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Identification of α-Tocotrienolquinone Epoxides and Development of an Efficient Molecular Distillation Procedure for Quantitation of α-Tocotrienol Oxidation Products in Food Matrices by High-Performance Liquid Chromatography with Diode Array and Fluorescence Detection

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ABSTRACT: The aim of this study was to investigate the most important oxidation products of α-tocotrienol (α-T3) along with other tocochromanols in lipid matrices and tocotrienol-rich foods. For this purpose, an efficient molecular distillation procedure was developed for the extraction of analytes, and α-T3-spiked and thermally oxidized natural lipids (lard and wheat germ oil) and α-T3-rich foods (wholemeal rye bread and oil from dried brewer's spent grain) were investigated through HPLC-DAD-F. The following α-T3 oxidation products were extractable from lipid matrices along with tocochromanols: α-tocotrienolquinone (α-T3Q), α-tocotrienolquinone-4a,5-epoxide (α-T3Q-4a,5-E), α-tocotrienolquinone-7,8-epoxide (α-T3Q-7,8-E), 7-formyl-β-tocotrienol (7-FβT3), and 5-formyl-γ-tocotrienol (5-FγT3). Recovery rates were as high as 88% and enrichment factors up to 124. The proposed method allows the investigation of α-T3Q, α-T3Q-4a,5-E, α-T3Q-7,8-E, 7-FβT3, and 5-FγT3 in small quantities (<0.78 μg/g) in lipid matrices, which is necessary for the investigation and analysis of the formation kinetics of these oxidation products in fat, oils, and tocotrienol-rich foods.

KEYWORDS: *α-tocotrienol, oxidation products, molecular distillation, HPLC, lipid matrix*

INTRODUCTION

Vitamin E is a generic term for naturally occurring tocopherols (T) and tocotrienols (T3), which are the most efficient fat-souble antioxidants in foods and biological systems and inhibit the autocatalytic lipid peroxidation process.[1] The four α-, β-, γ-, and δ-T vitamers and four α-, β-, γ-, and δ-T3 vitamers have equal polar chromanol rings, which vary in the number and position of methyl substituents in the chromanol ring. The only difference in the structures of T3 and T is in their isoprenoid chain: whereas T have a saturated isoprenoid chain, that of T3 contains three isolated double bonds.[2] Compared with T, T3 have been studied less; however, interest in T3 has increased enormously in recent years, after it was reported that T3 have special and unique health benefits. For example, only T3, but not T, inhibit cholesterol biosynthesis and have neuroprotective properties and special anticancer properties in cell culture.[3,4] Furthermore, α-T3 acts 40−60 times more efficiently than α-T as an antioxidant in liver microsomes.[5]

Good nutritional sources of T3 are crude palm oil (432−800 mg/kg)[6] from the tropics and certain cereal grains such as rye (*Secale cereale*; 22.7−43.4 mg/kg on a dry matter basis) and barley (*Hordeum vulgare*; 35.6−53.5 mg/kg on a dry matter basis). Calculated on a lipid basis, the T3 content in rye lipids varies from 841 to 1550 mg/kg and in barley lipids from 1000 to 1410 mg/kg.[7,8] A special attribute of food products derived from rye and barley is their high proportion of α-T3 (33−42 and 48−66% of the total tocochromanols, respectively[7,8]). This attribute may be nutritionally advantageous as α-T3 is the T3 vitamer with the highest oral bioavailability.[9] In palm oil, γ-T3 comprises 44% of the total tocochromanols[6] and is the prevailing T3 form. Brewer's spent grain is a byproduct of the brewing process; in a previous paper we concluded that oil from dried brewer's spent grain is a commercially viable source of T3, with up to 850.2 mg/kg total T3 in oil and up to 398.6 mg/kg of the α-T3 vitamer.[10]

During food processing and storage, the intact molecular structure of T3 vitamers can be changed by oxidative degradation, and T3 may lose their special and unique health benefits. α-T3 is the least thermostable T3 vitamer, with the highest rate of degradation,[11,12] so that the oxidation products of α-T3 are of particular interest. For example, α-T3 in rye wholemeal has been found to decrease by 80% after 12 months at room temperature,[12] but there is no information available on the chemical structures and physiological properties of the thereby resulting α-T3 oxidation products. However, the physiological properties of α-T oxidation products have been the topic of some studies: for example, it was found that after enzymatic reduction of α-tocopherolquinone (α-TQ), the resulting α-tocopherolhydroquinone can act as a cellular antioxidant again, and it has been suggested that α-TQ itself targets multiple pathogenic factors in Alzheimer's disease.[13] On the other hand, γ-tocopherolquinone is a highly active agent involved in cytotoxicity, apoptosis, and mutagenesis.[14,15]

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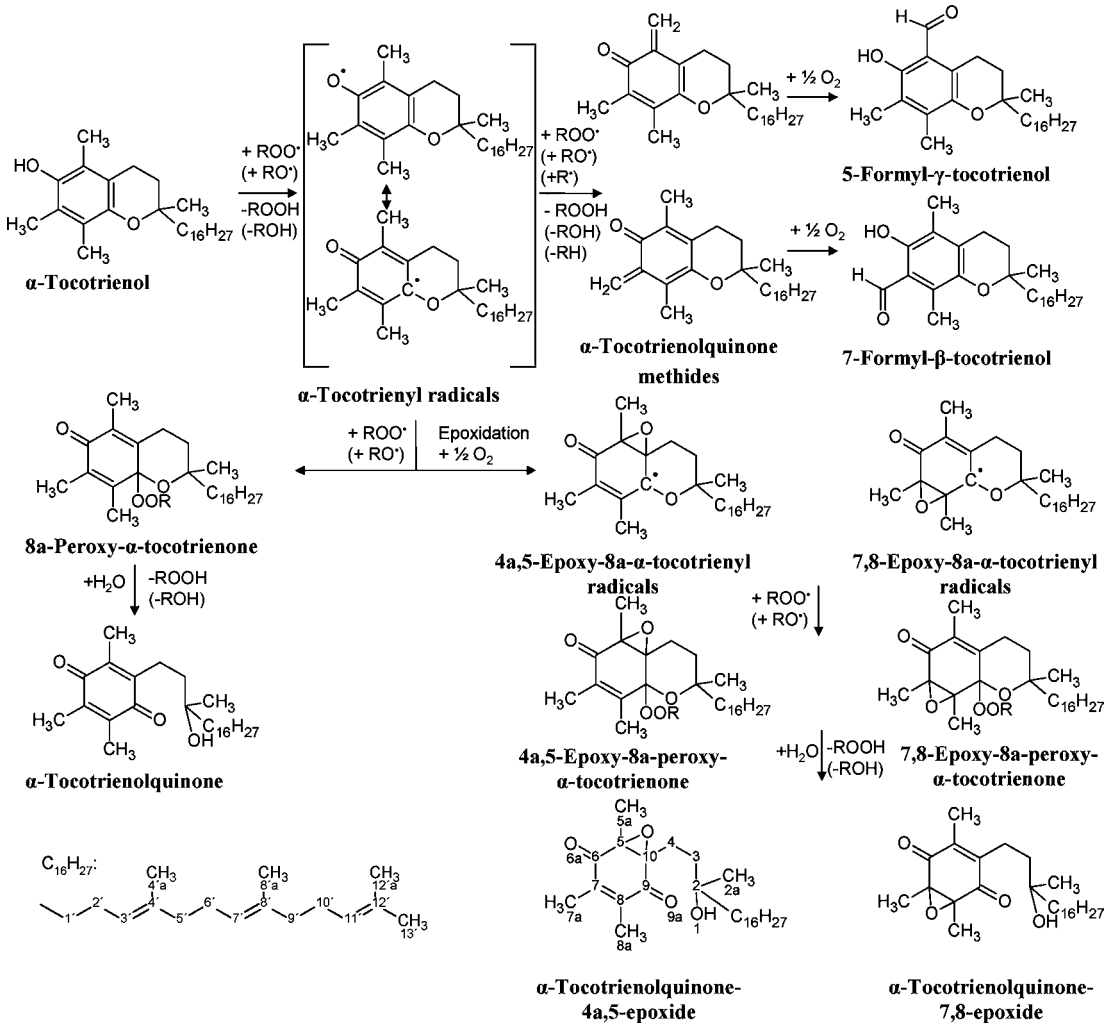

