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ORIGINAL

The Benefits of Ultra-High-Performance Supercritical Fluid Chromatography in Determination of Lipophilic Vitamins in Dietary Supplements

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Abstract

Nine tocopherol derivatives including four tocopherols, four tocotrienols, and one esterified form of α-tocopherol acetate were analyzed using ultra-high-performance supercritical fluid chromatography (UHPSFC) with diode array detection. The separation was carried out on BEH 2-EP column using shallow gradient elution of 1–4% methanol and CO_2 in 5 min. Complementary ultra-high-performance liquid chromatography method was developed using Ascentis Express F5 core–shell stationary phase and isocratic elution with 75% acetonitrile/methanol (1:1) and 25% water. This separation took 12 min that is the shortest liquid chromatography run in analysis of all tocopherol derivatives reported so far. However, our UHPSFC method resulted in more than two times faster analysis, and allowed direct injection of heptane solutions and extracts from liquid–liquid extraction. These features made UHPSFC a method of choice for subsequent analysis of dietary supplements containing different forms of vitamin E. Sample preparation methods were optimized for individual dosage forms of drops, capsules, tablets, and granulate.

Keywords Ultra-high-performance supercritical fluid chromatography · Ultra-high-performance liquid chromatography · Liquid–liquid extraction · Vitamin E · Dietary supplements · Method validation

Introduction

Vitamin E can be found in eight different forms in natural sources [1]. All of them contain a chromanol ring with a hydroxyl group in their structure [2, 3]. However, tocopherols have a saturated isoprenoid side chain, whereas tocotrienols contain a side chain with three *trans* double bonds [4]. The α, β, γ, and δ tocopherols as well as the α, β, γ, and δ tocotrienols can be distinguished based on the number and position of methyl groups on the chromanol ring, as shown in Fig. 1 [2].

The donation of one hydrogen atom from hydroxyl group leads to the reduction of free radicals [2, 3]. Therefore, it serves as a protection of unsaturated lipids from oxidation [3, 5, 6]. For this effect, hydrophobic side chain enabling penetration in biological membranes is also necessary [2, 6, 7]. Vitamin E deficiency is not common, but can cause several clinical problems [8]. In general, antioxidants should reduce oxidative stress in human body that can lead to atherosclerosis, arthritis, cancer, cardiovascular diseases, and inflammation [9].

Due to the beneficial effects of these compounds, an increasing number of dietary supplements containing antioxidants appeared on the market in the recent years. Owing to the growing interest in natural antioxidants, new products containing plant extracts, their blends, and/or isolated plant secondary metabolites occurred on the market [9]. However, in many countries, the control of dietary supplements faces important problems, such as lack of quality control, purity, and in some instances even lack of efficiency and safety. The absence of safety can often be attributed to the presence of

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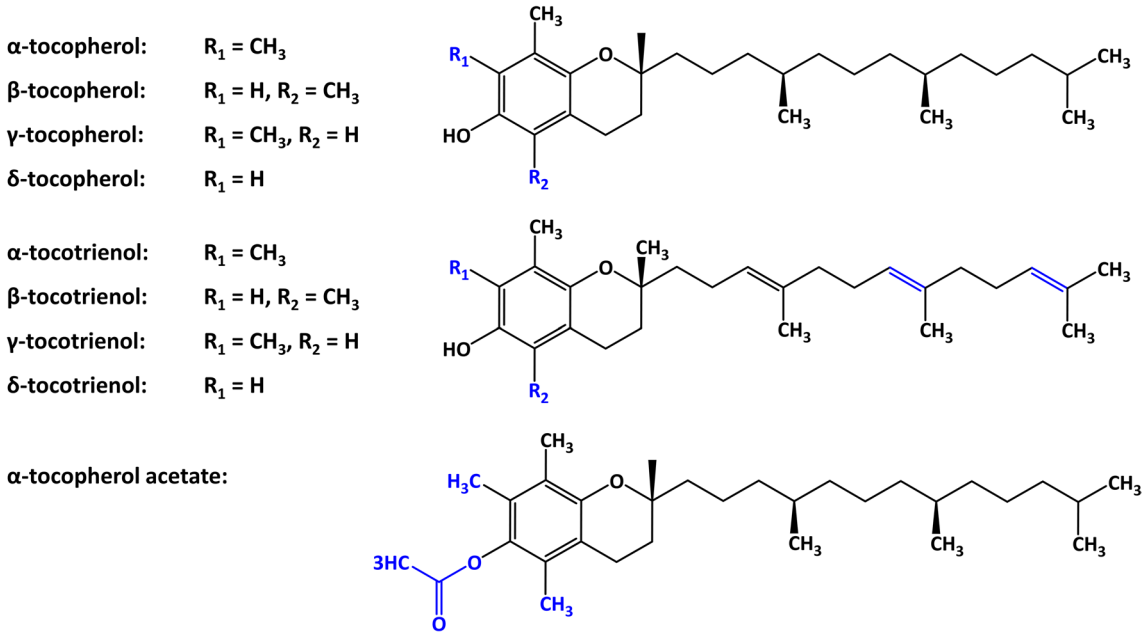

Fig. 1 Structures of tocopherols and tocotrienols present in dietary supplements

undeclared substances, i.e., product adulteration [10, 11]. The lack of efficiency is then related to under-dosing of active substance in the dietary supplement product [12]. Therefore, effective methods for the determination of these active compounds are required to ensure the desired quality of dietary supplements.

Currently, high-performance liquid chromatography (HPLC) is the gold standard for the determination of tocopherols [2] using normal phase (NP) as well as reversed phase (RP) modes, and isocratic or gradient elution with fluorescent (FLR), electrochemical, mass spectrometry (MS), and UV detection [2, 13]. Overall, all forms of tocopherols can be separated on NP columns, whereas it is challenging to separate β- and γ-positional isomers using RP stationary phases [2, 4] and these two often had to be determined together [13]. Due to the higher stability and lower susceptibility to oxidation, esterified forms of α-tocopherol, especially α-tocopherol acetate, are usually used as the source of vitamin E in dietary supplements, parenteral breeding, and cosmetics. The ester bond is hydrolyzed after digestion allowing the effect of α-tocopherol to be preserved [13].

