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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(4): 832-835

www.thepharmajournal.com
Received: 14-02-2019
Accepted: 16-03-2019

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Estimation of tocopherols by different extraction methods in underutilized seed oils

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Abstract
Tocopherols are the natural antioxidants present in plant oils. Their determination is affected by the technique and solvents used for their extraction. The aim of the study was to determine the tocopherol (α, γ and δ) content of two underutilized seed oils (avocado and flaxseed oil) and to see the effects of solvents on their extraction methods. The extraction of tocopherol was performed using two solvents namely, ethanol and methanol and the tocopherols (α, γ and δ) were identified and quantified using reverse phase UPLC (Ultra performance liquid chromatograph) equipped with Photo diode Array (PDA) detector at 295 nm. Results showed that the avocado oil contains high amount of α- Tocopherol (33.26ppm) whereas flaxseed oil was rich in γ- Tocopherol (84.58 ppm). The concentration of α, γ and δ tocopherols were found to be higher in the sample extracted with methanol compared to ethanol extracted samples for both the oils.

Keywords: Tocopherol, UPLC, solvents, avocado oil, flaxseed oil

1. Introduction
Tocopherols are natural lipid soluble antioxidants with high biological activity which accounts for chain-breaking antioxidant activity and oxidation preventive properties [1]. The generic term for tocopherols and tocotrienols is Vitamin E comprising of α, β, γ and δ tocopherols and α, β, γ and δ tocotrienol containing a 6-chromanol ring, methylated at varying degrees at the 5, 7, and 8 positions [2, 3]. The tocopherols are present in cereal grains, namely rice, oats, barley and wheat but they are abundantly found in vegetable and seed oils stored in several parts like fruits and oilseeds [4, 3].
The most active biological form of vitamin E is α-tocopherol which potentially protects the body against chronic and degenerative diseases by increasing the resistance of low density lipoprotein (LDL) to oxidation through radical chain-breaking [5, 6]. The γ-tocopherol on the other hand is the most potent free radical remover and decreases the platelet aggregation and has strong anti-inflammatory activity corresponding to inhibition of carcinogenesis [5, 7]. Fewer studies have been done on δ-tocopherol. Studies done by Isnardy (2003) [8] indicated fthe excellent antioxidant potential of δ-tocopherol in bulk oil triglyceride and its emulsion oxidized at low temperature. Wagner (2001) [9] reported that the antioxidant activity of δ-tocopherol was maximum at higher temperatures (100 °C and above).
Tocopherols are also responsible for preventing the non-enzymatic oxidation of unsaturated fatty acids by free radicals, hydrogen peroxide and reactive molecular oxygen and thus increase the shelf life of oil [10, 11] and improve the oxidative stability of oils [12].
The tocopherols distribution varies among different plant oils and their concentration differs considerably within a given type of oil [12]. Several methods have been reported in literature for the determination of tocopherol in oil using reversed phase high pressure liquid chromatography [13, 14, 15] using different extraction methods via. saponification, concentration and drying steps which may result in error in quantification due to degradation of tocopherols as they are heat and light sensitive.
The present study focuses on comparing two simple, rapid and sensitive methods for the extraction of tocopherols using reversed-phase ultra performance liquid chromatograph (UPLC) equipped with PDA detector for the identification and quantification of tocopherols which allows for improved speed and sensitivity in liquid chromatography.

2. Material and Methods
2.1 Chemicals
Two underutilized seed oils (Avocado oil and Flaxseed oil) used in this study were purchased from SNN Natural Products, Delhi, India and stored at 4°C until analysis α, γ and δ tocopherol

standards were obtained from Sigma-Aldrich, India. All chemicals and solvents used were of HPLC grade.

2.2 Instrumentation
The tocopherol (α, γ and δ) contents of oil samples were determined by a Acquity reverse phase UPLC (Ultra performance liquid chromatograph) H-Class (Waters, India) equipped with a Photo diode Array (PDA) detector at 295 nm. A reversed-phase column (Cortex C18, 100 mm × 2.1 mm; 1.6µm) was used for the separation of tocopherols, maintained at 35 °C. The mobile phase was consist of acetonitrile and methanol (50: 50, v/v), eluted at a flow rate of 0.5 mL/ min detected at 295 nm. The injection volume was 4 µL with a run time of 6 minutes.

