A Validated Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) Method for the Quantification of Gamma- Tocotrienol in Tocotrienol Rich Fractions of Crude Palm Oil

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Abstract

Background

Palm oil and its constituents have wide applications in food, cosmetics and pharmaceutical industries. The tocotrienol-rich fractions of crude palm oil have drawn greater research interest in recent years due to their potent health benefits. Therefore, reliable and validated analytical methods are essential for the quantification of tocotrienols.

Objective

This study aimed to develop a simple and economical RP-HPLC method for the quantification of gamma-tocotrienol in tocotrienol-rich fractions of crude palm oil.

Method

An Agilent HPLC system supplied with a Diode Array detector and an auto-injector system was used for the method development, and the wavelength was set 295 nm. A reversed-phase C18 column maintained at 30 °C using a mobile phase composition of methanol: water (95:05) at a flow rate of 1 mL/min was used for the analysis. The developed method was validated according to ICH guidelines.

Results and discussion

A symmetrical peak of gamma-tocotrienol was observed at 8.7 minutes with minimal peak tailing (between 0.76 and 0.78), and an acceptable resolution above 2.0. Excellent linearity was evident with R^2 values 0.9996 and 0.9991 for intra-day and inter-day respectively. The method demonstrated a high precision (%RSD values $\leq$ 5.8%) and accuracy (%RE<9.6%). The LOD and LOQ of gamma-tocotrienol were determined as 1.4 µg/mL and 4.2 µg/mL respectively. The system suitability studies indicate that the chromatographic parameters are well within the acceptable limit.

Conclusion

In conclusion, the developed RP-HPLC method is rapid, precise, stable and economical for the quantification of gamma-tocotrienol.

Keywords: TRF, gamma-tocotrienol, RP-HPLC, validation, ICH, system suitability.

INTRODUCTION

Vitamin E present in palm oil comprises 30% tocopherols and 70% tocotrienols [1]. Tocopherols and tocotrienols have structural similarities, and both contain a chromanol head group and a side chain attached at the second carbon position. It is the side chain which distinguishes both groups of compounds. The tocopherols possess a characteristic saturated phytyl tail whilst the tocotrienols (T3) have an unsaturated isoprenoid side chain. The tocotrienols are not commonly found in vegetable oils in large quantities, with the main exceptions being rice bran and corn oil. Crude palm oil (CPO) is rich in tocols than refined palm oil (RPO) because there are unintentional losses during the refining process. The amount of tocols in CPO range between 600-1000 ppm and in RPO this value falls to between 350 and 450 ppm [2]. Tocopherols and tocotrienols exist in four different forms, namely alpha (α), beta (β), gamma (γ) and delta (δ). The tocotrienol-rich fractions (TRF) of CPO contains >80% tocotrienols with gamma isomer (gamma-T3) (Figure 1) being the predominant compound. The abundance of alpha-tocopherol found in the human body may be the reason why scientists have tended to neglect the non-tocopherol vitamin E molecules as topics for basic research. Recent developments in tocotrienol research necessitate a serious reconsideration of this conventional perception that tocopherol is largely responsible for health benefits [3-4]. Currently, alpha-T3, gamma-T3, and delta-T3 have emerged as vitamin E molecules which appear to possess distinct roles in health and the prevention of disease [3]. It is thought that the polyunsaturated side chain of tocotrienols allows more efficient penetration through the cell membrane and therefore higher tissue concentrations can be attained as compared to the tocopherols [5]. Currently, many nanoformulations incorporating tocotrienols are in the development phase [6]. Hence reliable analytical methods are necessary for accurate drug quantification.