Several chromatographic methods have been developed for the determination of α-tocopherol in pharmaceuticals and medical food preparations. In general, the same conditions can be applied to both esterified and free forms leading to the same chromatography time in NP-HPLC and longer retention times of esters in RP-HPLC [13]. NP-HPLC with FLR detector was used for the analysis of α-tocopherol acetate alone [14] or together with γ- and δ-tocopherol [15]. The separations were also carried out using RP-HPLC with UV [16, 17] and MS [9] detection, non-aqueous RP-HPLC–UV [18, 19], gas chromatography with flame ionization detector [20], and micellar electrokinetic chromatography–UV [21] systems. Even though other compounds such as retinol, retinol acetate, vitamin A, and/or γ-tocopherol were analyzed simultaneously with α-tocopherol acetate, none of these methods separated α-tocopherol acetate together with all eight forms of vitamin E. Moreover, many of these methods required a long time for analysis preventing them from being useful as routine control methods. Only one paper reported separation of all eight vitamin E forms in biological samples in 15 min [22]. Considering sample preparation, several methods have been successfully used. Extraction in 2-propanol and hexane-ethyl acetate [14] and matrix solid-phase dispersion [15] was applied prior to NP-HPLC methods. Supercritical fluid extraction [19] and solvent extraction [9, 16] were used for the sample preparation before RP-HPLC. Liquid–liquid extraction (LLE) using hexane was carried out before non-aqueous RP-HPLC as well as GC method [18, 20]. SFC has recently shown great potential for analysis of tocopherols in clinical and food samples. SFC and/or ultra-high-performance supercritical fluid chromatography (UHPSFC) methods were developed for the determination of all eight forms of vitamin E in human serum in 4.5 min [23], in plant material in 6.2 min gradient [24], and in palm oil in 5.5 min [25], as well as for the determination of 4 tocopherols, 3 tocotrienols, and tocopherol acetate in soybean oil in 10 min [26]. Seven vitamin E derivatives were determined in plasma together with other

lipophilic vitamins and carotenoids within 8 min using UHPSFC–MS/MS and supported liquid extraction [27].

The aim of this work was to develop effective and fast analytical method including sample preparation and separation for the quality control of dietary supplements containing different forms of vitamin E. Due to the variability of dosage forms, different sample preparation procedures have been proposed and applied for the quality control of drops, capsules, tablets, and granulate dietary supplements.

Experimental Part

Chemicals and Reagents

Reference standards of tocopherols were ready-to-use solutions in hexane as follows: 100 mg mL^{-1} α-tocopherol (α-T, 99.9%), 50 mg mL^{-1} β-tocopherol (β-T, 99.0%), 25 mg mL^{-1} γ-tocopherol (γ-T, 97.3%), and 100 mg mL^{-1} δ-tocopherol (δ-T, 95.5%) and were provided by Sigma-Aldrich (Steinheim, Germany). Reference standards of tocotrienols were also ready-to-use 25 mg mL^{-1} solutions in ethanol as follows: α-tocotrienol (α-T3, 98%), β-tocotrienol (β-T3, 98%), γ-tocotrienol (γ-T3, 98%), and δ-tocotrienol (δ-T3, 98%) provided by Larodan Fine Chemicals (Malmö, Sweden). α-Tocopherol acetate (α-TA, 96%) was a viscous liquid obtained from Sigma-Aldrich (Steinheim, Germany).

Methanol (MeOH), ethanol (EtOH), isopropanol, acetonitrile (ACN), all of them LC–MS grade, hexane, heptane, and dichloromethane (DCM) in HPLC grade were obtained from Sigma-Aldrich (Prague Czech Republic). Hydrochloric acid (35% analytical grade) was purchased from Lach-Ner (Neratovice, Czech Republic). Carbon dioxide in purity > 99.995% was supplied by Messer, Prague, Czech Republic. Ultrapure water was prepared immediately prior to use using MilliQ reverse osmosis system (Millipore, Bedford, MA, USA).

Ultra-High-Performance Supercritical Fluid Chromatography

The ultra-high-performance supercritical fluid chromatography system Acquity UPC^2 (Waters, Milford, MA, USA) consisting of a binary pump, an autosampler, a column oven, a convergence manager, a PDA, and a QDa detectors were used. The separations and method validation were carried out using Acquity UPC^2 BEH 2-EP (100 × 3.0 mm i.d., 1.7 µm) column from Waters (Milford, MA, USA) kept at a temperature of 50 °C. The gradient elution using CO_2 and MeOH started with 0.5 min isocratic step at 1% MeOH, was followed with a linear increase to 4% MeOH within 0.5 min and another isocratic step at 4% MeOH for 2 min making the total gradient time 3 min. To equilibrate the column at the starting conditions, percentage of the modifier was reduced to 1% at 3.1 min and held for 2 min making the total analysis time 5 min. Flow rate was 2.0 mL min^{-1} and back pressure regulator (BPR) pressure 13.0 MPa (1885 psi, 130 bar). 1 µL of sample was used for injection. The autosampler temperature was kept at 5 °C. Analytes were detected and quantified using PDA detection and extraction of chromatograms at 290 nm. MS detection using single quadrupole was used for identity confirmation in more complex samples of dietary supplements. Coupling of QDa single quadrupole was achieved via dedicated splitting device and isocratic solvent manager (Waters, Milford, MA, USA) delivering methanol with 0.1% ammonium hydroxide as a make-up liquid at 0.3 mL min^{-1}.

