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A validated, rapid, simple and economical high-performance liquid-chromatography method to quantify palm tocopherol and tocotrienols

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ABSTRACT

A normal-phase high-performance liquid-chromatography (HPLC) method was developed and validated to determine and quantify α-tocopherol (T), α-, γ- and δ-tocotrienol (T3) in palm oil. The developed method was simple with direct sample injection into common ultraviolet (UV) detector and silica column. Analysis time was short (the last eluted peak ended at 8.8 min) and lesser mobile phase was consumed (0.8 mL/min). The relative standard deviation (RSD) was less than 7.11% for the response factor, coefficient of determination (R^2) was more than 0.998, peak resolution was more than 2.68, tailing factor was less than 1.35, percentage recoveries at three levels were in the range of 90.11–103.79%, limit of detection (LOD) was in the range of 100.28–175.24 μg/L and limit of quantification (LOQ) was in the range of 334.28–584.15 μg/L. The developed method was used to quantify the amount of the four major tocols in crude palm oil (CPO), refined palm oil (RPO), crude palm olein and stearin. Palm olein had 1.3 times more tocols than stearin and as much as 85.7% of the total tocols was recovered in palm olein. The potential of this method to elute the four T and four T3 isomers was also tested with resolution of more than 2.28.

1. Introduction

Vegetable oils, nuts, seeds and spices are food sources with high concentration of vitamin E (Saini and Keum, 2016; Vicente et al., 2011). Vitamin E or tocols are present in two forms: tocopherol (T) and tocotrienol (T3). They are fat-soluble antioxidants, each with a chromanol ring and a hydrophobic side chain (phytyl for T and isoprenyl for T3). The side chain of T is saturated while in T3, it is unsaturated due to the presence of three double bonds. Both T and T3 have four isomers, referred to as α-, β-, δ- and γ-, depending on the number and position of methyl group attached to the chromanol ring. The molecular structures of T and T3 are illustrated in Fig. 1.

Palm oil is the vegetable oil most consumed in the world. The two main palm oil exporters are Indonesia and Malaysia. Crude palm oil (CPO) is extracted from oil palm fruits (*Elaeis guineensis/tenera*) via mechanical screw-pressing. It is composed of approximately 1% of valuable minor constituents, which include vitamin E (tocols), carotenoids, glycolipids, sterols and squalene (Choo and Ab. Gapor, 1990). CPO contains 600–1000 ppm of tocols, which is composed of approximately 24% of T and 76% of T3. The four major tocols in palm oil are α-T, α-, γ- and δ-T3, which comprise of more than 95% of the total palm tocols (Ong, 1993). Various homologs of T can be commonly found in many vegetable oils while palm and rice bran oil are rich in T3s.

Both high-performance liquid-chromatography (HPLC) and gas chromatography (GC) can be used for the analysis of tocols. As opposed to HPLC, derivatization of the non-volatile tocols is

Abbreviations: AOAC, Association of Official Agricultural Chemists; AOCS, American Oil Chemists' Society; C_{30}, triacontyl; CDER, Center for Drug Evaluation and Research; CPO, crude palm oil; GC, gas chromatography; HPLC, high, performance liquid chromatography; ICH, The International Conference on Harmonization; LOD, limit of detection; LOQ, limit of quantification; OH, hydroxyl; PFP, pentafluorophenyl; PTFE, polytetrafluoroethylene; R^2, coefficient of determination; RPO, refined palm oil; RSD, relative standard deviation; T, tocopherol; T3, tocotrienol; UV, ultraviolet.

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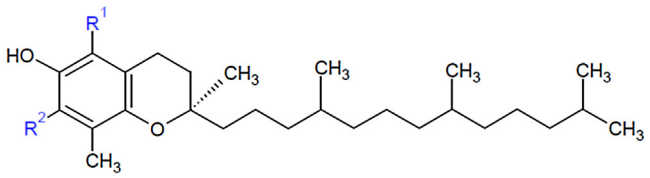
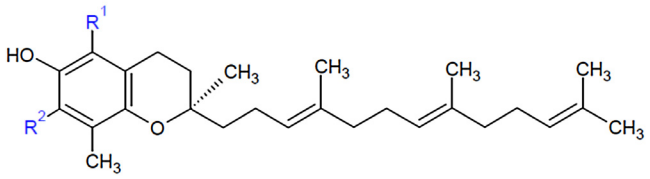


Tocopherol



Tocotrienol

Isomer	R¹	R²
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Fig. 1. Molecular structures of tocopherols (T) and tocotrienols (T3).

required before GC analysis (Cunha et al., 2006). The drawbacks of using GC include (i) pretreatment before injection, (ii) destruction of heat-sensitive T and T3 and (iii) longer analysis time (Puah et al., 2007). The use of GC is limited for tocols analysis because they are non-volatile and thermally-unstable compounds.

