Journal of Chromatographic Science 2012;50:97–101
doi:10.1093/chromsci/bmr032

Article

Determination of Vitamin E Isomers of Grape Seeds By High-Performance Liquid Chromatography–UV Detection

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A simple analytical method for the determination of vitamin E isomers in grape seeds by reversed-phase high-performance liquid chromatography with UV detection is described. The method is based on a solid–liquid extraction separation on an ODS column, and the analytes are monitored at 295 nm with a UV detector. Tocopherols are extracted in n-hexane and directly injected onto the column without using any purification step, such as saponification, prior to the separation and determination. The chromatographic separation of tocopherols is achieved in 12 min with a mobile phase that consists of n-hexane and isopropyl alcohol (99.99:0.01, v/v). The method is reproducible and accurate, with respect to demonstrating a relative standard deviation between 2.57% and 3.30% ($n = 10$, for 500 ng/mL) and a relative error between 0.84% and 6.54% ($n = 10$, for 500 ng/mL), respectively. The theoretical limits are estimated as 25 ng/mL for α-tocopherol, 43 ng/mL for γ-tocopherol, and 83 ng/mL for δ-tocopherols. The method is then applied for the determination of tocopherols in grape seeds grown in Turkey. The amounts of tocopherols are calculated by using the standard addition method.

Introduction

Vitamin E is a generic name used to describe a group of compounds (namely tocopherols and tocotrienols) that share a common structure with a chromanol head and an isoprenic side chain. They have four possible naturally occurring forms (α-, β-, γ-, and δ-tocopherols and tocotrienols) that differ in the number and the position of methyl groups attached to the chromanol head (1). They are lipid-soluble anti-oxidants synthesized only by photosynthetic organisms and extracted as oils from plant material (2–4). The biological activity of tocopherols and tocotrienols are mainly attributed to their antioxidant activity in inhibiting lipid peroxidation in biological membranes. α-Tocopherol is the most active form of vitamin E and accounts for almost all vitamin E activity in living tissue (5). The most widely accepted function of vitamin E is its chain-braking anti-oxidant activity. The α-tocopherol is capable of quenching free radicals, which protects phospholipids and cholesterol against oxidation and subsequent breakdown to potentially harmful chemically reactive products (6). α-Tocopherol has also been demonstrated to have beneficial effects on the oxidative and color stability of red meats, such as beef (7). The subject of natural anti-oxidants continues to captivate the interests of food and biomedical scientists because of the reports that diets rich in plant anti-oxidants derived from fruits and vegetables are associated with lower risks of coronary heart disease and cancer (4, 8). This vitamin is important for fruit itself because it protects unsaturated lipids from oxidation and allows for their conservation. Vitamin E plays the same role in the human body. There is much evidence that antioxidants from fruits and vegetables, such as tocopherols, play an important role in the prevention of cancer, inflammatory activities, and cardiovascular disease (9).

Several methods have been described in the literature for the determination of vitamin E, as tocopherols (10–18) and as both tocopherols and tocotrienols (19–26) in seeds, seed oils, leaves, flowers, fresh beans, spice, nuts, drinks, milk, oil, and meat. High-performance liquid chromatography (HPLC) is the technique of choice for determining the different tocopherols. Supercritical CO_2 has been used to improve the extraction of α-tocopherol from grape seeds (20, 27). The isolation of lipid-soluble vitamins in this type of sample generally involves alkaline hydrolysis of fatty material followed by extraction of the vitamins from the un-saponifiable material using organic solvents (10, 12, 19, 20). This extract is then injected into a chromatographic system for the separation and determination of the analytes.

The aim of this study was to establish a simple analytical method for the determination of tocopherols by using an HPLC–UV method with an ODS column. The extraction procedure and availability of the instrumentation make it useful in most analytical laboratories. The method was then applied for the determination of tocopherols in grape seeds grown in Turkey. Determination of tocotrienols was out of the scope of this study. In the extraction procedure, the saponification step, which is complex, time-consuming, and requires a lot of organic solvent, was not applied to the samples. The extracts were directly injected onto the HPLC column after a solid–liquid extraction (*n*-hexane extraction), without using any further purification step.

