Trade Science Inc.

December 2009 Volume 8 Issue 4

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 8(4) 2009 [580-584]

Determination of tocopherols and tocotrienols in tobacco seed oil by a normal-phase liquid chromatography

Marija A.Srbinoska[1,*], Vesna Gj.Rafajlovska[2]

[1]University St. Kliment Ohridski-Bitola, Scientific Tobacco Institute, Kicevska bb, 7500 Prilep, (REPUBLIC OF MACEDONIA)

[2]University Ss. Cyril and Methodius, Faculty of Technology and Metallurgy, Rudjer Boskovic 16, 1000 Skopje, (REPUBLIC OF MACEDONIA)

E-mail : srbinoska.marija@yahoo.com

Received: 31st August, 2009 ; Accepted: 10th September, 2009

ABSTRACT

Tocopherol and tocotrienol composition in the oil of four tobacco seed varieties from the Republic of Macedonia (NS-72, P-23, Y V 125/3 and Djebel 48) was determined. Oil content expressed as a yield of seed dry matter varied from 382 to 492 g kg^{-1}. A normal-phase HPLC method was optimized for the quantitative determination of tocopherols and tocotrienols in tobacco seed oil. Chromatographic separation was achieved using isocratic elution, LiChrosorb Si 60 column, 1.0 mL min^{-1} flow rate of hexane:dioxane:ethanol (97:3:0.1 v/v/v) as mobile phase and a fluorescence detector for effluent monitoring. The three tocopherols (α-, β-, δ-) and four tocotrienols (α-, β-, γ-, δ-) were present in all oil samples. The highest content of oil tocopherols and tocotrienols isomers is determined in Djebel 48 variety.

KEYWORDS

Tocopherols;
Tocotrienols;
Tobacco seed oil;
Normal-phase HPLC.

INTRODUCTION

The term tocochromanols is used to designate a family of related compounds, namely tocopherols and tocotrienols. The common structure, with a chromanol head and isoprenic side chain contribute to belong in the class of fat-soluble vitamins. Tocotrienols are distinguished from tocopherols by the presence of three unsaturations in the isoprenic side chain. Both, they have four naturally occurring forms (α-, β-, γ- and δ-) that differ in the number and position of methyl groups attached to the chromanol head[1].

Tocopherols have a strong antioxidant activity and increased oxidative stability of linoleic and oleic types of oil by 1.2 to 3 times[2]. The tocochromanol composition of plant seed oils can provide characteristic information in order to confirm taxonomical and phylogenetic relationships in the plant kingdom[3]. Tocopherols and tocotrienols posses a variety of potentially important biological activities, taken together, may be important in the future for the prevention and/or a treatment of cardiovascular diseases, cancer and neuronal degeneration[4].

The vegetable oils extracted from cereal grains, seeds, nuts and beans are the riches dietary sources of vitamin E. The distribution of tocopherols and tocotrienols in different plant oils varies greatly. Evaluation of the composition of fatty acids and triacylglycerol structure, as well as tocopherols, free fatty acids, and sterols content is of major importance for quality and

nutritional value of the oils[5,6].

For the determination of tocopherols and tocotrienols using HPLC, the most critical and time consuming step is the quantitative extraction of all their forms from seed samples. Traditional methods, for example percolation, exhaustive Soxhlet extraction, or direct extraction with boiling solvent under reflux are most often used[5,6,7].

A large number of published research papers have reported the separation and quantification of the tocopherols and tocotrienols from various seeds and oils[8,9,10]. This compounds are relatively non-polar and thus are chromatographed well using either normal-phase or reversed phase chromatography using different detectors[11,12]. The method of choice is the normal-phase HPLC and the fluorescence detector with silica column, most commonly used for tocopherol and tocotrienol analysis.

