Comprehensive Quantification of Palm Vitamin E by Normal Phase High Performance Liquid Chromatography

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INTRODUCTION

In 1922, embryologist H M Evans discovered tocopherols (TP) which are needed for human reproduction (Evans and Bishop, 1922; Evans *et al.*, 1974). More than 40 years later, the isolation of tocotrienol (T3) from latex was first reported by R A Morton (Danphy *et al.*, 1965). To date, vitamin E consists of T3 and TP. Together with TP, T3 provides a significant source of antioxidant activity in all living cells. This common antioxidant attribute reflects the similarity in chemical structure between T3 and TP, which differ only in their structural side-chain (T3 contains farnesyl while TP has a saturated phytyl side-chain). The common hydrogen atom from the hydroxyl (OH) group on the chromanol ring acts by scavenging the chain-propagating peroxyl free radicals. Depending on the location of the methyl groups on their chromanol ring, T3 and TP can be distinguished as four isomeric forms: alpha (α), beta (β), gamma (γ), and delta (δ). As TP was discovered much earlier compared to T3, TP has been more widely applied in human nutrition.

The term TP was used interchangeably with vitamin E, and αTP is the only form that is recognized to meet human nutritional requirements. With the advancement of T3 research, emerging evidence has revealed the antioxidant, anti-inflammatory (Wu *et al.*, 2008; Kuhad and Chopra, 2009), anti-angiogenic (Inokuchi *et al.*, 2003; Miyazawa *et al.*, 2004), anti-neurodegeneration (Khanna *et al.*, 2005b; Mazlan *et al.*, 2006), anti-hypercholesterolemic (Qureshi *et al.*, 2001; 2002) and anti-cancer (Yap *et al.*, 2008; 2009; Chang *et al.*, 2009) activities of T3. Apart from their common antioxidant and pro-fertility activities, the remaining clinical findings are not inherent in TP, making T3 a more important form of vitamin E. Thus, there is a need to differentiate between T3 and TP as a health-enhancing nutrient.

Various techniques have been reported for the analysis of vitamin E, including liquid chromatography and gas chromatography (Ruperez *et al.*, 2001). Liquid chromatographic analysis of vitamin E is often performed by normal (Kamal-Eldi *et al.*, 2000) and reversed phase (Ruperez *et al.*, 1999) high performance liquid chromatography (HPLC). The most significant advantage of using normal phase columns is the ability to separate the β and γ isomers of both T3 and TP. Normal phase systems show the elution of homologues in order of increasing polarity with separation based on the methyl substituents on the chromanol ring (Abidi, 2000). In addition, normal phase separations offer the advantages of operating with organic solvents that allow for high solubility of lipids and oil-based samples. Although reversed phase separations offer the advantages of easy equilibration of the mobile phases, reproducible chromatographic peak characteristics, compatibility with electrochemical detection and low

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volatility of mobile phase solvents, they cannot separate out all vitamin E isomers (γ and β). For gas chromatographic analysis, the sample is usually converted into trimethylsilyl derivatives to increase volatility and to prevent degradation of the analytes.

Despite extensive investigations into TP quantification, there is a lack of study on the method to quantify T3. The reasons are twofold: i) the lack of health awareness related to T3 (Ahn *et al.*, 2007); and ii) the absence of T3 analytical standards (Nagy *et al.*, 2007). Although quantifying T3 based on TP standards provides an estimation of total palm vitamin E purity, it overestimates the vitamin E purity as a result of higher UV absorption for αT3 at 290 nm. This overestimation is remarkably obvious when the vitamin E-rich sample is enriched with αT3. In addition, the linear range of vitamin E isomers has not been clearly determined by HPLC, let alone the less elusive α-tocomonoenol (αT1) (Yamamoto *et al.*, 2001; Ng *et al.*, 2004) in palm vitamin E concentrate.

In this work, we report a comprehensive method to quantify palm T3 and TP isomers using normal phase HPLC. The study proposes the use of the full vitamin E isomers as calibration standards and inclusion of αT1 (Yap *et al.*, 1999) as part of the total palm vitamin E content.

EXPERIMENTAL

T3 and TP Isomers, Chemicals and Reagents

The T3 and TP isomers, and palm tocotrienol-rich fractions (T30%, T50%, T70%, T90%) were purified from palm fatty acids distillate (PFAD) using molecular distillation and the Novasep® equipment. The feed was purchased from Kuala Lumpur Kepong Berhad. The purity of the vitamin E isomers was verified by the HPLC and gas chromatography (GC) percentage peak area. Final vitamin E isomer purity was determined based on the percentage peak area of GC. Sesamin 98% was a kind gift from Cactus Botanics Ltd (London, England). HPLC-grade n-heptane (Tedia), acetonitrile (Fluka), methanol (Fluka), and ethyl-acetate (Fisher Scientific Co.) were used throughout the study. Deionized water was purified by the MilliQ A10 system. Butylated hydroxytoluene (BHT), molecular-grade dimethyl sulphoxide (DMSO) and diethyl-ether were obtained from Sigma Aldrich (St Louis, MO, USA).

UV Spectrophotometry

The Agilent 8453 UV-visible spectrophotometer with a photodiode array (PDA) was used for the study of UV spectra, ranging from 200-500 nm, using a 1-cm cuvette.

Determination of High Performance Liquid Chromatography (HPLC) Parameters

The HPLC method was performed as a modification of the procedure reported previously (Yap *et al.*, 1999; Ng *et al.*, 2004). HPLC analysis was performed using the Agilent HPLC-1100 series on-line degasser connected to a HPLC 1100 series diode array detector (DAD). The monitoring wavelength selected was 290 nm. The following were the operating conditions evaluated: (1) LiChrospher Si60 column (250 mm x 4 mm I.D.; 5 μm), with a mobile phase of n-heptane:ethyl-acetate, (2) phenomenex luna silica column (250 mm x 4 mm I.D.; 15 μm), with a mobile phase of n-heptane:ethyl-acetate, (3) Zorbax ODS C18 column (250 mm x 2.6 mm I.D.; 5 μm), with a mobile phase of acetonitril:water, and (4) Develosil RPAQUEOUS C30 column (250 mm x 4.6 mm I.D.; 5 μm), with a mobile phase of methanol:water. The flow rate under isocratic separation was 1 ml min^{-1}, with a 10-μl injection volume. The monitoring wavelength selected was 290 nm.