2.3 Standard Preparation
Stock solutions with concentration of 100 ppm of each Tocopherol (α, γ and δ) standard were prepared in ethanol. Working standard solutions were prepared by diluting the stock standard solutions to obtain the concentrations of 1, 5, 10, 15, 20, 25 ppm. An external calibration was obtained by injecting these working standard solutions (1-25 ppm) on column. The standard curves (area versus concentration) for the tocopherols (α, γ and δ) were calculated by linear regression analysis and used for the quantification of tocopherols in oil samples.

2.4 Sample Preparation
The extraction was done using two solvents namely, ethanol and methanol according to the procedure described by Goossens and Marion, (2011) [16]. Briefly, the oils were diluted in two different solvents (ethanol and methanol) in ratio of 1:3 of oil to solvent in a centrifuge tube. The samples were vortexed for 10 minutes followed by centrifugation at 3000 rpm for 5 minutes in a centrifuge (Sigma, Germany). The samples were then filtered through a syringe filter of 0.45 µm pore size and stored in amber colored vials until analysis.

3. Result & Discussion
Fig 1 shows the tocopherol (α, γ and δ) concentration in avocado and flaxseed oil. The identification of tocopherols in underutilized seed oils was determined by comparing their retention times with that of standard tocopherols (α, γ and δ) which were about 4.12, 3.54 and 3.05 minutes respectively. The tocopherols were quantified using the calibration curves (Fig 2) of standard tocopherols by plotting peak area versus concentration. The curve showed the linear correlation between the standard tocopherol concentration and the peak area at 295 nm with the linear correlation coefficient of ($R^{2)}$ of 0.999 for all the standards. The tocopherol (α, γ and δ) content of oil samples extracted by using two different solvents (ethanol and methanol) is shown in Fig 3.

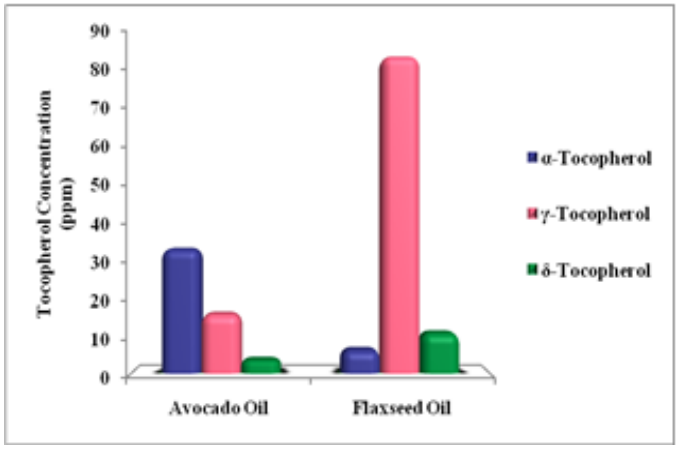


Fig 1: α, γ and δ Tocopherol concentration in Avocado and Flaxseed oil

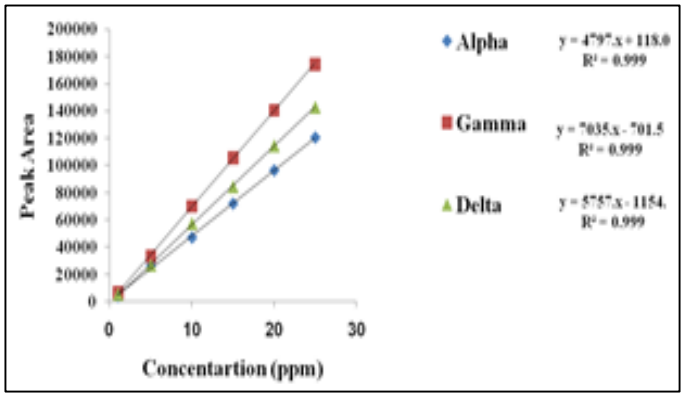
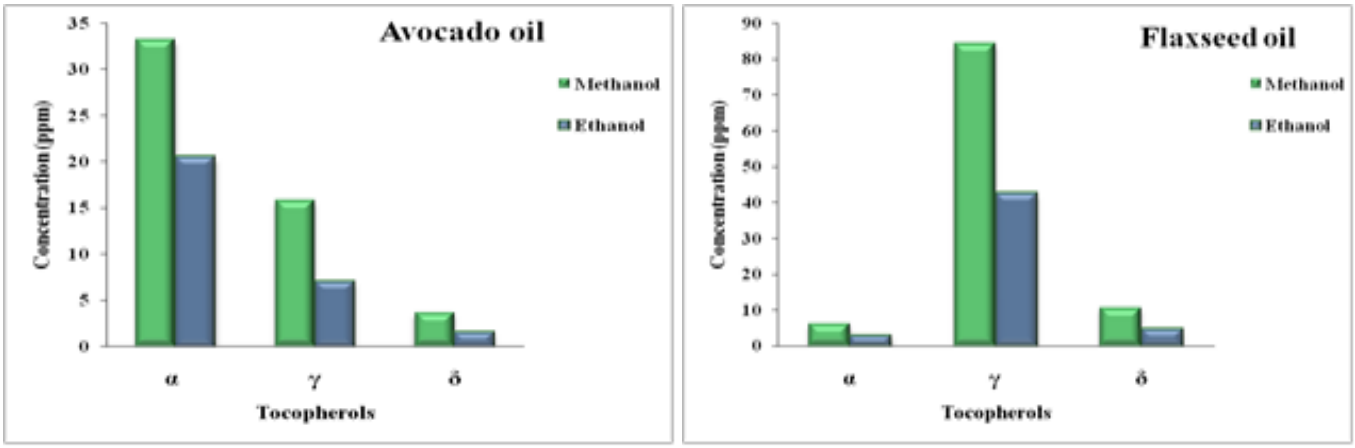


