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Separation of tocopherols by nano-liquid chromatography

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Abstract

Nanoliquid chromatography (nano-LC) was used for the separation of tocopherols (δ-, γ-, α-TOH), α-tocopherol acetate (α-TOH-Ac) and an antioxidant compound, namely butylated hydroxytoluene (BHT) used to prevent TOHs autoxidation. The separation was carried out in a fused silica capillary of 100 μm I.D. and 375 μm O.D. packed in our laboratory with RP_{18} silica stationary phase of either 5- or 3-μm diameter (23-cm long). The mobile phase was composed by mixtures of methanol (MeOH), acetonitrile (MeCN) and water. Typical analyses time for the separation of all the five components of the mixture were 6–9 min depending on the composition of the mobile phase. Efficiency and resolution were strongly influenced by the particle diameter and the highest R_s and N/m values were observed using 3-μm RP_{18} particles. Experiments performed with capillaries packed with 3-μm RP_{18} particles provided good limit of detection (LOD) and limit of quantification (LOQ) (for δ-, γ-TOH, α-TOH-Ac were 4 and 8 μg/ml, while for α-TOH were 6 and 10 μg/ml, respectively). The optimized method was applied to extracts of serum and pharmaceutical preparation containing α-TOH and α-TOH-Ac.

Keywords: Nano-LC; Tocopherols; Vitamin E; Drugs; Serum

1. Introduction

Tocopherols (TOHs) are compounds belonging to a class of phenol derivatives also named Vitamin E characterized by high hydrophobicity due to their chemical–physical properties. These compounds possess in their structure a chromanol head and a phytyl tail. The antioxidant activity of such vitamins is based on the ability of blocking the propagation of radical reactions triggered by the reactive oxygen species (ROS) [1]. Vitamin E, including TOHs as well as tocotrienol compounds are usually ingested with the normal diet and the analogs α- and γ-TOH having similar structures can be monitored in human blood

Abbreviations: TOHs, tocopherols; α-TOH-Ac, α-tocopherol acetate; Nano-LC, nano-liquid chromatography

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and tissues. It has been shown that α-TOH has the highest activity against lipid peroxidation induced by peroxyl radical [2] and is the most abundant TOH in vivo because selectively binds proteins [3]. Since TOH compounds are assumed in variable amount with the diet and can also be taken in pills [4], their concentration levels in plasma samples may be quite high.

Experiments carried out in both animal and in vitro showed that TOHs prevent the oxidation of lipoproteins and cellular lipid components involved in atherosclerosis and longevity [5,6]. The role of Vitamin E has been investigated in infection and neoplasia [7,8].

Analytical methods so far employed for the analysis of TOHs include high performance liquid chromatography (HPLC) (using both normal and reversed phase) [9–11], gas chromatography (GC) [12] and recently capillary electrochromatography (CEC) [13,14].

In the present paper, we investigated the potential of nano-LC for the separation of several TOHs compounds, namely δ-, γ-, α-tochoperol and α-TOH-acetate, in presence of an antioxidant compound (butylated hydroxytoluene, BHT).

The nano-LC technique was investigated by several groups for chromatographic separations in packed capillaries with small I.D. (10–150 μm) employing flow rates in the range 10–1000 nl/min [15–20]. The use of nano-LC can offer advantages over classical HPLC and among them the higher efficiency achievable and the lower consumption of mobile phase is very attractive because the reduced expenses for both solvents and their waste. On the contrary at the moment the instrumentation and the packed columns used are very expensive.