Determination of Palm Vitamin E Level by Normal Phase High Performance Liquid Chromatography (HPLC)

Standard stock solutions – 80 mg/25 ml of each vitamin E isomer – were prepared in n-heptane. Serial dilutions (10 000, 8000, 6000, 4000, 3200, 2400, 1600, 1200, 800, 600, 400, 200, 100, 50 ppm) for HPLC calibration and UV spectra were prepared from the standard stock solutions. Each calibration point was determined in triplicate. A typical calibration curve can be modelled as $Y = Mx + C$, where *Y* is the analyte concentration in ppm, *M* is the slope of the calibration curve, *x* is the HPLC peak area, and *C* is the intercept of the calibration curve at x=0. C is set at 0.

To test an unknown sample, a normal phase HPLC method was performed as a modification of the procedure reported previously (Yap *et al.*, 1999; Ng *et al.*, 2004). A sample of 10 ml was injected into the Agilent 1100 series HPLC system (Agilent, Santa Clara, CA, USA). The chromatographic separation was carried out by a LiChrospher Si60 (5 mm, 250 mm x 4 mm I.D.) analytical column. The

mobile phase used was a mixture of heptane:ethyl acetate (93:7, v/v) at a flow rate of 1.0 ml min^{-1}.

Quantification of Palm Vitamin E in Mice Fed with Tocotrienol-rich Fraction

Hypercholesterolemic ($Ldlr^{-/-}$) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were used throughout the experiment. Five-week-old mice were fed orally with 100 mg kg^{-1} day^{-1} of an emulsified palm tocotrienol-rich fraction (TRF) with or without 100 mg kg^{-1} day^{-1} of lignan sesamin. Another five-week-old mice were injected intraperitoneally with 100 mg kg^{-1} day^{-1} of palm TRF dissolved in DMSO. All mice were sacrificed after two weeks, and blood samples were collected through cardiac bleeding, together with vital organs (brain, lungs, heart, liver, spleen, kidneys). To isolate the serum, the blood samples were incubated at room temperature for 30 min, followed by centrifugation at 4400 rpm, at 4°C for 30 min.

Sera were thawed and sonicated in an ultrasonic bath (Lab Companion, Vernon Hills, IL, USA) for 5 min, followed by vortexing for 10 s. The 100 ml of serum were transferred into a IWAKI Pyrex glass tube (Jawa Tengah, Indonesia) containing 900 ml of water. For organ preparation, the tissues were homogenized in 1 ml of water using a borosilicate glass homogenizer (Fisher Scientific, Pittsburgh, PA), followed by transfer to a Pyrex glass tube. The 50 μl of γTP with purity 99% (200 mg of γTP dissolved in 50 ml of ethanol) were used as an internal standard solution, and was spiked into the mixture. The tube was vortexed for 10 s and sonicated for 2 min. The 5 ml of the butylated hydroxytoluene (BHT) solution (5 mg of BHT in 100 ml of diethyl ether plus 5 μl 6M HCl) were added into the tube to minimize oxidation of the target analytes. Liquid-liquid extraction was performed by vortexing vigorously for 10 s. After liquid-liquid extraction, the tubes were centrifuged at 4000 rpm for 5 min in a Heraeus Multifuge 3-SR centrifuge (Newport Pagnell, Buckinghamshire, UK). The 4.9 ml of the organic layer were transferred into another Pyrex tube. The extraction was repeated and the second organic layer was removed and pooled together with the first layer. The organic solution was evaporated using a Buchi rotavapor R-205 (Flawil, Switzerland), and the dried residue was reconstituted in 1.5 ml of heptane, filtered, and followed by HPLC analysis.

RESULTS AND DISCUSSION

Work-flow for Quantifying Palm Vitamin E

Figure 1a represents the work flow for the quantification of total palm vitamin E. First, the UV spectra and saturation limits for vitamin E were evaluated. Second, the separation resolution was investigated using normal and reverse phase columns. Finally, the impact of the T3 and TP calibration standards (*Figures 1b* and *1c*) on the quantification of total palm vitamin E content was investigated.

All nine vitamin E calibration standards were purified to ~95% using preparative liquid chromatography. Their purity was evaluated using GC and HPLC. Although vitamin E peaks were resolved successfully using HPLC, the impurities without UV absorbance at 290 nm resulted in an overestimation of vitamin E isomer purity based on the peak area percentage (*Figure 1b*). Thus, the GC peak area percentage was adopted for the quantification of single vitamin E calibration standards (*Figure 1c*) (Galli *et al.*, 2002).

Based on the chromatograms obtained, the five vitamin E constituents were successfully separated using normal phase HPLC. It was observed that the elution order for the six vitamin E constituents on normal and reverse phase columns were αTP>αT1>αT3>βT3>γT3>δT3 and δT3>γT3=βT3>αT3>αT1>αTP, respectively (*Figures 2a* and *2b*). The UV spectra for vitamin E indicated maximum absorption at 290-300 nm, and the absorption intensity saturated when the vitamin E analyte concentration exceeded 400 ppm (*Figure 2c*). A similar trend was observed for all the vitamin E analytes.

