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Study of Tocopherols Homologue in Needle of *Pinus tabulaeformis* Carr by High Performance Liquid Chromatography

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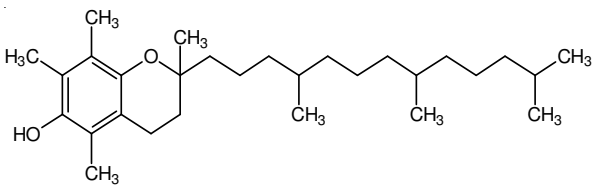
An HPLC method for separating tocopherol homologues using petroleum ether extraction mixed column purification pretreatment technique and methanol elution with in C_{18} column has been established. Results showed that the content of α,γ and δ-tocopherols are 5.40 ± 0.25, 1.18 ± 0.03 and 221.17 ± 20.15 mg/kg, respectively, in *Longnan pinus* needle and 3.16 ± 0.35, 3.09 ± 0.14 and 89.93 ± 22.76 mg/kg, respectively, in *Tianshui pinus* needle. The recovery rate is 74.6-89.7 % and the RSD is 1.5-5.6 %. The method is suitable for the homologous determination of tocopherols in samples of green plants, especially for the quantitative determination of easily oxidizable and low content tocopherols. The development of this method provides a theoretical basis for the understanding of the function and the application of *Pinus tabulaeformis* Carr.

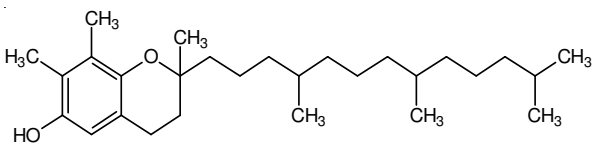
Key Words: Liquid chromatography, α,γ,δ-Tocopherols, *Pinus tabulaeformis* Carr.

INTRODUCTION

Pinus tabulaeformis Carr, one of the main tree species introduced to the arid area, is rich in nutrient components such as amino acid, volatile oil, water-soluble and fat-soluble vitamins and trace elements. Tocopherols, insoluble in water, soluble in various organic solvents, possess important physiological functions and, up to now, they are the only non-toxic natural antioxidant[1] found in oil or fat food. Tocopherols consist of 8 isomers. Among these isomers, α,γ,δ-tocopherol and γ-tocotrienol are of importance and biological significance[2]. Molecular structure of the 3 tocopherol homologues studied in this paper are shown as Fig. 1.



α-Tocopherol



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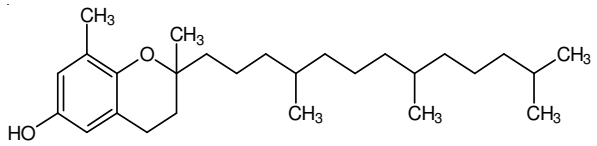

δ-Tocopherol

Fig. 1. Molecular structural formula of α,γ and δ-tocopherol homologues

Tocopherol homologues or isomers have a similar chemical structure but different physiological function. In the natural tocopherol homologue, the physiological activity rank is α,γ,δ-tocopherol. The order of antioxidatative property is δ,γ,α-tocopherol[2]. α-Tocopherol can be used in the fields of medicine, health products and cosmetics, but the other tocopherols can only be used as antioxidants in foods[3]. Many methods can be used to determine tocopherol, such as cerium sulphate method, spectrphotometry, fluorometry[4], gas chromatography[5,6] oscillopolarography, oscillopolarographic titration, liquid chromatography[5,7,8] near-infrared, spectrometry *etc*., but their sensitivity, accuracy and application scope are not the same and isomers can only be well separated by high performance liquid chromatography (HPLC). The working conditions of normal phase HPLC separation of plant samples are harsh[9], while in reversed phase HPLC there is less interference and many extraction and purification methods can be used[6-8]. At present, the commonly used pretreatment methods are using boiling isopropanol or saponification[6] since highly oxidizable

tocopherols affect the accuracy of the result. The objective of the study is to develop the solvent extraction and mixed column purification pretreatment to analyze green plant and foods samples for easily oxidizable and low content tocopherols.

EXPERIMENTAL

A Gilson 302, 303 HPLC with a variable wavelength detector, a chromatographic work-Station (Zhejiang University) and a rotary evaporator were used Polyamide fillers (200 mesh, Zhong Xie Joint Reagent Co., Beijing) were refluxed with alcohol for 2 times before use; silica (200 mesh) (Guang fu chemical Reagent Plant, Tianjin); methanol (chromatographic grade, ε-company, USA) and 30-60 ºC petroleum ether (AR) were used, The purity of α,γ,δ-tocopherol standards are 97.7, 99 and 97.6 %, respectively (SUPELCO,USA).