Figure 1. Proposed reaction pathway leading to the formation of the analyzed α-tocotrienol (α-T3) oxidation products: α-tocotrienolquinone (α-T3Q), α-tocotrienolquinone-4a,5-epoxide (α-T3Q-4,a5-E), α-tocotrienolquinone-7,8-epoxide (α-T3Q-7,8-E), 5-formyl-γ-tocotrienol (5-FγT3), and 7-formyl-β-tocotrienol (7-FβT3). R$^\bullet$, alkyl radical; RO$^\bullet$, alkoxy radical; ROO$^\bullet$, peroxy radical. The α-T3 oxidation products were identified by comparing retention times, UV−vis, and EIMS spectra with those of known standards.[16]

Unlike the relatively comprehensive literature on the oxidation products of α-T, studies on those of α-T3 are rare. The most comprehensive study on this topic was recently conducted by our working group, which investigated the oxidation products of α-T3 in different model systems.[16] For this purpose, we developed a reliable high-performance liquid chromatography (HPLC) method with diode array detection (DAD), fluorescence detection (F), and a particle beam interface (PBI) electron impact mass spectroscopy method (EIMS) to separate the most important oxidation products of α-T3 along with other tocochromanol vitamers. Furthermore, we sought to identify the as yet unknown oxidation products by EIMS, Fourier transform infrared spectroscopy, ^{13}C nuclear magnetic resonance spectroscopy (NMR), and ^{1}H NMR.[16] We identified the following most important oxidation products of α-T3: α-tocotrienolquinone (α-T3Q), two α-tocotrienolquinone epoxides (α-T3QEs), α-tocotrienol spirodimers and α-tocotrienol spirotrimers, α-tocotrienol dihydroxydimers, α-tocotrienolquinone dimers, and the aldehydes 5-formyl-γ-tocotrienol (5-FγT3) and 7-formyl-β-tocotrienol (7-FβT3).[16]

The generation of tocochromanol oxidation products, especially oxidation products of α-T, have chiefly been studied in model systems.[2,16] Obviously because of problems associated with the complex food matrices and comparatively low concentrations of the oxidation products formed, only a few papers describe the isolation and detection of T oxidation products formed in food under food processing or storage conditions.[17−21] Murkovic et al.,[17] Rennick and Warner,[18] and Verleyen et al.[20] studied the formation of α-TQ and α-tocopherolquinones epoxides (α-TQEs) in vegetable oils and model triacylglycerols under frying conditions. Other authors determined α-TQ and α-TQEs formed in beef and fish during storage at 4 and 10 °C.[22,23] Pirisi et al.[19] investigated α-TQ and 5-formyl-γ-tocopherol (5-FγT) formed during photolysis in olive oil and model triacylglycerols, and Gogolewski et al.[21] examined the formation of dimeric oxidation products of tocopherols in plant oils under storage conditions.

Measurement of tocochromanol oxidation products in food requires extraction and concentration prior to analysis by HPLC. The extraction or enrichment of α-T oxidation products from lipid matrices has thus far been accomplished by three different methods: (i) simple liquid extraction, mainly with methanol;[17,18,20] (ii) solid phase extraction on a silica SEP-PAK cartridge washed with hexane;[24] (iii) saponification;[25] or (iv) conventional distillation of fatty acid methyl esters of methylated triacylglycerols to yield a residue rich in T and T

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Table 1. ^{1}H NMR Spectroscopic Characteristics of α-Tocotrienolquinone-4a,5-epoxide and α-Tocotrienolquinone-7,8-epoxide and ^{13}C NMR Spectroscopic Characteristics of α-Tocotrienolquinone-4a,5-epoxide[a]

