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Original research

Non-aqueous reversed-phase liquid-chromatography of tocopherols and tocotrienols and their mass spectrometric quantification in pecan nuts

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delta-Tocopherol (PubChem CID: 92094)
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ABSTRACT

An easy and effective analytical method was developed for the simultaneous quantification of four tocopherols (Ts) and four tocotrienols (T3s) in three pecan nut cultivars (Stuart, Sioux, and Pawnee). The analytes were separated on a C_{30} column kept at 15 °C, under isocratic non-aqueous reversed-phase (NARP) conditions, in only 18 min and detected by atmospheric pressure chemical ionization–tandem mass spectrometry (APCI-MS/MS). The HPLC-APCI-MS/MS method was validated, according to the main FDA guidelines, and then applied for the characterization of the real samples. Analytes were extracted by cold saponification with recoveries greater than 87%. The limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.3–10 μg/100 mg and 1–30 μg/100 mg, respectively. Compared to other nuts, vitamin E composition of pecan nuts (Carya illinoinensis) has only partially been elucidated. Results have evidenced the prevalence of γ-forms for both Ts and T3 s and clear quantitative differences of the identified vitamers among the studied cultivars. The richest variety in vitamin E was Sioux with a total content of about ~ 32 mg/100 g wet weight, followed by Stuart (~16 mg/100 g) and Pawnee (~9 mg/100 g).

1. Introduction

Vitamin E consists of a group of eight tocochromanols, all of them of plant origin: four tocopherols (Ts) with a saturated isoprenoid chain, and four tocotrienols (T3s) with an isoprenoid chain containing three *trans* double bonds. The homologues Ts and T3 s are designated α, β, γ, and δ depending on number and position of the methyl groups on the aromatic ring (see Fig. 1); β and γ tocochromanols have two methyl groups and are positional isomers.

Vitamin E is incorporated into cellular membranes in which it plays an important antioxidant function. Both Ts and T3 s act as peroxyl radical scavengers and chain breakers of lipid peroxidation (Esterbauer et al., 1991). The antioxidant activity increases with the number of methyl groups in the phenolic ring; thus, α forms possess the highest antioxidant activity (α > β > γ > δ) and are very effective in contrasting reactive oxygen species (ROS). However, the unsubstituted C-5 position of γ-T makes it able to trap lipophilic electrophiles such as reactive nitrogen oxide species (RNOS) (Jiang et al., 2001), which are associated with chronic inflammation-related diseases (Cooney et al., 1993). It has also been verified that the antioxidant efficacy of T3 s in membranes is higher than that of Ts, in particular for the α-forms. The determining factors are: *i*) the more uniform distribution of T3 s in membrane bilayer; *ii*) the greater recycling activity of the tocotrienoxyl radicals; *iii*) the closer collocation of T3 s to the membrane surface

Abbreviations: BHT, butylated hydroxytoluene; CV, coefficient of variation; FDA, Food and Drug Administration; FL, Fluorescence; IS, internal standard; HPLC, –APCI, MS/MS, high performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry; LOQ, limit of quantification; LOD, limit of detection; MRM, multiple reaction monitoring; MTBE, methyl *tert*-butyl ether; NARP, non-aqueous reversed phase; NP, normal phase; QC, quality control; R^2, coefficient of determination; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; RP, reversed phase; T, tocopherol T; T3, tocotrienol

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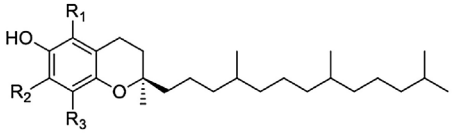
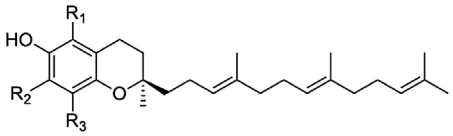
Vitamer	R1	R2	R3	Structure
α-Tocopherol (α-T)	-CH_3	-CH_3	-CH_3	
β-Tocopherol (β-T)	-CH_3	-H	-CH_3	
γ-Tocopherol (γ-T)	-H	-CH_3	-CH_3	
δ-Tocopherol (δ-T)	-H	-H	-CH_3	
α-Tocotrienol (α-T3)	-CH_3	-CH_3	-CH_3	
β-Tocotrienol (β-T3)	-CH_3	-H	-CH_3	
γ-Tocotrienol (γ-T3)	-H	-CH_3	-CH_3	
δ-Tocotrienol (δ-T3)	-H	-H	-CH_3	

Fig. 1. Structures of the eight vitamin E homologues.

(Packer et al., 2001).

The Dietary Reference Intake of vitamin E for adults is around 15 mg/day (Monsen, 2000). Vegetable oils and lipid-rich plant products are the main natural sources (Syväoja et al., 1986; Piironen et al., 1986); in particular, nuts and seeds cover up to 82% of the daily intakes of vitamin E (Bauernfeind, 1980). Traditionally, the most worldwide consumed nuts are hazelnuts, almonds and walnuts. Pecan is another kind of nut, which is native from North America and is distributed over a large geographic and climatic area, from the United States to Mexico (Thompson and Grauke, 1991; Hall, 2000). Its introduction in North Africa and Europe (Iberian Peninsula, and in Italy at the Botanical Garden of Palermo) was reported in the early 20th century. Regarding the vitamin E composition, pecans (Carya illinoinensis) have been the least studied of nuts, especially in relation to the main cultivars available on the American and European food market (Lee et al., 1998; Kornsteiner et al., 2006; Villarreal-Lozoya et al., 2007).