Further stationary phases including Acquity UPC^2 Torus Diol, Acquity UPC^2 Torus DEA, Acquity UPC^2 Torus 1-AA, and Acquity UPC^2 Torus 2-PIC (100 × 3.0 mm i.d., 1.7 µm) were tested during the method development as well as several other organic modifiers including ethanol and mixtures of MeOH/ACN (1:1) and EtOH/ACN (1:1).

Ultra-High-Performance Liquid Chromatography

The ultra-high-performance liquid chromatography system Acquity UPLC (Waters, Milford, MA, USA) was used. The isocratic elution separations were carried out using Ascentis Express F5 column (100 × 3.0 mm) packed with 2.7 µm core–shell particles and the mobile phase comprising 75% ACN/MeOH (1:1) and 25% water. Flow rate was 1.0 mL min^{-1}, temperature at 40 °C, the autosampler temperature 4 °C, and the injected volume 5 µL. Acquity PDA was used for detection at 290 nm.

Other columns used in method development included 100 × 2.1 mm i.d., 1.7 µm Acquity UPLC CSH C18, Acquity UPLC CSH PFP (pentafluoro phenyl), Acquity UPLC CSH phenyl, and Kinetex PFP (100 × 2.1 mm i.d., 2.7 µm) packed with core–shell particles.

Preparation of Standard Solutions

The stock standard solutions of tocopherols and tocotrienols for UHPSFC measurement were prepared by dilution of individual compounds with heptane to give a solution at a concentration of 1 mg mL^{-1} or by making a mixed solution diluted with heptane. These solutions were stored at − 20 °C and were prepared fresh every 2 weeks. Heptane was also further used as the dilution solvent. Stock solutions for standard addition approach during method validation had to be water miscible. Therefore, ethanol was used for dilutions in this case.

Preparation of Samples of Dosage Forms

Several different dosage forms of vitamin E are commercially available for vitamin E supplementation including drops, capsules, tablets, and granulate. They contain α-tocopherol acetate in most instances, but can also contain free α-tocopherol or a combination of various tocopherols and tocotrienols from plant extracts. In total, eight dietary supplements with vitamin E were tested. Their characteristics in terms of dosage form, vitamin E type, and declared content are shown in Table 1.

Preparation of Samples from Drops

Drops are very viscous solution of vitamin E in olive oil. Contents of the container were thoroughly homogenized and subsequently diluted with heptane using dilution factor of ten. Slow pipette draw rate was needed due to very high viscosity of the sample.

Preparation of Samples from Capsules

Gelatin capsules were filled with vitamin E in solution of a vehicle, typically olive oil. To release the content, the capsule was mechanically disrupted with scissors to allow the release of the content in heptane. Due to relatively high content of vitamin E in capsules, a large volume of solvent (10 mL) and 10 min shaking at 250 RPM using a mechanical shaker (Promax 1020, Heidolph Instruments, Schwabach, Germany) was used for quantitative transfer from the capsule.

Preparation of Samples from Tablets and Granulate

Each individual tablet was dissolved in 75% MeOH using a large vial with a cap. Ultrasonic bath (Bandelin Sonorex Digitech, Sigma-Aldrich, Prague, Czech Republic) was used for 10 min to promote complete dissolution. Subsequently, heptane was added for liquid–liquid extraction in the ratio sample/heptane 1:2. The mixture was shaken for 10 min at 250 RPM using mechanical shaker. The upper layer was collected for UHPSFC analysis.

Samples from granulate were prepared in a similar way. Approximately 0.5 g granulate was weighted precisely and dissolved in 0.5 mL water. Dissolution was straightforward and did not require sonication. MeOH (1.5 mL) was added to achieve 75% MeOH concentration. The sample was processed with the LLE including additional centrifugation step to allow better phase separation.

Method Validation

Method validation involved determination of accuracy, precision, selectivity, linearity, and system suitability test (SST) following International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) requirements [28]. Limits of detection and quantification were not evaluated, because they are not mandatory for assay methods.

Results and Discussion

Optimization of UHPSFC and UHPLC Methods

In our previous study, we reported usefulness of UHPSFC–MS for analysis of tocopherols and tocotrienols in human serum using BEH 2-EP stationary phase [23] which was in agreement also with another previously published study in food analysis [24]. However, α-TA was not among target analytes. Therefore, we had to incorporate it in the current method. With this aim, new stationary phases including Torus Diol, Torus DEA with diethylamine functionality, Torus 1-AA with 1-aminoanthracene functionality, and Torus 2-PIC with 2-picolylamine functionality were tested for selectivity of the separation of tocopherol and tocotrienol derivatives besides BEH 2-EP column. While tocopherols and tocotrienols possess very lipophilic structures with log *P* within 8–10, even higher value of log *P* equal to 12 is reported for α-TA. Thus, α-TA is likely to be an early eluting

Table 1 Characteristics of tested dietary supplements

No.	Product name	Dosage form	Manufacturer	Vitamin E type	Declared content (mg)
1	Natural vitamin E	Tablets	Favea	Plant extract	12
2	Vitamin E 100	Capsules	Zentiva	α-TA	100
3	Vitamin E 100	Capsules	Noventis	α-TA	100
4	Vitamin E 100 I.U	Capsules	Generica	α-TA	100
5	Evening primrose	Capsules	Vitaharmony	α-TA	5
6	Selzink	Tablets	PRO.MED	α-TA	31.5
7	Dologran	Granulate	Farmex EU SE	α-TA	3.6 mg/2 g
8	Vitamin E, liquid	Drops	Martinez Nieto	α-T	24 mg/1 mL

α-TA α-tocopherol acetate, *α-T* α-tocopherol

compound in UHPSFC and its elution may require very low percentage of organic modifier for retention.