analyte	spectroscopic characteristics
α-tocotrienolquinone-4a,5-epoxide (compound **12** in Figures 2 and 5)	^{1}H NMR data: δ 5.15 (m, 3H, 3′-, 7′-, and 11′-C<u>H</u>), 3.52 (s, 1H, 1-O<u>H</u>), 2.30 (m, 2H, 4-C<u>H</u>$_2$), 2.08 (m, 12H, 1′-, 2′-, 5′-, 6′-, 9′-, and 10′-C<u>H</u>$_2$), 1.99 (s, 6H, 7a-C<u>H</u>$_3$ and 8a-C<u>H</u>$_3$), 1.62 (s, 3H, 5a-C<u>H</u>$_3$), 1.62 (m, 12H, 4′a-, 8′a-, 12′a-, and 13′-C<u>H</u>$_3$), 1.56 (m, 2H, 3-C<u>H</u>$_2$), 1.29 (s, 3H, 2a-C<u>H</u>$_3$)
	^{13}C NMR data: δ 194.33 (6-<u>C</u>═O), 193.95 (9-<u>C</u>═O), 141.23 (7-C═<u>C</u>—C═O), 140.96 (8-C═<u>C</u>—C═O), 135.66 (4′-<u>C</u>), 135.08 (8′-<u>C</u>), 131.27 (12′-<u>C</u>), 124.38 (3′-<u>C</u>H), 124.09 (7′-<u>C</u>H), 124.05 (11′-<u>C</u>H), 72.37 (2-<u>C</u>—OH), 65.81 (5-<u>C</u>-epoxy), 63.46 (10-<u>C</u>-epoxy), 39.72 (1′-, 5′-, and 9′-<u>C</u>H$_2$), 31.60 (3-<u>C</u>H$_2$), 26.61 (10′-<u>C</u>H$_2$), 26.58 (6′-<u>C</u>H$_2$), 25.71 (13′-<u>C</u>H$_3$), 22.70 (2a-<u>C</u>H$_3$), 22.60 (2′-<u>C</u>H$_2$), 20.70 (4-<u>C</u>H$_2$), 17.70 (12′a-<u>C</u>H$_3$), 16.05 (8′a-<u>C</u>H$_3$), 16.03 (4′a-<u>C</u>H$_3$), 13.24 (5a-<u>C</u>H$_3$), 11.59 (7a-<u>C</u>H$_3$), 11.57 (8a-<u>C</u>H$_3$)
α-tocotrienolquinone-7,8-epoxide (compound **13** in Figures 2 and 5)	^{1}H NMR data: δ 5.16 (m, 3H, 3′-, 7′-, and 11′-C<u>H</u>), 3.51 (s, 1H, 1-O<u>H</u>), 2.38 (m, 2H, 4-C<u>H</u>$_2$), 2.05 (m, 12H, 1′-, 2′-, 5′-, 6′-, 9′-, and 10′-C<u>H</u>$_2$), 2.01 (s, 3H, 5a-C<u>H</u>$_3$), 1.62 (s, 6H, 7a-C<u>H</u>$_3$ and 8a-C<u>H</u>$_3$), 1.64 (m, 12H, 4′a-, 8′a-, 12′a-, and 13′-C<u>H</u>$_3$), 1.56 (m, 2H, 3-C<u>H</u>$_2$), 1.29 (s, 3H, 2a-C<u>H</u>$_3$)

[a]The carbon numbering is shown in Figure 1 (α-tocotrienolquinone-4a,5-epoxide). In $CDCl_3$, internal standard TMS; s = singlet, t = triplet, m = multiplet.

oxidation products.[26] One of the main difficulties of the enrichment of these analytes is that the oxidation products are thermolabile and undergo further degradation.

In our present study, we intended to extract α-T3 oxidation products from lipid matrices for the first time by molecular distillation to yield a highly concentrated sample solution. There has been thorough investigation of the extraction of T and T3 by molecular distillation of vegetable oils and fats and of oil deodorizer distillates.[27−29] Molecular distillation is an evaporation process very suitable for thermolabile compounds such as α-T3 and thus was expected also to be suitable for the corresponding oxidation products. In particular, the method is characterized by an extremly high vacuum, so that the analytes can be evaporated at low temperature.

The aim of this study was to investigate the chief oxidation products of α-T3 along with other tocochromanols in lipid matrices and tocotrienol-rich food. For this, an efficient molecular distillation procedure first had to be developed and validated. Second, we wanted to test the suitability of the newly developed sample preparation and analysis method by analyzing α-T3-spiked and thermally oxidized natural lipids (lard and wheat germ oil) and to investigate the kinetics of the degradation of α-T3 and the formation of α-T3 oxidation products in these two different natural lipid matrices at different temperatures. Third, we aimed to demonstrate the applicability of the method using α-T3-rich foods such as wholemeal rye bread and dried brewer's spent grain, the latter being a potential feedstock for the production of T3-rich extracts for nutritional purposes.[10] We were able to fulfill these aims and propose a reaction pathway for the formation of α-T3 oxidation products (Figure 1).

MATERIALS AND METHODS

Chemicals and Materials. The reference substances α-, β-, γ-, and δ-T3 and α-, β-, γ-, and δ-T (>97% purity) were provided by Davos Life Science (Singapore); α-TQ (>97% purity) was obtained from TCI Europe (Zwijndrecht, Belgium), whereas 5-FγT3, α-tocotrienolquinone-4a,5-epoxide (α-T3Q-4a,5-E), and α-tocotrienolquinone-7,8-epoxide (α-T3Q-7,8-E) were synthesized and purified by HPLC as previously described.[16] All solvents were of HPLC grade. *n*-Hexane and acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany); 1,4-Dioxane (not stabilized) was from Carl Roth (Karlsruhe, Germany), and *tert*-butyl methyl ether (TBME) was from Acros Organics (Geel, Belgium). Cold-pressed wheat germ oil, lard, and wholemeal rye bread were purchased from a local supermarket. Industrial-scale oven-dried brewer's spent grain was kindly provided by Leiber GmbH (Bramsche, Germany). The tocochromanols in wheat germ oil were removed by molecular distillation extraction before further use.

Characterization of Tocotrienolquinone Epoxides by ^{1}H and ^{13}C NMR. α-T3QEs were synthesized and two α-T3QEs were isolated by preparative HPLC as described in our previous publication.[16] The isolated α-T3QEs were characterized by ^{1}H NMR, and the α-T3QE that was formed in larger quantity was further investigated by ^{13}C NMR spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were obtained in deuterated chloroform with a Bruker DPX 400 MHz NMR spectrometer (Bremen, Germany). Tetramethylsilane was used as an internal standard, and the chemical shift of protons and carbons was expressed as δ values (tetramethylsilane = 0) (Table 1).

HPLC Analysis. The HPLC-DAD-F analysis of all eight tocochromanol vitamers and of the most important oxidation products of α-T3 was performed using our method described previously.[16] Briefly, this involves separation on a ProntoSil 120-5 Diol column (250 mm × 4 mm) and gradient elution with *n*-hexane, 1,4-dioxane, and TBME. Analytes were determined by fluorometry (λ (excitation) = 297 nm, λ (emission) = 330 nm, attenuation factor = 1024) and UV−vis (200−400 nm).

Further identification of the α-T3 oxidation products was performed by the HPLC-PBI-EIMS method previously described.[16] Fractionation of α-T3 oxidation products was carried out using the analytical HPLC-DAD-F system described above.

Extraction of α-Tocotrienol, 5-Formyl-γ-tocotrienol, 7-Formyl-β-tocotrienol, α-Tocotrienolquinone, α-Tocotrienolquinone-4a,5-epoxide, and α-Tocotrienolquinone-7,8-epoxide from Lipids by Molecular Distillation. Molecular distillation was performed with a KDL1 BASIC shortpath distillation system from UIC (Alzenau-Hörstein, Germany), which is a falling-film molecular distillation apparatus. The system was equipped with a short-path evaporator (surface area = 0.018 m^2) heated by a jacket with oil circulated from an oil bath (HE-4 julabo heating circulator; Julabo, Seelenbach, Germany). The internal glas condenser (surface area = 0.007 m^2) was tempered with circulated water (Thermo Haake Phoenix II heating circulator, Karlsruhe, Germany). The high-vacuum was achieved by a vacuum pump system consisting of a Vakuubrand RZ 2.5 rotary vane pump (Wertheim, Germany) and an AX-65 Varian oil diffusion pump (Lexington, MA, USA). In addition, the molecular distillation was equipped with a wiper basket assembly, a glass cryotrap, a graduated feed vessel also tempered with circulated water, and a discharge system consisting of two 100 mL receiver flasks for distillate and residue.

