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Alkaline saponification results in decomposition of tocopherols in milk and ovine blood plasma

M. Czauderna *, J. Kowalczyk

The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jablonna, Poland

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Abstract

Alkaline saponification of entire sample matrixes for quantification of α-, γ-, δ-tocopherols (α-T, γ-T, δ-T) and α-tocopherol acetate (α-TAc) was examined. High-performance liquid chromatography was used to measure α-T, γ-T, δ-T and α-TAc in tocopherol standard solutions, milk and ovine blood plasma. Saponification in the presence of vitamin C decreases the concentration of tocopherols, especially α-T and γ-T. The poor recovery of tocopherols is due to the decomposition of tocopherols in saponified standard solutions, milk or plasma. Saponification of samples in the presence of 2,[6]-ditertbutyl-*p*-cresol or flushed only with a stream of Ar resulted in a major decrease in the concentrations of α-T, γ-T, δ-T and α-TAc in comparison with saponification in the presence of vitamin C.

Keywords: Tocopherols; Alkaline saponification; HPLC

1. Introduction

Tocopherols, also known as vitamin E, consist of a group of isoprenoid compounds of plant origin, which were first described as essential micronutrients for normal fertility in rats [1–3]. Later studies have established a wide range of physiological functions for this vitamin, e.g. protecting cell membranes and LDL cholesterol, and indirect effects on blood cell regulation, connective tissue growth, inflammation and genetic control of cell division [3–6]. Vitamin E corresponds, in decreasing order of physiological activity, α-, β-, γ- and δ-tocopherols (α-T, β-T, γ-T and δ-T). Tocopherols are added during the manufacture of foods either to prevent lipid oxidation or to improve the vitamin E supply. The use of α-T (D and DL) as well as α-tocopherol acetate (α-TAc) for supplementing foods is permitted by law [7]. Moreover, fat-soluble vitamin E is one of the most effective natural antioxidants that protect, e.g. polyunsaturated fatty acids in foods from oxidation during storage. Unfortunately, tocopherols in foods also rapidly degrade themselves at high temperatures, during storage, or upon exposure of foods to daylight. Determination of vitamin E in foods like meat, milk, dairy products or physiological fluids (e.g. blood plasma) usually involves saponification [3,8–11].

Naturally occurring β-, γ- and δ-tocopherols and tocotrienols do not contribute toward meeting vitamin E requirements because (although absorbed) they are not converted to α-tocopherol by humans and they are poorly recognized by the α-tocopherol transfer protein in the liver [4]. Considering the above, we investigated the stability of α-T, δ-T and γ-T as well as α-TAc, which is usually used for supplementing foods. It is noteworthy that γ-T and β-T have the same chemical formula, differing only in the methyl group substitution pattern, and have similar relative antioxidant activities [4]. In line with previous reports [1,2,11], α-T, δ-T and γ-T as well as α-TAc were used in the study, as the native concentration of β-T in foods is usually below the limits of UV or fluorescence detection or significantly lower compared with the concentration of α-T and other tocopherols.

Therefore, the major aim of our current study was to examine the influence of typical alkaline saponification procedures on the decomposition of α-T, δ-T, γ-τ and α-TAc standards in processed solutions. High-resolution reversed-phase HPLC with fluorescence and photodiode array detectors was used for analysis of tocopherols in processed standard samples.

* Corresponding author. Tel.: +48 22 782 40 372; fax: +48 22 774 20 38.
E-mail address: m.czauderna@ifzz.pan.pl (M. Czauderna).

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2. Experimental

2.1. Reagents and standards

All chemicals used were of analytical-reagent grade (POCh; Gliwice, Poland), while α-, γ-, δ-tocopherols (α-T, γ-T, δ-T), α-tocopherol acetate (α-TAc), vitamin C and 2,[6]-ditertbutyl-*p*-cresol (BHT) were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (99.9%) and *n*-hexane (95%) were purchased from Lab-Scan (Ireland). Argon (Ar) was analytical-reagent grade (MULTAS, Poland) containing 3.7 ppm H_2O, 1.4 ppm O_2, 0.1 ppm H_2, 5.6 ppm N_2, 0.1 ppm CO, 0.1 ppm CO_2 and 0.1 ppm alkanes.

