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Journal of Chromatography A, 909 (2001) 215–223

JOURNAL OF CHROMATOGRAPHY A

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Determination of *RS*,*E*/*Z*-tocotrienols by HPLC

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Received 1 May 2000; received in revised form 17 October 2000; accepted 30 October 2000

Abstract

Synthetic α-tocotrienol was separated into four geometrical *E*/*Z* side chain isomers by preparative HPLC (permethylated β-cyclodextrin phase). The isolated isomers were resolved in ethylene glycol dimethyl ether, converted into the corresponding methyl ether using dimethyl sulfate, and the tocotrienol methyl ethers were extracted with *n*-hexane. A subsequent HPLC separation on a chiral phase (adsorbent cellulose derivated with 3,5-dimethyl phenyl carbamate) discriminates between the enantiomers of each *E*/*Z* side chain isomer, achieving the complete resolution of the eight occurring synthetic *RS*,*E*/*Z*-α-tocotrienols. The method can be shortened by omitting the preparative separation of the *E*/*Z* tocotrienol isomers prior to the chromatography on the chiral dimethyl phenyl carbamate phase. The simplified method achieved the following separation: *RS*,*E*/*Z*-α-tocotrienol separated into five peaks, *RS*,*E*/*Z*-β-tocotrienol into eight, *RS*,*E*/*Z*-γ-tocotrienol into six and *RS*,*E*/*Z*-δ-tocotrienol into eight peaks. The naturally occurring *R*,*E*-*E*-tocotrienol isomer could be identified within the synthetic *RS*,*E*/*Z*-isomers by co-chromatography with tocotrienol methyl ethers derived from natural sources, respectively.

Keywords: Tocotrienols; Vitamin E

1. Introduction

Vitamin E consists of eight biologically active vitamers: a family of four tocopherols (α-, β-, γ- and δ-) and the four structurally related tocotrienols (α-, β-, γ- and δ-). The latter differ from the tocopherols only by having three double bonds in the isoprenoid side chain.

Tocotrienols (T_3) show some very interesting physiological qualities, such as their high antioxidative potency in biological membranes, their anticancer properties and their effect as hypocholesterolemic agent [1–3]. Because of the health benefits of the tocotrienols, many synthesis methods have been suggested. As a matter of fact, a completely stereoselective synthesis could be applied successfully [4], but the practicable ways often lead to racemic products and *E*/*Z*[1] isomers [5–9]. Thus, a total of eight isomers (four racemic pairs of geometric *E*/*Z* isomers) can be present for each tocotrienol (Table 1). Contrary to this, naturally occurring tocotrienols exclusively possess the 2*R*,3′(*E*),7′(*E*)-configuration (Fig. 1). For this reason, the separation and identifi-

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[1]The term *E*/*Z* describes the sum of *Z*–*Z*-, *Z*–*E*-, *E*–*Z*- and *E*–*E*-tocotrienols.

0021-9673/01/$ – see front matter
PII: S0021-9673(00)01110-9

Table 1
The eight possible *RS*,*E*/*Z* isomers of each tocotrienol

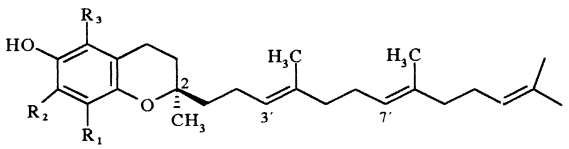
R configuration at position 2 of tocotrienols		*S* configuration at position 2 of tocotrienols	
Possible isomers	Short term	Possible isomers	Short term
2*R*,3′(*Z*),7′(*Z*)	*R*,*Z*–*Z*	2*S*,3′(*Z*),7′(*Z*)	*S*,*Z*–*Z*
2*R*,3′(*Z*),7′(*E*)	*R*,*Z*–*E*	2*S*,3′(*Z*),7′(*E*)	*S*,*Z*–*E*
2*R*,3′(*E*),7′(*Z*)	*R*,*E*–*Z*	2*S*,3′(*E*),7′(*Z*)	*S*,*E*–*Z*
2*R*,3′(*E*),7′(*E*)	*R*,*E*–*E*	2*S*,3′(*E*),7′(*E*)	*S*,*E*–*E*

cation of synthetic tocotrienol isomers is a necessary, but also challenging task.