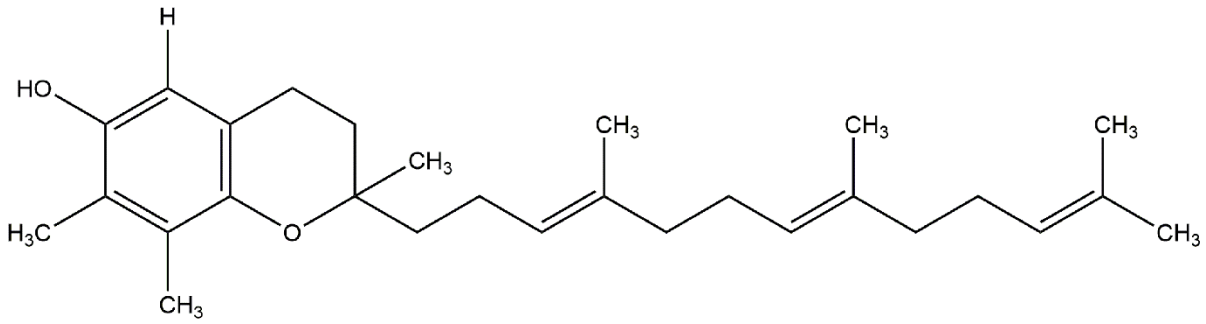

Figure 1. Chemical structure of gamma tocotrienol.

Although HPLC methods for the analysis of a majority of currently used drugs have been reported, it often remains necessary to be simple to use, and optimise these methods for precise quantification. Variations in

the mobile phase, column type and dimensions, and the HPLC system itself can all affect peak quality and retention time. Method validation ensures that the results obtained using the optimised methods are reproducible and reliable. Although many different techniques have been used to quantify tocotrienols, HPLC is the widely preferred method of choice given its reproducibility and convenience. Reports suggest that most HPLC methods for tocotrienol analysis were developed using normal phase column [7-8] and have not been validated adequately according to the recommended guidelines. Most of these methods give priority to linearity, detection or quantification limits; however, other important parameters such as system suitability, robustness, and stress degradation studies were not given sufficient importance. These parameters are of prime importance in the accurate quantification of drugs. In the TRF chromatogram, many adjacent peaks indicate tocopherol and tocotrienol isomers, only symmetrical and well-resolved peaks can facilitate a precise analysis. Therefore, in this study, we aimed to develop a reliable and economical RP-HPLC method for gamma-T3 analysis and validated the method according to the recommended guidelines [9].

2. MATERIALS AND METHODS

2.1. Materials

TRF and Gamma-T3 standards were obtained from ExcelVite Sdn Bhd, Perak, Malaysia. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific, UK. Hydrochloric acid and sodium hydroxide were purchased from R & M chemicals, UK, and the deionised water was collected from laboratory water purification system, ELGA Pure lab, USA.

2.2. Methods

2.2.1. Instrumentation and chromatographic conditions

Agilent HPLC system supplied with a Diode Array detector and an auto-injector system was used for the method development and validation. The Hypersil Gold C18, ODS (5 µm) column (250 mm × 4.6 mm) was maintained at 30 °C throughout the analysis. The method validation includes linearity, precision and accuracy, lower detection and quantification limit, robustness, system suitability and stability.

2.2.2. Linearity and range

To establish linearity, ICH 2005 recommends that a minimum of 5 different concentrations be evaluated. The stock solution of TRF and the gamma-T3 standard was made by dissolving in ethanol and DMSO (1mg/mL).

From the stock solution, a set of five different concentrations of TRF, ranging between 6.25 and 100 µg/mL were prepared by diluting with methanol. From each of the five concentrations, 30 µL sample was then injected into the system, and corresponding peak areas were noted. Calibration curves were generated using Microsoft Excel 2016 by plotting the area under the curve (AUC) determined using the Chemstation software against the sample concentration. Linearity was assessed by examining the regression coefficient (R^2) values obtained on intra-day and inter-day (n=5).

2.2.3. Precision and Accuracy

Precision expresses the closeness of measurements obtained by repeated sampling of a homogeneous solution under defined experimental conditions. The precision was determined for intra-day and inter-day on low, medium, and high concentration samples of TRF in the calibration range (n = 5), reported as relative standard deviation (% RSD).