HPLC is the primary analytical method to quantify tocols. The use of reversed-phase HPLC is usually unable to separate β- and γ-isomers even though some researchers achieved good separation using better columns such as pentafluorophenyl (PFP) and triacontyl (C_{30}) column (Górnaś et al., 2014; Knecht et al., 2015; Shammugasamy et al., 2015). In addition, the method requires sample preparation to be compatible with the polar mobile phase. The majority of the analytical methods, especially reversed-phase HPLC, involve sample preparation steps such as saponification, extraction, concentration and drying before analysis in order to separate tocols from the bulk of triglycerides. Through the extensive handling processes, degradation of tocols occurs due to heat and chemical attack. It was reported that the concentration of tocols reduced by approximately 20% with the application of saponification during sample preparation (Bonvehi et al., 2000; Górnaś et al., 2014). Thus, simple and direct injection after dissolution in suitable solvents is favored.

Several advantages of employing normal-phase HPLC are the ability to (1) separate β- and γ-isomers, (2) allow direct dissolution of oils in mobile phase, (3) tolerate high loads of lipids, and (4) provide a wide range of selectivity due to the introduction of different polar modifiers in the mobile phases (Eldin et al., 2000; Balz et al., 1993; Truedsson and Smith, 1981). Through normal-phase HPLC, Cunha et al. (2006) studied the performance of different detectors including florescence and diode array in series, ultraviolet (UV) and evaporative light scattering. It was reported that florescence had the best sensitivity with the lowest detection limits in the range of 0.1–7.2 ng/L.

Eldin et al. (2000) compared the use of silica, amino and diol columns to quantify all of the tocols including four Ts and four T3s. Amino column was found to be unstable while diol column had poor reproducibility. Silica column was reported to provide the best separation. In addition, most researchers integrated silica column with normal-phase HPLC with great selectivity (Abidi, 2000).

Research on both reversed- and normal-phase HPLC coupled with many different detectors continues till present in order to explore the potential use of new and existing set-ups (Abidi, 2000; Saini and Keum, 2016). Simultaneous determination of multiple groups of compounds is also possible with photodiode array detector. Andrés et al. (2014) and Gimeno et al. (2000b) quantified T and T3 along with carotenes at 292 and 450 nm, respectively.

From the literature, the implementation of HPLC to quantify tocols is not uncommon. However, most studies reported the elution of Ts because they are prevalent in many vegetable oils (Bakre et al., 2015; Gimeno et al., 2000a,b; Goossens and Marion, 2011). In recent years, the health benefits of T3 s have been extensively explored. These compounds act as regulators of cholesterol level, neuroprotective agents against stroke and inhibitors of fats accumulation in the liver (Gopalan et al., 2014; Magosso et al., 2013; Nesaretnam et al., 1995; Theriault et al., 1999). T3 was reported to be more effective than T due to their unsaturated chain, which facilitates cell penetration and is highly antioxidative (Jiang, 2014; Suzuki et al., 1993). α-T3 was reported as having more than three times the in vitro free radical scavenging activity of α-T (Chen and Bergman, 2005). The order of antioxidant activities of T3 is γ > δ > α, and γ-T3 has twice the antioxidant effect of α-T3 (Lin, 2011).

Since the supply of pure individual T3 standards is scarce, T3 s are frequently quantified based on the response of their respective T homologs (Cerretani et al., 2010; Hidalgo et al., 2006; Posada et al., 2007; Mitei et al., 2009; Schwartz et al., 2008). The approach was recommended in the official American Oil Chemists' Society (AOCS) method to use T in the absence of T3 standards. The approaches to use only α-T and individual authentic T3 standards were compared and discussed by Ng and Choo in 2012. It was found that the results deviated by as much as 40% when only α-T was used as the standard for calibration. Thus, the technique was highly inaccurate due to the structural differences of T and T3.

Method validation is crucial to ensure that the developed analytical method is precise, reliable, accurate and robust. All methods should be validated before implementation. Most papers focused on method optimization with or without method validation. Linearity, repeatability, recovery, limit of detection (LOD) and limit of quantification (LOQ) have been commonly reported in the presence of method validation. However, both the procedure and criteria differ largely among the published works. To date, there is no one paper found to discuss on the methodology of method validation with the general acceptance limit given for each criteria. Furthermore, important peak characteristics such as peak asymmetry have never been reported before and the majority omitted peak resolution. These criteria are equally important to evaluate the performance of the method to yield symmetrical peaks for better peak area determination, and to ensure that the compounds are fully resolved from each other (not overlapping peaks). In this paper, guidelines for method validation and acceptance criteria as recommended by the Center for Drug Evaluation and Research (CDER) (United States), International Conference of Harmonization (ICH) and Association of Official Agricultural Chemists (AOAC) (United States) are discussed to evaluate the method developed (Yin, 2011).

The aim of this study was to develop and validate a normal-phase HPLC method using UV detector and silica column to quantify the four major tocols (α-T, α-, γ-, and δ-T3) in several palm oil fractions. In this paper, calibration was conducted using individual authentic external standards of T3 to ensure the