.1 – 0.9 μg ml^{-1}. The results indicated that the detector has sufficient sensitivity for the method where it provides a good margin for the identification at the lowest concentration of vitamin E.

Conclusion

In summary, UPLC has been proved to be an effective technique for analyzing Toc and TCT in plasma samples. The analysis required shorter time to complete (2.50 min), which is two to three time faster as compared to currently reported HPLC methods [13,15]. This system which used 1.7-μm particles provides significantly more resolution while reducing run times and improves sensitivity for the analyses of plasma sample.

The condition of chromatography must be selected to allow complete baseline separation and able to quantitate all the vitamin E isomers. This method was validated by linearity, precision, accuracy, recovery and limits of detection and quantification. This method is low in cost where the liquid-liquid extraction sample preparation allows fast quantitation of the vitamin E constituents from various samples.

In conclusion, analytical performance of the method used in this study is satisfactory.

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