Accuracy of a method is determined from the closeness of the measurements with the true value and is often also referred to as trueness. Intra-day and inter-day accuracy was assessed by analysing the TRF samples of high, medium and low concentrations (n = 5). The percentage relative error (% RE) was calculated by comparing the measured concentration with that of the real concentration [9].

$$\% \text{ RE} = \frac{\text{Measured concentration - Actual concentration}}{\text{Actual concentration}}$$

Equation (1)

2.2.4. Limit of detection (LOD) and limit of quantification (LOQ).

ICH guidelines outline mainly three approaches for the determination of LOD and LOQ. Those approaches are either based on the signal to noise ratio, visual inspection or using the calibration curves [9]. In our experiment, data obtained from calibration curves were used for the LOD and LOD determinations. Firstly, from the calibration curves constructed from 5 different concentrations of TRF, the slope and intercept values were measured (n=5), and the LOD and LOQ were calculated using the following equations (Eqs. 2 and 3):

$$\text{LOD} = \frac{3.3\ \sigma}{S}$$

Equation (2)

$$\text{LOQ} = \frac{10\ \sigma}{S}$$

Equation (3)

Where, ‘S’ is the slope of the calibration curves, and ‘σ’ the standard deviation of the y-intercept values obtained from the regression lines.

2.2.5. System suitability studies

System suitability evaluation is an integral part of the validation of any analytical method, and it helps ensure the accuracy of the analysis. Major parameters included the capacity and tailing factor, resolution and theoretical plate number [10].

2.2.6. Robustness

The robustness of this method was assessed by applying slight deliberate changes in the chromatographic conditions such as flow rate (± 0.1 mL), mobile phase composition (± 2.0%), column temperature (± 2.0 °C), and wavelength (± 5.0 nm).

2.2.7. Stress degradation studies

These studies can help identify the degradation products of compounds, if any, and help determine compounds' stability under different experimental conditions [11].

2.2.7.1 Acid and alkali degradation studies

The degradation studies were carried out by exposing 1 mL of TRF stock solution to the same volume of 0.1 M HCl or 0.1 M NaOH in volumetric flasks for 30 minutes followed by immediate neutralisation with the same molar concentration either NaOH or HCl [12]. The volume was made up to 10 mL with methanol and diluted suitably to obtain concentrations in the calibration range. Samples were then quantified using the same method and system, and the values obtained were compared with the reference concentration (n=4).

2.2.7.2 Heat and UV degradation

For heat stability, TRF stock solution (10 mL) was held at a temperature of 70°C in a water bath for 30 minutes. Another clear glass sampling vial containing the same volume of stock solution (10 mL) was also exposed to UV light (253 nm) in a biological safety cabinet for 30 minutes. After the heat and UV exposure, samples (n=4) were diluted suitably with methanol, and the TRF concentration was determined. The percentage degradation of gamma-T3 was assessed by making a comparison with the untreated standard solution of the same concentration.

2.8. Stock solution stability

The TRF stock solution was stored at room temperature (25 °C) and also in a refrigerator (4°C) for 24 hours. The solution was diluted so as to reach a concentration in the calibration range (100 µg/mL), and the AUC at zero and 24 hours was determined using the method described earlier.

3. RESULTS AND DISCUSSION

3.1. Optimisation of the mobile phase

For tocotrienol analysis, reports suggest a mobile phase consisting of acetonitrile and water [13] and a combination of acetonitrile: methanol: water [14-15]. Considering the economy and the convenience of using a binary mobile phase, we have employed methanol: water as the mobile phase. The mobile phase composition was optimised after many attempts using varying proportions. The proportion of methanol was varied between 90% and 95%, whilst the proportion of water ranged between 0 and 10%. It was observed that increasing the proportion of water leads to a prolonged retention time. Although a shorter run time was possible using higher proportions of methanol, a reasonable percentage of the aqueous phase is beneficial for a better resolution. Ali et al. reported a similar mobile phase composition for the simultaneous estimation of TRF isomers and simvastatin revealed a poorer peak separation on increasing the aqueous composition (about 25%), also reported a peak tailing with increasing water content [16]. However, the study highlights a quicker elution of simvastatin with 95% methanol and 5% water, suggesting a gradient elution technique for the simultaneous estimation. An optimal retention time at 8.7 minutes with an acceptable resolution was obtained with the mobile phase composition of 95:05 (methanol: water) at a flow rate 1.0 mL/min, column temperature 30 °C and 295 nm as the detection wavelength. The chromatogram of gamma-T3 (standard) and the TRF was presented in Figure 2. The peaks at 7.7 and 9.6 minutes (Figure 2 b), were corresponding to delta and alpha-T3 respectively, and the delta-tocopherol and gamma-tocopherol were observed at 12.8 and 15.3 minutes respectively.

