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# Simple HPLC Analysis of Tocopherols and Cholesterol from Specimens of Animal Origin

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An improved saponification method followed by original isocratic high-performance liquid chromatography (HPLC) with photodiode detection (996 PAD, Waters) and/or fluorescence detection (474 Waters) for simultaneous analysis of cholesterol (CHOL) and δ-, α-, γ-tocopherols (δ-T, α-T and γ-T; forms of vitamin E) has been described. The method involved direct saponification of sample solutions flushed with a stream of argon, in the presence of vitamin C, followed by isocratic liquid chromatographic elution (Nova Pak $C_{18}$ column, 4 μm, 300 × 3.9 mm, I.D., Waters) and photodiode detection (UV) at 205 nm and/or fluorescence monitoring ($\lambda_{ex}/\lambda_{em}$ = 290/327 nm). Reversed-phase HPLC analyses have revealed that the optimum separation of CHOL and tocopherol from endogenous substances in biological samples can be obtained using the mobile phase containing 17% propan-2-ol and 83% of acetonitrile (v/v) at the flow rate of 1.5 mL $min^{-1}$. Applying isocratic elution with UV monitoring at 205 nm and fluorescence detection, δ-T, α-T, γ-T and CHOL were eluted after 6.19 ± 0.09, 7.01 ± 0.08, 7.79 ± 0.08 and 14.7 ± 0.2 min, respectively. UV detection at 205 nm assured better detector responses for all tocopherols compared to other wavelengths. Detailed investigations have proven that alkaline saponification at 80°C for 15 min followed by isocratic chromatographic elution and UV and fluorescence detection enable simple and satisfactory simultaneous analysis of CHOL and δ-, α-, γ-tocopherols in specimens of animal origin.

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Opisano poprawioną metodę jednoczesnego oznaczania cholesterolu oraz δ-, α- i γ-tokoferoli (trzy formy witaminy E). Metoda polega na zmydlaniu próbki i następnie zastosowaniu oryginalnej, izokratycznej, wysokosprawnej chromatografii cieczowej z detekcją wykorzystującą fotodiodę (996 PAD Waters) oraz z detekcją fluorescencyjną (474 Waters). Zmydlanie próbki prowadzono w roztworze mieszanym strumieniem argonu w obecności witaminy C. Inne warunki onaczanie: kolumna Nova Pak $C_{18}$ 4 μm, 300 × 3,9 mm I.D., Waters; detekcja w nadfiolecie – 205 nm; fluorescencja: $\lambda_{ex}/\lambda_{em}$ = 290/327 nm. W układzie odwróconych faz najlepsze rozdzielenie otrzymano w przypadku fazy ruchomej o składzie objętościowym: propan-2-ol – 17% i acetonitryl – 83%. Po rozdzieleniu piki δ-, α- i γ-tokoferoli oraz chloroform pojawiły się w czasach: 6,19 ± 0,09, 7,01 ± 0,08, 7,79 ± 0,08 and 14,7 ± 0,2 min. Najlepszą detekcję UV, dla wszystkich tokoferoli, otrzymano przy długości fali 205 nm. Stwierdzono również, że ługowanie alkaliczne w 80°C przez 15 min umożliwia prostą, jednoczesną chromatograficzną analizę tokoferoli w próbkach pochodzenia zwierzęcego.

The frequency incidence of cholesterol-induced artherosclerosis, obesity, cancer and cardiovascular diseases has increased in economically developed western-countries as an effect of changes in human diet and lifestyle. Meat and meat products provide one third to one half of daily-recommended cholesterol intake (300 and 225 mg per day for man and women, respectively; World Health Organization) [1–3]. Clinical and epidemiological studies have shown that cholesterol intake is directly associated with a higher risk of hypercholesterolemia and obesity, conditions that predispose to some chronic diseases of the circulatory system. Furthermore, the contents of cholesterol oxidation products (oxysterols) in beet or pork, possessing carcinogenic, mutagenic and cytotoxic properties, strongly depend on cholesterol concentrations in meat [5–7]. Indeed, cholesterol is oxidized in almost alike way to phospholipids or unsaturated fatty acids. Cholesterol esters and oxysterols in food are usually formed during grilling, frying, cooking or even in frozen meat as a consequence of the oxidation process. It is clear, from the discussion above, that the adequate level of antioxidants can decrease the risk of oxysterols formation as well as other oxidation products of unsaturated fatty acids (UFA). Vitamin E (α-tocopherol, α-T) is one of the most important naturally occurring primary antioxidants. It plays a very important role in cholesterol and UFA protection from oxidative modification, what may have deleterious effects on the human health (*e.g.* artherosclerosis) [6, 7]. Naturally occurring tocotrienols and β-, γ-, δ-tocopherols (β-T, γ-T, δ-T) do not contribute toward meeting vitamin E requirements because they are not converted to α-tocopherol in humans and they are poorly recognized by the α-tocopherol transfer protein in the liver [8, 9]. Considering the above, there is a need for the procedure for determination of α-T, δ-T and γ-T, which is usually used for supplementing feedstuff or human foods.