The seed of tobacco (*Nicotiana tabacum* L.) contains thin walled cells rich in oil. The oil content ranges from 33 to 40% of the total seed mass[13,20]. Tobacco seed oil is free from nicotine and its physical and chemical properties were comparable to other vegetable and edible oils[13]. For its usage as alimentary oil, the raw tobacco seeds oil must be first refined. The fatty acid composition of this oil is dominated by linoleic, oleic, palmitic and stearic fatty acids plus much less proportion of linolenic, palmitoleic, eicosenoic and heptadecanoic acids[13,15]. There are limited data of tocopherols and tocotrienols content in tobacco seed oil.

In the present investigation is studied the content of seed oil and composition of tocopherols and tocotrienols in tobacco varieties from the Republic of Macedonia. A normal-phase HPLC method has been optimized for the simultaneous determination of tocopherol and tocotrienol isomers in crude tobacco seed oil. In addition, Soxhlet extraction was working out to maximize total tocochromanols extraction yield.

EXPERIMENTAL

Plant material

Mature seeds of tobacco varieties P-23, NS-72, Djebel 48 and Y V 125/3 were collected in 2008 year, from the plants grown in the experimental field of the Scientific Tobacco Institute from Prilep, Republic of Macedonia. They were stored in an well-aired dark place for 30 days, than packed in paper bags and stored at room temperature.

In the tobacco seeds 5.7% moisture content was determined by drying on 105°C, to constant mass achievement[14].

The seeds were dried at 40°C for 4h using a ventilated oven, up to a moisture content of about 5 %. The dried seeds were grounded using Retsch ZM1mill (Germany), 0.25 mm sieve.

Reagents and standards

HPLC-grade *n*-hexane, 1,4 dioxane, ethanol and butylated hydroxytoluene (BHT) were purchesed from Merck (Damstadt, Germany).

Tocopherols (α-, β-, γ-, δ-) and tocotrienols (α-, β-, γ-, δ-) purchased from Supelco (Buchs, Switzerland) were HPLC-grade purity. Individual stock standards of all eights isomers were prepared in *n*-hexane, flushed with nitrogen and stored at 20°C, protected from the light. A stock standards mixture prepared in *n*-hexane was used in preparation of working standard mixture solution. Each standard solution was chromatographed in triplicate and calibration plots were constructed. Calibration plots were linear in range of 2.5-50 $\mu g\ mL^{-1}$ for all the tocopherol's and tocotrienol's isomers.

Extraction procedure

A 10 g ground tobacco seeds (0.0001 g accurately weight, 0.25 mm particles size) were extracted by *n*-hexane, using Soxhlet procedure[14]. To prevent oxidation of the analytes during extraction step 0.01% BHT was added, as is recommended from the several authors[5,8].

After 8h extraction (18-22 cycles/h), the extract was transferred to a tarred round flask and concentrate to dryness with vacuum rotary evaporator (Corning, UK) during 1h (50ºC, atm. pressure). The flask was than cooled in a dessicator and weighted. The steps of drying, cooling and weighting were repeated until the differences between two consecutive weights were smaller than 2 mg. The yield of crude oil estimated to the dry seed weight (d.m.) was taken to represent the oil content in seeds. Under the conditions of Soxhlet extraction method, BHT gives no interferences with any eluting compound.

Full Paper

The oil sample (1 g) was weighted accurately into 10 mL volumetric flask, diluted in *n*-hexane, filtered through a 0.2 x 10^{-3} mm disposable filter disk and than directly injected in the HPLC system.