For molecular distillation, 30 g lipid samples were inserted into the feed vessel. Distillation was carried out at a system pressure of 0.001 mbar, an evaporator temperature of 210 °C (lard) or 180 °C (wheat germ oil, wholemeal rye bread extract, and brewer's spent grain extract), and a feed vessel and condenser temperature of 70 °C. The feeding flow rate was 0.3 g/min, and the roller wiper speed was fixed at 500 rpm. After distillation, the residue and the distillate fractions were weighed. The yields of the distillate fractions obtained from 30 g of lard at 210 °C were 0.386 ± 0.026 g (180 °C, 0.078 ± 0.004 g), and from 30 g of depleated wheat germ oil at 180 °C, 0.188 ± 0.035 g (210 °C, 1.183 ± 0.031 g). The distillate was transferred into a 5 mL volumetric flask and filled to volume with *n*-hexane. The sample

Table 2. Statistical Parameters of the HPLC-F[a,b] (Tocochromanols) and HPLC-DAD[a,b] (α-Tocotrienol Oxidation Products) Method Validation

analyte	linear equation	r	SD (μg/mL)	VC (%)	IDL[c] (μg/mL)	IDC[c] (μg/mL)	IQL[c] (μg/mL)
α-tocopherol	$y = 1.630x - 0.017$	0.997	0.286	6.19	0.360	0.721	1.25
α-tocotrienol	$y = 1.904x + 0.208$	0.995	0.312	7.64	0.445	0.889	1.53
β-tocopherol	$y = 2.935x - 0.051$	0.995	0.354	8.30	0.451	0.901	1.55
β-tocotrienol	$y = 4.329x + 0.412$	0.995	0.366	7.91	0.460	0.921	1.58
γ-tocopherol	$y = 2.913x + 0.340$	0.996	0.337	7.29	0.424	0.848	1.47
γ-tocotrienol	$y = 3.150x - 0.069$	0.993	0.442	9.56	0.557	1.113	1.89
δ-tocopherol	$y = 2.045x + 0.024$	0.994	0.408	8.56	0.523	1.047	1.79
δ-tocotrienol	$y = 3.239x + 0.068$	0.996	0.339	7.33	0.426	0.853	1.47
5-formyl-γ-tocotrienol	$y = 1.174x + 0.025$	0.999	0.366	4.72	0.383	0.733	1.30
α-tocopherol quinone	$y = 5.531x + 0.940$	0.999	0.350	4.52	0.367	0.766	1.36
α-tocotrienolquinone-4a,5-epoxide	$y = 2.664x + 0.333$	0.999	0.415	2.55	0.512	1.024	1.81

[a]Each data point represents the mean of four determinations ($n = 4$). [b]Linearity is confirmed in the range 1.25−20 μg/mL (tested by residual analysis and comparison with an calibration function second order). [c]DIN 32645:2008-11.[30]

solution was centrifuged at 15000*g* for 10 min, and the supernatant was filtered through a Whatman GF/A glass fiber filter (1.6 μm) (Carl Roth) and finally used for HPLC analysis. The optimum evaporator temperature was determined using spiked lipid matrices (concentrations are given under Method Validation and Recovery Rates). The optimum evaporator temperature was defined to be the highest possible temperature (between 180 and 210 °C) that generates the highest recovery rates of tocochromanols and α-T3 oxidation products in the distillate and the lowest percentage of interfering substances.

Method Validation and Recovery Rates. Nine stock solutions of 1000 μg/mL each were prepared containing one of the following substances: α-, β-, γ-, or δ-T3; α-, β-, γ-, or δ-T; or α-TQ. The stock solutions of 5-FγT3 and α-T3Q-4a,5-E contained 500 μg/mL and were prepared from the purified synthesized standards. All stock solutions were prepared in ethanol. Working solutions were prepared in *n*-hexane and used for creating calibration curves. The four working solutions for fluorescence detection contained 1, 2.5, 5, and 10 μg/mL of α-, β-, γ-, and δ-T3 and of α-, β-, γ-, and δ-T. The five working solutions for UV−vis diode array detection contained 1.25, 2.5, 5, 10, and 20 μg/mL of α-TQ, 5-FγT3, and α-T3Q-4a,5-E. As α-T3Q, α-T3Q-7,8-E, and 7-FβT3 were not available as reference substances, α-T3Q was quantitated using the calibration curve derived from α-TQ; α T3Q-7,8-E was quantitated using the calibration curve derived from α T3Q-4a,5-E; and 7-FβT3 was quantitated using the calibration curve derived from 5-FγT3, due to their related absorption maxima.

The sensitivity of the HPLC method was evaluated by determining the instrument limit of detection (IDL), the instrument detection capability (IDC), and the instrument limit of quantitation (IQL) according to the DIN 32645:2008-11 calibration function method.[30] The calibration curves were tested for outliers and homoscedasticity. All statistical validation parameters were calculated using Valoo 2.3[31] (Table 2).

For determination of recovery rates and enrichment factors, samples of lard ($n = 4$) and depleted wheat germ oil ($n = 4$) were spiked with analytes at the concentrations given in Table 3. The spiked concentrations were chosen to simulate T3-rich barley oil, such as can be extracted from brewer's spent grain.[10] By applying the molecular distillation method, tocochromanol contents were also determined in the plain lipid matrices (Table 3). The recovery rates of the analytes were expressed in percentages to allow comparison of the amount of each analyte obtained after distillation (minus the analyte's amount in the plain lipid matrices) with the spiked amount of each analyte present before distillation. The enrichment factors represent the factors of increase of each analyte's concentrations in spiked fat (present before distillation) compared to the analyte's concentration in the distillate. It was calculated by dividing the content of each analyte in the distillate by the sum of spiked content of each analyte (present before distillation) and the analyte's contents of the plain lipid matrices. Furthermore, we did not observe the formation of α-T3 oxidation products (artifacts) caused by the molecular distillation process.