Total cholesterol determination is usually done by chromatographic or enzymatic methods. The commonly used is gas chromatography [1], however there also some HPLC methods are popular as well [8, 10–12]. Many of chromatographic methods for determination of cholesterol include a laborious saponification and extraction, and a chromatographic run lasts *ca* 25 min. Cholesterol, α-T, δ-T and γ-T can be analyzed directly and simultaneously, because tocopherols and cholesterol are non-polar compounds, which absorb in the ultraviolet (UV) range.

Considering the above, we have developed direct saponification and isocratic liquid chromatography (HPLC) procedures with selective photodiode array detection (DAD) for simultaneous determination of α-T, δ-T and γ-T and cholesterol (CHOL) in liver, meat, and blood plasma of sheep and laboratory rats..

## EXPERIMENTAL

### Reagents and standards

Vitamin C, cholesterin (CHOL) (99+ %; cat. number: 36,279-4), α-, δ- and γ-tocopherols (α-T, δ-T and γ-T) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (99.9%) and propan-2-ol (iso-propanol; 99.8%) were purchased from Lab–Scan (Ireland). Rectified ethanol and KOH were obtained from Fluka (Buchs, Switzerland). Water used for the preparation of chemical reagents was prepared using an Elix™ water purification system (Millipore, Canada). Argon of analytical 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 was used. All other chemicals were of analytical grade and were purchased from POCh (Gliwice, Poland).

### Chromatographic equipment

An alliance separation module (model 2690, Waters) equipped with a Waters 996 photodiode array detector (DAD) and a Waters 474 fluorescence detector (FD) was used for HPLC investigations. A Nova Pak analytical $C_{18}$ column (4 μm, 300 × 3.9 mm, I.D., Waters) was accompanied by a guard-column of 10 × 6 mm, I.D. (Nova Pak, Waters) containing $C_{18}$ pellicular packing material (degrade themselves 30–40 μm).

An autosampler was thermostated at 7°C. DAD was operated in the UV range from 195 to 390 nm with a measurement frequency of 1 spectrum $s^{-1}$ and a spectral resolution of 1.2 nm. Development of the isocratic elution program, collection of chromatograms, peak integrations and peak purity analyses were performed using a Pentium III computer (Compaq) and Millennium 2001 software (version 2.15). CHOL peak purity analyses were carried out in the UV range from 200 to 215 nm, while tocopherol peak purity analyses were performed in the UV range from 200 to 215 nm and from 280 to 305 nm.

Fluorescence detection (FD) was performed at the excitation wavelength ($\lambda_{ex}$) of 290 nm and emission wavelength ($\lambda_{em}$) of 327 nm (at a gain 10). CHOL, α-T, δ-T and γ-T in standards and biological materials were UV-monitored at 205 nm, whereas α-T, δ-T and γ-T in all samples were determined simultaneously applying fluorescence detection (FD; $\lambda_{ex}/\lambda_{em}$ = 290/327 nm).

All isocratic HPLC analyses were performed at a flow rate of 1.5 mL $min^{-1}$. Chromatographic separations were performed using the mobile phase composed of propan-2-ol in acetonitrile (17:83, v/v). The measurement were carried out at the ambient temperature of 21–24°C, while the analytical column was kept at 26°C. The α-T, δ-T and γ-T peaks in UV (at 205 nm) and FD ($\lambda_x/\lambda_{em}$ = 290/327 nm) chromatograms and the CHOL peak in UV (at 205 nm) chromatograms were identified on the basis of the retention times of the standards injected separately and by adding standards solutions to the processed biological samples. The α-T, δ-T, γμ-T and CHOL standards were dissolved in the mobile phase. The CHOL and tocopherol peaks in the assayed biological samples were also identified on the basis of retention times the UV spectra of CHOL and tocopherol standards.