2.2. Chromatographic equipment

An alliance separation module (model 2690, Waters) with a Waters 996 photodiode array detector (DAD) and Waters 474 fluorescence detector (FD) was used for the development of HPLC methods. An autosampler was thermostated at 7 °C. DAD was operated in a UV range from 195 to 330 nm with a measurement frequency of 1 spectrum/s and spectral resolution of 1.2 nm; γ-T, δ-T, α-T and α-TAc were UV monitored in 294, 298, 298 and 286 nm, whereas the fluorescence detection (FD) was at the excitation wavelength (λ_{ex}) of 290 nm and emission wavelength (λ_{em}) of 327 nm (at a gain 10). An analytical Nova Pak C_{18} column (4 μm, 300 × 3.9 mm, i.d., Waters) was fitted with a pre-column of 10 × 6 mm, i.d. (Nova Pak, Waters) containing C18 pellicular packing material (degrade themselves 30–40 μm).

The elution was performed at a flow-rate of 2 ml/min. The elution conditions were: for 1 min isocratic flow of acetonitrile and water (92:8, v/v), followed by a linear gradient of acetonitrile in water (from 92 to 94% in 15 min). Afterwards, the analytical column should be cleaned for 10 min in 100% acetonitrile. The column was re-equilibrated for 10 min in acetonitrile and water (92:8, v/v). The ambient temperature was 21–24 °C, while the analytical column was kept at 40 °C.

The α-T, γ-T, δ-T and α-TAc peaks in UV and FD chromatograms were identified on the basis of the retention times of saponified tocopherol standards and standards dissolved in ethanol as well as by adding these standards to assayed samples. The tocopherol peaks in the assayed samples were also identified on the basis of the UV spectra of tocopherol standards (Fig. 1E).

2.3. Saponification and extraction of tocopherol standards from processed solutions

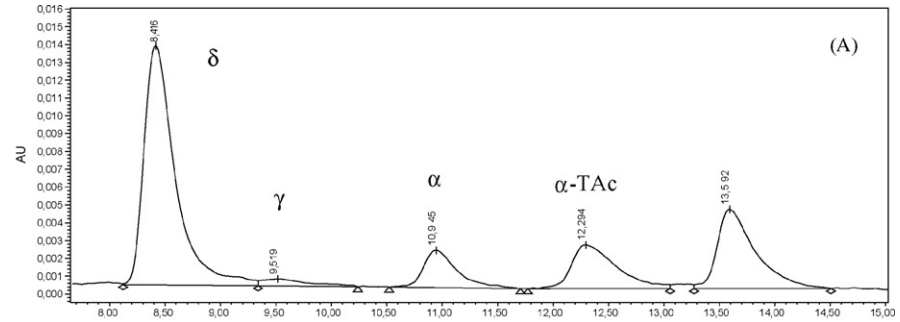
In a 10 ml glassware screw-capped Pyrex tube, 1 ml of ovine blood plasma, cow milk or 0.1 ml of ethanolic solution of standards of δ-T, γ-T, α-T and α-TAc (each ∼1 mg) in 1 ml of water was mixed with 2 ml of ethanol, 0.5 ml of methanol, 1 ml of aqueous vitamin C (20%, w/w), 0.3 ml of aqueous KOH (50%, w/w) and 0.7 ml of water. Alternatively, 0.5 ml of methanol with or without BHT (20 mg/ml methanol) and 1 ml of water were added instead of 0.5 ml of methanol and 1 ml of aqueous vitamin C (20%, w/w). The resulting mixture containing 1 ml of the vitamin C solution or 0.5 ml of methanol with or without BHT was only flushed for ∼5 min with a stream of Ar (11–13 ml/min). The obtained solution in a tightly closed tube was vigorously mixed and heated at 80 °C for 20 min, next cooled for 10 min at room temperature. Twenty microlitres of the resulting solution of processed tocopherol standards were injected directly onto the HPLC column. Tocopherols from saponified milk or blood plasma samples were extracted two times, using 4 ml of heptane each time. The heptane layers were then combined and the organic solvent was removed under a gentle stream of Ar. The tocopherol residue was reconstituted in 0.5 ml of heptane and then 20–50 μl of the resulting solution (0.5 ml) were injected onto the HPLC column.