Racemic tocopherols originating from chemical synthesis have been in use as dietary supplements or for pharmaceutical application since a long time, but, so far not the tocotrienols. Many publications deal with the different bioavailability and vitamin E-activity of tocopherol stereoisomers [10–14] whereas equivalent studies in the case of the biological active tocotrienols can be realized only now, by using the new HPLC method presented here.

Recently, we introduced a HPLC method using a β-cyclodextrin phase that for the first time allows a baseline separation of the four racemic *E*/*Z* side chain isomers of separately injected tocotrienols [15]. This analytical HPLC method can be combined with preparative HPLC steps and is suitable for the determination of *E*/*Z* tocotrienols in complex matrices, as demonstrated in another publication [16].

A reliable analysis of synthetic tocotrienol isomers must allow for the identification of racemic products. But until now, an analytical method for the separation of *RS* tocotrienols was still lacking. We therefore developed an efficient HPLC method, which is a necessary tool in the HPLC determination of racemic tocotrienol products. Moreover, this new type of vitamin E-analysis has to be considered if any thorough physiological investigations about the properties of synthetic tocotrienol isomers shall be performed.



2R,3′*(E)*,7′*(E)* (R,*E*-*E*)-tocotrienol

	R_1	R_2	R_3
α-tocotrienol	CH_3	CH_3	CH_3
β-tocotrienol	CH_3	H	CH_3
γ-tocotrienol	CH_3	CH_3	H
δ-tocotrienol	CH_3	H	H

Fig. 1. Structures of tocotrienols.

2. Experimental section

All solvents were HPLC-grade and were purchased from Merck (Darmstadt, Germany) unless otherwise stated. The water used was Millipore 18 MΩ grade.

2.1. Chemicals

rac-α-tocotrienol, *rac*-β-tocotrienol, *rac*-γ-tocotrienol, *rac*-δ-tocotrienol were obtained from Merck with a purity certified each 100% (HPLC). Approximately 50 mg of the standards were initially diluted in 25 ml 2-propanol. Further dilution was made in acetonitrile or *n*-hexane, depending on the procedure they were needed for. The derivatization of tocotrienols to the methyl ether was performed with ethylene glycol dimethyl ether from Sigma (Deisenhofen, Germany), potassium hydroxide from Merck, dimethyl sulfate in analytical grade from Merck and double distilled *n*-hexane. 60 g of the potassium hydroxide were added to 100 ml water in order to prepare a potassium hydroxide solution.

2.2. Separation of α-tocotrienol E/Z isomers prior to derivatization

Two×250 μl of a solution of α-T_3 (approx. 2.0 g/l) was separated into *E*/*Z* isomers by preparative HPLC on a permethylated β-cyclodextrin (β-PM) phase, as described earlier [15]. The elution order is first *RS,Z-Z-*, second and third *RS,Z-E-* and *RS,E-Z-* or the reverse and *RS,E-E*-α-T_3 as last eluting isomer. Peaks containing α-T_3-isomers were accordingly collected and pooled, and the solvents were evaporated. Finally the fractions were concentrated by a stream of nitrogen and the residuals were stored in an overall of 1.5 ml of 2-propanol. Provided, that the fractions were collected without loss of *E*/*Z* isomers, the portion of each isomer can be estimated to be one fourth of the total amount of α-T_3.

2.3. Separation of α-tocopherol, α-, γ- and δ-tocotrienol from palm oil prior to derivatization

A solution of raffinated, hardened palm oil in *n*-hexane (100 g/l) was dried with sodium sulfate and filtered through two folded filters. The content of vitamin E in the palm oil was determined before, according to the analytical HPLC method (diol phase) as described earlier [16]: α-tocopherol 100 mg/kg, α-T_3 143 mg/kg, γ-T_3 101 mg/kg, δ-T_3 46 mg/kg and traces of β-T_3. Three×100 μl portions of the palm oil solution were fractionated into α-tocopherol, α-, γ- and δ-T_3, using the semi-preparative HPLC method (diol phase) as published before [15]. The collected and accordingly pooled eluates were each evaporated and the residues were used for derivatization.