Materials and Method

Reagents

All chemicals were of chromatographic and analytical reagent grade. α-, γ-, and δ- tocopherols were purchased from Sigma (St. Louis, Steinheim, Germany), and *n*-hexane and isopropyl alcohol were from Merck (Darmstadt, Germany). Ultrapure de-ionized water, which was used for cleaning all glassware and other equipment throughout the experimental work, was purified by a Millipore ultrapure water purification system to a specific resistance of 18 MΩcm or greater (Merck, Millipore, Molsheim, France). The stock standard solution, containing 500 μg/mL tocopherols, was prepared in hexane, and the standards used were prepared by dilution of the appropriate volume of the stock in colored bottles and stored at −20°C. Samples (21 different kinds of grapes) were obtained from Manisa Viniculture Research Institute.

Instrumentation

HPLC analysis was performed on a PerkinElmer series reverse phase HPLC system (PerkinElmer, Norwalk, CA, USA), which consisted of a microprocessor controlled PerkinElmer model 200 eluent delivery pump and a fixed wavelength PerkinElmer model 200 UV-vis spectrophotometer detection system. Samples were injected via a Rheodyne Model 7725i injector valve fitted with a 50-μL constant volume injector loop. Separation was achieved on a 25 cm × 4.6 mm, i.d. ODS column with 5 μm octyldecylsilane packing material maintained at room temperature. Data collection and handling were carried out by PerkinElmer software (TotalChrome v6.2.1).

Procedure

Chromatographic procedure

The chromatographic determination of tocopherols was based on peak areas of tocopherols monitored at 295 nm with a UV detector. Quantification of tocopherols in samples was carried out by using the standard addition method. The mobile phase was a mixture of 99.99% hexane and 0.01% isopropyl alcohol, and the flow rate was 1.5 mL/min. Standards and samples were injected via a 50-μL injector loop, and separation was performed on an ODS column. It is known that the concentration of tochopherols, especially gamma-T and delta-T, are low. Therefore, to detect these compounds, either a higher injection volume or higher sample weight should be utilized. A higher injection volume does not require any extra work, organic solvent, or time consumptions. In contrast, a higher sample weight requires a higher volume of the organic solvent used and extraction time. For that reason, instead of increasing the sample weight, a 50 μL injection volume, which is more economical when considering the amount of organic solvent and time needed, was preferred and chosen for this study.

Preparation of standard curve

The stock standard solution containing 500 μg/mL tocopherols was prepared in pure *n*-hexane. The standard solutions of 250, 500, 750, and 1000 ng/mL for each tocopherol, which were used to obtain the calibration graphs, were prepared by dilution of the stock to an appropriate concentration in n-hexane in colored bottles. Triple injections were made for each concentration, and the average peak areas were calculated from the triple injections. The calibration graph was worked out by using the average peak areas as a function of concentrations.

Samples preparation and extraction

The grape seeds were initially separated from the plump of the grapes, collected in a plastic bag, and then stored in deep freezer at −20°C. Prior to the extraction, the grape seed samples were taken out from the deep freezer and left at room temperature for 24 h for drying. They were then ground in a mortal-and-pestle to have homogenized samples. The dried and homogenized samples were accurately weighed 3.0000 g (± 0.0010 g, three parallel samples were weighed for each sample) and were transferred into a two-necked glass balloon. The tocopherols in the grape seeds were extracted with 25 mL *n*-hexane for 1 h on a stirring plate in a dark and nitrogen-rich atmosphere. The dark and nitrogen-rich atmospheric conditions were necessary to prevent the loss of tocopherols by oxidation from light and oxygen. The nitrogen-rich atmosphere was created by passing nitrogen gas through a two-necked glass balloon during the extraction. The mixture was filtered through filter paper. The residual was then extracted for second time with 25 mL of *n*-hexane and filtered. These two extracts were combined in a 50-mL volumetric flask and the volume was completed to 50 mL with *n*-hexane. Further extraction (third time extraction with n-hexane) did not improve the recovery and the amount of the tocopherols extracted from the grape seeds. Two successive extractions were sufficient to take all of the tocopherols into the extraction solvent. The extract was then transferred into a brown glass bottle and stored at −20°C, until to analysis.