HPLC analysis of tocopherols and tocotrienols

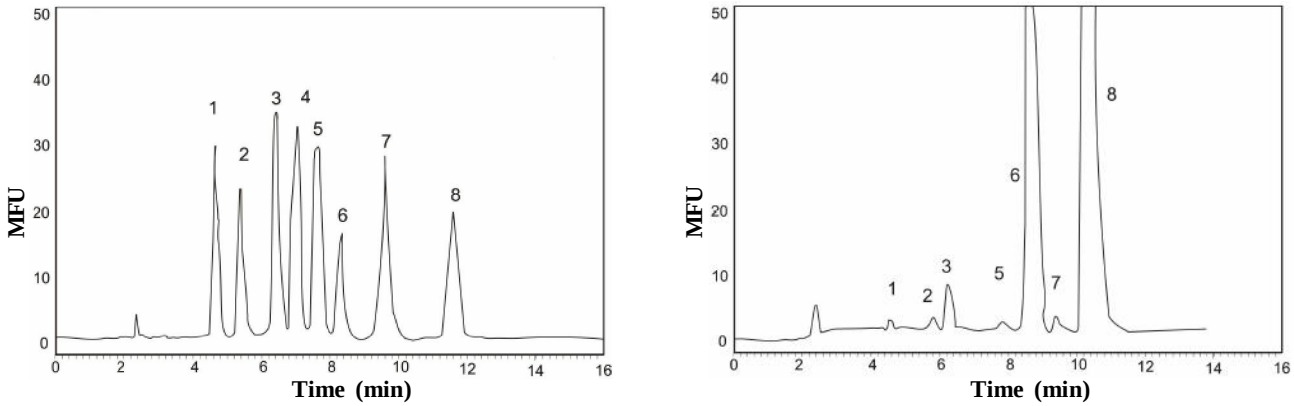
The normal-phase HPLC system consisted of a LDC Milton Roy Constant Metric III metering pump (Riviera Beach, Florida) equipped with an fluorescence detector RF 530 (Kyoto, Japan), Marathon 810 autosampler (Spark Holland, Emmen, The Netherlands) and LiChrosorb Si 60 (150mm × 4.6mm, 5µm) normal-phase column from Merck (Darmstadt, Germany) operating at room temperature.

The analyzed components were detected at an emission wavelength of 330nm and 295nm excitation wavelength. Isocratic elution was performed with mixture of solvents *n*-hexane:1,4 dioxane:ethanol (97:3:0.1, v/v/v) at flow rate 1.0 mL min^{-1}, and the injection volume was 20 µL. The mobil phase was de-gassed by sonification, 10 min.

The concentrations of tocopherol and tocotrienol isomers in oil samples were calculated in g kg^{-1} of oil weight using average peak area compared between standard and samples, after triplicate injections.

Each oil samples were chromatographed in triplicate and quantification was carried out using external standard calibration. Tocopherols and tocotrienols peaks from oil samples were identified by comparing their retention time to those of standards.

A chromatogram of a mixture of standard solutions of tocopherols and tocotrienols is shown on Figure 1.



1. α-tocopherol; 2. α-tocotrienol; 3. β-tocopherol; 4. γ-tocopherol; 5. β- tocotrienol; 6. γ- tocotrienol; 7. δ- tocopherol; 8. δ- tocotrienol

Figure 1 : Chromatogram of a standard solutions of tocopherols and tocotrienols (A) and of tobacco seed oil (B)

The retention times, reproducibility and correlation coefficient of the analyzed mixture standards of tocopherols and tocotrienols using HPLC method are given in TABLE 1.

TABLE 1 : Analytical characteristics of the reported method

Component	Retention time - Rt (min)	Reproducibility - CV (%, n=6)	Linearity ranges (µg mL^{-1})	Correlation coefficient - R^2
α-tocopherol	4.650	0.73	0.25-0.45	0.9990
α-tocotrienol	5.370	0.65	1.00-40.00	0.9985
β-tocopherol	6.433	0.52	0.50-25.00	0.9981
γ-tocopherol	7.003	0.39	30.00-90.00	0.9986
β- tocotrienol	7.633	0.37	0.50-25.00	0.9983
γ- tocotrienol	8.349	0.77	0.10-5.00	0.9981
δ- tocopherol	9.568	0.61	30.00-90.00	0.9988
δ- tocotrienol	11.604	0.48	0.10-5.00	0.9993

RESULTS AND DISCUSSION

The quantity of oil obtained by the extraction of different tobacco seeds varieties expressed as an oil yield is given in TABLE 1. The seeds of all investigated tobacco varieties have been found rich in oil (399.7-460.4 g kg^{-1} d.m.). Tobacco seeds of Y V 125/3 variety are characterized with high oil yield in comparison to the oil yield of other analyzed tobacco seeds. It can be established that our results for oil content of tobacco seed are in accordance with the data from previous published reports[13,20].