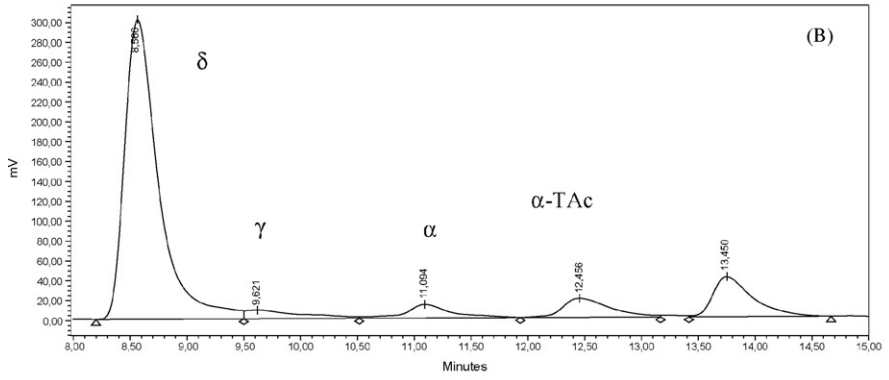
3. Results and discussion

The major aim of this work was to examine the alkaline saponification methods [3,8–11] in order to investigate the influence of these saponification procedures on the decomposition of tocopherols commonly present in foods and physiological fluids like blood plasma. Vitamin C and BHT were used as antioxidants as these compounds are typically added to processed tocopherol solutions [3,10,4,12]. On the other hand, hydroquinone cannot be recommended due to its carcinogenic and mutagenic properties and its dangerous effect on the environment.

Saponification in KOH solution [10,11] makes tocopherols removable in the case of meat, milk, dairy product, infant formula as well as blood plasma samples [3,10,11]. Therefore, in the current study usually used alkaline saponification in KOH in the presence of vitamin C as an antioxidant was examined using δ-T, γ-T, α-T and α-TAc commonly found in foods. Fig. 1D shows the typical UV chromatogram obtained for non-saponified tocopherol standards in the mixture of reagents used for the saponification in the presence of vitamin C. To decrease system pressure and retention times without loss of resolution, all separations were performed on a column at a temperature of 40 °C using a binary gradient program. The use of these chromatographic conditions resulted in excellent peak shapes, which were close to symmetrical even with analyte elution times from 10 to 13 min.

As expected, δ-T, γ-T, α-T and α-TAc possess a relatively high absorbance band in the longer UV range with absorbance maxima at 298 ± 0.2, 299.2 ± 0.2, 294.4 ± 0.2 and 286.1 ± 0.3 nm, respectively (Fig. 1E). As shown in Fig. 1A, B and D, δ-T, γ-T, α-T and α-TAc peaks having retention times of 8.8 ± 0.4, 9.5 ± 0.2, 10.9 ± 0.2 and 12.3 ± 0.2 min respectively, were absent from the blank when HPLC with UV (in the range from 240 to 310 nm) and fluorescence detection was used. Fortunately, as can be seen from Fig. 1B, selective fluorescence detection (FD) ($\lambda_{ex}/\lambda_{em}$ = 290/360 nm) can also be used for determination of δ-T, γ-T, α-T and α-TAc. However, exhaustive quantitative analyses of UV chromatograms have demonstrated that alkaline saponification at 80 °C for 20 min (compare Fig. 1A and D) resulted in decreasing the concentrations of all tocopherol standards compared with non-processed tocopherol standards (Table 1). Moreover, detailed analysis of UV and FD chromatograms revealed that 4 h storage of the saponified tocopherol standard solution decreased the concentration of γ-T and α-T,