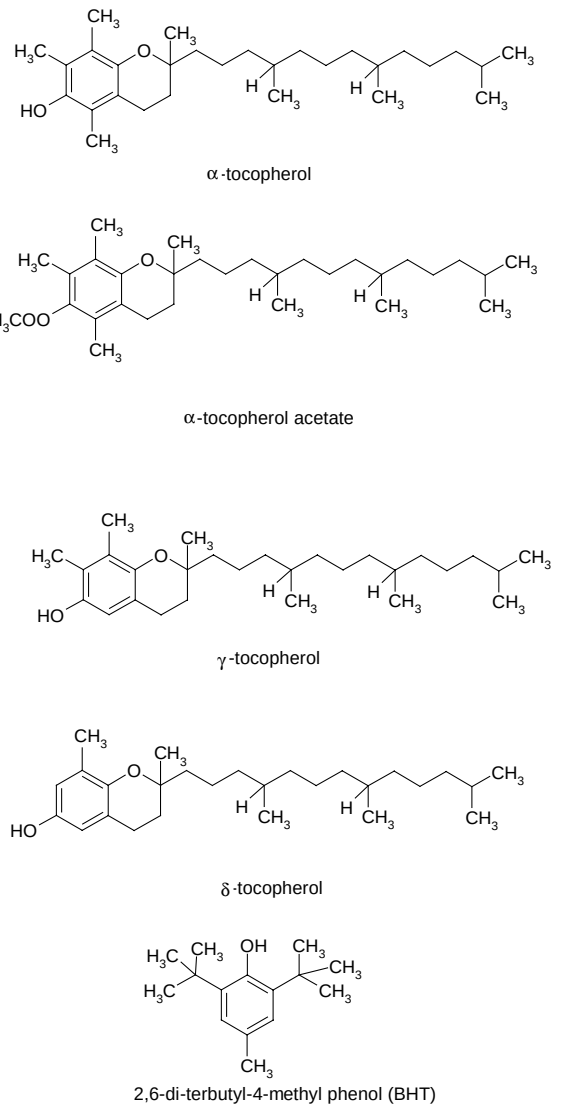

Fig. 1. Chemical structures of studied compounds.

2. Experimental

2.1. Reagents and chemical

Methanol (MeOH) and acetonitrile (MeCN) were purchased from BDH (Poole, UK). δ-, γ-, α-TOH, α-tocopherol acetate (α-TOH-Ac) and butylated hydroxytoluene (BHT) were from Sigma (St. Louis, MO, USA) (for their chemical structures see Fig. 1). All chemicals were of analytical grade and used as received. Mobile phases were daily prepared by mixing the appropriate volumes of organic solvents and water. Stock solutions of standard samples were prepared in hexane (10 mg/10 ml); an exact volume of stock solution was dried under nitrogen and dissolved in the appropriate volume of a mixture MeCN/MeOH (70:30 v/v) and injected for the nano-LC analysis.

2.2. Sample preparation

Vitamin E was extracted from human serum with hexane as previously described [14]. α-Tocopherol acetate was extracted from a commercial nutritional

preparation in a similar way used for the extraction of serum. Briefly three tablets of nutritional preparation were accurately weighed, finely ground to a powder and thoroughly mixed; an aliquot of 303.4 mg was taken and supplemented with n-hexane (2 ml × 5 times) for extraction. The fractions of hexane extract were collected and 1.5 ml solution was dried under a nitrogen stream and the solution reconstituted with 1.5 ml of MeCN–MeOH (70:30 v/v).

2.3. Instrumentation

Experiments were carried out using a LC 10 Perkin Elmer pump delivering the mobile phase in a constant-pressure mode (5–35 MPa); the pump was connected with a 10 cm × 1 mm I.D. stainless steel column containing the mobile phase and than to a modified reodyne injector.

Fused silica capillaries, 100 μm I.D. × 375 μm O.D., were purchased from Composite Metal Services (Hallow, Worcestershire, UK) and packed in our laboratory with LiChrospher 100 RP_{18} 5-μm particles (Merck, Darmstadt, Germany) or with ChromSpher three C_{18} 3-μm particles (Varian, Darmstadt, Germany); the slurry was prepared suspending the stationary phases in methanol and acetone, respectively and the pumping solvent was water.

The packing procedure was similar to that previously used. Briefly one end of the capillary was connected to a mechanical temporary frit and packed with the stationary phases for about 40 cm, pumped for 30 min with distilled water and the two frits prepared. The capillary was cut at the desired length, equilibrated with the mobile phase; the outlet frit was covered with a layer of epoxy resin.

The capillaries were 66-cm long, 23-cm packed and the distance between the first frit and the detector window was 56 cm. Detection was done on column at 205 nm with a Spectra 100 photometric absorbance detector (Thermo Separation Products, S. Jose, CA, USA). The data from the detector were acquired with a Shimadzu CR5A Chromatopac integrator (Kyoto, Japan).