2.4. Separation of α-tocotrienol from ewe's milk fat prior to derivatization

The extraction of milk fat from ewe's milk (approx. 0.6 mg α-T_3/1000 g milk), its saponification, the extraction of the unsaponifiable lipids and the isolation of α-T_3 by semi-preparative HPLC was carried out as described recently [16].The isolated α-T_3-fractions were pooled, evaporated and the residue was used for derivatization.

2.5. Derivatization of tocopherols and tocotrienols to the corresponding methyl ether

Riss et al. [17] developed a method for the derivatization of tocopherols to their methyl ether. This method was adopted to a great extent concerning the reagents, but simplified with regard to the apparatus.

2.5.1. Method I

An amount of solution, containing approx. 10 μg tocopherols, tocotrienols or tocotrienol side chain isomers, was transferred into an 1.3 ml ambered screw-cap vial. The solvent was evaporated under nitrogen and the residue was dissolved in 50 μl ethylene glycol dimethyl ether. During the whole procedure, the solution was stirred by a small magnetic stirring bar. Twenty five (25) μl potassium hydroxide solution (100 ml water plus 60 g potassium hydroxide) were added dropwise. The vial was flushed with nitrogen and 30 μl dimethyl sulfate were dripped inside. The vial was closed and after 1 h at ambient temperature, a further 15 μl dimethyl sulfate were added. After an additional 2 h at ambient temperature, the solution was evaporated by a stream of nitrogen, 100 μl water were added and the methyl ether derivative was extracted twice with 500 μl *n*-hexane. The pooled *n*-hexane phases were then used for HPLC determination.

2.5.2. Method II (scale up operation)

Compared to method I, the total yield of methyl ethers can be increased by using a double amount of vitamers, reagents and solvents. The reaction was performed in a larger vial and the methyl ethers were extracted three times with a total amount of 3 ml *n*-hexane.

2.6. Chromatography on a chiral phase

Chromatographic separation was achieved on a Chiralcel OD-H column (250×4.6 mm, 5 μm particle size, adsorbent cellulose derivated with 3,5-dimethyl phenyl carbamate) from Daicel (distributed by Mallinckrodt Baker, Griesheim, Germany) at ambient temperature. A 20 μl sample loop was used. The mobile phase was a solution of 0.05% 2-propanol in isohexane (v/v) and the flow-rate was 1.0

ml/min. The apparatus consisted of a L-7100 chromatography pump (Merck Hitachi, Darmstadt, Germany) with a L-7480 fluorescence detector (Merck, Hitachi). Chromatograms were recorded at an emission wavelength of 330 nm (excitation wavelength 295 nm).

3. Results and discussion

3.1. HPLC separation of RS,E/Z-tocotrienols

Following the preparative isolation of the geometric isomers, the complete chiral HPLC resolution of the eight existing *RS,E/Z* isomers could be achieved as demonstrated in the case of α-tocotrienol-methylether (α-T_3-ME). Omitting the preparative step on a cyclodextrin phase, the isomers of the tocotrienol derivatives also could be separated to a great extent.

The separation of *E/Z*-α-T_3 isomers into its *RS* enantiomers succeeded under isocratic elution conditions on a chiral cellulose carbamate derivative HPLC column. The method involved the isolation of *E/Z* isomers by separation of the four fractions each of them containing a pair of α-T_3 enantiomers. This step was followed by the methylation of the separated *E/Z*-α-T_3 to yield α-tocotrienol-methyl ether (α-T_3-ME) and finally the chiral separation. By comparison of the results with a chromatogram of α-T_3-ME from natural sources, the *R,E-E*-isomer could be identified. The chromatograms of the racemic *E/Z*-α-T_3-ME are given in Fig. 2. The retention times of enantiomers of *Z-Z*-α-T_3-ME differed more than six min. Even more distinct appeared the separation of *E/Z*-α-T_3 (derived from the second peak of β-PM method). The enantiomers of the other *E/Z*-α-T_3 (derived from the third peak of β-PM method) eluted in an interval of 3 min, and also the fourth pair of *E-E*-α-T_3-ME enantiomers was baseline separated. By co-injection with a derivative of natural α-T_3, the second signal of *E-E*-α-T_3-ME could be identified to be *R,E-E*-α-T_3-ME. In Fig. 2c and d a third peak arose. This remarkable observation can be hypothetically explained to be caused by a degradation product of the before eluting enantiomer, respectively. The signal occured and increased in intensity with progressive use and storage of the derivated sample solution. Besides, the peak area of the late eluting enantiomer is equal to the peak area of the earlier enantiomer. This finding is consistent with the assumption that the chemical synthesis produces the enantiomers in equal proportions. In any case this interesting phenomenon should have to be clarified by additional experiments in order to confirm potential differences in enantiomer stability.