(A)
δ
γ
α
α-TAc
AU
8,416
9,519
10,945
12,294
13,592


(B)
δ
γ
α
α-TAc
mV
Minutes
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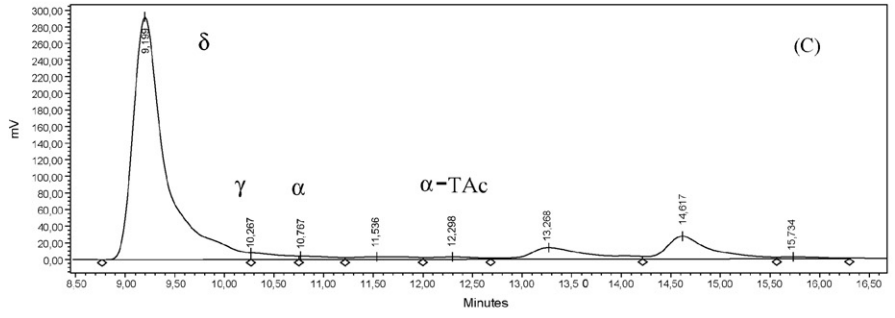
9,621
11,094
12,456
13,450


(C)
δ
γ
α
α−TAc
mV
Minutes
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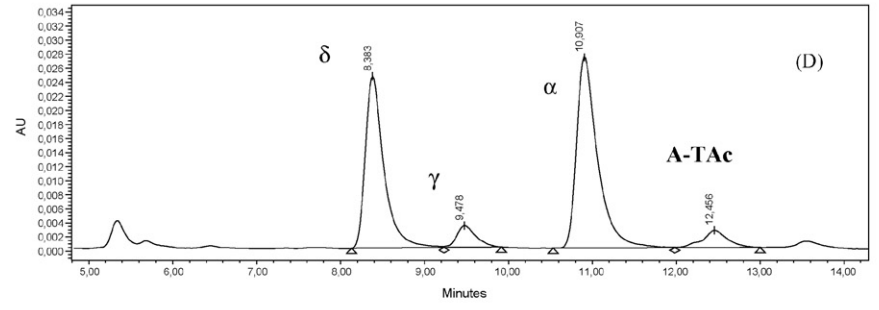
12,298
13,268
14,617
15,734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δ
γ
α
A-TAc
AU
Minutes
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10,937
12,466

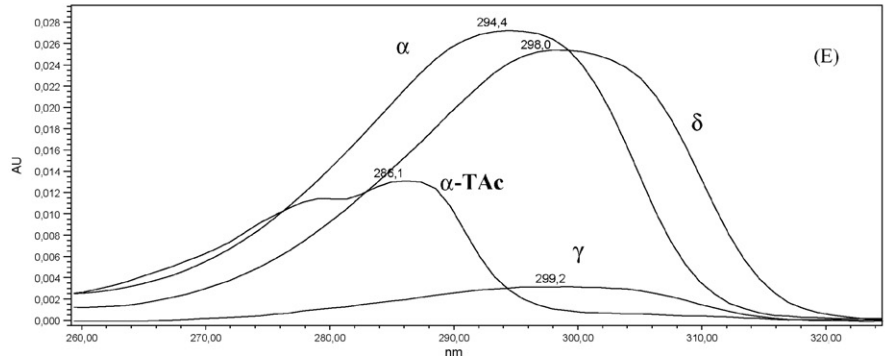
(E)
α
δ
α-TAc
γ
AU
nm
294,4
298,0
286,1
299,2

