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Countercurrent chromatographic isolation and purification of alpha-tocomonoenol from the vitamin E extract of palm oil

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Abstract {später}

A new Vitamin E homologue, α -tocomonoenol was detected in palm oil, but was not isolated in large amounts and with high purity so far. Here we present an easy and fast method to isolate α -tocomonoenol from Vitamin E rich nutrient capsules with countercurrent chromatography (CCC). With the solvent system *n*-hexane – acetonitrile – benzotrifluoride (10:6.5:3.5, v/v/v) about 30 mg α -tocomonoenol with a purity of 75 % could be isolated from 1 g crude sample. Column chromatography with 20 % deactivated silica gel and *n*-hexane – ethyl acetate (95:5, v/v) was performed to gain 5.6 mg α -tocomonoenol with a purity of 99.5%. Determination of the purity was performed by GC/MS and structural verification was done by NMR spectroscopy. The only remaining impurity was found to be the marine derived form of α -tocomonoenol (MDT) by NMR analysis which was so far not described in palm oil.

Introduction

Vitamin E is the name for a group of eight different substances consisting of a chromane backbone which is substituted with one or more methyl groups and one hydroxyl group on the aromatic part as well as a C₁₆ hydrocarbon and a methyl group on C-2. The so-called tocopherols can be distinguished by number and positions of the methyl groups on the 6-chromanol (O₁–C₈) moiety (α , β , γ and δ) as well as by either zero (tocopherols) or three double bonds (tocotrienols) on the C₁₆-hydrocarbon (**Fig. 1a,b**) [1]. The radical chain breaking antioxidant function of vitamin E leads to ameliorative effects especially on cardiovascular diseases [2]. Interestingly the biological activity differs from tocopherol to tocopherol [1]. While α -tocopherol (α -T) has the highest antioxidant capacity *in vivo*, tocotrienols were found to have anti-inflammatory effects, prevent arteriosclerosis and inhibit tumor promotion in mice [1, 3–5]. Typically, tocopherols are dominant in most plants and were therefore discovered about 40 years before the discovery of tocotrienols in palm oil [1, 6]. Today, palm oil is still one of the very few known natural sources with significant amounts of tocotrienols [7, 8].

Interestingly, palm oil was also the source in which Japanese researchers described an additional tocopherol derivative with one double bond on C-2 of the C₁₆ hydrocarbon, which was named α -tocotrienol (α -T₁) (**Fig. 1c**) [9]. This finding was verified by the estimation of the concentration of α -T₁ in palm oil with the help of the response factor of α -tocopherol because the target compound was not available as standard [8, 10]. Subsequently, α -T₁ was also qualitatively detected in roasted pumpkin seed oil from Slovenia and in sunflower oil [11, 12]. Evaluation of the effectiveness of α -T₁ has not been provided, most likely due to lack of a reference standard. However, another α -tocotrienol isomer was identified in marine samples and thus termed marine derived tocotrienol (MDT, **Fig. 1d**) [13, 14]. A study on the bioavailability of MDT and α -T₁ in liver cells of mice (fed with deodorization scum formed from tuna oil) indicated 49% (MDT) and 30% (α -T₁) of the bioactivity of α -T [15].

The goal of our study was the isolation of α -T₁ from a palm oil source by countercurrent chromatography (CCC). CCC is a chromatographic technique first described by Ito in the late 1960s [16] and since then successfully used for the isolation and purification of various natural and synthetic compounds from mixtures [17]. In CCC the liquid stationary phase is held in a hollow tube wound around a coil, which is fast rotating around a central axis, while the liquid mobile phase is pushed through [16]. The absence of a solid stationary phase in CCC leads to several advantages, i.e. high sample loads could be injected in a CCC system without time consuming sample preparation steps [16, 18]. To circumvent the isolation of the tocopherol fraction from crude palm oil we selected vitamin E dietary supplementary capsules made from palm oil according to the label. The product was claimed to contain ~0.1 g tocotrienols per g.

2. Materials and methods

2.1. Sample

Dietary supplementary capsules enriched in vitamin E were purchased from an internet store. According to information provided by the manufacturer, vitamin E of these capsules was enriched from the pulp of the oil palm *elaeis guineensis*.