General procedure

Standard solution: 2.5, 3.1 and 8.9 mg of γ,α,δ-tocopherols, weighed by the decrement method, were dissolved in 10 mL of methanol, respectively and preserved and sheltered from light. Based on the sample content, solutions were diluted to the required concentrations when use.

Sample pretreatment: The needle of Longnan *Pinus tabulaeformis* Carr, collected from the grown forest in autumn-winter and that of Tianshui collected from the younger forest in spring, were ground into powder of 100 mesh. A 10 g sample was extracted with freshly distilled petroleum ether for many times. The extract was then dried in a rotary evaporator under nitrogen atmosphere and transferred to a 2 mL volumetric flask with methanol.

Sample purification: The mixed purification column was obtained by wet filling 7/8 of the effective volume of the glass column with polyamide and 1/8 of that with silica in turn. The pretreated sample was then added to the mixed column, eluted with methanol of 20 times of column volume. The eluent was collected at a flow rate of 2 mL/min purged with nitrogen gas and concentrated to 2 mL with methanol and then preserved and sheltered from light.

Detection method

Chromatographic conditions: Column: Hopsil ODS, 5 μ, 4.6 mm × 250 mm; mobile phase: 100 % methanol; detection wavelength: 300 nm, 0.2AUF; injection volume: 20 μL; flow rate: 1.0 mL/min.

RESULTS AND DISCUSSION

Optimal conditions for extraction and purification of tocopherols: Many methods have been reported for the extraction and purification of tocopherols **1-9**. *Pinus tabulaeformis* Carr is an evergreen plant rich in chlorophyll. Chlorophyll and tocopherol can be extracted by organic solvents. γ-Tocopherol is the main existing form and α-tocopherol is easily oxidizable and its content is very low in plant. The purification of these products is difficult. Methods reported by Yao[9] cannot meet the demand of the detection of samples rich in chlorophyll, while in the saponification method with the addition of antioxidant, pigment interference exists and it is difficult to detect the very low content and easily oxidizable α-tocopherol during the saponification. The mixed column purification developed in the current study not only eliminates the pigment interference but also avoids the oxidation of α-tocopherol and γ-tocopherol. According to the special properties of the experimental object, good purification effect was obtained by the use of a mixed column with a small amount of coarse-grained silica and polyamide. The ratio of the filler and the filling technique are the key to obtain the purification efficiency. After screening, the optimum ratio of polyamide and silica is 1:8, which gives the best recovery (Table-1).

TABLE-1
CONDITIONS FOR SPIKED SAMPLE OF STANDARD γ-TOCOPHEROL

Filler ratio (silica/polyamide)	Recovery (%)	RSD (%)
1/2	68.3	8.3
1/4	71.9	6.5
1/6	77.6	4.3
1/8	85.5	4.1
1/10	79.5	3.6

Selection of detection wavelength: Tocopherol homologues have similar chemical structure which can be detected at the range of 265-292 nm. By scanning the homologue under the range of 260-310 nm, it is found that 300 nm is the optimal detection wavelength.

Results of mixed standard and samples: Strong polar disruptors existed in the samples drift the flowing out of the peak. Peak positions of tocopherols are defined by adding a low content standard. Results are shown in Figs. 2-4 and Table-2.

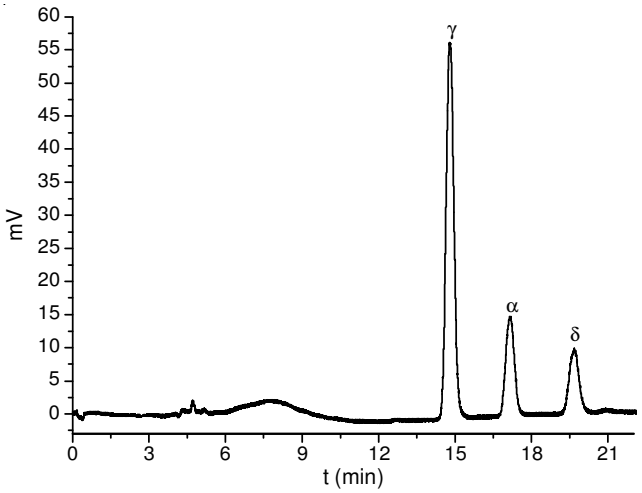

Fig. 2. Chromatogram of γ,α,δ-tocopherol standard

Linear relationship and detection limit: Under the experimental conditions determined by this method, the linear equation and correlation coefficient were obtained by the mass concentration and peak area of the mixed standard solution, with different concentrations with signal-to-noise ratio as the detection limit, the results obtained are shown in Table-3.