Table 1
Effects of the solution composition and saponification conditions on the concentrations of δ-T, γ-T, α-T and α-TAc processed samples[a]

Tocopherol	Solution composition and processing conditions				
	Without vitamin C, KOH and saponification[b]	Without KOH and saponification[c]	Without saponification[d]	HPLC analysis 5 min after saponification at 80 °C for 20 min	HPLC analysis 4 h after saponification[e] at 80 °C for 20 min
δ-T					
UV = 298 nm	293,930	286,880	307,700	264,490	281,800
FD = 290/360 nm	7,012,320	6,802,740	7,502,170	6,239,350	6,602,480
γ-T					
UV = 298 nm	65,630	61,370	48,580	11,800	6,400
FD = 290/360 nm	1,235,640	1,174,660	1,021,210	305,700	86,210
α-T					
UV = 294 nm	636,310	597,660	452,950	44,490	3,150
FD = 290/360 nm	4,499,270	4,143,850	3,267,330	379,800	124,720
α-TAc					
UV = 286 nm	289,600	264,190	195,960	68,710	47,720
FD = 290/360 nm	185,450	161,950	304,200	573,480	490,560

[a] 100 μl of δ-T, γ-T, α-T and α-TAc standards (each ∼1 mg) in ethanol were processed.
[b] Final solvent composition: 3 ml of H_2O, 2 ml of ethanol and 0.5 ml of methanol; tocopherols were directly HPLC analyzed.
[c] Final solvent composition: 2.0 ml of H_2O, 2 ml of ethanol, 0.5 ml of methanol and 1 ml of aqueous vitamin C (20%, w/w).
[d] Final solvent composition: 1.7 ml of H_2O, 2 ml of ethanol, 0.5 ml of methanol, 1 ml of aqueous vitamin C (20%, w/w), 0.3 ml of aqueous KOH (50%, w/w).
[e] Final solvent composition: 1.7 ml of H_2O, 2 ml of ethanol, 0.5 ml of methanol, 1 ml of aqueous vitamin C (20%, w/w), 0.3 ml of aqueous KOH (50%, w/w); HPLC analysis was performed after 4 h of storage at temperature at 7 °C (see Fig. 1C).

in particular, and increased the number and the concentration of unidentified species (Fig. 1C). Surprisingly, fluorescence detection showed that the peak area corresponding to α-TAc in saponified tocopherol standard solution is greater compared with the one found in non-saponified standard solutions (Table 1).

Alkaline saponification was also evaluated by analyzing recoveries of δ-T, γ-T, α-T and α-TAc added to cow milk and bovine blood plasma. The obtained results also demonstrated a marked decrease in the concentrations of all added tocopherols compared with the concentrations of non-saponified tocopherol standards in the mixture of reagents used for the saponification in the presence of vitamin C. This alkaline saponification especially decreased the recoveries of γ-T and α-T added to milk, i.e. the recovery percentages obtained were: 25–32 and 10–15%, respectively. Moreover, endogenous components present in milk or plasma and the unidentified species formed considerably disturbed identification and integration of the peaks corresponding to δ-T, γ-T, α-T and α-TAc.

In contrast, the use of BHT, a commonly used effective antioxidant, caused a considerable decrease of the concentrations of δ-T (−55%), γ-T (−35%), α-T (−30%) and α-TAc (−25%) in saponified tocopherol standards and tocopherols added to cow milk compared with saponification in the presence of vitamin C. The quantifications of tocopherols in saponified samples were performed using UV and fluorescence detection simultaneously.