The analyzed tobacco seed oil samples contained predominantly tocopherol derivatives (54 % of the total tocochromanol content), whereas the tocotrienol derivatives were more less represented in all the samples. At the same time, some differences were observed among the relative proportion of total tocopherol and total tocotienol in oil from different seed varieties (TABLE 2). The high tocochromal content in oil (2.372 g kg^{-1}),

and content of tocopherols and tocotrienols, respectively of 1.274 and 1.098 g kg^{-1} is determined in Djebel 48 variety.

TABLE 2 : Total tocopherols and tocotrienols (g kg^{-1}) in oil and tobacco seeds oil content (g kg^{-1} d.m.)

Tobacco variety	Oil content	Total tocopherols	Total tocotrienols	Ratio of tocopherol/ tocotrienol
NS-72	399.7	1.017	0.880	1.16
P-23	427.0	0.902	0.746	1.21
Y V 125/3	460.4	1.079	0.912	1.18
Djebel 48	408.2	1.274	1.098	1.16

The extraction procedure and the normal-phase HPLC employed in this study gave an excellent separation of different tocopherol and tocorienols vitamers in shortest possible time, which provide a fast and reliable method for these determinations.

Normal-phase HPLC has been found capable of separating isocratically all the eight tocopherol and tocotrienol and it has been utilized in determining the distribution of these compounds in wide variety of oils, fats, and foodstuffs. The oil sample was dissolved in *n*-hexane and injected without concentration on a silica column[5,6].

Several silica columns and solvents for mobile phases have been used with normal-phase HPLC[5,8,16,17]. Hexane with small quantities of more polar solvent is the most frequently used mobile phase in the analysis of tocopherols using normal-phase HPLC. In the present work, the used mobile phase was a mixture of *n*-hexane, ethanol and 1,4-dioxane in proportion of 3 % (v/v/v), being the organic polar modifier tested. The fluorescence detector was selected for quantification purposes since it provide a higher sensitivity than the variable-wavelength absorbance detector[5,10,16,19,20].

The successful separation and identification of the tocopherol and tocotrienol isomers enable their quantification. The quantification of the isomers in tobacco seed oil was carried out using external standard calibration. A linear relationship between the concentration of the compounds and the fluorescence detector response was obtained under assayed conditions (TABLE 1). The use of fluorescence detector provides high sensitivity and selectivity.

For each compound, a 6-level calibration curve was constructed using the peak-area ratio between the tocopherol and tocotrienol isomers *versus* concentration of the standards μg mL^{-1}. The calibration curves were obtained by triplicate determination for each level used. The correlation coefficients were ranged from 0.998 to 0.999.

The estimated content of tocopherol and tocotrienol isomers are given in TABLE 3.

TABLE 3 : Tocopherol and tocotrienol content of tobacco seed oil (g kg^{-1})

Composition	Tobacco variety			
	NS-72	P-23	Y V 125/3	Djebel 48
α-tocopherol	0.011	0.007	0.009	0.012
β-tocopherol	0.034	0.025	0.066	0.058
δ- tocopherol	0.972	0.870	1.004	1.204
α-tocotrienol	0.081	0.105	0.098	0.158
β- tocotrienol	0.019	0.014	0.020	0.021
γ- tocotrienol	0.708	0.533	0.712	0.827
δ- tocotrienol	0.072	0.094	0.082	0.092

The three tocopherols (α-, β-, δ-) and four tocotrienols (α-, β-, γ-, δ-) were present in all oil samples. δ-tocopherol was the major compound, ranging from 0.870 to 1.004 g kg^{-1}. Unsaturated α-tocotrienol was found to be in those seed oils too. The presence of γ-tocopherol was not confirmed allowing us to think that this compound is present in traces or is degraded during extraction of seeds.

The Djebel 48 tobacco seed oil of is characterized with the highest content of all determined tocopherols and tocotrienols.

The results obtained are different in comparison with the data reported in other investigations, owing to the tobacco variety[20].