Relationship between Vitamin E Isomer Concentration and High Performance Liquid Chromatography (HPLC) Peak Area and Determination of Calibration Curves

Although a single vitamin E isomer can be resolved efficiently by GC, the number of interfering peaks for a sample containing multiple vitamin E isomers is higher than that by HPLC (Lodge *et al.*, 2000). Therefore, HPLC is preferred for the quantification of palm vitamin E samples. To this end, it is required first to calibrate the HPLC peak area and the vitamin E analyte concentration for each vitamin E isomer. *Figure 3* shows the relationship between the two aforementioned calibration parameters, indicating a non-linear relationship when the concentration of the vitamin E isomer exceeded 1600 ppm. As each isomeric form of T3 and TP has a different UV absorbance at 290 nm (*Figure 4a*) (Tan and Br-

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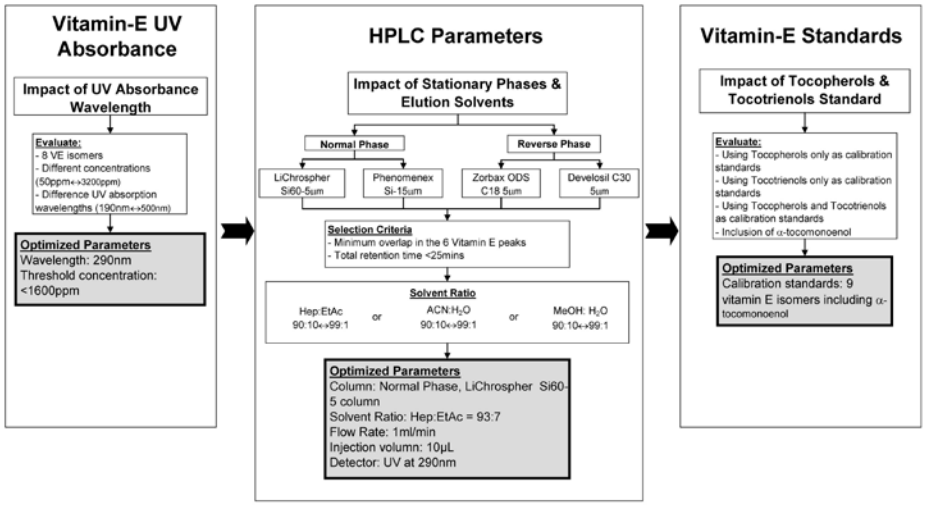

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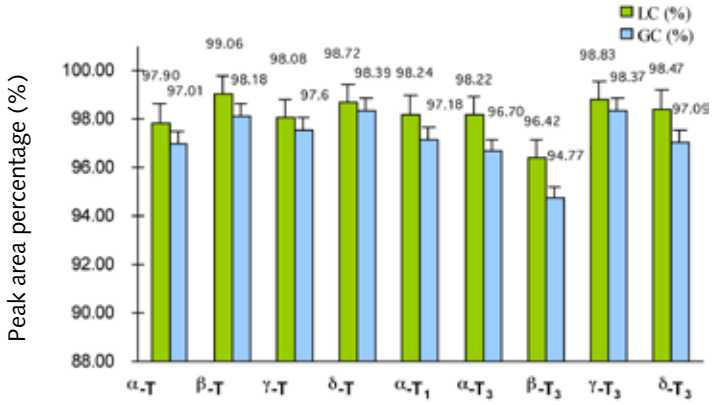

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Vitamin-E Isomers	Purity of standards (%)
α-T	97.01
β-T	98.18
γ-T	97.6
δ-T	98.39
α-T_1	97.18
α-T_3	96.70
β-T_3	94.77
γ-T_3	98.37
δ-T_3	97.09

Figure 1. (a) Work-flow for quantifying palm tocotrienols (T3) and tocopherols (TP) content. The study was divided into three stages: i) UV spectra of palm vitamin E; ii) operating parameters for high performance liquid chromatography (HPLC), and iii) analytical standards of vitamin E. (b) Vitamin E standards were purified by preparative liquid chromatography. Their purity was evaluated as the peak area percentage using HPLC and gas chromatography (GC). (c) The lower GC purity values represent the concentration of vitamin E calibrating standards.

zuskiewicz, 1989; Lee *et al.*, 2003), it is therefore necessary to establish the calibration curve for each isomeric form of vitamin E (*Figure 4b*). As a result of non-linearity above the threshold concentration (*Figure 3*), the calibration curves were established for analyte concentrations of less than 1600 ppm. Thus, when testing a palm vitamin E sample, it should be noted that the concentration of each vitamin E isomer should not exceed 1600 ppm.

Quantifying Palm Tocotrienol-rich Fractions (TRF)

To validate the proposed methodology, we evaluated four palm TRF (palm vitamin E 30%, 50%, 70% and 90%) based on different calibration standards. *Figure 5a* indicates that quantifying palm vitamin E content based on TP standards alone will overestimate the overall purity. In contrast, quantifying palm vitamin E content based on T3 standards alone will underestimate the overall purity. *Figure 5a* indicates that palm vitamin E purity based on T3 and TP standards gave the most representative purity contents for T3 and TP. It is worth noting that the concentration of α-tocomonoenol (Yamamoto *et al.*, 2001; Ng *et al.*, 2004) was higher than that of β-T3 in the tocotrienol-rich test samples (*Figure 5b*). Thus, it may be included as part of vitamin E content in palm oil.