Saponification mixture was prepared freshly each day; it contained 11% of KOH m/v in rectifies ethanol–distilled water mixed solutions (57:43, v/v).

The limits of detection ($L_D$) were calculated at a signal-to-background ratio of 3. The limits of quantification ($L_Q$) were defined as 10 × the background under a peak [13].

**Direct saponification and extraction of cholesterol and tocopherols from animals' tissues**

For alkaline saponification, 0.4–0.8 g of a homogenized tissue of sheep and rats (blood plasma, muscles, livers and intermuscular fat tissue) was placed in a screw-cap tube, to which 220–250 mg of ascorbic acid and 7.3 mL of freshly prepared saponification mixture were added. The resulting mixture was flushed for 2–3 min with a stream of argon (11–13 mL $min^{-1}$). The processed mixture should be protected from light. The tube with the resulting mixture was placed for 15 min in a shaking water bath of 80°C. After saponification, the tubes were cooled to ≈ 20°C. After cooling, 3 mL of hexane and 1.5 mL of distilled water were added. Finally, the content of the tube was rigorously vortexed and the upper hexane layer was collected in a vial. Afterwards, the organic solvents were removed under a stream of argon. It was recommended to protect the obtained residues from light and to store them at *ca* –20°C until HPLC analyzed. For HPLC analysis of α-T, δ-T, γ-T, and CHOL a residue in the tube was re-dissolved in 0.5 mL of propan-2-ol. 10–30 μL of this solution were injected onto a reversed-phase HPLC column.

The recovery experiments for tocopherols and CHOL in assayed biological materials were performed just before saponification of biological samples.

## RESULTS AND DISCUSSION

Our previous studies [15, 16, 18] and current exhaustive investigations using the new saponification procedure and HPLC analysis have demonstrated that the stability of δ-T, α-T and γ-T in the processed standards and biological samples depend upon the presence of the oxygen/air in these solvents and storage temperature of the samples injected onto HPLC column. In line with our previous study [16, 19], we improved the saponification procedure. Originally, saponification was performed in the presence of only witamin C [9]. To better prevent oxidation of tocopherols and CHOL, we used vitamin C again and simultaneously deaerated the saponified solvent with a stream of argon. We recommend careful flushing of the saponified samples with a stream of argon and protecting the processed samples from light. Moreover,

the residue re-dissolved in propan-2-ol should be stored at –20°C under argon and protected from the light until HPLC analysis.

We examined the elution effectiveness of the mobile phases containing various concentrations of propan-2-ol (from 50 to 17%) in acetonitrile to fractionation of α-T, δ-T, γ-T and CHOL in muscles, liver and blood plasma of sheep and rats. A detailed chromatographic analyses with UV (at 205 nm) or FD ($\lambda_{ex}/\lambda_{em}$= 290/327 nm) detection showed that the optimum separation of CHOL and tocopherol from endogenous substances in all assayed biological samples was achieved only under the following conditions: the processed sample residue was re-dissolved in propan-2-ol and afterwards eluted with the mobile phase containing 17% of propan-2-ol and 83% of acetonitrile (v/v) and at the flow rate of 1.5 mL $min^{-1}$. Moreover, extraction of α-T, δ-T, γ-T and CHOL from hydrolysates only with hexane, without re-dissolving a residue in propan-2-ol, followed by HPLC analysis, resulted in overlapping of the peaks of tocopherols and CHOL with those of unidentified species present in the asayed biological samples.

The main advantages of the proposed isocratic liquid chromatographic method include optimal solvent consumption and relatively low system pressure (*i.e.* up to 20.0 ± 0.1 MPa). The use of the transparent mobile phase allowed for the simultaneous analysis of tocopherols and CHOL applying the fluorescence detection and multiple UV wavelengths in DAD (Fig. 1C). Moreover, the improved purification procedure of the saponification product resulted in better selectivity of the analyses of tocopherols and CHOL compared to the method based on extraction of the saponification product only with hexane [10]. Indeed, extraction with hexane followed by re-dissolution of the obtained residue in propan-2-ol resulted in the decreased background fluctuation and removal of some unidentified species, which co-eluted with the assayed tocopherols and CHOL. As expected, CHOL and α-T, δ-T, γ-T were substantially retained on the long reversed-phase $C_{18}$ column. Moreover, all tocopherols and CHOL were markedly distinct from the matrix present in biological samples were eluted faster than CHOL and tocopherols. Furthermore, the combination of the photodiode array detection (DAD) and fluorescence detection (FD) increased the credibility of identification of α-T, δ-T, γ-T in the assayed biological materials as well as assured satisfactory separation of tocopherols from endogenous substances present in the samples. As expected, the peaks of α-T, δ-T, γ-T and CHOL were absent from the blank signal, when the proposed isocratic chromatographic elution, photodiode array detection (at 205 and 296 nm) and fluorescence detection (FD) were used.