2.2. Organic solvents and chemicals

Acetonitrile (99.9%) was purchased from Th. Geyer (Renningen, Germany). Methanol (>99.8%) and *n*-hexane (>95%) were from VWR Chemicals (Darmstadt, Germany). Pyridine, ethyl acetate (both distilled before use), trifluorotoluene (benzotrifluoride, BTF, >99%) and silica gel 60 were from Sigma-Aldrich (Steinheim, Germany). Deuterated chloroform (CDCl₃, 99.8%) was from Deutero (Kastellaun, Germany) and toluene (99.9%) was from Carl Roth (Karlsruhe, Germany). The trimethylsilylating reagent (99% *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane) was from Supelco

(Bellefonte, PA, USA). The internal standard for GC/MS measurements, 5 α -cholestane (purity >98%), was from Acros Organics (Geel, Belgium).

2.3. Sample preparation

Exposure to light was omitted whenever possible. Samples were stored in amber glass vials and laboratory lights were turned off during the day. When the use of white glass could not be avoided, it was wrapped with aluminum foil. About 20 vitamin E capsules (each ~300 mg) were cut with a knife and the viscous liquid inside was squeezed into a 50 mL tube which contained 10 mL *n*-hexane. After addition of ~10 mL water the tube was vigorously shaken and centrifuged for 5 min at 4000 rpm. Then, the upper (organic) phase with the analytes was separated with a Pasteur pipette and the solvent was removed by a gentle stream of nitrogen at 40 °C. The crude extract (~6 g) was stored in the refrigerator at 4 °C until use.

2.4. Gas chromatography with mass spectrometry (GC/MS)

An aliquot of the crude extract (section 2.3) was measured on a 6890/5973 GC/MS system (Agilent Technologies, Santa Clara, CA, USA), equipped with a 30 m HP-5MS column (0.25 mm internal diameter, 0.25 μ m film thickness) as recently described in detail [19]. The temperature program started at 55 °C (1 min), was raised with 20 °C/min to 255 °C by 1.5 °C/min to 283 °C and finally by 15 °C/min to 300 °C which was held for 5 min (total run time 35.8 min) [19]. All other GC measurements were performed with a second 6890/5973 GC/MS system equipped with a cool on-column inlet (Hewlett-Packard/Agilent, Waldbronn, Germany), a pre-column (2 m, 0.53 mm i.d., deactivated with 1,3-diphenyl-1,1,3,3-tetramethyldisilazane; BGB Analytics, Bökten, Switzerland) and a 15 m, 0.25 mm i.d. capillary column coated with 0.1 μ m film thickness dimethylpolysiloxane (Rtx-1; Restek, Bellefonte, PA, USA), which was previously described in detail [12]. Only the GC oven program was slightly different: It started for 1 min at 60 °C, followed by a ramp of 10 °C/min

until the final temperature 320 °C, which was held for 14 min (total run time: 39 min). All samples were silylated prior to GC/MS analysis as recently described in detail [12].

2.5. Countercurrent chromatography (CCC)

Separations were performed with a Quickprep MK8 system (AECS Downend, UK) equipped with four separable coils (each about 120 mL) which were located in two different bobbins [20]. A perfectly balanced system was obtained by using two coils (2.1 mm internal diameter stainless steel tubing) located in different bobbins, namely coil 2 (122 mL) in bobbin 1 and coil 3 (114 mL) in bobbin 2, which were connected with PTFE tubing, resulting in a total volume of 236 mL. The temperature was maintained at 25 °C by external cooling and the rotor speed was set to 870 rpm (maximum value). The effluent of the centrifuge was continuously monitored by a Flash 10 diode array detector (DAD; Ecom, Praha, Czech Republic) set at $\lambda = 290$ nm [10, 21]. After each CCC experiment, the whole system was flushed with methanol.

2.6. CCC procedure

The solvent system was created by mixing *n*-hexane, acetonitrile and BTF (10:6.5:3.5, *v/v/v*) in a 2.5 L separatory funnel. After about 1 h equilibration time, the two phases were separated and degassed by ultrasonication. The selected coil system was first filled with the upper (stationary) phase. Thereafter, rotation (870 rpm) was started and the lower (mobile) phase was pumped into the system in the head-to-tail mode at 4 mL/min. The stationary phase retention (S_f value) was measured by collecting the effluent of the system in a graduated cylinder. When displacement of the stationary phase stopped and the CCC/UV baseline at $\lambda = 290$ nm was stable, the measurement was started by the injection of ~1 g crude extract (section 2.3) dissolved in 5 mL upper and 5 mL lower phase. The CCC/UV chromatogram showed five distinct peaks, and the fourth was initially tested to be α -T₁ by GC/MS. Based on the UV response at 290 nm, this peak was collected as one fraction (77.5 min – 92.5 min) in a 100 mL

pear shaped flask. The CCC fraction was liberated from solvents by means of a rotary evaporator (40 °C, 200 mbar).