Similarly, the saponification of tocopherol solutions flushed with a stream of Ar (i.e. without addition of BHT or vitamin C) resulted in decreased concentrations of δ-T (−10%), γ-T (−40%), α-T (−45%) and α-TAc (−70%) in comparison with saponification in the presence of vitamin C. The detection of tocopherols in saponified standard solutions was performed using UV detection at 295 nm. According to these results, fluorescence detection also revealed similar changes in the concentrations of δ-T, γ-T and α-T. Surprisingly, fluorescence monitoring ($\lambda_{ex}/\lambda_{em}$ = 290/327 nm) of tocopherols in effluents revealed a significant increase of the peak area corresponding to α-TAc (310%) in HPLC-analyzed saponified samples flushed with a stream of Ar compared with saponification in the presence of vitamin C. As can be seen from UV and FD chromatograms, the presence of unidentified species disturbed accurate quantification of all δ-T, γ-T, α-T and α-TAc, especially 8 h after storage of saponified samples flushed with Ar (i.e. without BHT or vitamin C).

Poor recoveries of α-T and γ-T, due to significant decomposition of these particular forms of vitamin E, markedly decreased the sensitivity of vitamin E determination in foods or blood plasma using alkaline saponification in the presence of vita-

Fig. 1. Parts of typical RP-chromatograms for the standards of δ-T, γ-T, α-T and α-TAc using a binary gradient program (injection volumes: 20 μl). (A) Chromatogram A: UV detection at 295 nm (RP-HPLC analysis 5 min after saponification in the presence of vitamin C). (B) Chromatogram B: fluorescence detection (RP-HPLC analysis 5 min after saponification in the presence of vitamin C). (C) Chromatogram C: fluorescence monitoring 4 h after saponification of γ-T, α-T and α-TAc standards (a storage temperature: 7 °C). (D) Chromatogram D: RP-HPLC analysis of non-saponified of δ-T, γ-T, α-T and α-TAc standards in the solvent containing 1.7 ml of H_2O, 2 ml of ethanol, 0.5 ml of methanol, 1 ml of aqueous vitamin C (20%, w/w), 0.3 ml of aqueous KOH (50%, w/w); UV detection at 295 nm. (E) The UV spectra and maximum values (nm) of UV bands corresponding to δ-T, γ-T, α-T and α-TAc standards presented in chromatogram D. Letters δ, γ, α, α-TAc indicate δ-T, γ-T, α-T and α-TAc, respectively.

min C [10,11]. Therefore, α-T, the form commonly found in milk or infant formula, can only be determined using fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 290/327 nm), whereas γ-T as well as other tocopherols were below the limit of quantification defined as 10 times the background under a peak. Unfortunately, no tocopherols could be quantified using UV detection because the concentrations of δ-T, γ-T and α-T were below the limit of detection defined as three times the background under a peak.

On the other hand, our recent RP-HPLC method without saponification permitted rapid quantification of δ-T, γ-T and α-T in milk and infant formula using UV detection [11]. Moreover, RP-HPLC without saponification exhibits applicability for determination of α-TAc commonly added to milk or infant formula, as this form of vitamin E is less susceptible to oxidation [1,11]. Obviously, methods of analyzing vitamin E without the saponification of assayed samples permit quantification of α-TAc as well as δ-T, γ-T and α-T in milk and infant formula also using reversed- or normal-phase HPLC with fluorescence or/and UV detection [1,2,11]. Therefore, more research is needed to develop a gentle saponification method for the sensitive and accurate HPLC quantification of δ-T, γ-T and α-T as well as α-TAc in milk, meat and products derived from domestic animals.

4. Conclusions

The current study examined the alkaline saponification commonly recommended for RP-HPLC determination of tocopherols in foods and blood plasma. The results show that KOH saponification at 80 °C for 20 min in the presence of vitamin C is the more accurate procedure for quantifying tocopherols in food compared with saponification with or without BHT. However, saponification in the presence of BHT or vitamin C resulted in a marked decrease of the sensitivity of vitamin E determination. Therefore, further research is required to develop a more versatile saponification method for determination of tocopherols, especially for routine analysis of tocopherols in meat as well as milk, infant formula, and dairy products.

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