Quantifying Palm Vitamin E in Animals Fed with Tocotrienol-rich Fractions (TRF)

The optimized solvent extraction protocol (Schultz *et al.*, 1997; Lodge *et al.*, 2000) for vitamin E was adopted to investigate whether the current HPLC methodology (*Figure 6a*) can be integrated for routine vitamin E analyses of biological samples from pre-clinical and clinical studies. As an internal standard (IS), 0.2 mg of γTP was added either directly to the homogenized sample before solvent

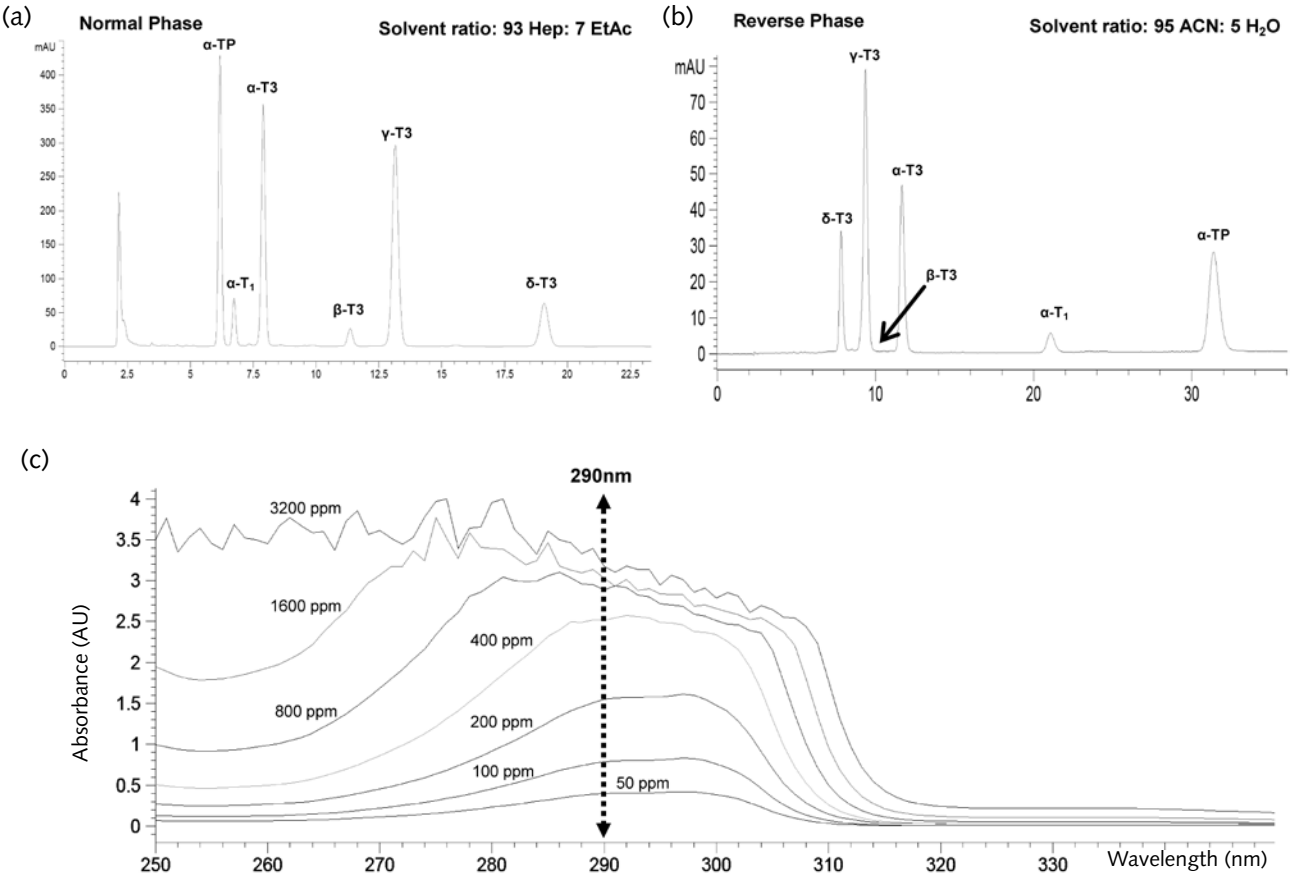
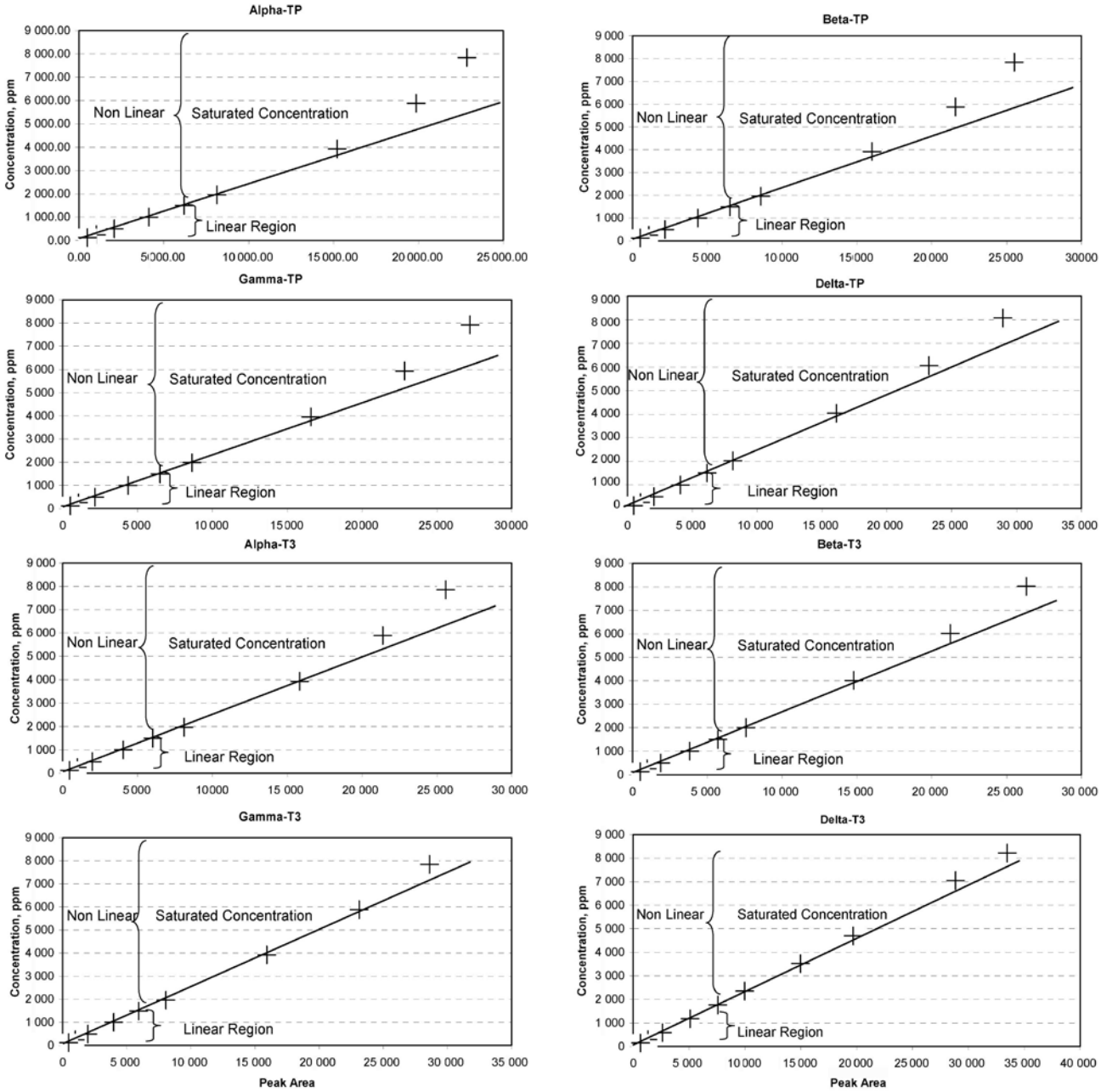