UHPSFC with Torus chemistries and BEH 2-EP column has shown itself as a powerful technique enabling separation of all nine derivatives of vitamin E (Fig. 2). Using simple isocratic elution without any optimization with the mobile phase containing 3% of MeOH and CO_2, a full baseline separation was obtained with Torus 2-PIC in less than 4 min. Two tested stationary phases, namely, Torus DEA and BEH 2-EP, enabled efficient separation of eight derivatives and one coelution was monitored at a similar analysis time. Difficulty of the separation of the critical pairs of β- and γ-isomers of both tocopherols (peaks 3 and 4) and tocotrienols (peaks 6 and 7) was not generally confirmed under UHPSFC conditions. These isomers separated well using three tested stationary phases with the best resolution observed with BEH 2-EP. Overall, the worst results were obtained using Diol stationary phase and 1-AA stationary phase. The latter stationary phase was designed to provide good retention for non-polar analytes due to the non-polar functionality. Although the retention capability was confirmed, the separation selectivity was poor demonstrated

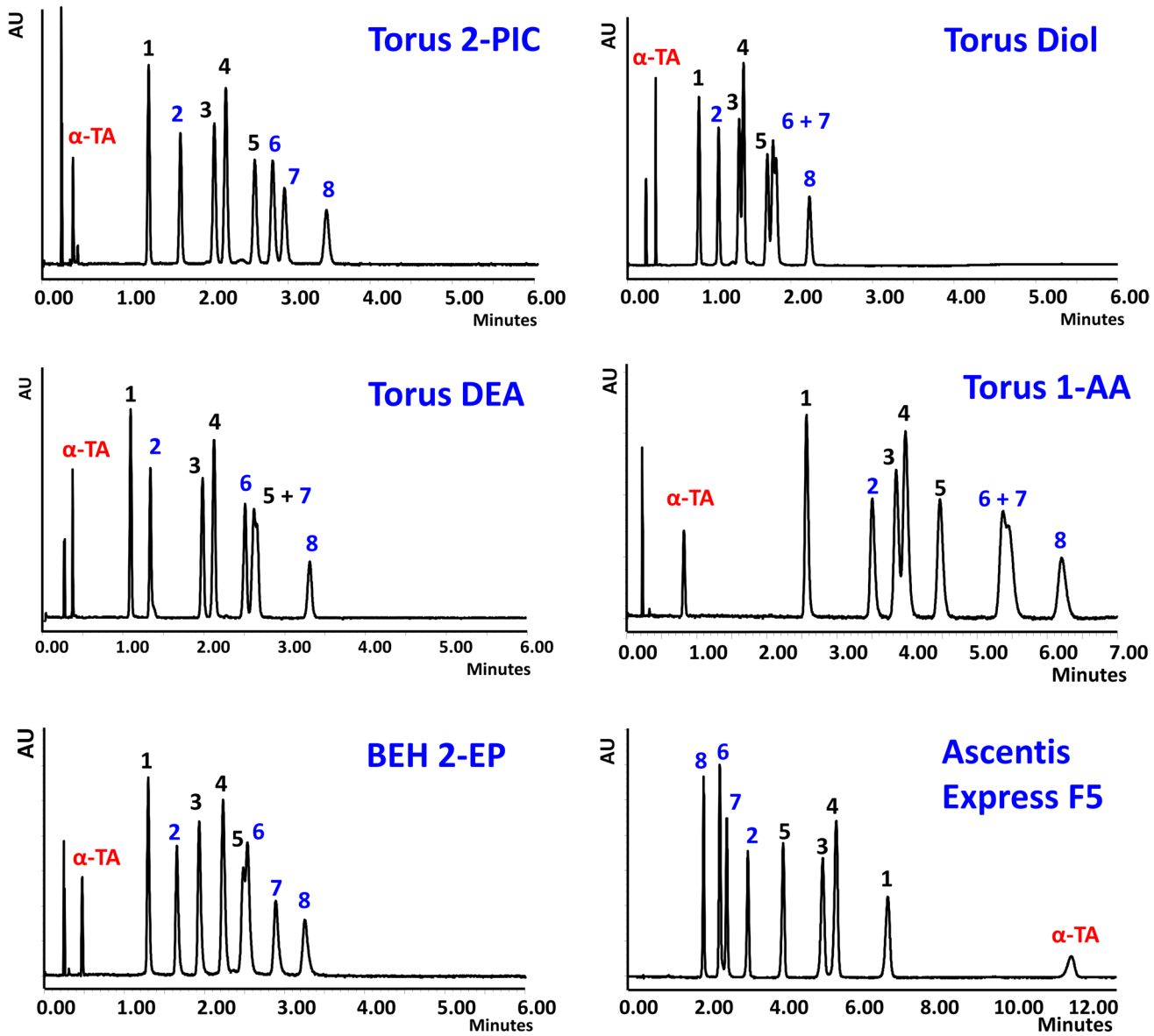

Fig. 2 Separation of nine tocopherol derivatives in UHPSFC and UHPLC. UHPSFC separations were carried out using 5 columns including Torus 2-PIC, Torus diol, Torus DEA, Torus 1-AA, and BEH 2-EP using isocratic elution with the mobile phase containing 3% MeOH/CO_2, flow rate 2 mL min^{-1}, 50 °C, and 13 MPa. UHPLC was carried out using Acentis Express F5 column and isocratic elution with mobile phase ACN/MeOH (1:1) and water 75:25, flow rate 1.0 mL min^{-1}, 40 °C. (1) α-tocopherol, (2) α-tocotrienol, (3) β-tocopherol, (4) γ-tocopherol, (5) δ-tocopherol, (6) β-tocotrienol, (7) γ-tocotrienol, (8) δ-tocotrienol