3. Results and discussion

TOHs are compounds belonging to phenol class characterized by a relatively high hydrophobicity due to the side groups on the aromatic ring and to the relatively long aliphatic chain, consequently they cannot be dissolved in aqueous solvent. Such type of compounds were separated and analyzed by CEC employing fused silica capillaries packed with RP_{18} silica particles and eluting with polar organic mobile phase (acetonitrile-methanol) containing ammonium acetate [14].

Based on our previous work in CEC we selected an MeCN–MeOH (70:30 v/v) mixture as mobile phase for the separation of δ-, γ-, α-TOH, α-TOH-Ac and BHT standard mixture.

Fig. 2 shows the chromatogram of the separation of the five analytes by nano-LC. As can be observed the separation was obtained in a relatively short time (less than 4.5 min), however the baseline resolution of α-TOH, α-TOH-Ac was not achieved.

In order to find the optimum experimental conditions we studied the effect of the mobile phase composition changing the MeCN/MeOH ratio (in the range 100:0–0:100% MeCN–MeOH). Retention time and ln *k* of BHT were not influenced remarkably by the changing of the mobile phase while for the other ana-

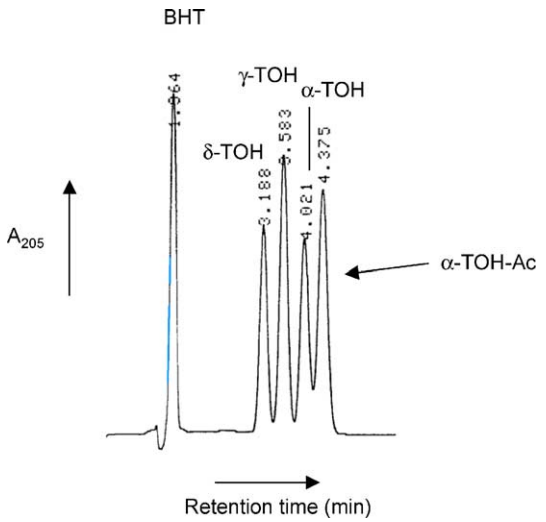

Fig. 2. Chromatogram of the separation of δ-, γ-, α-TOH, α-TOH-Ac and BHT obtained by nano-LC in a fused silica capillary packed with RP_{18} silica stationary-phase 5-μm particles. Capillary 100 μm I.D. × 66 cm (length), 23 cm packed and the distance between the first frit and the detector window was 56 cm. Mobile phase MeCN–MeOH (70:30 v/v) delivered at 17 MPa constant. Samples were at concentration of 80 μg/ml except for BHT that was 50 μg/ml; volume injected about 40 nl. Detection at 205 nm.

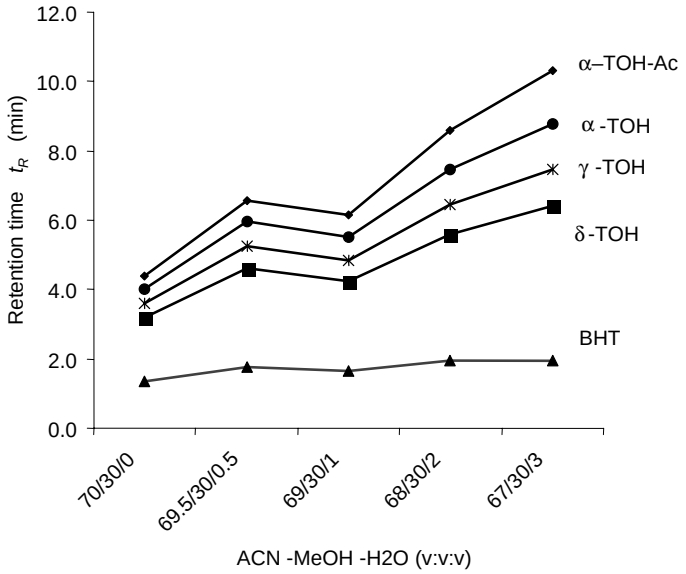

Fig. 3. Effect of water concentration on retention time of studied compounds. Experimental conditions as reported in Fig. 1 and text.

lytes t_R decreased by increasing MeOH concentration up to 70% and then raised. ln *k* decreased by increasing MeOH concentration. Decreasing the MeCN concentration caused an increase of α-TOH/α-TOH-Ac resolution while the other analytes lost partly the separation from each other (results not shown).