The sensitivity of the molecular distillation method was estimated by determining the method limits of detection (MDL) and quantitation (MQL) according to the DIN 32645:2008-11 blank value method.[30] For this purpose, samples of lard ($n = 6$) and depleted wheat germ oil ($n = 6$) were spiked with 1 μg/g 5-FγT3, α-T3Q, α-T3Q-4a,5-E, and tocochromanols. Analytes were extracted by molecular distillation and measured by HPLC-DAD-F. All statistical validation parameters were calculated using Valoo 2.3.[31]

Thermal Oxidation of α-Tocotrienol in the Lipid Matrices of Lard and Wheat Germ Oil. In our study, we used naturally tocochromanol-low lard and tocochromanol-depleted wheat germ oil as lipid matrices. For depletion of wheat germ oil, tocochromanols were extracted by molecular distillation and the distillation residue was used for further processing. A total of 90 mg of α-T3 was added to 100 g of each lipid matrix and stirred constantly for 5 min at 50 °C. The actual tocochromanol contents of the spiked lipid matrices were determined by HPLC-DAD-F. Thermal oxidation of the spiked lipids was performed using the Metrohm Rancimat 679 (Herisau, Switzerland). Six 15-g aliquots of the spiked samples were transferred into glass tubes and heated to 130 °C (spiked lard) or 90 °C (spiked wheat germ oil), with an air flow of 15 L/h for 1, 3, 4, and 6 h (spiked lard) or 3, 6, and 10 h (spiked wheat germ oil). The Rancimat induction periods of the lipid matrices were 4.28 ± 0.25 h for spiked lard (130 °C) and 4.68 ± 0.22 h for spiked wheat germ oil (90 °C). After the oxidation process, three samples of the same type were combined, transferred into 50 mL brown glass vessels, purged with nitrogen, sealed with screw caps, and stored in a freezer (−20 °C) until molecular distillation.

Data were statistically analyzed by analysis of variance and statistical significance. The selected significance level was $\alpha = 5\%$. Variance analyses were calculated using Valoo 2.3.[31]

Application of the Method to Wholemeal Rye Bread and Dried Brewer's Spent Grain. Wholemeal rye bread samples ($n = 2$) were dried under extreme conditions in a drying oven for 5 h at 100 °C to simulate toasting and to maximize the formation of oxidation products. The toasted bread was ground with an IKA Basic Analytical Mill A 11 (Staufen, Germany). Dried brewer's spent grain samples ($n = 2$) were used without further sample preparation. In batches of 500 g, the samples were suspended in 3 L of *n*-hexane. Overall, 1000 g of wholemeal rye bread and 500 g of dried brewer's spent grain were extracted for each sample. The suspension was shaken at room temperature for 3 h, and subsequently the supernatant was decanted through a filter paper. The extraction procedure was repeated twice with the residue. The combined extraction solvents were dried over anhydrous sodium sulfate, and the organic solvent was transferred into a round-bottomed flask and evaporated using a vacuum rotary evaporator at a temperature below 40 °C. The viscous brown oil obtained from the wholemeal rye bread (16.2 ± 2.31 g) or dried

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Table 3. Recovery Rates and Enrichment Factors of Tocochromanols and α-Tocotrienol Oxidation Products from Lard and Wheat Germ Oil, Determined after the Molecular Distillation Process (Temperature Applied during Molecular Distillation: Lard, 210 °C; Wheat Germ Oil, 180 °C)

		lard[a]					wheat germ oil[a]				
analyte	spiked contents, c_S (mg/kg)	contents in plain lipid matrices, c_{PL} (mg/kg)	contents in distillates of spiked samples, c_D (g/kg)	recovered contents in spiked lipids, c_{SL} (mg/kg)	recovery rate[b] (%)	enrichment factor[c]	contents in plain lipid matrices, c_{PL} (mg/kg)	contents in distillates of spiked samples, c_D (g/kg)	recovered contents in spiked lipids, c_{SL} (mg/kg)	recovery rate[b] (%)	enrichment factor[c]
α-tocopherol	10.05	25.44	2.50 ± 0.19	32.81 ± 1.51	73.4	70.4	98.25	13.38 ± 1.06	105.93 ± 7.92	76.5	124
α-tocotrienol	122.13	<MDL	6.84 ± 0.34	90.07 ± 3.16	73.8	56.0	2.33	13.25 ± 7.82	100.56 ± 2.23	80.4	106
β-tocopherol	10.49	<MDL	0.59 ± 0,07	7.89 ± 0.66	75.3	56.6	22.84	3.92 ± 0.06	31.13 ± 2.09	79.1	118
β-tocotrienol	12.16	<MDL	0.67 ± 0.04	8.95 ± 0.58	73.6	55.4	3.06	1.83 ± 0.04	13.80 ± 1.03	88.3	120
γ-tocopherol	9.18	<MDL	0.48 ± 0.05	6.42 ± 0.49	69.9	52.7	4.92	1.55 ± 0.14	11.88 ± 1.50	75.8	110
γ-tocotrienol	43.67	<MDL	2.39 ± 0.12	31.62 ± 0.81	72.4	54.7	<MDL	5.01 ± 0.56	37.76 ± 2.02	86.5	115
δ-tocopherol	9.06	<MDL	0.40 ± 0.04	5.44 ± 0.60	60.0	44.5	<MDL	0.99 ± 0.09	7.41 ± 0.04	81.8	109
δ-tocotrienol	17.33	<MDL	0.92 ± 0.04	12.38 ± 0.52	71.5	53.3	<MDL	1.93 ± 0.26	14.50 ± 1.27	83.7	112
α-tocopherol quinone	99.54	<MDL	5.31 ± 0.34	64.85 ± 9. 37	65.2	53.3	<MDL	10.69 ± 0.05.	79.57 ± 2.57	79.9	107
5-formyl-γ-tocotrienol	13.51	<MDL	0.76 ± 0.06	10.28 ± 0.59	76.1	56.4	<MDL	1.37 ± 0.06	10.67 ± 0.98	79.0	101
α-tocotrienolquinone-4a,5-epoxide	25.41	<MDL	1.72 ± 0.18	21.43 ± 1.76	84.3	67.6	<MDL	2.29 ± 0.11	18.83 ± 1.10	75.3	90.0

[a]Each data point represents the mean of four determinations ($n = 4$). [b]Recovery rate = $(c_{SL} - c_{PL})/c_S \times 100$, expressed in percentage to allow comparison of the amount of each analyte obtained after distillation (minus the analyte's amount in the plain lipid matrices) with spiked amount of each analyte present before distillation. [c]Enrichment factor = $c_D/(c_{PL} + c_S)$, factor of increase of analyte's concentration in spiked fat (present before distillation) compared to concentration in the distillate.

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brewer's spent grain (47.9 ± 4.64 g) was transferred into a 50 mL brown glass vessel, purged with nitrogen, sealed with a screw cap, and stored in a freezer (−20 °C) until molecular distillation.