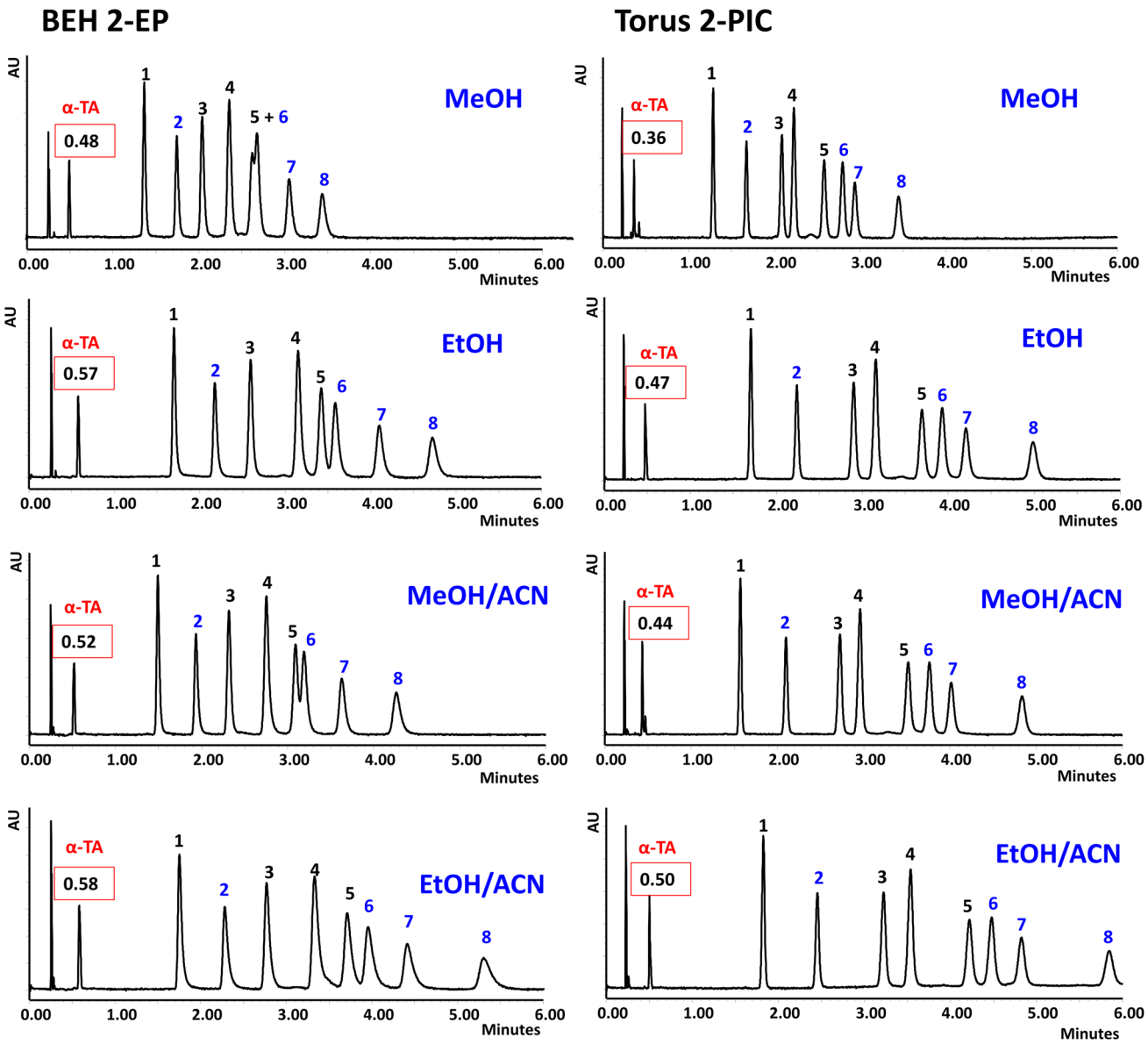

Fig. 3 Effect of organic modifier on separation using Torus 2-PIC and BEH 2-EP and isocratic elution with the mobile phase containing 3% organic modifier/CO_2, flow rate 2 mL min^{-1}, 50 °C, and 130 bar. (1) α-tocopherol, (2) α-tocotrienol, (3) β-tocopherol, (4) γ-tocopherol, (5) δ-tocopherol, (6) β-tocotrienol, (7) γtocotrienol, (8) δ-tocotrienol

with two coelutions, one full and one partial, that were attributed to the critical pairs of β- and γ-isomers. These coelutions were obtained despite the longest retention resulting in analysis time of 7 min exceeding that found for other tested stationary phases. Actually, these conditions exhibited some similarity with problematic separation of tocopherols under some RP-HPLC conditions. Separation using Torus diol column also led to two coelutions including the critical pairs of β- and γ-isomers. However, this happened at a generally shorter retention of the target analytes.

Finally, an important feature of our newly developed method was also the retention of α-TA, which was very poorly retained using most of the tested stationary phases featuring a retention time of about 0.35 min. The best retention of α-TA was observed when using BEH 2-EP and Torus 1-AA column. Torus 2-PIC and BEH 2-EP were then selected for further optimization, since they enabled the baseline separation of all derivatives in case of the former and both relatively good selectivity and retention of α-TA in case of the latter (Fig. 2).

To tackle the problem of limited retention of α-TA, organic modifiers with lower elution strength were tested including EtOH, as well as mixtures of MeOH/ACN (1:1) and EtOH/ACN (1:1). Their use further increased the

retention and in some cases also resolution among individual peaks of tocopherols and tocotrienols, but had a very small effect on retention of α-TA while using isocratic elution with 3% organic modifier. Figure 3 shows a comparison of effect of organic modifier on retention and separation using Torus 2-PIC and BEH 2-EP column. Indeed, while the retention of α-TA increased from 0.36 min to 0.50 when using EtOH/ACN (1:1), the retention of the last peak almost doubled. This apparently resulted in prolonged analytical run. Slightly better results were observed using BEH 2-EP stationary phase, as well as the improvement in separation of critical pair of peaks of δ-T and β-T3 (Fig. 3).