Results and Discussion

Optimization of the methods

The chromatographic system was first conditioned by flowing the mobile phase through the column until obtaining a constant pump pressure and absorbance reading. After that, the chromatographic system was optimized for the composition of the mobile phase, which consisted of *n*-hexane and isopropyl alcohol. *n*-Hexane, including isopropyl alcohol between 0.001 and 2% (v/v), was studied for the separation of the tocopherol standards. The reason for using isopropyl alcohol in *n*-hexane was to adjust the polarity of the mobile phase. Experimental studies demonstrated that the analysis time was too short at a higher isopropyl alcohol percentage (2%) because higher isopropyl alcohol percentages increase the polarity of the mobile phase and decrease the distribution coefficient of the tocopherols due to increasing the solubility of tocohperols in the mobile phase. At lower isopropyl alcohol percentages (0.001%) in the mobile phase, the analysis time was too long because lower percentages decrease the polarity of the mobile phase and increase both the distribution coefficient of the tocopherols and the interaction between the stationary phase and tochopherols, due to decreasing the solubility of the tocopherols in mobile phase. Consequently, *n*-hexane with 1.5% of isopropyl alcohol was chosen as the optimum mobile phase composition for the separation of tocopherols standards. Nevertheless, the separation of tocopherols in real samples was not satisfactory with this mobile phase including 1.5% isopropyl alcohol in *n*-hexane because the tocopherols and the co-extracted compound were eluted together by the mobile phase, and their peaks were overlapped. Therefore, a second optimization work was necessary to separate the tocopherols and the co-extracted compound peaks, in which the grape seed extracts were used instead of tocopherols standards. Experimental results show that 0.01% of isopropyl alcohol was a sufficient amount to keep the polarity of the mobile phase in optimum condition to separate the tocopherols and the co-extracted compounds peaks, and the remains of the interaction between the tochopherols and the stationary phase lower than those of the tocopherols and the mobile phase.

Figure 1 shows the chromatogram of the tocopherol at 1.5 mL/min flow rate of the mobile phase. The analysis time

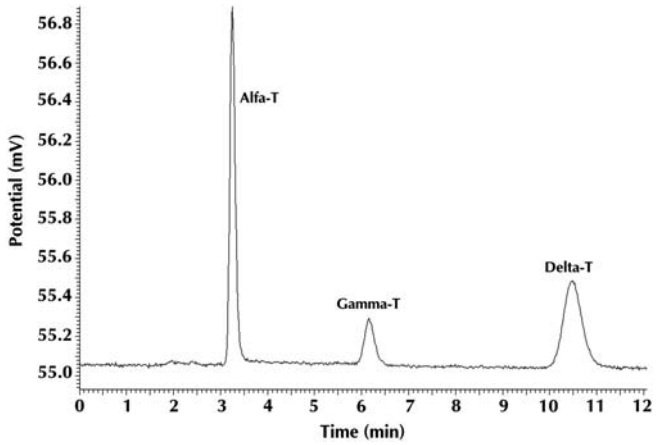

Figure 1. The chromatogram of the standard tocopherols, separated on ODS column with 99.99% *n*-hexane and 0.01% isopropyl alcohol mobile phase at 1.5 mL/min flow rate.

was less than 12 min at the optimum conditions. Although tocopherols contain four isomers (α-, β-, γ-, and δ-tocopherol), only α-, γ-, and δ-tocopherol were analyzed. It is important to note that the positional β- and γ-tocopherol isomers cannot be completely resolved by reversed-phase HPLC (9, 12, 24). Generally, β-tocopherol levels in grape seed samples are lower than those of γ-tocopherol. Only in a few cases, the β-tocopherol concentration is important with respect to that of the γ-tocopherol (1, 12). For these reasons, β-tocopherol was not included in the standard solutions, and the peak around 6.5 min was evaluated as γ-tocopherol in this study.

The linearity of the method was studied with the solutions of pure tocopherols standards. Series standard solutions containing the three tocopherols were prepared over a wide concentration range (250, 500, 750, and 1000 ng/mL). Three replicated injections were made to ensure that accurate responses were generated. The average peak areas were used to obtain the calibration graph that was plotted using the peak areas as a function of concentrations. The linearity of the method was determined by plotting a straight line through the data points. A linear calibration curve of $y = 11523.00x + 172.95$ with a correlation coefficient (R) of 0.997 for α-tocopherol and $y = 8406.40x + 516.03$ with a 0.998 for γ-tocopherol and $y = 11743.00x + 1651.40$ with a 0.999 for δ-tocopherol were obtained at 295 nm. The linearity of the method for the determination of three tocopherols was very good.

Reproducibility and accuracy of the method were studied by injection of the standard solution of 500 ng/mL of α-, γ-, and δ-tocopherols in the same solution. The reproducibility and the accuracy of the method were measured from 10 replicated injections. The relative standard deviations (RSDs) of the method were 2.57%, 3.30%, and 2.88% for reproducibility, and relative errors were 6.54%, 5.60%, and 0.84% for accuracy of α-, γ-, and δ-tocopherols. The reproducibility was also studied by injecting the same samples 10 times. The RSD obtained for the samples were within those of the standards.