Fig. 2e shows the chromatogram of α-T_3-ME, omitting the preparative separation of *E/Z* isomers prior to methylation. The eight *RS,E/Z*-α-tocotrienols were divided into five peaks, as could be expected from Fig. 2a–d. The ratios of peak areas were 18:5:24:19:27:7, considering the supposed degradation product.

In order to asses the efficiency of this new method, the synthetic β-, γ- and δ-T_3 references have been investigated as well, which also consisted of the eight possible *RS,E/Z*-isomers, respectively. By comparison with the other vitamers, *RS,E/Z*-β-T_3-ME (Fig. 3) showed very long retention times from up to 42 min. Besides, there were nine peaks in the chromatogram, but the fourth was thought to be no isomer, due to its low intensity. *RS,E/Z*-γ-T_3-ME (Fig. 4) retarded much less and eluted already within 10 min. The last eluting peak represented the naturally occurring *R,E-E*-isomer derivative, as could be deduced from Fig. 7. Although the peak was very symmetrical, a co-elution with another isomer could not be excluded, since the total number of theoretically expected isomers of *RS,E/Z*-γ-T_3-ME was not fully separated. The chromatogram of *RS,E/Z*-δ-T_3-ME (Fig. 5) exhibited the separation of all existing eight isomers. The optimum separated last substance could be identified to be the methyl ether of the naturally occurring *R,E-E*-δ-T_3 (Fig. 7). This for the derivatization of tocotrienols adapted method also allowed the splitting of *all-rac*-α-tocopherol into the 2*R* and 2*S* stereoisomers (Fig. 6). The separation pattern looked like the one that could be achieved by Riss et al. [17], who used 100% *n*-hexane as eluent. But as an advantage, the analysis time was shortened by a half. By co-chromatography with α-tocopherol-ME derived from natural sources, we confirmed *RRR*-α-tocopherol-ME to produce the fourth peak.

Also matrices with a very low content of vitamers were suitable for the determination of *RS,E/Z*-toco-