Fig 2: Calibration curve of Standard Tocopherols (α, γ and δ)



(a) (b)

Fig 3: Tocopherol (α, γ and δ) content of Avocado oil (a) and Flaxseed oil (b) extracted in Methanol and Ethanol

The results showed that the avocado oil contains higher amount of α- Tocopherol followed by γ- Tocopherol and δ- Tocopherol whereas flaxseed oil was rich in γ- Tocopherol followed by α- Tocopherol and δ- Tocopherol. Both the oils

exhibited lower amounts of δ- Tocopherol. Similar results have been reported by Jorge (2015) and Chun (2006) [17, 18] whereby the study demonstrated that the α- Tocopherol content was higher than γ- Tocopherol and δ- Tocopherol in avocado oil. Tanska (2016) [19] also found the similar results showing dominance of γ- Tocopherol in flaxseed oil.

However the concentration of tocopherols in both the oils varied with solvent used for the extraction. In avocado oil, the concentration of α- Tocopherol, γ- tocopherol and δ- tocopherols were found to be higher in the sample extracted with methanol (33.26 ppm, 15.88 ppm and 3.62 ppm respectively) compared to the sample extracted in ethanol (20.47 ppm, 7.04 ppm and 1.58 ppm respectively). Similarly, the methanol extracted samples of flaxseed oil showed higher concentration of α- Tocopherol, γ- tocopherol and δ- tocopherols (6.32 ppm, 84.58 ppm and 10.8 ppm respectively) compared to the sample extracted in ethanol (3.04 ppm, 42.62 ppm and 4.81 ppm respectively).

The α tocopherol content of methanol extracted avocado oil reported by Peraza-Magallanes (2017) [20] was found in the range of 32.28-50.66 ppm which was comparable to the value reported in present study (33.26 ppm). Chun (2006) [18] had reported α, γ and δ tocopherol in the range of 18.1-26.6 ppm, 1.3-6.9 ppm and 0.2-0.3 ppm respectively which were comparatively lower than the values obtained in the present study except for ethanol extracted α- Tocopherol. Results depicted by Manaf (2019) [21] showed the amount of γ and δ tocopherol (0.28-4.18 ppm and 0.04-0.08 ppm respectively) were lower but α- Tocopherol (32.42-44.98 ppm) was higher than the results obtained in the present study.

On comparing the α, γ and δ tocopherol values of flaxseed oil with the values reported by Rafałowski (2008) and Matthäus (2017) [22, 23], it was found that the α and γ tocopherols were much lower in present study while concentration of δ tocopherol was higher. On the other hand values reported by Tanska (2016) [19] showed higher value of δ tocopherol (36.4 ppm) than the present study. The variation seen in the tocopherol content could be due to the variation of solvents used, incomplete extraction and degradation of tocopherols during storage and higher temperature of storage [24].

4. Conclusions

The present study concludes that UPLC method used in the present study for the determination of tocopherols (α, γ and δ) is an effective tool for the separation and quantification of tocopherol in the oils. Thus providing a sensitive, rapid and selective determination of tocopherols (α, γ and δ). Further the type of solvent used for the extraction of tocopherol (α, γ and δ) from oils have an effect on the yield of the types of tocopherols.

5. Acknowledgments

The authors are thankful to NIFTEM for providing support and fellowship to carry out the research work. The authors would also like to thank Mr. Yogesh Sharma for his technical assistance.

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