The theoretical limit of detection of the method was calculated to be approximately 25 ng/mL for α-tocopherol, 43 ng/mL for γ-tocopherol, and 83 ng/mL for δ-tocopherols for a signal-to-noise ratio of 3:1.

Recovery studies were performed by using the spiked samples. The grape seeds used for the recovery studies were first extracted in *n*-hexane (twice) and dried at room temperature for 1 h. After that, the grape seeds were spiked with tocopherol standards in a known volume and in three different concentrations. The same extraction procedure was then applied, and the extracts were analyzed. The recovery for the tocopherols for three different concentrations was between 91% and 102%. The recovery results show that the extraction procedure applied is adequate and minimized the loss of tocopherols during the extraction step.

All of the experimental data given herein shows that the HPLC–UV method with the ODS column is rapid, accurate, reproducible, and can be applied for the determine tocopherols in real samples.

Determination of tocopherols in grape seeds

Prior to the determination of tocopherols with the proposed method, the tocopherols must be extracted from the grape seeds. Grape seeds contain 15% oil on average, and for that reason, an extraction procedure described in literature was followed. In this method, tocopherols were extracted with n-hexane, and then a saponification step was applied to separate tocopherols from the fatty acids. The saponification step takes a long time, uses a high amount of organic solvent and heat, and causes a significant loss of tocopherols. The loss of tocopherols from heat have been described in the literature and are significant (28). For these reasons, it was questioned whether to separate the tocopherols peaks from the fatty acid peaks with an ODS column in an optimum mobile phase composition or not, if the saponification step was bypassed? Therefore, the extraction was only made with *n*-hexane, and the extract was directly injected onto the ODS column. The Figure 2 shows the chromatogram of the tocopherol extracts with and without saponification of the same grape seed. Tocopherols peaks are well separated from the fatty acid peaks and do not interfere with the separation of tocopherols with the proposed extraction method; thus, the saponification step is not needed. It is believed that peaks around 2, 3.7, and 8.1 min retention times belong to the co-extracted fatty acids or tocotrienols, such as oleic and linoleic acids, which have been reported to be the main composition of fatty acids of grape seed (21) or α-, γ-, or β-tocotrienols. No attention was paid to these peaks, because they do not interfere with the tocopherols peaks. If Figure 1 and 2 are evaluated together, the differences between the retention times of the gamma-T and the delta-T for the standards and the real samples can be noticed. The differences in the retention times of the gamma-T and the delta-T are about 0.5 and 1.2 min for the standard and real samples, respectively, and increases with the analysis time. This is due to the column pressure. Although exactly the same experimental conditions are applied, somehow the column pressure shows different pressure between days. This situation does not present a serious problem because identification is made by the standard addition method and, most importantly, all tocopherols for the real samples and after the standard addition were eluted in exactly the same retention times in Figure 2.

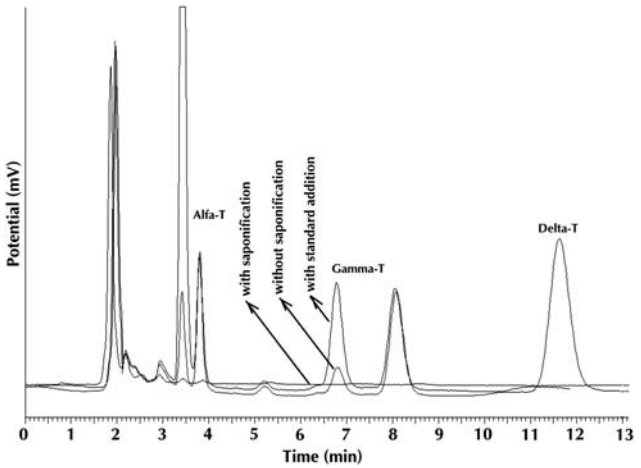

Figure 2. The overlaid chromatogram of grape seeds extracts with and without saponification of the samples and standard additions of tocopherols to the extract obtained without saponification.