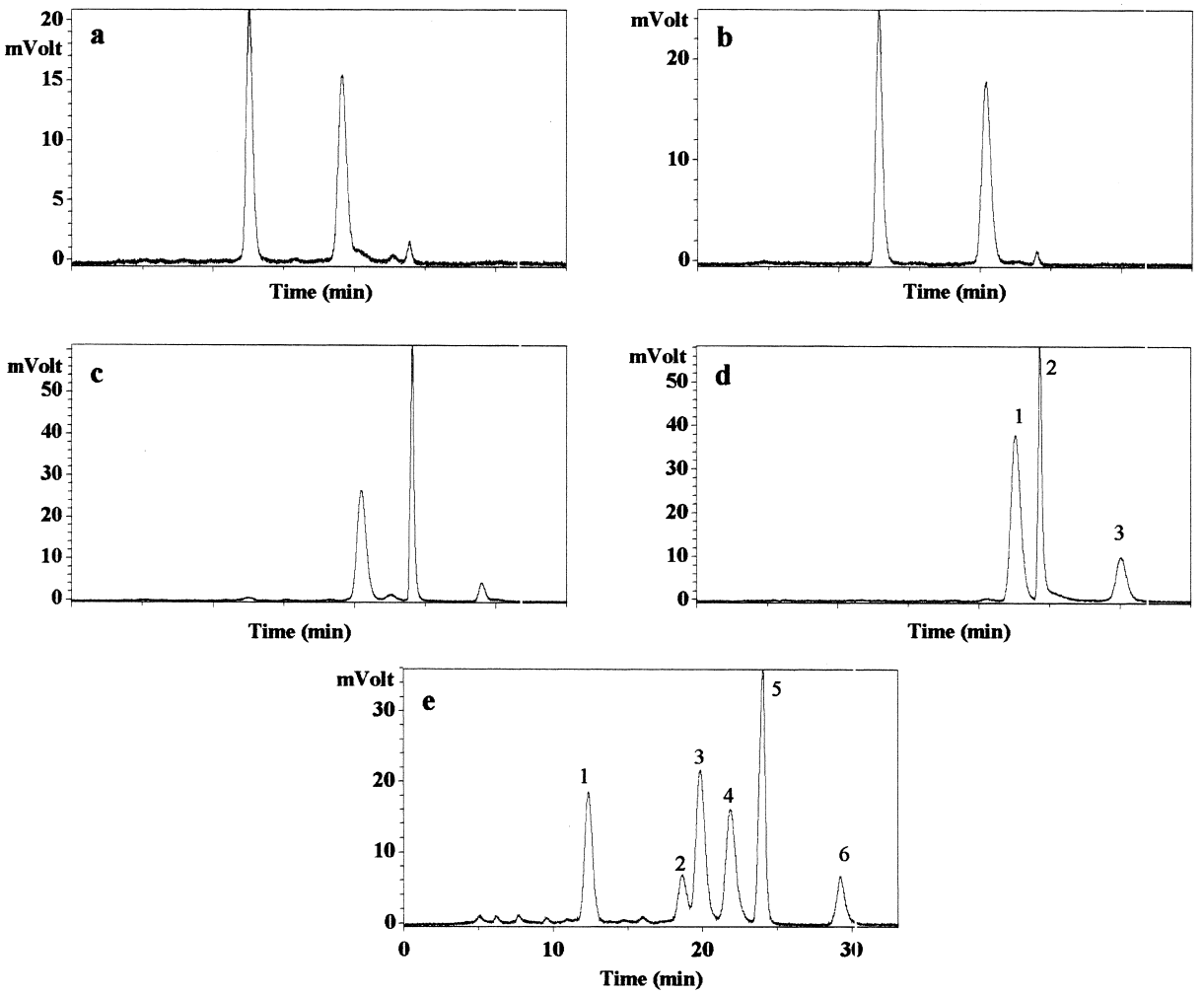


Fig. 2. a–e. Separation of *E*/*Z*-α-T_3-ME (method II) into their enantiomers by chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. (a) Pair of enantiomers of *RS,Z-Z*-α-T_3-ME. (b) Pair of enantiomers of *RS,E*/*Z*-α-T_3-ME (derived from the second peak isolated by β-PM phase). (c) Pair of enantiomers of *RS,E*/*Z*-α-T_3-ME (derived from the third peak isolated by β-PM phase); the last eluting substance is supposed to be a degradation product of the second eluting enantiomer. (d) 1=*S,E-E*-α-T_3-ME; 2=*R,E-E*-α-T_3-ME; 3=presumably degradation product of 2. (e) *RS,E*/*Z*-α-T_3-ME omitting the separation of side chain isomers by preparative HPLC; 1=two diastereomers of *Z-Z*- and *E*/*Z*-α-T_3-ME (derived from the second peak isolated by β-PM phase); 2=one enantiomer of *Z-Z*-α-T_3-ME; 3=two diasteromers of *E*/*Z*-α-T_3-ME (derived from the second and third peak isolated by β-PM phase); 4=*S,E-E*-α-T_3-ME; 5=*R,E-E*-α-T_3-ME coeluting with a diastereomer of *E*/*Z*-α-T_3-ME (derived from the third peak isolated by β-PM phase; 6=presumably degradation product of *R,E-E*-α-T_3-ME.

trienols. Therefore, this chiral method was supplemented with a saponification step and the extraction of the unsaponifiable matter during the sample preparation procedure, in order to enrich small amounts of vitamers before methylation. An example is demonstrated in Fig. 8: α-T_3 has been isolated from ewe's milk and the methylation product successfully investigated on the chiral carbamate derivative HPLC-phase. The main peak has been identified to be *R,E-E*-α-T_3-ME by co-injection with *RS,E-E*-α-T_3-ME (not shown).