The chromatogram of tobacco seed oil (Figure 2B) shows a good baseline separation of the all tocopherol and tocotrienol isomers. It illustrates six identifiable peaks which correspondent to α-tocotrienol (4.895 min), α-tocotrienol (5.705 min), β-tocopherol (6.210 min), β-tocotrienol (7.725 min), γ-tocotrienol (8.619 min), δ-tocopherol (9.266 min) and δ-tocotrienol (10.338 min). The total analysis time was less than 12 min.

The proposed method in this work is a fast and reliable, allowing number of tocopherol and tocotrienol isomers determinations of in oil matrices to be carried out in one day.

Full Paper

CONCLUSIONS

The Soxhlet extraction procedure and the normal-phase HPLC employed and optimized in this study gave an excellent simultaneous separation of different tocopherol and tocotrienol isomers in tobacco seed oil.

The results obtained in the analysis of tobacco seed oil point to the existence of apparent differences in realtion to tocochromanols composition among tobacco varieties.

Nevertheless, more studies should be made in order to evaluate if the tocochromanol profile can be useful to discriminate different varieties of tobacco seeds, years of productions/or geographical origins.

REFERENCES

[1] R.R.Eitenmiller, J.Lee; 'Vitamin E, Food Chemistry Composition, and Analysis', Dekker; New York, **(2004)**.

[2] A.Kamal-Eldin; Eur.J.Lipid Sci.Technol., **108**, 12 **(2006)**.

[3] E.Bagci, L.Bruehl, K.Aitzetmuller, Y.Altan; Nord.J.Bot., **22**, 6 **(2008)**.

[4] A.Theriault, J.T.Chao, Q.Wang, A.Gapor, K.Adeli; Clin.Biochem., **32**, 309 **(1999)**.

[5] V.R.Preedy, R.R.Watson; 'The Encyclopedia of vitamin E', CABI, **(2007)**.

[6] L.M.L.Nollet; 'Food analysis by HPLC', Dekker, New York, **(2000)**.

[7] R.E.Majors; Trends in sample preparation, LC-GC, **19**, 11 **(2001)**.

[8] J.S.Amaral, S.Casal, D.Torres, R.M.Sebra, B.B.Oliviera; Anal.Sc., **21**, 1545 **(2005)**.

[9] G.Panfili, A.Fratianni, M.Irano; J.Agric.Food Chem., **52**, 14 **(2003)**.

[10] P.Gama, S.Casal, B.Oliviera, M.A.Ferreira; J.Liq.Chromatogr.Relat.Technol., **23**, 19 **(2000)**.

[11] A.Cert, W.Moreda, M.C.Pérez-Camino; J.Chromatogr.A., **881**, 131 **(2000)**.

[12] F.J.Rupérez, D.Martín, E.Herrera, C.Barbas; J.Chromatogr A., **935(1-2)**, 45 **(2001)**.

[13] N.Frega, F.Bocci, L.S.Conti, F.Testa; JAOCS, **68**, 1 **(1991)**.

[14] AOAC, 'Official methods of analysis' (16th ed.). DC: Association of Official Analytical Chemists; Washington, **(1995)**.

[15] M.Zlatanov, N.Menkov; Tutun/Tobacco, **52(9-10)**, 287 **(2002)**.

[16] J.Chun, J.Lee, L.Ye, J.Exler, R.R.Eitenmiller; J.Food Compos.Anal., **19**, 196 **(2006)**.

[17] A.Kamal-Eldin, S.Gorgen, J.Petterson, A.Lampi; J.Chromatogr.A, **881(1-2)**, 217 **(2000)**.

[18] W.D.Pocklington, A.Dieffenbacher; Pure Appl.Chem., **60(6)**, 877 **(1988)**.

[19] F.Manan; Journal of Islamic Academy of Sciences, **7(1)**, 34 **(1994)**.

[20] M.Zlatanov, M.Angelova, G.Atnonova; Bulgarian Journal of Agricultural Science (BJAS), **13**, 539 **(2007)**.