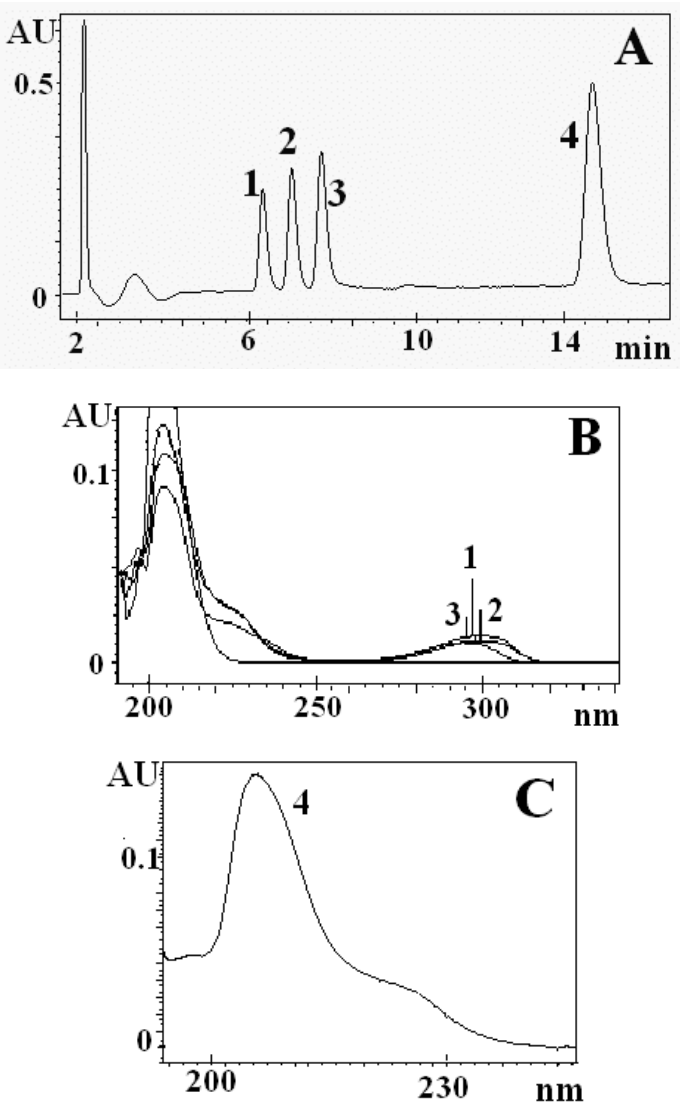


**Figure 1.** Typical chromatograms of tocopherols (δ-T, α-T, γ-T) and cholesterol (CHOL) standards and muscle sample of sheep origin after the direct alkaline saponification and isocratic elution (AU – the absorption unit). Peak identification: (1) δ-T; (2) α-T; (3) γ-T; (4) CHOL. A – processed δ-T, α-T, γ-T and CHOL standards (each of tocopherol – 0.20 μg; CHOL – 2.1 μg); 10 μL were injected onto the column; UV detection at 205 nm; B – UV spectra of δ-T, α-T and γ-T; UV spectrum identification: (1) δ-T; (2) α-T; (3) γ-T; C – UV spectrum of CHOL; spectrum identification: (4) CHOL; D – a typical part of a chromatogram of saponified muscle (*M. longissimus dorsi*) of sheep; UV detection at 205 nm; 10 μL were injected onto the column; E – a typical part of a chromatogram of a saponified *femoral* muscle of rats spiked with δ-T, α-T and γ-T standards (each of tocopherol – 5 μg); fluorescence detection – $\lambda_{ex}/\lambda_{em}$ = 290/327 nm. 5 μL were injected onto the column. (Continuation on the next page)