2.7. Column chromatography

Column chromatography with deactivated silica as adsorbens was performed according to Hammann *et al.* [22, 23]. Silica gel 60 (~10 g) was first dried for 8 h at 110 °C and then deactivated with 20 w% water and shaken for at least 15 min. The slurry of 5 g deactivated silica gel and ethyl acetate was transferred into a glass column (1 cm inner diameter). To remove ethyl acetate, the column was flushed four times with ~20 mL *n*-hexane. The residue (30 mg) of CCC fraction 77.5 min – 92.5 min (section 2.6) was liberated from solvents, taken up in 1 mL *n*-hexane and placed onto the column. Silica fraction 1 was gained with (i) 30 mL *n*-hexane, then the elution solvent was changed to (ii) 40 mL *n*-hexane – ethyl acetate (99:1, *v/v*) (silica fraction 2). Both silica fractions were separately collected in 100 mL pear shaped flasks. Silica fraction 3 was eluted with 50 mL *n*-hexane – ethyl acetate (95:5, *v/v*) and the effluent was collected in ten subfractions of 5 mL each (silica fractions 3-1 to 3-10). Finally, silica fraction 4 was flushed with 40 mL ethyl acetate which was also collected in a 100 mL pear shaped flask. All silica fractions were evaporated to dryness and the residue was transferred into pre-weighed 1.5 mL vials with a small volume of *n*-hexane. After removing the solvent by a gentle stream of nitrogen, the vials were weighed again and aliquots of the residue (~0.05 mg) were silylated and subjected to GC/MS analysis (section 2.5).

2.8. Shake-flask procedure

About 10 mL of the solvent system *n*-hexane – acetonitrile – trifluorotoluene (10:6.5:3.5, *v/v/v*) was mixed and equilibrated. Then, the phases were separated by means of a separatory funnel. About 1 mg crude extract (section 2.3) was placed in a 6 mL vial and 1 mL of each phase of the solvent system was added. After vigorous shaking the phases were allowed

to settle (30 min) before 250 μ L of upper and lower phase were removed and transferred into two different 1.5 mL screw cap vials. The solvent was removed by a gentle stream of nitrogen at 40 °C. The residue was silylated, re-dissolved in 1 mL *n*-hexane and 5 α -cholestane was added as internal standard before GC/MS analysis (section 2.5). $K_{U/L}$ values of tocotrienols (δ -, γ - and α -T₃), α -T₁ and α -T were calculated by dividing the peak areas (corrected by the peak area of 5 α -cholestane) of the substances in the upper phase by the corresponding peak areas in the lower phase.

2.9. NMR spectroscopy

¹H NMR spectra of purified α -T₁ were recorded with a Varian Inova 300 MHz (Varian, Darmstadt/Germany) in CDCl₃. NMR data was processed using the Spinworks software (4.0.5, K. Marat, University of Manitoba, Winnipeg/Canada, 2014).

3. Results and discussion

3.1. GC/MS analysis of the silylated vitamin E extract

The major four abundant tocopherol peaks detected in the silylated capsule oil could be identified by GC/MS to be three silylated tocotrienols (α -, γ -, and δ -T₃) and silylated α -tocopherol (α -T) (**Fig. 2a**), while the small peak in front of silylated γ -T₃ showed the same mass spectrum and was tentatively identified as β -T₃ which is known to occur in traces in palm oil [8, 10]. Besides two silylated sterols (campesterol and stigmasterol, peaks 1 and 2 {geändert damit man nicht mit a und b durcheinanderkommt} in **Fig. 2a**), the sample additionally featured α -T₁ which eluted ~1 min after silylated α -T₃ and ~2 min before silylated α -T from the GC/MS system (**Fig. 2a**). GC/MS analysis of silylated α -T₁ showed the molecular ion at m/z 500 (which is 2 u below silylated α -T and thus featured one additional double bond) as well as the abundant

substituted tropylium cation at m/z 237 (**Fig. 2b**) which verified the presence of three methyl substituents in the aromatic system (while m/z 223 would be characteristic for two methyl groups as in the case of silylated β - and γ -tocools and m/z 209 for one methyl group as in the case of silylated δ -tocools) [24]. Although GC/MS was unsuited to assign the position of the double bonds in tocools, literature data of palm oil [8, 10] produced evidence that the double bond of α -T₁ in palm oil was located on the antepenultimate carbon of the alkyl chain (i.e. between C11' and C12' [8, 10]), as shown in **Fig. 2b**. GC/MS analysis also indicated that α -T₁ contributed with ~3% to the total tocools in the sample, which agrees well with the reported range for palm oil [8, 10]. With the capacity of the present CCC system (~1 g crude oil can be injected), each injection would provide ~30 mg α -T₁. Hence, a further enrichment of α -T₁ before CCC was not required.