Scheme 1 summarizes the steps that are required to determine α-T_3 stereoisomers from complex matrices.

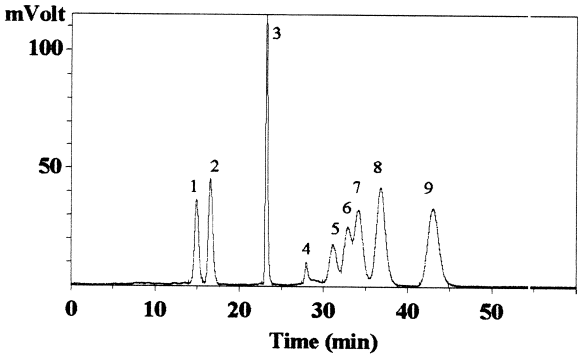


Fig. 3. *RS,E/Z*-β-T_3-ME (method II) was separated into nine peaks but the fourth is supposed to be no isomer; chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection.

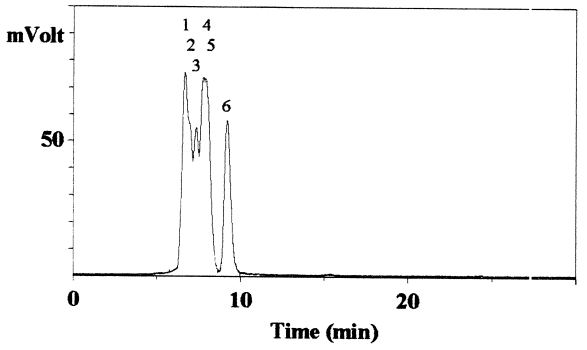


Fig. 4. *RS,E/Z*-γ-T_3-ME (method II) was discriminated into six peaks; chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. 6=*R,E-E*-γ-T_3-ME.

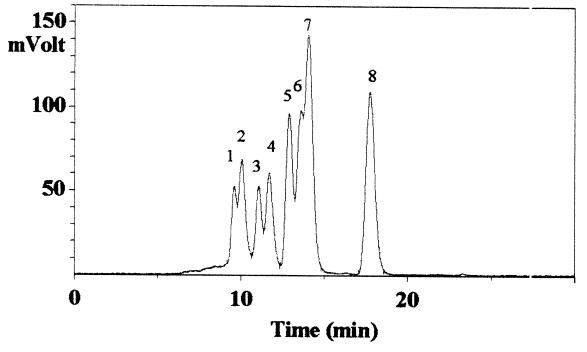


Fig. 5. *RS,E/Z*-δ-T_3-ME (method II) was fully resolved into eight stereoismers; chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. 8=*R,E-E*-δ-T_3-ME.

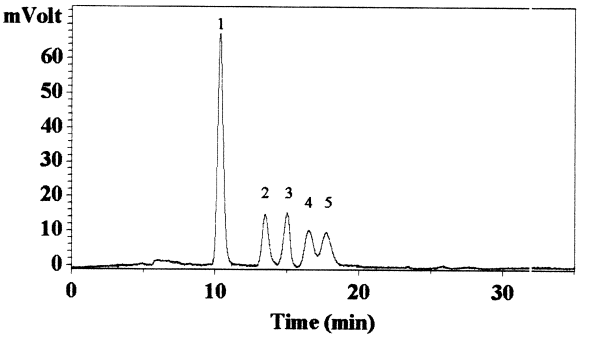


Fig. 6. *all-rac*-α-tocopherol-ME (method II); chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. 1=*SSR, SSS, SRS* and *SRR* [17]; 2=*RSS* [17]; 3=*RRS* [17]; 4=*RRR*; 5=*RSR* [17].