Figure 2. (a-b) Typical chromatograms of palm vitamin E using normal phase and reverse phase columns. The normal phase column separated all palm vitamin E peaks without overlap. In contrast, merged βT3 and γT3 peaks were observed during the reverse phase column separation. Hep, EtAc and ACN represents n-heptane, ethyl-acetate and acetonitrile, respectively. (c) UV spectra for VE isomer (e.g. *αTP) was investigated between the concentrations 50 ppm and 3200 ppm. Maximum absorption wavelength was determined to be 290-300 nm, and the absorbance intensity was saturated when αTP concentration exceeded 400 ppm.*

extraction, or immediately prior to derivatization. The latter sample yielded a γTP peak area that was then taken as 100% rate of recovery (*Figure 6b*). Any deviation from this peak area value represented a difference in vitamin E recovery.

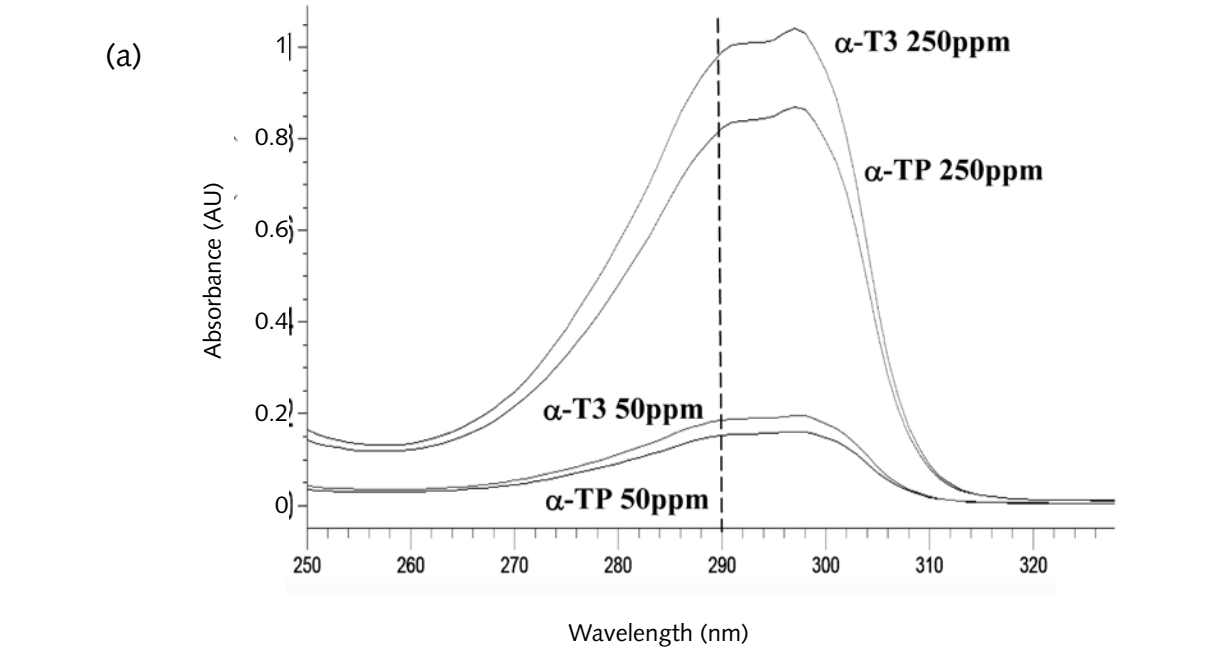
In the hypercholesterolemic mice receiving 100 mg kg^{-1} day^{-1} of palm TRF via intraperitoneal (i.p.) injection, all palm vitamin E isomer peaks were detected consistently in sera and vital organs. The profile of the vitamin E isomers extracted was similar to that found in TRF feed. *Figure 6c* shows that the vitamin E deposition in liver, heart, kidneys, spleen and lungs was ≥0.19 mg g^{-1} at the end of the two-week treatment. Although the brain cells are primarily fatty substance (myelin) that may act as a storage compartment for vitamin E (Schafer and Overvad, 1990), low TRF deposition (0.0268 mg g^{-1}) was found in these tissues following intraperitoneal TRF administration.