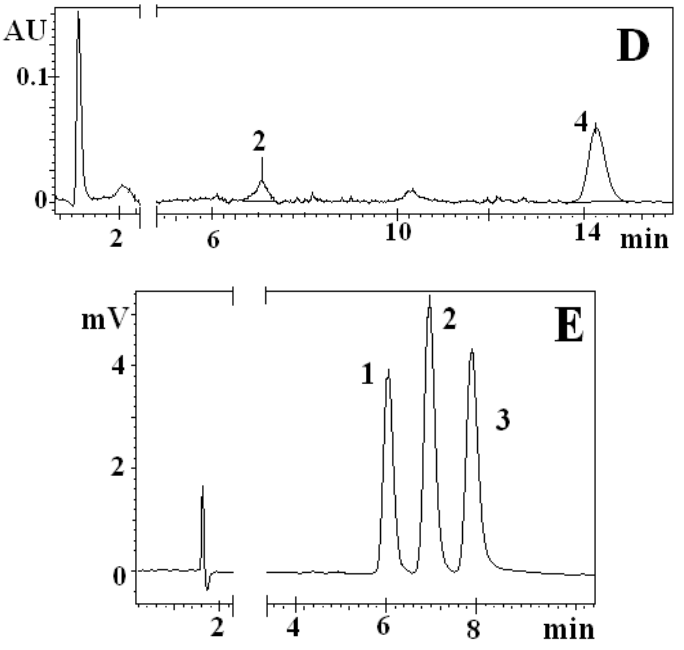


**Figure 1.** (Continuation)

Following the methodology proposed in this work, cholesterol and lipid-soluble tocopherols from animals tissues were subjected to direct saponification, without pre-extraction of fat. Figure 1D depicts typical chromatograms of CHOL in sheep muscles. As illustrated in Figure 1, the isocratic elution composed of propan-2-ol–acetonitrile mixture (17:83, v/v) provided satisfactory separation of α-T, δ-T, γ-T and CHOL in standards (Fig. 1A) and in muscle samples (Fig. 1D and E). Detailed chromatographic analyses showed that the proposed isocratic chromatographic method with UV and fluorescence detections provided satisfactory peak shapes of α-T, δ-T, γ-T and CHOL, close to symmetrical in the standards (Fig. 1A) and all assayed biological samples (*i.e.*: liver, fat tissues, blood plasma of sheep and rats). In the proposed new chromatographic method with UV detection at 205 nm the α-T, δ-T, γ-T and CHOL were eluted after 6.19 ± 0.09, 7.01 ± 0.08, 7.79 ± 0.08 and 14.7 ± 0.2 min, respectively. Thus, the results of performed chromatographic analyses of α-T, δ-T, γ-T and CHOL prove that the applied new isocratic elution program and long $C_{18}$ column assure successful separation of these compounds from the interfering species present in the assayed biological materials. The proposed high-resolution HPLC column and photodiode and/or fluorescence detections are suitable for selective simultaneous analysis of tocopherols and CHOL in biological samples without pre- or post-column derivatization.

In line with our previous studies [15, 16], it seemed reasonable to analyse α-T, δ-T and γ-T in milk or blood plasma using direct liquid-liquid extraction with hexane [19] or chloroform [20], respectively (*i.e.* tocopherol separation without prior saponification). Finally, the organic solvent was removed under a stream of argon [19]. The resulting residue in the tube was re-dissolved in 0.5 mL of propan-2-ol. Finally, determination α-T, δ-T and γ-T in the resulting solution was carried out using the proposed isocratic chromatographic method with UV monitoring at 205 nm and/or fluorescence detection (*i.e.*: $\lambda_{ex}/\lambda_{em}$ = 290/327 nm). Subsequently, the content of CHOL in milk and blood plasma was determined using the proposed saponification method followed by new isocratic chromatography with UV detection at 205 nm.

**Reliability of direct saponification procedure and isocratic HPLC method**

Evaluation of the improved saponification method and HPLC quantification was performed. For this purpose, the purity of the analytical peaks (Fig. 1B and C) [18] corresponding to CHOL, α-T, δ-T and γ-T in muscles, liver, blood plasma and fat tissue of sheep and rats was analysed. From the chromatographic separations and purity peak analyses (Millennium 2001 software; version 2.15) [18] it was found that the purity of CHOL peak was 96 ± 3%, while the purities of α-T, δ-T and γ-T peaks were 98 ± 1%, 96 ± 2% and 98 ± 2%, respectively. Considering the above results it seems that the peaks corresponding to CHOL, α-T, δ-T and γ-T in all assayed biological samples were pure in the UV range from 200 to 215 nm, *i.e.*, devoid of interferences due to potential co-elution peaks of unidentified species absorbing at 205 nm.