In general, liquid chromatographic techniques are the golden standard for the speciation of tocols. However, so far, the elucidation of their natural distribution in nuts as well as other matrices have mainly been hindered by the difficulties in resolving the β- and γ isomers, which have often been determined globally. The literature has reported not many methods related to the separation of all eight homologous of vitamin E. Some of them were based on Normal Phase (NP) chromatography (Cunha et al., 2006; Pinheiro-Sant'Ana et al., 2011; Kua et al., 2016). This is a chromatographic mode particularly selective towards geometric and positional isomers, which are usually well-resolved on siliceous phases based on their different steric fitting with the adsorption sites. However, NP has two main limitations: *i*) it is subject to phenomena of tailing; *ii*) the mobile phase is not ideal for MS detection. In fact, the most used mobile phases contain hexane and low percentages of alcohols or ethers which do not favour either the electrospray or Atmospheric Pressure Chemical Ionization (APCI). On the other hand, selectivity and sensitivity of MS detection is crucial when complex matrices such as food and biological samples have to be studied. Compared to NP, Reversed Phase (RP) offers a series of advantages that include the use of more appropriate mobile phases for MS detection, greater robustness of chromatographic columns, reproducibility of the retention times, faster conditioning and better peak shape. For these reasons, several RP-HPLC methods were developed on C_{18} columns (Thompson et al., 1980; Stöggl et al., 2005; Barba et al., 2011). Unfortunately, the standard microparticulate C_{18} phases fail to separate the β- and γ-forms of T_S and T3 s because their retention is very similar. However, recently, the partial resolution of the two pairs of isomers was achieved on a conventional C_{18} column, thermostatically controlled at 7 °C, using a mobile phase composed by water and isopropanol (Irakli et al., 2012). The low flow rate, which was set at 0.3 mL/min to avoid high backpressure values, was responsible for a separation time longer than 1 h. A complete resolution was achieved on non-alkyl RP columns such as pentafluorophenyl (PFP) columns (Lanina et al., 2007; Viñas et al., 2014; Wong et al., 2014; Górnaś and Siger, 2015; Knecht et al., 2015) and naphthalethyl (π-NAP) columns (Shammugasamy et al., 2013). The good performance offered by both these stationary phases is due to the relative rigidity of the PFP and π-NAP groups which provides superior selectivity for analytes with similar solubility but different size and shape. Although C_{30} is another kind of stationary phase suitable for separations of geometric and positional isomers, there is only two methods based on it (Saha et al., 2013; Knecht et al., 2015). The method of Knecht et al. (2015) was able to separate all vitamin E homologues in 45 min, keeping the C_{30} column at 18 °C, employing a flow rate of 0.5 mL/min and using a mobile phase composed by water, methanol and methyl-t-butyl ether (MTBE). A similar mobile phase was used also for the separation on the Core Shell PFP column (Knecht et al., 2015). However, the mobile phase used for both columns is neither completely ideal for HPLC (MTBE tends to form bubbles) nor totally suitable for the analysis of most of real samples. In fact, in the majority of cases, Ts and T3 s are extracted from lipid-rich matrices whose analysis requires a purge step at the end of the chromatographic run to wash the column.

The major aim of this work has been the development of a simple and fast non-aqueous reversed-phase (NARP) chromatographic method for the separation and quantification of Ts and T3s. Actually, since not all vitamin E forms have the same function and biological potency, the study of their natural distribution is of special interest and can provide valuable information on the nutritional value of foods. This is especially important for T3s, which are the least studied forms due to the limited availability and high cost of the authentic standards. The needed sensitivity and selectivity to detect the minor components and to characterize complex matrices such as nuts were provided by the APCI-tandem mass spectrometry (MS/MS). To the best of our knowledge, this is the first application of a HPLC-APCI-MS/MS method to the speciation of vitamin E homologues in different pecan cultivars (Sioux, Stuart and Pawnee) to assess differences in individual and total content of these precious antioxidants.

2. Materials and methods

2.1. Chemicals and materials

α-T (all-rac-α-tocopherol; CAS Number 10191-41-0), β-T (all-rac-β-tocopherol; CAS Number 148-03-8), γ-T ((+)-γ-tocopherol; CAS Number 54-28-4), δ-T ((+)-δ-tocopherol; CAS Number 119-13-1) and α-T-d_6 (ring-5,7-dimethyl-d_6; CAS Number 113892-08-3) were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy). α-T3 (D-α-tocotrienol; CAS Number 58864-81-6), β-T3 (D-β-tocotrienol; CAS Number 490-23-3), γ-T3 (D-γ-tocotrienol; CAS Number 14101-61-2), and δ-T3