In contrast, in the mice fed orally with 100 mg kg^{-1} day^{-1} of palm TRF (*Figure 6d*) for two weeks, all palm vitamin E isomers were detected consistently in their sera and vital organs. Although the palm vitamin E deposition in lungs, heart, liver, kidney and spleen (≥0.0423 mg g^{-1}) was higher compared to deposition in the brain tissue (0.0118 mg g^{-1}) at the end of the two-week treatment, the overall vitamin E deposition following oral administration was significantly lower compared to by i.p. injection. It is worth noting that the γδT3 compositions extracted from brain, liver and lungs were remarkably low compared to the TRF feed (Lee *et al.*, 2003). This observation was possibly due to the preferential catabolism of γδT3 (You *et al.*, 2005; Sontag and Parker, 2007) (shorter biological half-lives) as a result of the high CYP4F2 enzyme level in the liver and kidneys (Su *et al.*, 2004; Sontag and Parker, 2007). The CYP4F2 is the first



Note: TP represents tocopherols; T3 represents tocotrienols.
HPLC-high performance liquid chromatography.

Figure 3. Relationships between vitamin E isomer concentration and HPLC's peak area were determined to be non-linear when the concentration of vitamin E exceeded 1600 ppm.



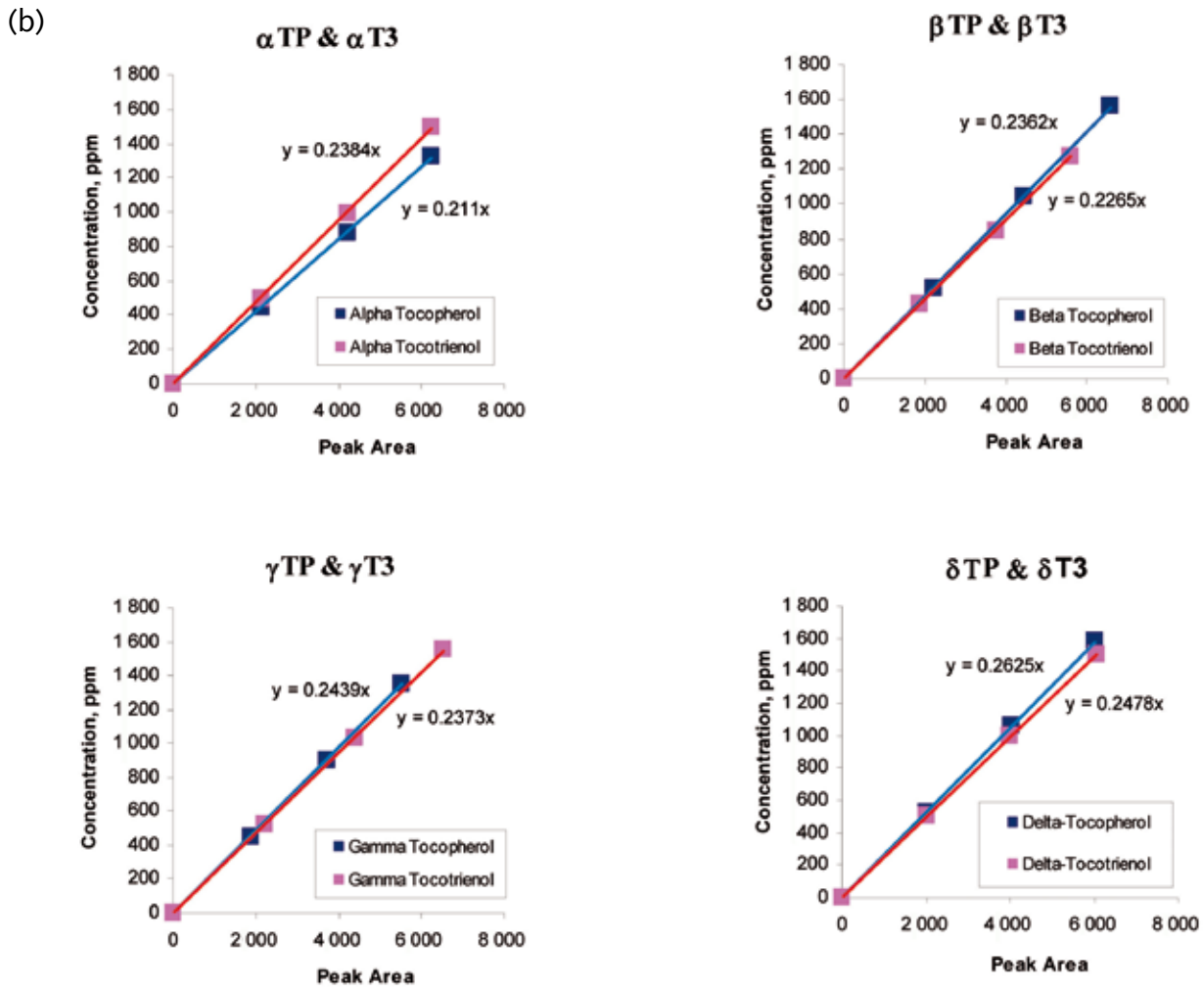
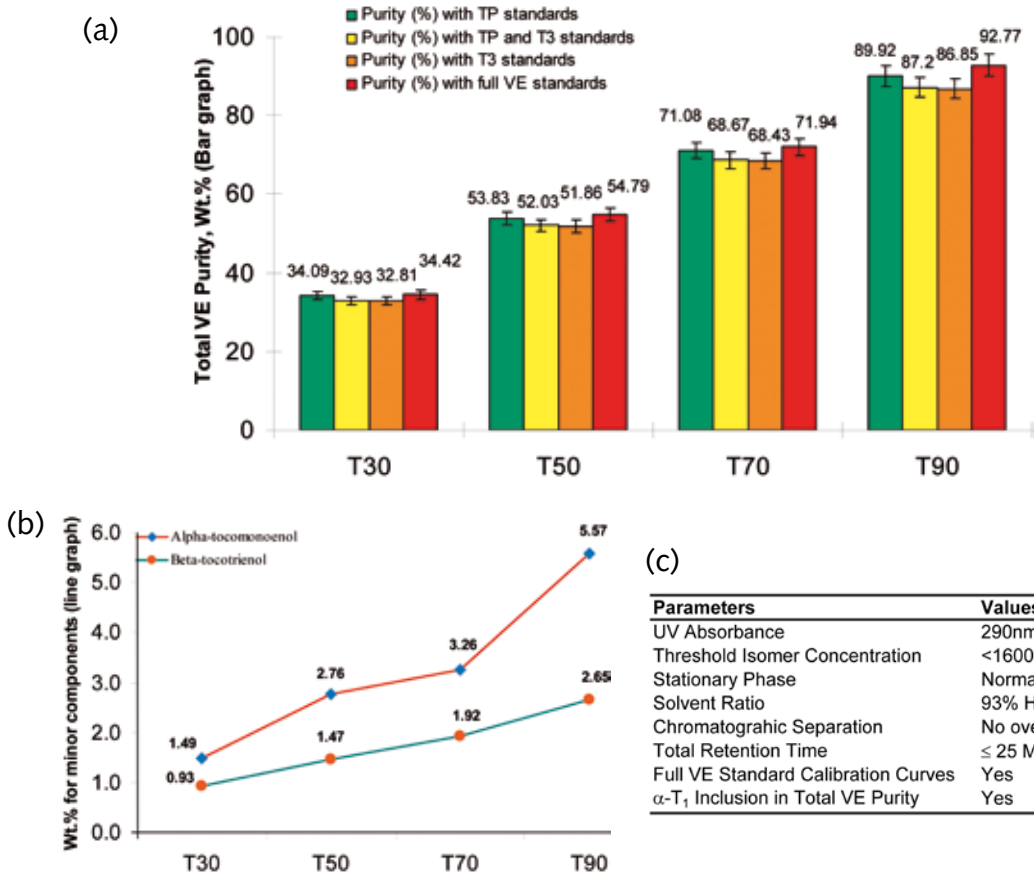