Using lower percentage of organic modifier would be a possibility to increase retention of α-TA. However, lower elution strength of such mobile phase resulted in similar results as in case of lower elution strength organic modifiers. Indeed, the elution pattern was similar to that of MeOH/ACN, but the retention was substantially longer. Finally, the problem has been solved using gradient elution that started at a very low percentage of organic modifier corresponding to 1% MeOH and keeping a short isocratic hold to allow α-TA to be retained on BEH 2-EP column. Following elution was then accomplished using 4% organic modifier to facilitate elution of tocopherols and tocotrienols that exhibit a lower log *P* coefficients compared to α-TA.

UHPLC method for separation of nine forms of vitamin E was also developed to compare both the state-of-the art approaches. Tested stationary phases involved CSH with C18, PFP, and phenyl moiety, as well as two columns packed with pentafluorophenyl core–shell particles. The difficulty to separate the β- and γ-isomers in RP-HPLC conditions was confirmed when using column packed with media containing CSH chemistries despite the analytical runs even longer than 30 min. Several mobile phase combinations including mixtures of MeOH and water, ACN/MeOH and water, as well as the addition of isopropanol were tested. The poorest selectivity was observed using CSH C18 column even at an analysis time of 60 min, while columns with phenyl moieties produced better results at shorter analysis times. The baseline separation of all nine derivatives was achieved using Ascentis Express F5 column with 75% ACN/MeOH (1:1) and 25% water as the mobile phase in isocratic mode (Fig. 2). Similar separation featuring a longer retention times and worse efficiency was achieved using Kinetex PFP column. The analytical run on Ascentis Express F5 required 12 min and demonstrated complementary selectivity to developed UHSFC method. Despite being the best results reported so far for UHPLC separation of all nine tocopherol derivatives, UHPSFC enabled the separations more than two times faster. Therefore, it was used in the further experiments.

Optimization of Sample Preparation from Capsules

Gelatin capsules are made of a soft gel material enabling delivery of oil-based liquid active compounds. Therefore, their dissolution in extracting solvent such as MeOH or heptane was impossible. Preliminary experiments using 0.1 mol L^{-1} HCl as the model gastric acid allowed the dissolution of the capsule. However, the recovery obtained after LLE from HCl solution was inconsistent and lower that 80%. Thorough disintegration of the capsule using scissors, then rinsing the scissors in a large volume of the extracting solvent, and 10 min shaking on mechanical shaker allowed release of the oil content from the capsule with α-TA and the quantitative determination.

Optimization of Sample Preparation from Tablets and Granulate

Tablets and granulate samples were treated with LLE to selectively extract non-polar vitamin E and to remove the ballast compounds from the dosage form matrix. Hexane, heptane, and their mixtures with DCM at a ratio of 8:2 and 9:1 were selected for extraction due to the high lipophilicity of the target analytes. Two solvents including water and MeOH were tested as potential dissolution media for tablets and granulate, respectively. This selection was limited by immiscibility with extraction solvents allowing complete phase separation. α-TA was used as the model analyte for this optimization. Figure 4 demonstrates that MeOH was more convenient dissolution medium providing overall higher recoveries except when MeOH:DCM (9:1) was used as the extraction solvent. The reason probably resulted for the better solubility of lipophilic analytes in MeOH. LLE using mixtures of hexane and heptane with DCM produced in general low recoveries in a range of 41–69%, again except when heptane:DCM (9:1) was used for extraction from MeOH. In general, the best results were obtained with extraction of α-TA from MeOH solution in heptane (Fig. 4). The repeatability of the extraction procedure was very good, as demonstrated with the error bars.

Practical test with the tablet and granulate samples revealed that MeOH is not the most convenient solvent for the granulate sample. Further experiments were carried out to find alternative dissolution solvent. We found that the solubility of granulate was promoted in the presence of water. Therefore, the final procedure for granulate involved dissolution in water and subsequent addition of MeOH in a volume to achieve a percentage of 75% MeOH in water. This solvent was suitable for all tablets and granulates, and was used in method validation procedure.

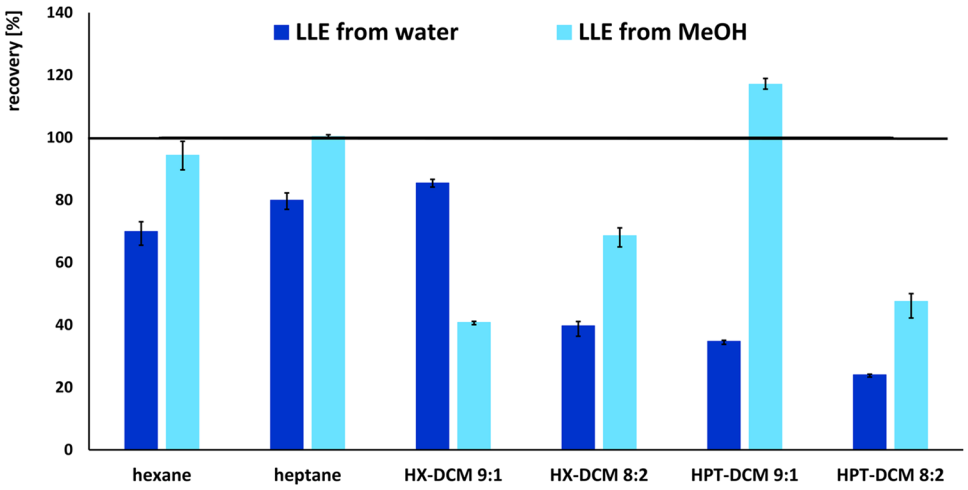

Fig. 4 Optimization of liquid–liquid extraction procedures using water and methanol as the polar phase and a variety of non-polar solvents as extractants. *HX* hexane, *HPT* heptane, *DCM* dichloromethane

Method Validation

First, SST and calibration range were evaluated for individual analytes. While the linearity was confirmed in a range of 0.25–10 µg mL^{-1} for tocopherols and tocotrienols with correlation coefficient ≥ 0.9994, extended range was needed for evaluation of α-TA in dietary supplements due to its generally higher content (see Table 1). Therefore, linearity range for α-TA was defined as 0.25–50 µg mL^{-1} with correlation coefficient ≥ 0.9998. SST results provided repeatability < 0.70% RSD and < 2.0% RSD for retention time and peak area, respectively. Method selectivity was confirmed by the inspection of blank analyses and using PDA spectra purity function.