In order to obtain the baseline resolution of the five analytes, we supplemented the mobile phase with low concentration of water and decreased the MeCN content. Fig. 3 shows the effect of mobile phase composition on t_R of studied compounds. As can be observed the increase of water concentration (associated with a decrease of MeCN concentration) caused an increase of retention time for all analytes except for BHT. This behavior is typical of RP_{18} stationary phases. Baseline resolution of the five analytes was achieved at water concentrations higher than 1% (see Fig. 4).

In order to increase the efficiency a new capillary (with the same I.D.) was packed with a RP_{18} stationary phase with 3-μm particles of different origin than those used in the above-discussed experiments. Due to the smaller diameter of the particles, we increased the constant pressure applied at 34 MPa in order to achieve the separation of the same standard mixture in comparable analysis time. As expected the column packed with the stationary phase with particles of smaller diameter exhibited higher efficiency eluting with the same mobile phase. Table 1 shows the reduced plate heights obtained analyzing the standard mixture using two columns packed with two different stationary phases and eluting with the same mobile phase.

The effect of the water concentration in the mobile phase mixture on retention time, ln *k* and resolution of analytes was studied by increasing the percentage of

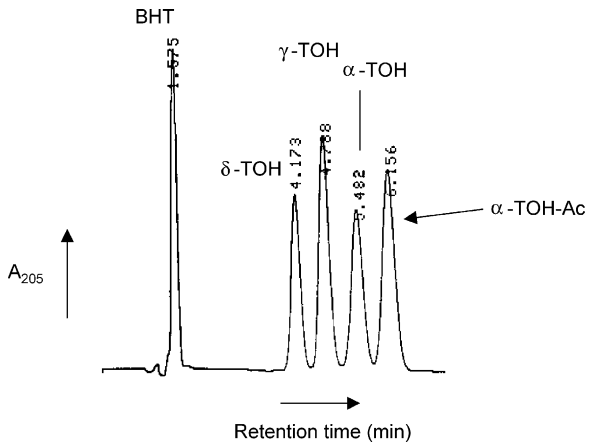

Fig. 4. Nano-LC baseline separation of BHT, δ-, γ-, α-TOH and α-TOH-Ac. Mobile phase: MeCN–MeOH-H_2O (69:30:1 v/v/v). Capillary column packed with 5-μm particles. For other experimental conditions see Fig. 1 and text.

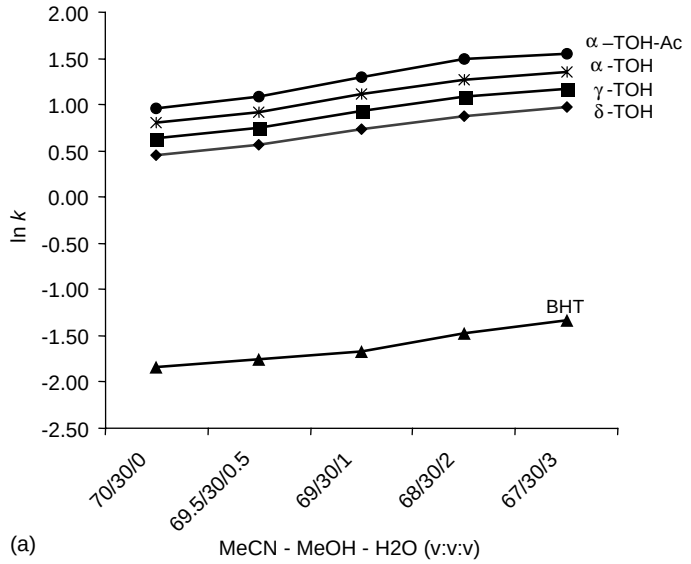

Fig. 5. Effect of water concentration present in the mobile phase used for the separation of TOHs, α-TOH-Ac and BHT standard compounds on (a) natural logarithm of retention factor and (b) resolution of α-TOH-Ac/α-TOH. Capillary packed with 3-μm particles. Elution at constant pressure of 34 MPa, approximate flow rate and injected volume sample, 240 nl/min and 60 nl, respectively. For other experimental conditions see text.