Figure 4. (a) The α form of tocotrienol (T3) and tocopherol (TP) have different absorbance at 290 nm. For the α form, T3 has higher absorbance at 290 nm compared to its corresponding TP counterpart. (b) Calibration curves for each vitamin E isomer were evaluated within the linear region (concentration ≤1600 ppm). Except for the α form, TP isomers have higher absorbance at 290 nm compared to their corresponding T3 counterparts.



(c)

Parameters	Values
UV Absorbance	290nm
Threshold Isomer Concentration	<1600ppm
Stationary Phase	Normal Phase, LiChrosper Si60-5
Solvent Ratio	93% Heptane : 7% Ethyl Acetate
Chromatograhic Separation	No overlap between Palm VE peaks
Total Retention Time	≤ 25 Minutes
Full VE Standard Calibration Curves	Yes
α-T_1 Inclusion in Total VE Purity	Yes

Figure 5. (a) Vitamin E purity of the four tocotrienol-rich fractions (TRF) were evaluated based on tocopherol (TP) and tocotrienol (T3) standards. Full standard refers to the vitamin E purity inclusive of α-tocomonoenol (αT1). It was found that estimating TRF based on pure tocopherol standards alone resulted in inflated vitamin E purity. (b) Proportion of αT1 content in TRF was higher than βT3. Thus, it is recommended that αT1 be included in palm total vitamin E. (c) Summary of high performance liquid chromatography (HPLC) parameters for palm vitamin E quantification.

enzyme catalysing the degradation of vitamin E, in particular the γδ-form of T3 and TP. In addition, another plausible explanation for the low γδT3 bioavailabiliy is the competition between T3 and TP for the TP-transport protein (TTP) that primarily transports aTP and has a low affinity for γδT3 (Khanna *et al.*, 2005a).

Although the inhibition of vitamin E catabolism by lignan sesamin has been previously demonstrated using a liver cell line (HepG2) (Sontag and Parker, 2002), co-administration of TRF (100 mg kg^{-1} day^{-1}) and lignan sesamin (100 mg kg^{-1} day^{-1}) through oral feed was unable to either enhance *in vivo* vitamin E deposition, or prevent *in vivo* degradation of γδT3 (*Figure 6e*). Consequently, the low oral bioavailability of γδT3 in this study (*Figures 6d* and *6e*) may provide an explanation for the several failed studies investigating the cholesterol-lowering property of palm vitamin E in human subjects (Wahlqvist *et al.* 1992; Mensink *et al.*, 1999; O'Byrne *et al.*, 2000). Nonetheless, the possibility of using lignan sesamin to prevent γδT3 degradation in human subjects remains to be elucidated.

CONCLUSION

A method to separate palm vitamin E isomers using normal phase HPLC was developed successfully. The linear range for individual palm vitamin E isomers was <1600 ppm at a UV absorbance of 290 nm. The elution solvent was optimized at a 93:7 n-heptane:ethyl-acetate mixture for an elution time of 25 min. All vitamin E isomers (T3, TP and αT1) should be employed as calibration standards for accurate estimation of palm vitamin E content. The αT1 may be included as part of the palm vitamin E members. Finally, the method developed herein is rapid and accurate for the quantification of vitamin E content in palm TRF and biological samples containing palm vitamin E.

ACKNOWLEDGEMENT

This work was supported by a research grant from Kuala Lumpur Kepong Berhad to Davos Life Science Singapore.