RESULTS AND DISCUSSION

Identification of α-Tocotrienolquinone-4a,5-epoxide and α-Tocotrienolquinone-7,8-epoxide. In our previous publication,[16] we identified, among others, two α-T3QEs as α-tocotrienol oxidation products and characterized them by means of HPLC-DAD-F and HPLC-PBI-EIMS. However, it was not possible to determine the exact structures (position of epoxy group) with these methods. For further evidence for the exact identity of the α-T3QEs, information on their structures was obtained by ^{1}H and ^{13}C NMR (Table 1). For this, compounds **12** and **13** (numbering refers to Figures 2 and 5) were isolated by preparative HPLC. The ^{1}H NMR spectrum of compound **12** revealed an aliphatic hydroxyl proton with a chemical shift at 3.52 ppm and that of compound **13** a chemical shift at 3.51 ppm, indicating the opening of the chromanol ring. The chemical shifts of the methyl groups at positions 5a, 7a, and 8a of the quinone ring are crucial for distinguishing the two α-T3QEs. Epoxidation of the quinone ring causes the loss of a double bond, which leads to a stronger shielding and lower chemical shift of the protons of the adjacent methyl groups. Compound **12** showed one singlet signal with a chemical shift at 1.99 ppm with an intensity corresponding to six protons and one singlet signal with a chemical shift at 1.62 ppm with an intensity corresponding to three protons. In comparison, compound **13** showed the same singlet signals with the same chemical shifts but with inverse intensities. This indicates that compound **12** is α-T3Q-4a,5-E and compound **13**, α-T3Q-7,8-E. The olefinic multiplet signals at 5.17−5.12 ppm (compound **12**) and 5.19−5.12 ppm (compound **13**) are characteristic of the isoprenoid chain (3′, 7′, 11′) of T3.

The ^{13}C NMR spectrum of compound **12** confirmed the *p*-quinone structure by showing the presence of two conjugated carbonyl carbons with chemical shifts at 194.33 (position 6) and 193.95 ppm (9) and two unsaturated carbons in conjugation with the carbonyl carbons with chemical shifts at 141.23 (7) and 140.96 ppm (8). Important are the chemical

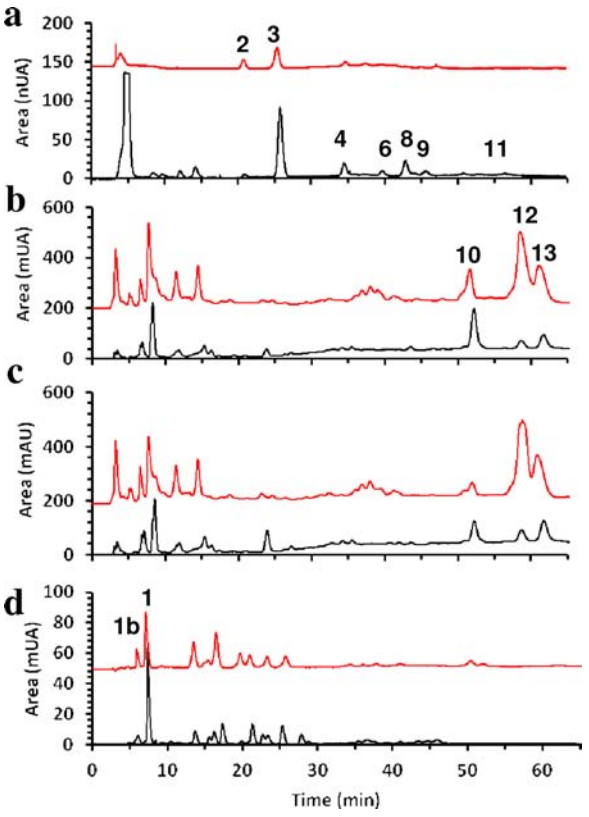

Figure 2. Normal-phase HPLC-DAD-F chromatograms of depleted wheat germ oil (upper, red) and lard (lower, black) spiked with α-tocotrienol, after thermal oxidation under Rancimat conditions (10 h at 90 °C and 6 h at 130 °C, respectively) and extraction of analytes by molecular distillation, recorded at λ (excitation) = 297 nm and λ (emission) = 330 nm (a), 268 nm (b), 274 nm (c), and 389 nm (d). Compounds: **1b**, 7-formyl-β-tocotrienol; **1**, 5-formyl-γ-tocotrienol; **2**, α-tocopherol; **3**, α-tocotrienol; **4**, β-tocopherol; **6**, β-tocotrienol; **8**, γ-tocotrienol; **9**, δ-tocopherol; **10**, α-tocotrienolquinone; **11**, δ-tocotrienol; **12**, α-tocotrienolquinone-4a,5-epoxide; **13**, α-tocotrienol-quinone-7,8-epoxide.

Table 4. Method Detection Limit (MDL) and Quantitation Limit (MLQ) for the Determination of Tocochromanols and α-Tocotrienol Oxidation Products from Spiked Lard and Wheat Germ Oil, after the Molecular Distillation Process (Lard, 210 °C; Wheat Germ Oil, 180 °C)[a,b]

	lard		wheat germ oil	
analyte	MDL (μg/g)	MQL (μg/g)	MDL (μg/g)	MQL (μg/g)
α-tocopherol	1.54[c]	4.63[c]	2.37[c]	7.13[c]
α-tocotrienol	0.373	1.12	0.282[c]	0.847[c]
β-tocopherol	0.274	0.822	1.57[c]	4.71[c]
β-tocotrienol	0.194	0.581	0.289[c]	0.867[c]
γ-tocopherol	0.227	0.682	0.372[c]	1.12[c]
γ-tocotrienol	0.405	1.21	0.089	0.268
δ-tocopherol	0.219	0.655	0.108	0.325
δ-tocotrienol	0.309	0.928	0.407	1.22
5-formyl-γ-tocotrienol	0.125	0.376	0.077	0.230
α-tocopherolquinone	0.067	0.200	0.043	0.129
α-tocotrienolquinone-4a,5-epoxide	0.259	0.779	0.181	0.542

[a]Concentrations of analytes in spiked lipid matrices were 1 μg/g unless otherwise mentioned in the text. Prior to tocochromanol analysis by HPLC-F, sample solutions (5 mL) were diluted 1:10. [b]Each data point represents the mean of six determinations (n = 6). MDL and MQL were assessed according to DIN 32645:2008-11.[30] [c]These data points were obtained from nonspiked matrices and calculated on the basis of the low tocochromanol contents occuring in the plain lipid matrices.

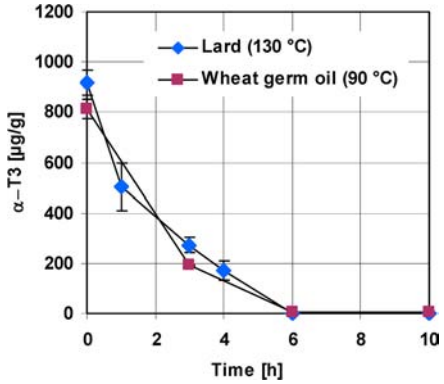

Figure 3. Decrease in α-tocotrienol (α-T3) in spiked lipid matrices during thermal oxidation under Rancimat conditions.