3.2. CCC fractionation of the vitamin E extract and GC/MS analysis of silylated fractions

The resulting CCC-UV chromatogram (290 nm) obtained from the injection of ~1 g capsule oil featured the three tocotrienols (δ -T₃ < γ -T₃ < α -T₃), followed by α -T₁ and α -T (**Fig. 3a**) which was verified by GC/MS of collected fractions at the corresponding peak maxima. The elution times corresponded very well with calculated elution times based on the *K*-values determined in shake flask experiments (section 2.8) (**Table 1**). Accordingly, application of CCC in head-to-tail mode eluted tocools with decreasing number of double bonds (T₃ < T₁ < T) while additional methyl groups on the aromatic part of the 6-chromanol moiety increased the CCC retention time (δ -T₃ > γ -T₃ > α -T₃). This elution order resembled the one of reversed phase high performance liquid chromatography (RP-HPLC) [8] while normal phase HPLC eluted the compounds in reversed order [10].

Collecting the effluent at the α -T₁ peak according to the CCC/UV-signal (77.5 min – 92.5 min, 310-374 mL, **Fig. 3a**) provided 30.7 mg α -T₁ with a purity of ~75%. Unfortunately, collection of small CCC fractions (0.5 min or 2 mL) throughout the elution range of α -T₁ did not improve the purity because the purest subfractions only contained ~80% α -T₁. Next to several minor impurities, the remaining share mostly originated from β -T and γ -T which were identified by GC/MS with the help of authentic standards (**Fig. 3b**). The present data published by Hammann *et al.* [25] enabled to develop a general CCC elution scheme for tococools. While the CCC elution increased with increasing number of methyl groups on the 6-chromanol moiety ($\alpha < \beta/\gamma < \delta$), it decreased with the number of double bonds on the C₁₆ hydrocarbon ($T < T_1 < T_3 < T_4$) (**Fig. 4**). These reversed trends led to co-elutions in form of α -T₃ with γ -T₁ and δ -T as well as γ -T₄ with δ -T₃ (**Fig. 4**) in the vitamin E fraction enriched from rice bran oil [25].

Unfortunately, other CCC solvent systems for the separation of these co-eluting tococools were not readily available. Testing of some known solvent systems like the least polar HEMWat system -7 [26] and the toluene system (*n*-hexane – acetonitrile – toluene, 45:45:10, *v/v/v*) recently used to separate polybrominated dibenzofurans (PBDFs) [27] did not lead to separation of α -T₁ from the reported impurities (data not shown).

3.3. Purification by column chromatography

Purification of α -T₁ was performed with column chromatography using deactivated silica as adsorbens (section 2.7). Injection of one CCC fraction with α -T₁ (~30 mg, purity ~75%, section 3.1) eluted α -T₁ and other tococools into silica fraction 3 (50 mL *n*-hexane – ethyl acetate, 95:5, *v/v*) while most other impurities predominantly eluted into the more polar silica fraction 4. Only a small share was also found in silica fraction 3. In the next step, silica fraction 3 was thus divided into 10 sub-fractions of 5 mL (section 2.7). GC/MS analysis of the silylated fractions showed that α -T₁ eluted first from silica gel, followed by the impurities in the sample.

As a consequence, silica fractions 3-1 to 3-5 (0-25 mL) provided 5.6 mg α -T₁ with a purity of 99.5% (**Fig. 5a**, **Fig. S1**). Only traces of another compound co-eluted into this fraction. According to GC/MS this was an isomer of α -T₁ which eluted ~0.1 min earlier from the GC column (**Fig. 5b**).