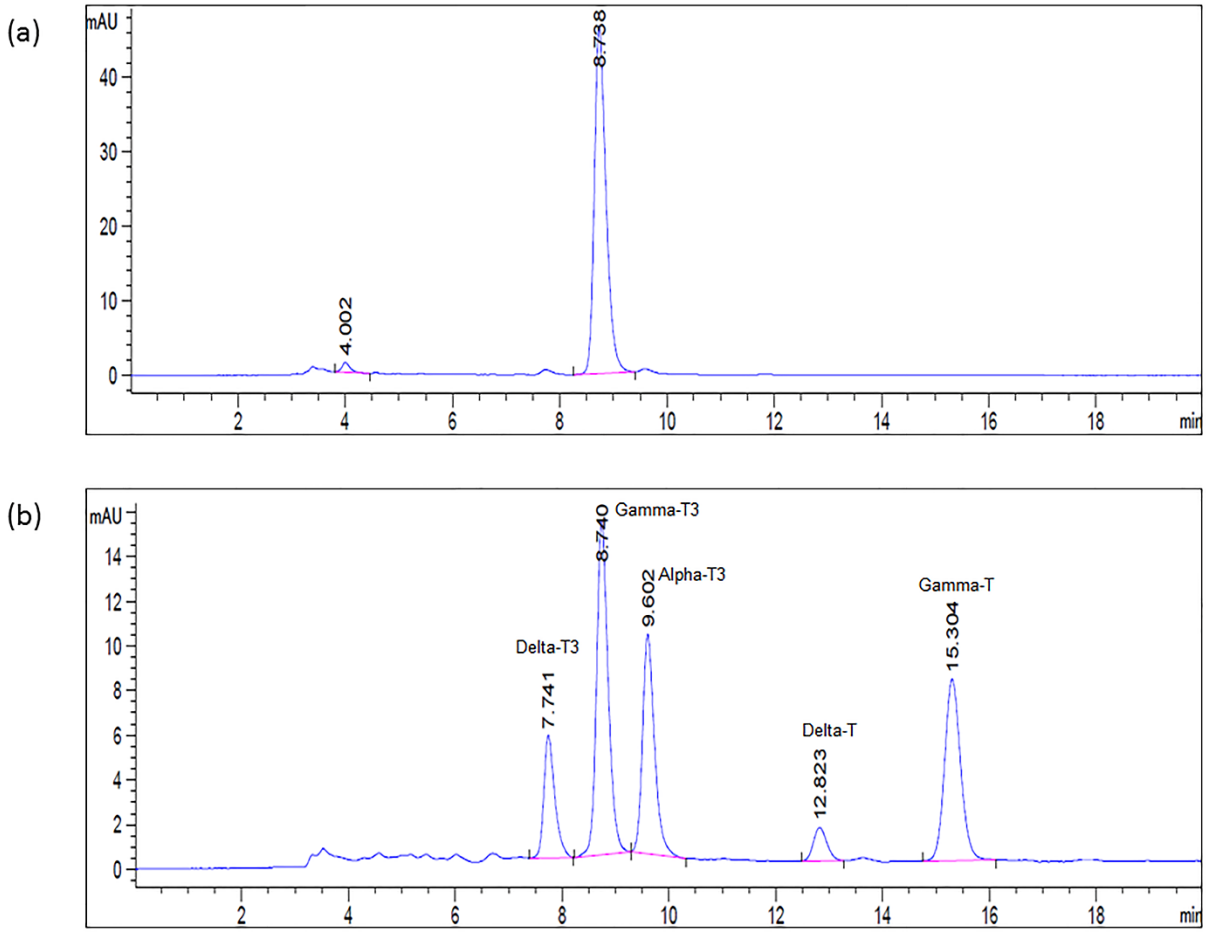

Figure 2. HPLC chromatograms showing the retention time of gamma-T3 at 8.7 minutes from the analytical standard (a), and the TRF mixture (b).