Accuracy and precision: Using known content *Pinus tabulaeformis* Carr sample as the matrix, the recovery was tested with 3 tocopherol homologues of different concentrations. The results obtained with 7 repeated determinations for each sample indicated the average recovery is 74-89 % with a

TABLE-2
CONTENTS AND PER CENT OF TOCOPHEROLS IN SAMPLES OF DIFFERENT AREAS

Sample	α-Tocopherol		γ-Tocopherol		δ-Tocopherol	
	Content (mg/kg)	%	Content (mg/kg)	%	Content (mg/kg)	%
Longnan sample	5.40 ± 0.25	2.4	1.18 ± 0.03	0.5	221.17 ± 20.15	97.1
Tianshui sample	3.16 ± 0.35	3.3	3.25 ± 0.14	3.4	89.93 ± 22.76	93.3

TABLE-3
REGRESSION EQUATION, LINEAR RANGE AND DETECTION LIMIT

Compound	Regression equation	Correlation coefficient	Linear range (μg/mL)	Detection limit
α-Tocopherol	$Y = 7.166 \times 10^4X + 1.282 \times 10^5$	0.9961	0.012-158.5	2.50 ng/mL
γ-Tocopherol	$Y = 9.527 \times 10^3X + 1.329 \times 10^5$	0.9968	0.13-150	0.26 ng/mL
δ-Tocopherol	$Y = 1.528 \times 10^4X - 5.014 \times 10^4$	0.9985	0.6-260	0.03 μg/mL

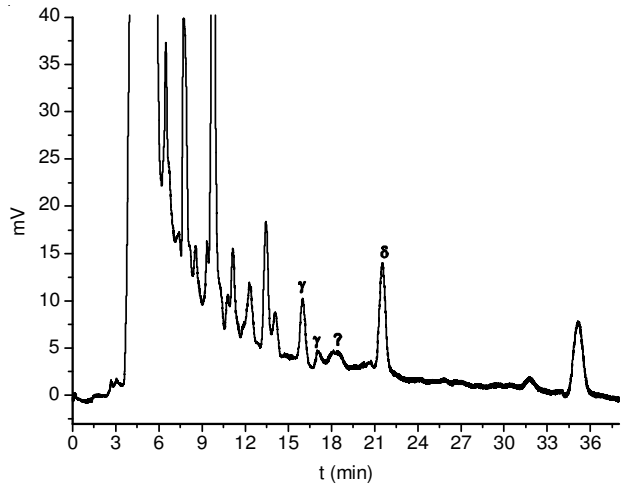

Fig. 3. Chromatogram of Tianshui sample

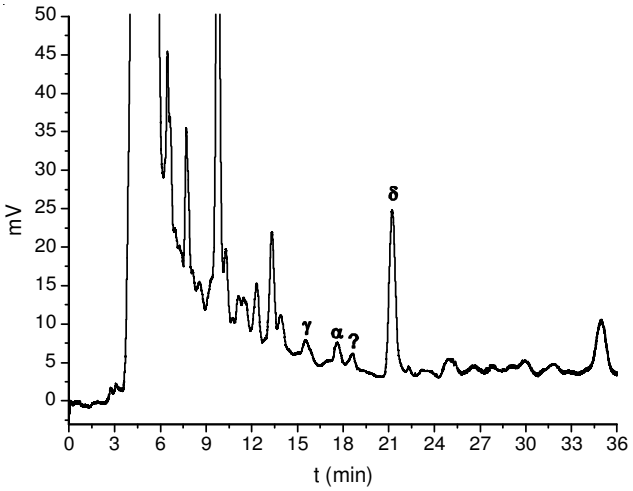

Fig. 4. Chromatogram of Longnan sample

RSD of 0.9-5.6 %, suggesting that results are in accordance with the methodology (Table-4). The sample of *Pinus tabulaeformis* Carr contains a lot of polar substance and the retention time shifts. Thus it can be determined by the addition of a low concentration standard (Table-5).

In present study, we only have the standard product and chromatograms of α,γ,δ-tocopherol. However, in the chromatograms of the samples (Figs. 3 and 4) are seen 4 separate peaks whether or not the fourth peak is β-tocopherol awaits further verification.

As shown in Table-2, the contents of α,γ,δ-tocopherols in the Longnan sample (grown trees) amount to 2.4, 0.5 and 97.1 %, respectively, of the total tocopherols, while in the Tianshui sample (young trees), these are 3.3, 3.4 and 93.3 %, respectively. Since δ-tocopherol has strong antioxidative activity and the contents of δ-tocopherol in the grown forest (221 mg/kg) are much higher than that in the young forest (89 mg/kg). Pinus needle of *Tabulaeformis* Carr of grown trees can be used as a natural antioxidant in food processing.