3.4. Structural verification of isolated α -T₁

The position of the double bond of the (free) α -T₁ isolate was determined by ¹H-NMR. Due to the three methyl groups and the hydroxyl group on the aromatic part of the 6-chromanol, α -T₁ does not show resonances between 6 and 7 ppm. Next to the many unresolved signals high-field in the aliphatic range (<2.5 ppm) [8, 28], tocomonoenols feature resonances in the olefinic range (~4.5–6 ppm) [8, 29]. The α -T₁ isolate featured the expected complex pattern in the aliphatic range along with one triplet at $\delta = 5.12$ ppm (³J = 7 Hz) (**Fig. 5d**). This verified that the double bond system featured one proton with two isochronic vicinal hydrogens that is in accordance with the structure shown in **Fig. 1c** (double bond between C11' and C12' and vicinal coupling with the methylene protons on C10'). However, these structural pre-requisites are also fulfilled with the double bond being located on C3', C4', C7' and C8' position. Interesting in this context is that the GC/MS spectrum of silylated α -T features a characteristic fragment ion at *m/z* 459 ([M-43]⁺) which results from the elimination of the terminal three carbons. Of the structural variants discussed above, the proposed silylated α -T₁ isomer with the double bond on C11' is the only one which cannot produce a characteristic [M-43]⁺ fragment ion in the GC/MS. In agreement with that [M-43]⁺ at *m/z* 457 was not detected in the isolate (**Fig 2b**). Instead, the GC/MS of silylated α -T₁ showed a fragment ion at *m/z* 444 which may be formed by elimination of the four terminal carbons ([M-55]⁺) and further stabilized by loss of one hydrogen ([M-C₄H₇-H]⁺) (**Fig 2b**). Hence, both ¹H-NMR and GC/MS verified a double bond on C11' in α -T₁ as shown in **Fig. 2b**, which is in accordance with literature reports [8].

Likewise more detailed NMR analysis of δ -T₁ recently detected in kiwis also suggested that the double bond was located on C11' [29]. Altogether, 5.6 mg α -T₁ were obtained from 1 g raw material with a purity of 99.5%. {Dieser Satz passt irgendwie nicht zur Strukturbestimmung in diesem Kapitel – sollte das nicht eher am Ende des vorigen Kapitels stehen ?}

3.5. Structure of the trace impurity in the α -T₁ isolate

The ¹H NMR spectrum of α -T₁ additionally showed two small singlets in the olefinic range at 4.67 ppm and 4.69 ppm at ~0.5% of the intensity of the signal at 5.12 ppm (**Fig. 5c**). These signals were attributed to the impurity noted by GC/MS in the α -T₁ isolate (**Fig. 5c**). Since couplings were not observed both protons must be located on the terminal carbon C13' which is additionally involved in a double bond (between C12' and C13'). Only in this configuration protons cannot couple because C12' is bearing a methyl group (and no protons) [13, 15]. Interestingly, the structure ascertained by NMR is the one of the so-called marine derived tocomonoenol (MDT, **Fig. 1d**) previously detected in fish and marine samples [13, 14, 30]. In retrospect, the low concentrations of MDT would not have allowed its detection in the capsule oil before the CCC enrichment (~0.5% in the α -T₁ isolate are corresponding with 0.015% MDT in the capsule). Hence it remained unclear if MDT is a native ultra-trace compound of palm oil (and potentially other plant oils as well) or an artefact formed during the enrichment step (during production and storing of the vitamin E capsule, or during the sample treatment in this study). For instance, isomerization of MDT to α -T₁ was observed upon heating to 250 °C [15]. Irrespective of its origin, the detection of the ultra-trace compound MDT was only possible after CCC fractionation which highlights the potential role of CCC in the discovery of trace compounds in samples as previously shown by the example of sterols [31].

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Captions to Figures

Figure 1. General structure of (a) tocopherols (T), (b) tocotrienols (T₃), (c) C12'-tocomonoenols (T₁) and (d) C13'-tocomonoenols (marine derived tocomonoenol, MDT).

Figure 2. (a) GC/MS chromatogram (full scan mode, DB-5) of the silylated content of the analyzed vitamin E supplement capsule and (b) mass spectrum of silylated α -tocomonoenol (α -T₁) with inserts of the structure of the molecular ion and the fragment ion at m/z 237.

Figure 3. (a) CCC/UV chromatogram ($\lambda=290$ nm) of the tocols in the analyzed vitamin E supplement capsule with marking of the fraction collected for the isolation of α -T₁ (77.5 – 92.5 min) and (b) GC/MS spectrum of the marked CCC fraction after silylation.

Figure 4. CCC elution scheme of tocols using the BTF solvent system with T = tocopherols, T₁ = tocomonoenols, T₃ = tocotrienols and T₄ = tocotetraenols recently detected as artefacts in rice bran oil capsules [25].

Figure 5. GC/MS chromatogram of (a) isolated C12'- α -tocomonoenol (α -T₁) and (b) an enlarged excerpt visualizing the minor impurity C13'- α -tocomonoenol (MDT) and excerpts of the olefinic region of the ¹H-NMR spectrum of (c) MDT and (d) α -T₁.