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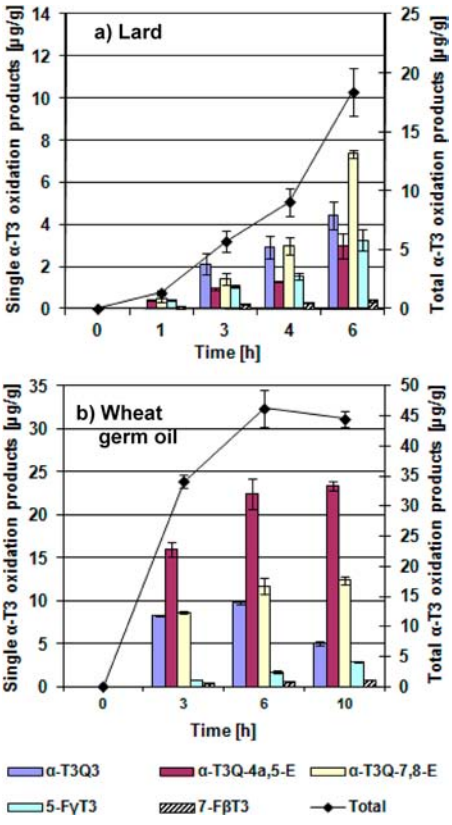

Figure 4. Formation of α-tocotrienolquinone (α-T3Q), 5-formyl-γ-tocotrienol (5-FγT3), 7-formyl-β-tocotrienol (7-FβT3), α-tocotrienolquinone-4a,5-epoxide (α-T3Q-4a,5-E), and α-tocotrienolquinone-7,8-epoxides (α-T3Q-7,8-E), in spiked lipid matrices during thermal oxidation under Rancimat conditions: (a) lard (130 °C); (b) wheat germ oil (90 °C).

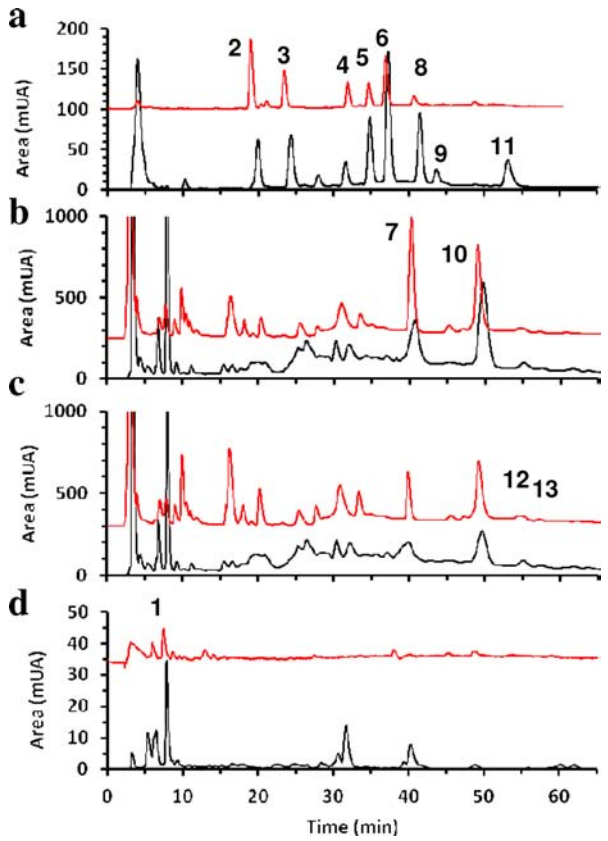

Figure 5. Normal-phase HPLC-DAD-F chromatograms of wholemeal rye bread (upper, red) and dried brewers's spent grain (lower, black), after *n*-hexane extraction and further extraction of the analytes by molecular distillation, recorded at λ (excitation) = 297 nm and λ (emission) = 330 nm (dilution 1/5) (a), 268 nm (b), 274 nm (c), and 389 nm (d). Compounds: (**1**) 5-formyl-γ-tocotrienol; (**2**) α-tocopherol; (**3**) α-tocotrienol; (**4**) β-tocopherol; (**5**) γ-tocopherol; (**6**) β-tocotrienol; (**7**) α-tocopherolquinone; (**8**) γ-tocotrienol; (**9**) δ-tocopherol; (**10**) α-tocotrienolquinone; (**11**) δ-tocotrienol; (**12**) α-tocotrienolquinone-4a,5-epoxide; (**13**) α-tocotrienolquinone-7,8-epoxides.

shifts at 65.81 (5) and 63.46 ppm (10), which belong to the carbons bonded with the epoxy group. Finally, compound **12** was identified as 4a,5-α-T3QE. In conclusion, the ^{1}H NMR and the ^{13}C NMR spectral data of α-T3Q-4a,5-E and α-T3Q-7,8-E showed good correspondence to published data for their tocopherol analogues, α-TQ-4a,5-E and α-TQ-7,8-E.[17,32] Nevertheless, this is the first report on ^{1}H NMR spectroscopic data of α-T3Q-7,8-E and α-T3Q-4a,5-E and ^{13}C NMR spectroscopic data of α-T3Q-4a,5-E.

HPLC Method Validation. For validation of the newly developed HPLC-DAD-F method,[16] the following statistical parameters were investigated: correlation coefficient (r), process standard deviation (SD), variation coefficient (VC), linearity, IDL, IQL, and IDC. As displayed in Table 2, the HPLC method shows for all studied analytes first-order calibration curve equations with very good r for a concentration range of 1−10 μg/mL (α-, β-, γ-, and δ-T3 and α-, β-, γ-, and δ-T) and 1.25−20 μg/mL (5-FγT3, α-TQ, and α-T3Q-4a,5-E). The linearity of the calibration curves was evaluated by comparing the residual standard deviation of the first-order calibration equation with that of the second-order calibration equation. Furthermore, the calibration data were free of outliers as calculated by F and t tests and showed homoscedasticity. The process VC for all analytes was <10%.

HPLC techniques have been widely applied in the analysis of tocopherols, and tocopherol oxidation products are usually detected by UV−vis (DAD), F, MS, or electrochemical detection.[33−38] Detection of tocochromanol oxidation products such as α-TQ, α-TQ-7,8-E, α-TQ-4a,5-E, and the corresponding 8a-(peroxy)-α-tocopherones by means of UV−vis or DAD is less sensitive than electrochemical detection. However, UV−vis detection using a DAD makes it possible to detect a wide range of different oxidation products and provides spectroscopic data for the analytes in a single run. Compared with the HPLC-MS techniques, a simple UV−vis detection using a DAD is less costly and more easily manageable. In addition, this method allows nondestructive determination of the analytes and thus leaves the analytes available for further investigation.