3.2. Linearity

Calibration curves for gamma-T3 were constructed from both the inter-day and intra-day data (n=5). The R^2 values of intra-day and inter-day linearity were 0.9996 and 0.9991 for respectively. A slightly lower R^2 value observed in the interday linearity could be due to the instrumental variation on different days. Similar linearity was reported by Tsochatzis et al. using acetonitrile :methanol: i-propanol (40:55:05) as mobile phase at a flow rate of 0.8 mL/minute in the detection concentration range between 1-40 µg/mL [14].

3.3. Precision and Accuracy

The precision and accuracy were tested by analysing the TRF samples at low (6.25 µg/mL), medium (50 µg/mL) and high (100 µg/mL) concentrations (Table 1). The method demonstrated a low %RSD value (≤ 5.8%) confirm excellent precision. The accuracy was determined with respect to the relative error (%RE). The accuracy at the low concentration was calculated as 8.8 %RE (inter-day) and 9.6 %RE (intra-day). Comparatively higher accuracy

was seen at the high concentration of TRF (100 µg/mL). This could be possibly due to the minimal experimental errors due to dilution at higher concentrations.

Table 1. Intra-day and inter-day precision and accuracy of the HPLC method using low, medium and high concentration samples of gamma-T3) (n=5).

Gamma-T3 concentration (µg/mL)	Intra-day		Inter-day	
	Precision (% RSD)	Accuracy (%RE)	Precision (% RSD)	Accuracy (%RE)
6.25	5.81	-9.6%	4.95	-8.8 %
50	3.32	5.2%	3.83	4.5%
100	1.56	-1.40%	3.31	-1.1%

3.4. Limit of detection and limit of quantification

The LOD and LOQ of gamma-T3 were calculated as 1.4 µg/mL and 4.2 µg/mL respectively. Ali et.al. reported an HPLC method for the simultaneous estimation of simvastatin and TRF with the LOD and LOQ as 7.5 and 20 µg/mL respectively [16]. A much lower LOD (0.02 µg/mL) was reported by another research group with the mobile phase composition methanol: water (97:3) using a fluorescence detector. However, system suitability studies were not reported in their report. Fluorescence detectors are much more sensitive than DAD detectors, and higher methanol composition in the mobile phase may also have been attributed to the low LOD reported. As discussed earlier, peak resolution might affect at higher methanol compositions (>95%).

3.5. System suitability studies

The system suitability parameters such as capacity factor, resolution, theoretical plate number, peak symmetry and signal to noise ratio (S/N) at three concentrations are shown in Table 2. An excellent resolution above 2.0 and a capacity factor of ≥2.5 was seen for the peak corresponding to gamma-T3 satisfying the criteria for a good separation. The 'N' was found to be >7950 for all of the three concentrations selected and the S/N ratio was found to increase with an increase in the drug concentration, that was 31.7 and 252.2 for 6.25 and 100 µg/mL respectively. Symmetrical peaks were observed with minimal peak tailing between 0.76 and 0.78. All these system suitability parameters were well within ICH and CDER guidelines [9-10].

Table 2. System suitability studies at low, medium and high concentration samples of gamma-T3 (n=5).