The accuracy of the procedure was assessed in recovery studies. For recovery analyses, the standards of CHOL, α-T, δ-T and γ-T were added in the known two amounts to liver, muscles and inter-muscular fat tissues of sheep. The recoveries, asseyed by spiking muscle samples with two different concentrations of CHOL and tocopherols, are shown in Table 1. As expected, the recovery percentages (R%) were satisfactory (*ca* 100%) for all compounds added to muscle samples (Tab. 1) as well as to liver and fat tissues (data not showed). For direct saponification procedure and isocratic HPLC method the following parameters were also determined: limits of detections, coefficients of variation (C.V.), calibration equations, correlation coefficients (r) and standard errors of the slope (Tab. 1). In order to check the linearity of the detector responses to CHOL, α-T, δ-T and γ-T, linear regression equations were defined. As expected, in the studied concentration ranges the relationships between the peak area ratios ($S_n$) of CHOL, α-T, δ-T and γ-T and the amounts of these compounds (y, μg) were linear, with the correlation coefficients above 0.990.

**Table 1.** Recoveries (R%; mean ± SD) of δ-tocopherol (δ-T), α-tocopherol (α-T), γ-tocopherol (γ-T) and cholesterol (CHOL) standards added to muscles of sheep. These standards were added to the muscles[1] at low[2] and high[3] levels (μg mL$^{-1}$). UV detection (UV) was at 205 nm for δ-T, α-T, γ-T and CHOL, while fluorescence detection (FD; $\lambda_{ex}/\lambda_{em}$ = 290/327 nm) for only δ-T, α-T and λ-T. Detection limits ($L_D$), correlation coefficients (r), standard errors in slope (SES) and linear regression lines standards of CHOL, δ-T, α-T and γ-T

| Species | Detection | Recovery R% Low level[2] | Recovery R% high level[3] | Inter-assay C.V.[4] % | Intra-assay C.V.[5] % | Detection limit, $L_D$[6] μg mL$^{-1}$ | Calibrations | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Equation[7] | r | SES |
| CHOL | UV | 99.8 ± 12.1 | 104.5 ± 11.1 | 4.1 | 2.7 | 0.39 | $y(\mu g) = 2.65 \times 10^{-6} S_n - 0.04$ | 0.9999 | $1.12 \times 10^{-8}$ |
| δ-T | UV | 99.6 ± 4.4 | 100.5 ± 4.4 | 2.9 | 2.2 | 0.35 | $y(\mu g) = 5.17 \times 10^{-6} S_n - 0.10$ | 0.9915 | $2.41 \times 10^{-8}$ |
| | FD | 97.1 ± 1.9 | 101.4 ± 5.0 | 3.1 | 1.4 | 0.14 | $y(\mu g) = 2.42 \times 10^{-7} S_n - 0.04$ | 0.9925 | $1.37 \times 10^{-9}$ |
| α-T | UV | 98.5 ± 6.4 | 101.0 ± 4.6 | 3.0 | 2.5 | 0.34 | $y(\mu g) = 5.10 \times 10^{-7} S_n - 0.004$ | 0.9999 | $1.93 \times 10^{-9}$ |
| | FD | 97.2 ± 2.5 | 104.3 ± 4.5 | 2.2 | 2.1 | 0.16 | $y(\mu g) = 2.27 \times 10^{-7} S_n - 0.002$ | 0.9999 | $1.21 \times 10^{-9}$ |
| γ-T | UV | 101.1 ± 6.7 | 102.1 ± 4.2 | 2.4 | 2.1 | 0.41 | $y(\mu g) = 3.65 \times 10^{-7} S_n - 0.02$ | 0.9995 | $1.38 \times 10^{-9}$ |
| | FD | 103.4 ± 11.4 | 103.7 ± 11.3 | 2.5 | 1.7 | 0.37 | $y(\mu g) = 4.16 \times 10^{-7} S_n - 0.01$ | 0.9997 | $1.96 \times 10^{-9}$ |

[1] *M. longissimus dorsi* – meat of sheep origin.