In the study, the tocopherol sample from Longnan was collected from grown forest in the winter season, while, the tocopherols sample from Tianshui was collected from young forest in the spring season. As shown in Figs. 3 and 4, the similar system's α,γ,δ of the tocopherols from the grown forest shall take the proportion of 2.4, 0.5 and 97.1 % of the whole content, but the rate of younger tocopherols is only 3.3, 3.4 and 93.3 %, which means that δ-tocopherol form with antioxidant capacity exist. The content of the similar tocopherols under different growing period can be exchange for each other. The high content of the high-activity γ-tocopherol, form from the younger period has shown the promoting function of γ-tocopherol. In the respect of the growing shown as Fig. 2.

Tocopherols which have physiological activity, are different in animals and plants. In animals, α-tocopherol possess the function of promoting the growth, while in the latter, γ-tocopherol plays the role. From Table-2, it is shown that the contents of α-tocopherol in the two samples are 2.4 and 3.3 % repectively, its stable existence in plants is probably related to antiageing and enhancing immunity. γ-Tocopherol in samples of Longna and Tianshui accounts for 0.5 and 3.4 %, respectively, showing a notable difference between old and young forests.

Conclusion

An HPLC method for the determination of contents of tocopherol homologues in green phants, using petroleum ether extraction and mixed column purification as the pretreatment techinque, has been established. The method eliminates the interference of pigments which have similar polarity with tocopherols and other substances with strong polarity. The method is reliable, repeatable, satisfactory in separation with a high recovery. The method is suitable to determine the low content and easily oxidizable tocopherol homologue in green plants.

TABLE-4
RECOVERY AND RSD (n = 7) FOR SAMPLES ADDED 3 TOCOPHEROL HOMOLOGUES

Sample	From Tianshui			From Longnan		
Parameters	Added standard (μg/g)	Recovery (%)	RSD (%)	Added standard (μg/g)	Recovery (%)	RSD (%)
α-Tocopherol	1.5	87.3	3.2	3.0	79.3	3.3
	3.1	89.7	2.5	5.4	83.1	1.5
	5.0	80.6	2.1	10.0	76.7	0.9
γ-Tocopherol	1.0	86.3	4.5	0.6	81.6	3.5
	2.5	83.4	3.7	1.2	84.5	3.1
	5.0	74.8	2.5	2.5	77.2	3.6
δ-Tocopherol	45.0	85.3	2.2	110	83.9	3.1
	89.0	74.6	2.7	220	76.7	3.3
	150.0	75.6	5.6	330	78.6	4.2

TABLE-5
RETENTION TIME AND PRECISION OF PEAK AREA (n = 7)

Homologue	Standard				Sample			
	tr (min)	RSD (%)	A	RSD (%)	tr (min)	RSD (%)	A	RSD (%)
α-Tocopherol	17.147	3.2	351032	2.5	17.320	4.0	61195	3.9
γ-Tocopherol	14.795	4.8	1141698	3.1	15.240	3.6	56675	4.5
δ-Tocopherol	19.673	5.1	249544	3.8	20.762	3.8	620123	3.6

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REFERENCES

1. M.X. Yuan, X.N. Huang, S.Y. Han, Y. Xi and H.B. Weng, *J. Biol.*, **22**, 4 (2008) *(Chinese)*.
2. J.Y. Wang, S.G. Zhu and C.F. Xu, Biochemistry, Higher Education Press (M-CHIP), Beijing, pp. 11/438-11/439 (2004).
3. G.H. Li, R.Y. Liu and J. Li, *J. Henan Univ. Technol. (Nat. Sci. Ed.)*, **30**, 6 (2009).
4. X.Y. Dong and Y.W. Yang, *Phys. Testing Chem. Anal. B*, **44**, 1 (2008).
5. W.M. Ma, X.H. Lin, W.D. Ma and Y.F. Zhou, *Sci.-Tech. Inform. Develop. Economy*, **19**, 4 (2009).
6. B.H. Yao, Study on Purification of Natural Tocopherols by Column Chromatography, Master Dissertation, Xi'an University of Technology, The, China (2007).
7. X.Y. Gao and W. Chen, *Henan Sci.*, **27**, 11 (2009).
8. C.F. Liu, Y.H. Liu, Y.L. Wang, W. Han, N.H. Deng and J.C. Li, *Prog. Modern Biomed.*, **9**, 1 (2009) *(Chinese)*.
9. Y. Luo and C.J. Sun, *Modern Prevent. Med.*, **36**, 5 (2009) *(Chinese)*.