Table I
Tocopherols Content of Grape Seeds Grown in Turkey

Name of grapes	α-Tocopherol (μg/g)	γ-Tocopherol (μg/g)	δ-Tocopherol (μg/g)
Ag Uzumu	9.03	–*	–*
Alfons	19.14	–*	–*
Aliconte Bauched	22.40	1.39	–*
Besni Uzumu	36.42	1.72	–*
Bogazkere	4.39	–*	–*
Cabernet Sabinon	11.05	2.64	–*
Calkarasi	18.03	–*	–*
Hamburg Misketi	31.06	–*	–*
Horoz Karasi	33.47	2.20	–*
Italyan Uzumu	2.31	–*	–*
Kalecik Karasi	27.79	–*	–*
Kardinal	35.19	–*	–*
Kizlar Tahtasi	15.50	1.67	–*
Kores Uzumu	22.73	–*	–*
Merlot	26.00	–*	–*
Okuzgozu	24.22	–*	–*
Pembe Germe	3.93	–*	–*
Razaki	7.20	–*	–*
Senirkent	25.63	–*	–*
Syrah	14.75	10.02	–*
Trakya Ilkeren	28.35	–*	–*

* – =Under the detection limits

In Figure 2, three chromatograms of the same grape seed are overlaid. One chromatogram belongs to the grape seed extract obtained with the extraction procedure (without saponification, middle line), one to the same grape seed extract obtained with saponification (lowest line), and the other to the standard of the tocopherols (highest line). According to the graphs in Figure 2, most of the tocopherols have been lost during the saponification step, most likely by the oxidation or decomposition by heat or adsorption on saponifiable material of tocopherols. For the extract obtained without saponification, the α- and γ-tocopherols peaks are obvious. After the saponification step, the α-tocopherol peak is very little, and γ-tocopherol peak is not visible.

Under carefully chosen conditions, such as the organic solvent, the ethanol concentration, and the level of lipids in the saponification media and adding some antioxidants, such as butylated hydroxytoluene, ascorbic acid, and pyrogallol (each one alone or combination) the losses of tocopherols could be overcome (1, 29–31). Nevertheless, the losses of tocopherols could be overcome; the saponification is time and organic solvent consuming and complex. Consequently, the proposed extraction procedure without saponification minimizes the losses of tocopherols, time, and organic solvent consuming.

Twenty-one different types of grape seeds, which were cultivated in Turkey, were extracted with the proposed extraction procedure and analyzed by the HPLC-UV method with an ODS column. The tocopherol content of the seeds of the 21 different kinds of grapes are given in Table I. Although all grape seeds contain α-tocopherol, only six of the grape seeds contained γ-tocopherol. δ-Tocopherol could not be detected in the seeds because δ-tocopherol is the least abundant isomer of vitamin E and was probably under the detection limit of the method. As seen in the Table I, α-tocopherol is the major tochopherols in the all samples analyzed, ranging from 2.31 μg/g (in Italyan uzumu) to 36.42 μg/g (in Besni uzumu). γ-Tocopherol was found in a low level, ranging from 1.39 μg/g (in Alicante Bauched) to 10.02 μg/g (in Syrah). Only a few studies were found on the tocopherol content in grape seed in the literature because most of the studies published were focused on grape seed oil. The content of α-tocopherol is in good agreement with the results given in the literature for grape seeds; in one study it was between 0.8 mg/100 g to 2.4 mg/100 g (25), and in another study it was between 20.51 mg/kg to 34.01 mg/kg (32), although the origins of the grape seeds analyzed are different. However, it has been reported that grape seed oils containing α-tocopherols ranged from 7.76 to 27.2 mg/100 g oil (20), from 18 to 229 mg/kg oil (21), and from 32.8 to 57.8 mg/100 g oil (26), which are more or less 20 times higher than those of the grape seed analyzed in this study. This is an expected result, because tocopherols have been concentrated in grape seed oils.

These results could be a useful guide to consumers who wish to consume tocopherol-rich grapes with their seeds, as a good contributor to a balanced intake of vitamin E. The results also provide important information about the amount of tocopherols in grape seed grown in Turkey.

Conclusion

It is proven that the extraction and the chromatographic methods presented herein are suitable for the routine determination of tocopherols in grape seeds and can be applied to the determination of tochopherols in other materials. The extraction procedure included only *n*-hexane extraction without saponification, saving an enormous amount of solvent, time, analysis steps, and preventing the loss of tocopherols. With the exception of δ-tocopherol, which could not be detected, the direct *n*-hexane extraction and the chromatographic separation showed to be efficient in achieving high repeatable extraction and analysis for all samples. The most abundant form of tochopherol in grape seeds is the α-tocopherol, ranging from 2.31 to 36.42 μg/g. The differences in the amount of tochopherols are significant, and this information can be used is an easier way to differentiate grape varieties.

Acknowledgements

The authors are grateful to Scientific Research Projects Department, University of Celal Bayar for their financial support.

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