Determination of accuracy and precision was carried out using the standard addition approach. Samples of individual dosage forms were spiked with stock standard solutions of target compound/s at 80%, 100%, and 120% concentrations of the declared content. Validation of drops and capsules' dosage forms was straightforward and could be carried out directly, while LLE used for tablets and granulate was further optimized to confirm that either 100% MeOH or 75% MeOH can be used as polar phase representing sample medium for LLE treatment. Figure 5 shows a comparison of accuracy and precision determined at three different concentration levels in three replicates as required by ICH [28]. The results confirm that both methods assure accuracy within the limit 100 ± 5% and precision < 5% RSD when using Selzink tablets.

Detailed validation results for Selzink tablets and vitamin E drops are presented in Table 2 as selected examples of different types of dosage forms.

Application of UHPSFC-PDA Method on Measurement of Dietary Supplements

All measured samples of standards and dietary supplements were injected in heptane, which is a good solvent (1) due to good solubility of tocopherol derivatives, (2) suitable injection solvent in UHPSFC, and (3) no need for evaporation after LLE. This feature simplifies the sample preparation, decreases the time of operation, and allows selective isolation of tocopherol derivatives from the solid dosage forms. Chromatographic profiles of selected dietary supplements are shown in Fig. 6 and confirm symmetric peak shapes for all analytes.

The results of determination of vitamin E content in individual dietary supplements are shown in Table 3 as an average of six different samples' analysis. Tested dietary supplements contained three different forms of vitamin E including α-TA, α-T, and an extract from plant containing tocopherols and tocotrienols. Eight vitamin E derivatives were confirmed in the plant extract containing tablet. α-T, α-T3, and γ-T3 were the most abundant components in the extract, while only δ-T was not present. All tested dosage forms contained declared type of vitamin E. No degradation of α-TA was observed in these dosage forms. Six of the tested dosage forms met rigorous criteria 100 ± 5% for the content of active compound required for the regulated pharmaceuticals. Two dosage forms, granulate and one of the capsules, contained even higher amount of α-TA than declared. These were low vitamin E amount containing dosage forms; thus, the risk of overdose was not relevant. Higher content found in the two formulations can be result of the relatively low price of α-TA as the active ingredient. Despite the lack of

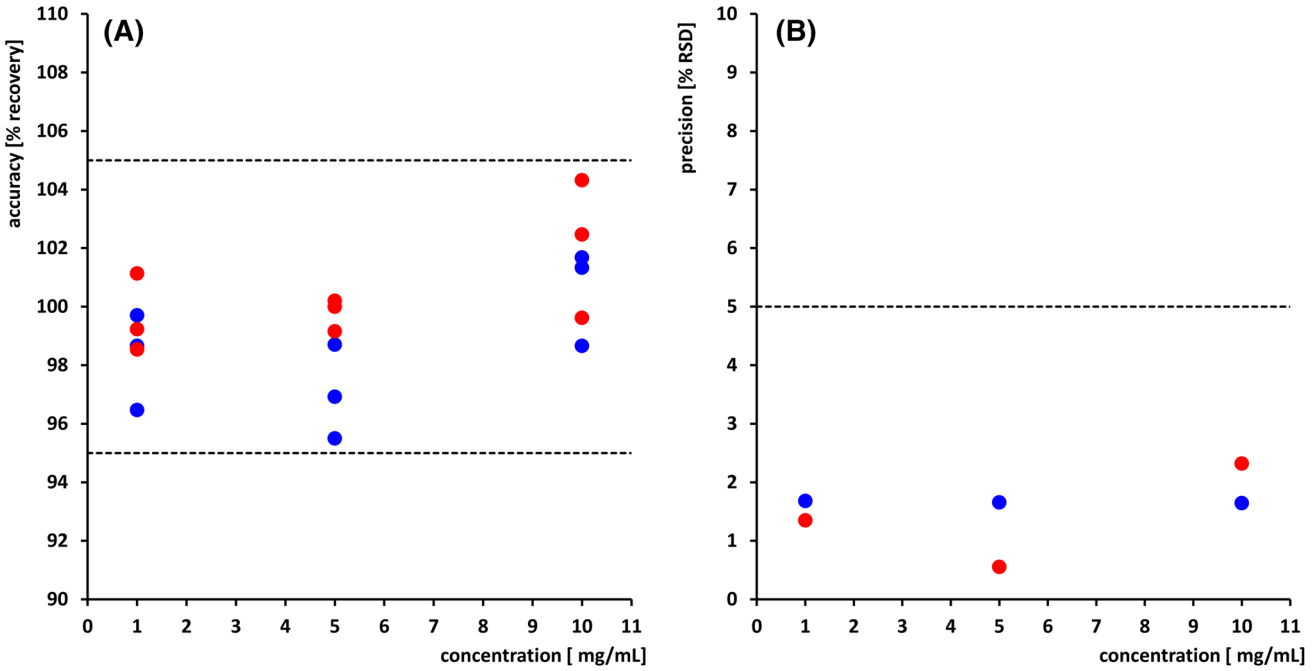

Fig. 5 Comparison of validation parameters of accuracy (**a**) and precision (**b**) for liquid–liquid extraction from 100% methanol (blue dots) and 75% methanol (red dots)

Table 2 Validation results for Selzink tablets and natural vitamin E drops

	Natural vitamin E, drops			Selzink tablets		
Validation level	80%	100%	120%	80%	100%	120%
Content (mg)	1.92	2.40	2.88	25.20	31.50	37.80
Content found sample 1	1.91	2.45	2.89	24.75	31.00	37.66
Content found sample 2	1.92	2.46	2.82	25.37	31.36	37.11
Content found sample 3	2.00	2.51	2.95	24.86	30.32	36.90
Method accuracy	101.7	102.9	101.1	95.9	99.6	100.0
Method precision	2.5	1.4	2.2	1.3	1.7	1.1

the quality control procedures in manufacturing of dietary supplements, good quality of tested vitamin E preparations was confirmed.