Gamma-T3 concentration (µg/mL)	Capacity factor (k')	Resolution (R_s)	Theoretical plates (N)	Tailing factor (T)	S/N ratio
6.25	2.5 ± 0.008	2.37 ± 0.21	8255.6 ± 395.8	0.78 ± 0.02	31.74 ± 4.8
50	2.5 ± 0.008	2.64 ± 0.06	8116.0 ± 307.2	0.77 ± 0.02	220.5 ± 26.7
100	2.5 ± 0.008	2.61 ± 0.04	7952.2 ± 158.1	0.76 ± 0.01	252.2 ± 47.8

3.6. Robustness

Robustness of the developed method was tested by making deliberate changes in the chromatographic conditions. A small reduction in flow rate (1.0 to 0.9 mL/min), leads to a prolonged retention time (8.7 to 11.5 minutes). Similarly, an increased flow rate of 1.1 mL/min resulted in a shorter retention time (8.0 minutes) which was expected. Other parameters such as the peak symmetry, resolution, and the capacity factor were within the acceptable limits. There were no significant differences seen in the peak characteristics with the change in wavelength to 290 nm or 300 nm. However, minor changes in the column temperature showed significant differences in the retention time and the capacity factor (Table 3). Similarly, when the mobile phase composition was changed to 93:07, resulted in a prolonged retention time (8.7 to 14.2 minutes) having a high capacity factor (4.6) and resolution (3.2). On the other hand, a shorter retention time (7.8 minutes) was observed when increased the methanol composition (97:03). Although, the run time was reduced, due to the high proportion of organic phase, peak overlapping occurred. The resolution reduced from 2.6 to 1.99 with an increased peak tailing to 0.71. Maintaining a reasonable aqueous content in the solvent mixture may result in enhanced peak symmetry and resolution. Hence, by considering the peak characteristics, run time and the cost-effectiveness; the selected chromatographic conditions were most suitable for the quantification of gamma-T3.

Table 3. HPLC method Robustness for gamma-T3 analysis on modifying the chromatographic conditions (n=3).

Chromatographic conditions		**Retention Time (min) R_t**	**Capacity factor (k')**	**Resolution (Rs)**	**Tailing factor (T)**
Flow rate (mL/min)	0.9	11.53 ± 0.06	3.17 ± 0.03	2.65 ± 0.02	0.79 ± 0.01
	1.1	8.00 ± 0.04	2.54 ±0.04	2.64 ± 0.09	0.78 ± 0.02
Wavelength (nm)	290	8.65 ± 0.02	2.43 ± 0.01	2.42 ± 0.03	0.81 ± 0.02
	300	8.64 ± 0.01	2.42 ± 0.01	2.43 ± 0.02	0.80 ± 0.01
Mobile phase composition (methanol: water)	93:07	14.18 ± 0.08	4.67 ± 0.01	3.23 ± 0.06	0.80 ± 0.017
	97:03	7.84 ± 0.05	2.19 ± 0.04	1.99 ± 0.04	0.71 ± 0.02
Column temperature (ºC)	28	11.10 ±0.02	3.43 ± 0.03	2.67 ± 0.06	0.80 ± 0.01
	32	8.44 ± 0.04	2.47 ± 0.05	2.60 ± 0.07	0.77 ± 0.01

3.7. Stress degradation studies and stock solution stability

The stress degradation studies showed that the TRF stock solution was stable against heat and acid; however, significant degradation was observed when exposed to strong alkali and UV light (Figure 3). Although vitamin E is generally known to be a stable compound, the unesterified form of tocopherol and tocotrienol has shown to affect stability due to the free phenolic group [17].