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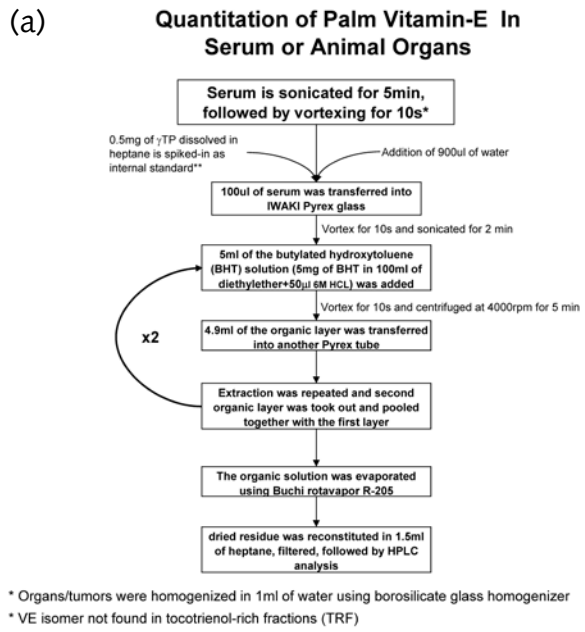
(a)



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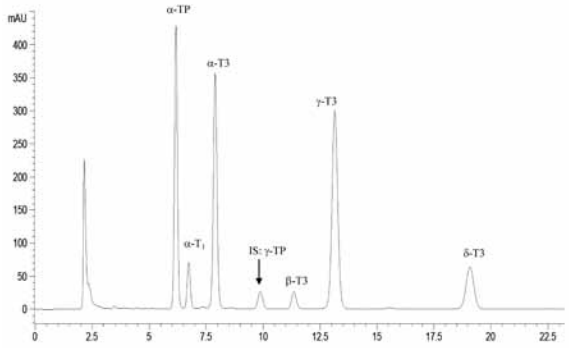

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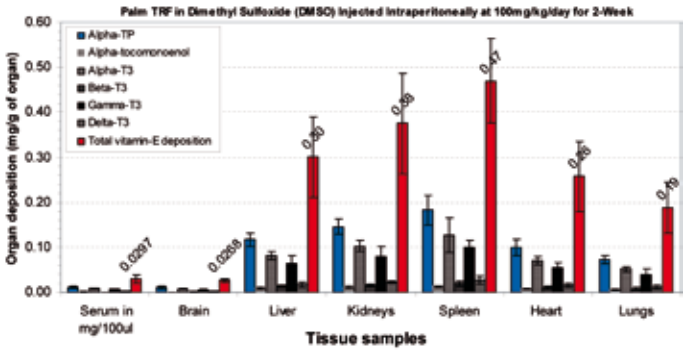

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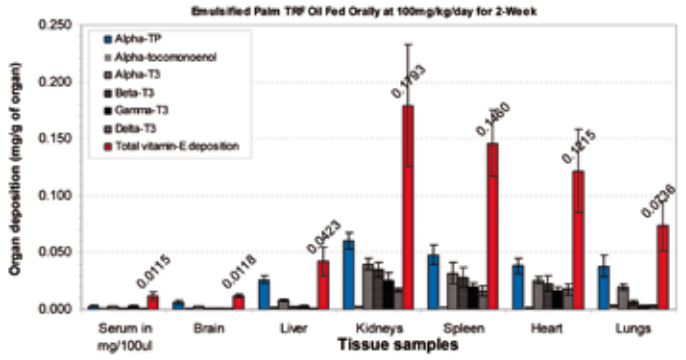

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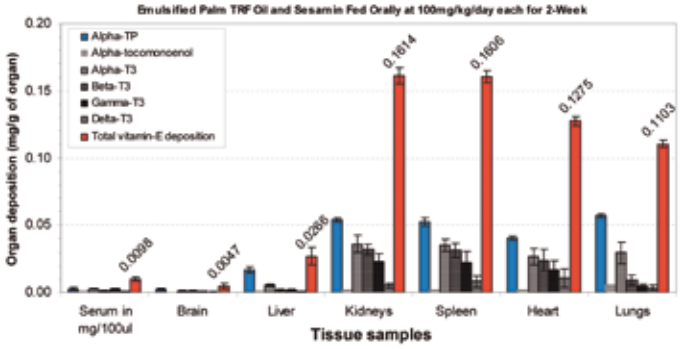

Figure 6. (a) Protocol to extract and quantify palm vitamin E deposition in the blood serum and vital organs of animals fed with tocotrienol-rich fractions (TRF) for two weeks. (b) Typical high performance liquid chromatography (HPLC) chromatogram of palm vitamin E extracted from serum/organs. 'IS' represents the internal standard (γTP) spiked into homogenized liquid before the solvent extraction protocol. (c) In the hypercholesterolemic mice receiving 100 mg kg^{-1} day^{-1} of palm TRF via intra-peritoneal (i.p.) injection, all palm vitamin E isomer peaks were detected consistently in sera and vital organs. The profile of the vitamin E isomers extracted was similar to that found in TRF feed. The vitamin E deposition in liver, heart, kidneys, spleen and lungs was ≥0.19 mg g^{-1} at the end of the two-week treatment. (d) In the mice fed orally with 100 mg kg^{-1} day^{-1} of palm TRF for two weeks, all palm vitamin E isomers were detected consistently in their sera and vital organs. Although the palm vitamin E deposition in lungs, heart, liver, kidney and spleen (≥0.0423 mg g^{-1}) was higher compared to deposition in the brain tissue (0.0118 mg g^{-1}) at the end of the two-week treatment, the overall vitamin E deposition following oral administration was significantly lower compared to that by intraperitoneal (i.p.) injection. (e) In the mice fed orally with TRF (100 mg kg^{-1} day^{-1}) and lignan sesamin (100 mg kg^{-1} day^{-1}), the in vivo *vitamin E deposition remained comparable to that of the mice fed with only 100 mg kg^{-1} day^{-1} of palm TRF.*

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