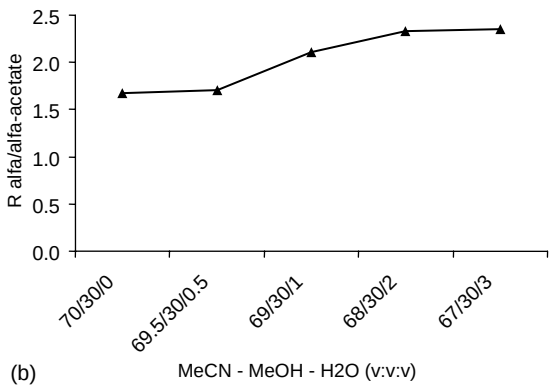

Fig. 5. (*Continued*).

water in the range 0–3% and decreasing the content of MeCN. The retention time of TOH and TOH-Ac increased with increasing the concentration of water while t_R of BHT was not influenced by the water content. Fig. 5a shows the linear increase of ln *k* by increasing the water percentage in the mobile phase. Besides the effect was studied at a restricted change of solvent composition, the variation was typical of a reversed phase interaction. Fig. 5b depicts the in-

Table 1
Reduced plate heights of the studied TOHs and TOH-Ac

Silica column type (μm)	Reduced plate heights			
	δ-TOH	γ-TOH	α-TOH	α-TOH-Ac
5	21.21	17.21	18.45	16.65
3	15.13	12.41	13.62	12.67

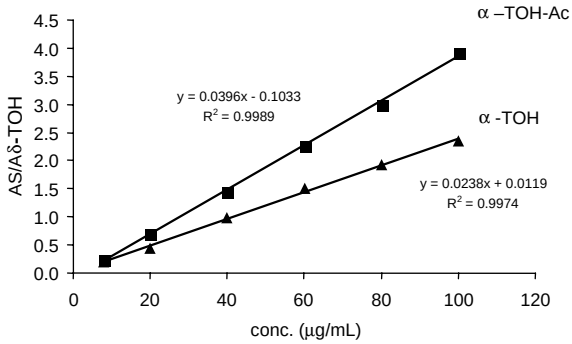

Fig. 6. Calibration curves for α-TOH-Ac and α-TOH. Separation performed in a capillary packed with 3-μm particles; mobile phase MeCN–MeOH (70:30 v/v).

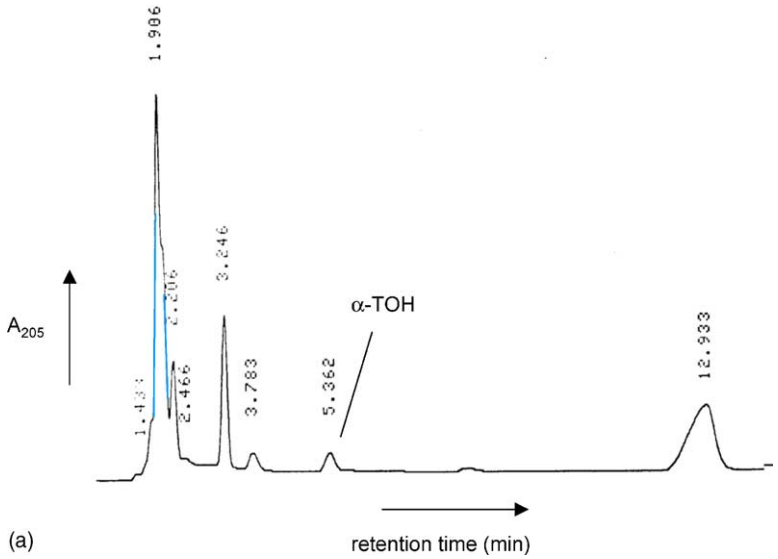

Fig. 7. Nano-LC of extract of (a) human plasma (b) commercial nutritional preparation. Capillary packed with RP_{18} 3-μm particles; mobile phase MeCN–MeOH (70:30 v/v). For other experimental conditions see text.