[2] Low level of δ-T, α-T, γ-T and CHOL standards: 10.37, 6.71, 6.71, and 21.3 μg mL$^{-1}$, respectively; n = 7–9 (n – number of replicates).

[3] High level of δ-T, α-T, γ-T and CHOL standards: 55.2, 35.7, 35,7 and 113.6 μg mL$^{-1}$, respectively; n = 6–7.

[4] Inter-assay C.V. values (%) [14] based on three muscle samples repeated three times (processing and injection).

[5] Intra-assay C.V. values (%) for repeated injections based on three muscle samples each with three injections.

[6] $L_D$ were determined in chromatographic analyses of δ-T, α-T, γ-T and CHOL standards in propan-2-ol.

[7] $S_n$ and y(μg) are the peak areas and the amounts of CHOL, δ-T, α-T and γ-T (μg) in assayed samples, respectively; the ranges of the analysed amounts of standards are: CHOL: 1.4–20.1 μg, while δ-T, α-T and γ-T: 0.09–1.81 μg, respectively.

Reproducibility and reliability of the proposed methods were assessed by performing replicate saponifications, extractions and injections of muscle and fat tissue samples (*i.e.* inter-assay C.V.). As it can be seen from the inter-assay C.V. values (2.2–4.1%) summarized in Table 1, the proposed method offers satisfactory precision of CHOL, α-T, δ-T and γ-T determinations. Moreover, low values of intra-assay CV (2.7–1.4%) for CHOL and tocopherols demonstrate satisfactory precision and sensitivity of the proposed isocratic chromatographic method. As expected, the values of intra-assay C.V. and limit of detections ($L_D$) obtained in HPLC method with fluorescence detection for tocopherols were lower than the C.V. and $L_D$ values obtained by HPLC with UV detection at 205 nm.

The obtained results and recorded isocratic chromatographic runs show that determination of tocopherols by HPLC with fluorescence detection provided better selectivity, precision and sensitivity in comparison with HPLC with UV detection. In comparison to the isocratic chromatographic method with UV detection (especially at 205 nm), the proposed isocratic chromatographic method with fluorescence detection assured better separation of α-T, δ-T and γ-T from endogenous substances in the assayed biological materials as well as significantly reduced teh background under the tocopherols peaks. On the other hand, UV detection at 205 nm assured better detector responses for all tocopherols compared to the responses obtained in the UV detection at the absorbance maxima of α-T, δ-T and γ-T (*i.e.*: at 298, 299 and 295 nm, respectively; Fig. 1B). The ratios of the peak areas of α-T, δ-T and γ-T in the standards monitored at 205 nm and at 298, 299 and 295 nm were 8.18 ± 0.07, 7.93 ± 0.12 and 11.47 ± 0.39, respectively. Considering the above results, we claim that the proposed HPLC method with UV detection at 205 nm is more sensitive than other HPLC methods with UV detection at longer wavelengths [15–17].

## CONCLUSION

The proposed simple HPLC method enables simple, accurate and precise analysis of CHOL as well as simultaneous determination of α-T, δ-T and γ-T, which are usually used as food supplements. The results have shown that KOH saponification at 80°C for 15 min in air- and light-protected solvents in the presence of vitamin C (antioxidant that protects tocopherols and CHOL from oxidation) is a reproducible procedure for simultaneous quantification of CHOL and tocopherols in specimens of animal origin. As it can be seen from the chromatographic runs of α-T, δ-T and γ-T and CHOL, the proposed simple isocratic HPLC method is more successful tool for separation of these compounds in comparison to the fractionation accompanying gradient liquid chromatography. The use of long $C_{18}$ column and photodiode array detection at 205 nm resulted in better selectivity and sensitivity of determination of CHOL

and tocopherols compared to other liquid chromatographic methods with UV detection at longer wavelengths. The UV detection at longer wavelengths (*i.e.* at the absorbance maxima of tocopherols) is typically used in other HPLC methods for quantification of tocopherols. So, the proposed method with selective UV detection at 205 nm and fluorescence detections offered the more sensitive analytical tool for simultaneous determination of CHOL and tocopherols in comparison with earlier methods. Our exhaustive investigations have demonstrated that the new procedure based on direct saponification of the assayed sample solutions flushed with a stream of argon in the presence of vitamin C and using a widely available high-resolution $C_{18}$ column and direct photodiode detection and/or sensitive fluorescence monitoring is suitable for selective analysis of CHOL and tocopherols in specimens of animal origin.

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