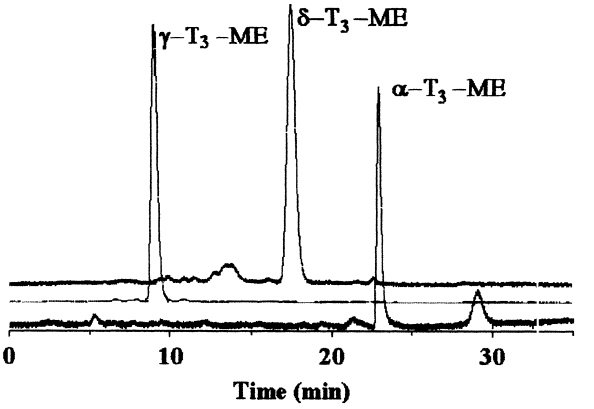


Fig. 7. *R,E-E*-T_3-ME (method I) derived from palm oil; chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. The tocotrienols have been isolated by semi-preparative HPLC (diol phase) and afterwards converted to their methyl ether.

3.2. Influence of derivatization on the HPLC separation of enantiomers

Vecci and coworkers [18] already discussed the influence of different O-substituted *all-rac*-α-tocopherols on the HPLC separation, using a home-made chiral phase ((+)poly(triphenylmethylmethacrylate) coated on silica) and an acetonitrile/water (9:1, v/v) eluent. Neither this, nor another working group reports on a HPLC separation of tocopherol stereoisomers without derivatization [13,14,19]. A method using a cellulose carbamate derivative phase was published in 1991 for the first time [20],

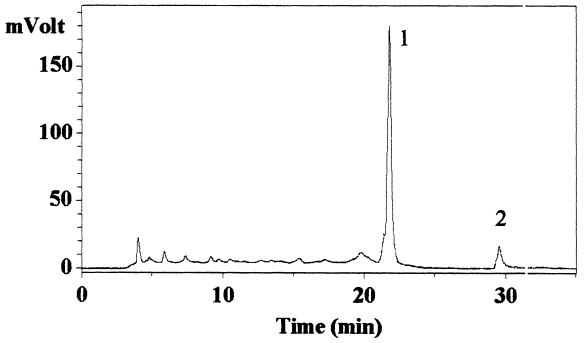


Fig. 8. α-T_3-ME derived from ewe's milk, chiral HPLC on a cellulose carbamate derivate phase (Chiralcel OD-H) and fluorescence detection. The α-T_3 has been enriched from ewe's milk fat by saponification and extraction of the unsaponifiable lipids. It has been isolated by semi-preparative HPLC (diol phase) and afterwards converted to its methyl ether (method I) 1=*R,E-E*-α-T_3-ME,2=probably degradation product of 1.

followed by a detailed description of the chiral separation of methyl ether derivatives [17]. Also tocotrienols have to be converted prior to separation as confirmed by own investigations. Non-methylated tocotrienols were scarcely retarded and left the column in a broad peak with large tailing (25 min). In the case of cellulose derivative phases, enantioresolution depends upon inclusion into shape-selective chiral cavities within the polymer network [21]. Obviously, the highly polar hydroxyl group of tocotrienols give rise to nonstereoselective binding between the solute and the carbamate site of the stationary phase. Thus, derivatization is used to block these unspecific associations and provides the analyte molecule with groups capable of interacting with the chiral cellulose stationary phase.

3.3. Influence of the mobile phase on the HPLC separation of RS,E/Z-α-T_3-ME

The choice of mobile phase is one of the main influencing factors for chiral recognition in this stationary phase. According to the manufacturers specification, hexane/2-propanol and hexane/ethanol mixtures are allowed as eluent in any proportions. 2-propanol acts as a strong displacer of the α-T_3-ME, and isohexane is a comparatively poor competitor for the insertion into the chiral cavity or for the binding to achiral sites nearby the cavity. Using 100% isohexane, a resolution of six broad peaks of *RS,E/Z*-α-T_3-ME could be achieved within 60 min, whereas a mobile phase of isohexane/2-propanol (90:10, v/v) caused the elution of all eight stereoisomers without retention.