Conclusions

Developed UHPSFC-PDA method for the determination of nine forms of vitamin E including four tocopherols, four tocotrienols, and tocopherol acetate was found to be applicable for quality control of dietary supplements with vitamin E. Fast and efficient separation was achieved using BEH 2-EP column with gradient elution and enabled two times faster analysis compared to the state-of-the art UHPLC method. Sample preparation procedures were adapted to the available dosage forms. Simple dilution was sufficient for oil-based liquid dosage forms represented by drops and capsules filled with liquid vitamin E. Liquid–liquid extraction was optimized for tablet containing vitamin E in solid state. Non-polar nature of vitamin E requires use of non-polar solvents that cannot be directly injected into RP-HPLC system. Possibility of direct injection of heptane solutions after dilution and LLE is an inherent benefit of UHPSFC method and was clearly demonstrated in this study.

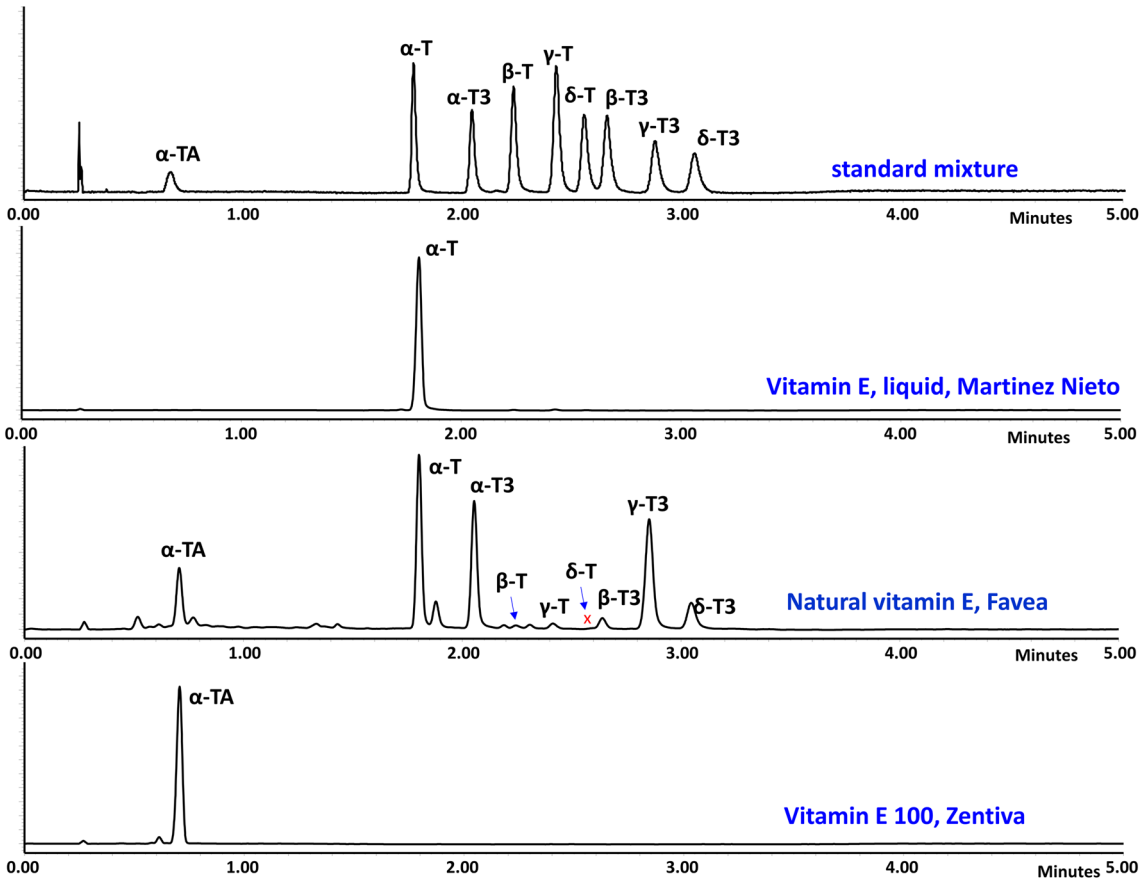

Fig. 6 Chromatograms of dietary supplements in comparison with the standard mixture analyzed on BEH 2-EP stationary phase using gradient elution from 1 to 4% of MeOH in 3 min, flow rate 2 mL min^{-1}, 50 °C, and 130 bar

Table 3 Summary of vitamin E content found in individual dietary supplements

No.	Product name	Declared content (mg)	Found content (mg)	% of found vs. declared	RSD (%)
1	Natural vitamin E	12	11.5	95.5	4.1
2	Vitamin E 100	100	102.6	102.6	1.8
3	Vitamin E 100	100	100.3	100.3	2.3
4	Vitamin E 100 I.U	100	101.5	101.5	2.5
5	Evening primerose	5	6.9	138.3	0.8
6	Selzink	31.5	31.8	101.0	3.4
7	Dologran	3.6 mg/2 g	117.1	117.1	2.5
8	Vitamin E, liquid	24 mg/1 mL	102.1	102.1	2.4

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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