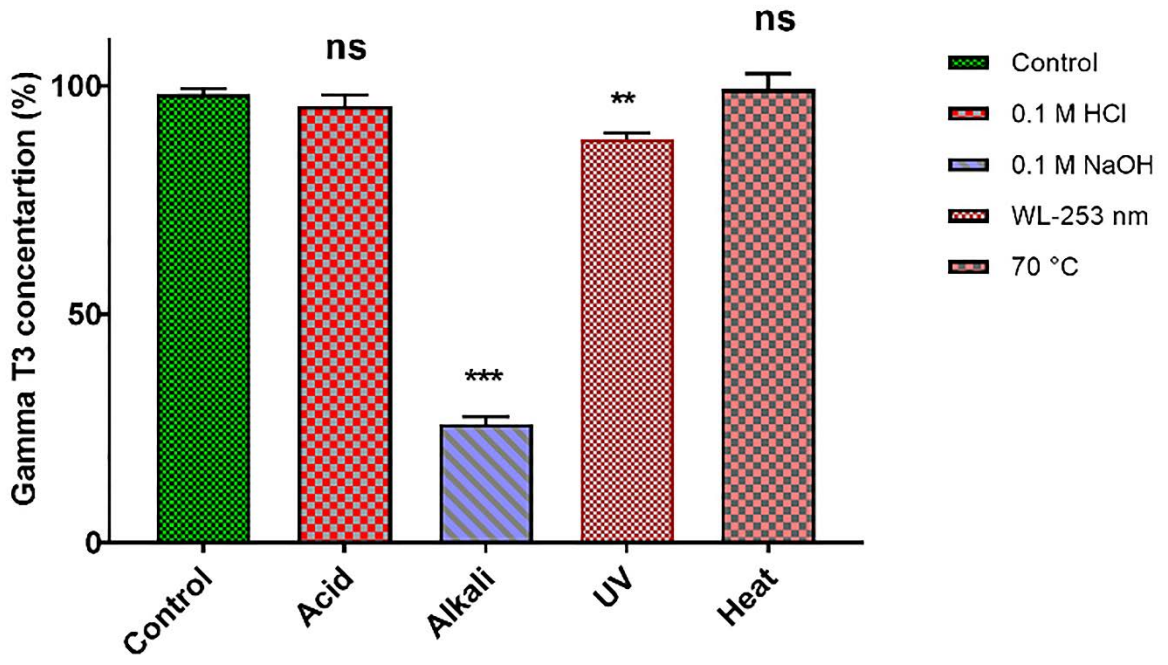

Figure 3. Percentage degradation of gamma-T3 under various stress conditions. Mean ± SD, n=3. (*** $p < 0.001$, ** $p < 0.002$, ns- not significant).

The stock solution stability study was performed at 25 °C and at refrigerator temperature. The amount of gamma-T3 remained in the stock solution after 24 hours at 25 °C and at 4-8°C was found to be 100.20 ± 0.39% and 100.57 ± 0.55% respectively. This clearly indicates the stability of gamma-T3 in the stock solution and the dilution medium at different storage conditions.

Palm tocotrienol is an approved food additive in many countries and is Generally Regarded As Safe (GRAS) by USFDA [18]. Therefore, tocotrienols have a lot of potential in the dietary supplement industry, and precise analysis is crucial. The most commonly used techniques for determining tocols in food matrices are HPLC and gas chromatography (GC). However, the derivatization process in GC can decompose compounds. Tocotrienols are stable in HPLC conditions and are easy to solubilize in the required solvents. Furthermore, RP-HPLC uses polar solvents rather than organic solvents used in normal phase HPLC. Cunha et al. reported the determination of tocopherols and tocotrienols in olive oil using various detectors, recommending DAD and fluorescent detection systems in RP-HPLC further support this study [19]. The developed method is simple, economical, and has further potential in precisely analysing tocotrienols in other food supplements and pharmaceuticals.

CONCLUSION

A rapid RP-HPLC method for gamma-T3 analysis was developed using binary solvents system and validated for linearity, precision, accuracy, and stability. The method utilizes cost-effective solvents such as methanol and water, which made the analysis economical. The technique is simple to perform, rapid and robust. The method validation demonstrated accuracy and precision in the acceptable range and is well suited for quantifying gamma T3 from crude palm oil. This RP-HPLC method can also be applied for determining tocotrienols in other food matrices.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

The authors would like to express their gratitude to the Faculty of Science, University of Nottingham Malaysia, for the financial support provided to this research project.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONFLICT OF INTEREST

None.

ACKNOWLEDGEMENTS

We appreciate ExcelVite Sdn. Bhd. (Perak, Malaysia) for providing TRF and tocotrienol standards.

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