3.4. Estimation of the detection limit of RS,E/Z-α-T_3-ME

The calibration graph for *RS,E/Z*-α-T_3-ME was obtained under the optimum conditions shown in

Step 1: Enrichment of α-T_3 from samples with low α-T_3 content by extraction of the lipid fraction, saponification and extraction of the unsaponifiable lipids
↓
Step 2: Isolation of α-T_3 from sample solution with known α-T_3 contents by semi-preparative HPLC (diol phase)
↓
Step 3: Isolation of the four *E/Z*-α-T_3 side chain isomers (RS,*Z-Z*-, RS,*Z-E*-, RS,*E-Z*-, RS,*E-E*-α-T_3) by preparative HPLC (permethylated β-cyclodextrin phase)
↓
Step 4: Conversion of RS,*E/Z*-α-T_3into RS,*E/Z*-α-T_3-ME
↓
Step 5: Chiral resolution of racemic RS,*E/Z*-α-T_3-ME into enantiomers
a: RS,*Z-Z*-α-T_3-ME → R,*Z-Z*-α-T_3-ME + S,*Z-Z*-α-T_3-ME
b: RS,*Z-E*-α-T_3-ME → R,*Z-E*-α-T_3-ME + S,*Z-E*-α-T_3-ME
c: RS,*E-Z*-α-T_3-ME → R,*E-Z*-α-T_3-ME + S,*E-Z*-α-T_3-ME
d: RS,*E-E*-α-T_3-ME → R,*E-E*-α-T_3-ME + S,*E-E*-α-T_3-ME

Scheme 1.

Fig. 2e and described in the Experimental section. It was determined by adding the peak area of all separated isomers and plotting the sum (y) against the analyte concentration in the test solution (x). The fluorescence detector response was linear over the tested range of 1.2–12 mg/l and the equation for the calibration graph ($n=6$) was $y=6.061\ x+2.277$ (r value>0.99). When 24 ng (i.e. 1.2 mg/l) *RS,E/Z*-α-T_3-ME were resolved into their geometric and racemic compounds using the described chiral HPLC method, the separated isomers caused a signal intensity 3–5 times higher than the baseline noise. The detection limit of the single isomers therefore could be estimated to be 1/4–1/8 (3–6 ng), as determined for the sum of the isomers.

On the basis of these data the calibration graphs of the isomers that do not coeluate could also be determined. Knowing the ratios of peak areas the equation for one enantiomer of *Z-Z*-α-T_3-ME was $y=1.095\ x+0.021$ and for S,*E-E*-α-T_3-ME it was $y=6.081\ x+0.432$.

In summary, it can be said that the presented chiral HPLC method achieved resolution of the synthetic tocotrienol stereoisomers for the first time. As demonstrated in the case of α-T_3-ME, all *E/Z* isomers could be discriminated into their enantiomers. The *R,E-E* isomer mixed with the eight occurring synthetic isomers could be identified by comparison with the corresponding tocotrienol derivative from a natural source. A semi-preparative HPLC method (diol phase) was required for complex matrices in order to isolate the α-, β-, γ- and/or δ-T_3 from the lipid fraction, or, if necessary because of low concentration, from the unsaponifiable matter. A preparative HPLC step (β-PM phase) followed to obtain the four *E/Z* isomers separately. Afterwards, every single one of the eight methylated *RS,E/Z*-α-T_3 could be identified by means of a chiral cellulose carbamate derivate phase.

4. Conclusion

In comparison to the conventional tocotrienol assays, this new method as described above offers major improvements. The chiral recognition makes it possible to follow the bioavailability and the endogenous transport of synthetic tocotrienol preparations. Thus, physiological studies in the manner of *all-rac*-α-tocopherol can now be realized. Even performing the chiral analysis without the previous preparative separation of the geometric side chain isomers, the following conclusion can be drawn: several peaks indicate a synthetic preparation, that contains more than the single natural *R,E-E*-T_3. This time-saving variant is appropriate whenever it is sufficient to identify the presence of any *RS,E/Z*-tocotrienols. Also accompanying stereospecific steps in a chemical synthesis of tocotrienols, this new method can be very helpful.

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