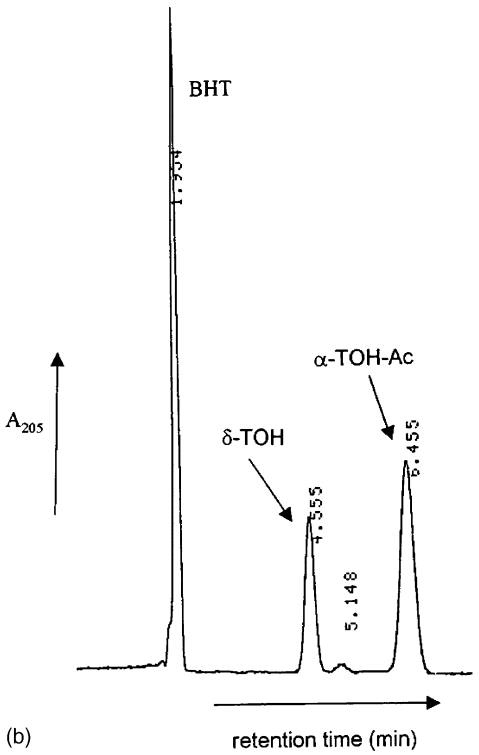

Fig. 7. (*Continued*).

crease of α-TOH/α-TOH-Ac resolution by increasing the water concentration (decrease of MeCN content).

It is noteworthy to mention that these two analytes were baseline resolved ($R > 1.5$) also in absence of water (this was not observed with the capillary packed with 5-μm particles). This can be due to either the higher efficiency achieved using the column packed with 3-μm particles or the slightly different nature of the reversed phase employed. Based on the above described results the mobile phase MeCN–MeOH (70:30 v/v) was used for further experiments.

The repeatability of the method was verified analyzing the mixture of the five compounds ($n = 7$) using the packed capillary column with 3-μm particles and eluting with MeCN–MeOH mixture (70:30 v/v). Good results were obtained for retention time (STD, 1.78, 0.95, 0.84, 1.08 and 0.69% for BHT, δ-, γ-, α-TOH and α-TOH-Ac, respectively). Less satisfactory results were observed for peak area (10–15%) that improved when considering the peak area ratio ($A_S/A_{\alpha\text{-TOH}}$) (STD, 2–3%). The inter-day repeatability for retention time ($n = 5$) was in the range 5–6%.

Limit of detection (LOD) and limit of quantification (LOQ) (S/N=3 and 10, respectively) were measured analyzing standard mixtures at different concentrations and eluting with the mobile phase

composed of MeCN–MeOH (70:30 v/v) providing the complete separation of the five compounds in the shortest analysis time. The LOD and LOQ for δ-, γ-TOH, α-TOH-Ac were 4 and 8 μg/ml, respectively while for α-TOH were 6 and 10 μg/l, respectively.

It has been reported that α-TOH is injected with diet and present in human serum, therefore, considering that we demonstrated the possibility to use capillary electrochromatography (CEC) for the analysis of such Vitamin, we tried to apply nano-LC to the analysis of this compound in serum. Additionally it was reported that some esters of α-TOH (e.g., acetate, nicotinate, succinate), are more stable than those of the parent compound because less susceptible to oxidation, are currently used, e.g. added to nutritional supplements as source of Vitamin E [17].

Therefore the calibration curves were studied only for α-TOH and α-TOH-Ac in the concentration range 10–80 μg/ml. As can be observed in Fig. 6 good linearity was observed for the two studied analytes with good correlation.

The applicability of the optimized nano-LC method to the analysis of samples containing Vitamin E is demonstrated in Fig. 7a and b where two extracts (human serum and pharmaceutical preparation, respectively) were injected. The α-TOH and α-TOH-Ac present in the analyzed real samples were confirmed by their retention time and by spiking the injected mixture with the standard compounds.

4. Conclusions

The separation of three TOHs, α-TOH-Ac and BHT (an antioxidant compound) was achieved by nano-LC in fused silica capillaries of 100-μm I.D. packed with reversed phase silica particles of either 5 or 3 μm. As expected the column packed with 3-μm particles exhibited the highest efficiency and resolution of the studied compounds. Baseline resolution of the mixture was achieved using both types of stationary-phase 3- and 5-μm particles eluting with a mixture of MeCN–MeOH (70:30 v/v) and MeCN–MeOH-water (69:30:1 v/v/v), respectively. The optimized nano-LC method can offer advantages over classical HPLC ones such as fast analysis time, reduced volumes of mobile phases and therefore low costs and low pollution. The sensitivity of the method was sufficient to apply the optimized method to a biological and a pharmaceutical sample due to the relatively high content of Vitamin E or its derivative and to the liquid–liquid extraction method used. Further study will be carried out in our laboratory in order to improve the detection limit, e.g. using a mass spectrometer detector and/or on line pre-concentration step.

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