In our study, the IDL of the tocochromanols and the oxidation products of α-T3 were similar to those found previously for tocochromanols and the oxidation products of α-T by HPLC-UV−vis.[34,35] Our aim was to develop a simple and reliable HPLC-DAD-F method for the separation and determination of all eight tocochromanols along with the oxidation products of α-T3, and we consider the newly developed method to be sufficiently sensitive and suited for that purpose.

Extraction of α-Tocotrienol and α-Tocotrienol Oxidation Products from Lipid Matrices Using Molecular Distillation. Extraction of tocochromanols and α-T3 oxidation products from lipid matrices is important to obtain sample

Table 5. Tocochromanols and α-Tocotrienol Oxidation Products in Toasted Wholemeal Rye Bread and Dried Brewer's Spent Grain[a]

analyte	concn in toasted wholemeal rye bread (μg/g)	concn in dried brewer's spent grain (μg/g)
α-tocopherol	16.0 ± 0.170	24.2 ± 0.651
α-tocotrienol	6.73 ± 0.245	20.0 ± 0.652
β-tocopherol	3.35 ± 0.048	2.84 ± 0.299
β-tocotrienol	3.92 ± 0.022	19.6 ± 0.225
γ-tocopherol	3.33 ± 0.027	20.8 ± 1.27
γ-tocotrienol	1.16 ± 0.016	15.5 ± 0.229
δ-tocopherol	<MDL[b]	3.02 ± 0.244
δ-tocotrienol	0.855 ± 0.062	15.1 ± 0.652
5-formyl-γ-tocotrienol	<MQL[c]	0.837 ± 0.042
α-tocotrienolquinone	1.21 ± 0.047	8.32 ± 0.695
α-tocopherolquinone	0.964 ± 0.023	4.00 ± 0.695
α-tocotrienolquinone-4a,5-epoxide	<MQL	<MQL
α-tocotrienolquinone-7,8-epoxide	<MQL	<MQL

[a]Each data point represents the mean of four determinations (n = 2). [b]MDL, method detection limit. [c]MQL, method quantitation limit.

solutions with sufficiently high concentrations of analytes for HPLC determination. With this experiment, we intended to confirm the suitability of molecular distillation for the extraction of α-T3 oxidation products from lipid matrices. For this, we spiked lard and depleted wheat germ oil with T, T3, and α-T3 oxidation products (5-Fγ T3, α-TQ, and α-T3Q-4a,5-E). The tocochromanol pattern and the spiked concentrations were chosen to simulate an oxidized tocotrienol-rich natural lipid derived from barley, that is, dried brewer's spent grain.[10]

The extraction of T and T3 from vegetable oils and fats, and from oil deodorizer distillate,[27−29] by molecular distillation has been studied thoroughly. In general, the concentrations of the T and T3 in the distillate increase with increasing evaporator temperature and decreasing feed flow rate. Posada et al.[28] reported that tocochromanols evaporate in good yields at a temperature of 160 °C and a feed flow rate of 0.1 g/min, and Martins et al.[27] found that an evaporator temperature of 180 °C is optimal for the separation of tocochromanols from triacylglycerols and diacylglycerols. Molecular distillation is also a viable procedure for the enrichment of tocochromanols from oil deodorizer distillate;[27−29] however, in this case a second molecular distillation step is necessary due to the high proportion of highly volatile components such as free fatty acids.

We found that molecular distillation of wheat germ oil and lard with evaporator temperatures of 180 and 210 °C, respectively, a feed vessel and condenser temperature of 70 °C, and a feed flow rate of 0.3 g/min were optimal to maximize the yield of evaporating analytes while minimizing interfering volatile components. Under the optimized distillation conditions, spiked lard and wheat germ oil were distilled in a single run, and distillates were analyzed by HPLC. Concentrations of analytes in the distillate and enrichment factors (Table 3) were determined to be sufficiently high, confirming the effective extraction of tocochromanols and oxidation products from lipid matrices for subsequent HPLC analysis. Under these extraction conditions, recovery rates of all analytes were between 60.0% (δ-T) and 88.3% (β-T3), and the enrichment factors of all analytes were between 44.5 (δ-T) and 124 (α-T). The lower enrichment factors of the analytes in lard were due to the higher evaporator temperature, which led to a higher proportion of interfering volatile components in the distillate. The recovery rates of the tocochromanols found in our experiment are comparable to those determined in other studies involving molecular distillation or solvent extraction of tocochromanols.[18,20,27,29] However, no information on the enrichment factors or the recovery rates of the tocopherol oxidation products is given for the conventional extraction procedures for tocopherol oxidation products such as solvent extraction, solid phase extraction, or saponification.[17,21,23,24,26] Some authors assumed recovery rates similar to those determined for T.[18,20] Applying trypsin incubation and solvent extraction, Faustman et al.[22] determined recovery rates of internal deuterated standards of α-T, α-TQ, α-TQ-4a,5-E, and α-TQ-7,8-E to be 95−111%. Furthermore, no data from the literature are available for the extraction efficiency of tocochromanol oxidation products by molecular distillation. It should be noted that Verleyen et al.[26] distilled under conventional conditions (0.5 mbar, 135 °C) the methyl esters of oleic acid after methylation to obtain a residue rich in α-T, α-TQ, and α-TQ-4a,5-E. Furthermore, we proved the stability of tocochromanols during molecular distillation using a sample that was spiked exclusively with tocochromanols: no oxidation products originating from distilled tocochromanols were determined in the distillate. The oxidation products are also assumed to be stable, as the proportions of analytes do not significantly differ before and after the distillation process.

Additionally, we determined the sensitivity of the entire method (comprising molecular distillation and HPLC-DAD-F determination of analytes) and obtained the MDL and MQL. Values were evaluated for extraction using molecular distillation and analysis of spiked lard and wheat germ oil (concentrations of 5-Fγ T3, α-T3Q, α-T3Q-4a,5-E, and spiked tocochromanols in lipid matrices = 1 μg/g) in six replicates according to the DIN 32645:2008-11 blank value method[30] (Table 4). The analytical method allows detection and quantitation of the α-T3 oxidation products in lard or wheat germ oil in concentrations below 0.26 and 0.78 μg/g, respectively. No information on comparable MDL and MQL was found in the literature. Because of the naturally occurring tocochromanols in the plain lipid matrices (α-T in lard and α-T, α-T3, β-T, β-T3, and γ-T in wheat germ oil), the MDL and MQL of these analytes were obtained from nonspiked matrices and calculated on the basis of the low tocochromanol contents occurring in the plain lipid matrices.

Thermal Oxidation of α-Tocotrienol in Lipid Matrix. The newly developed molecular distillation method was applied to investigate the degradation of α-T3 and the simultaneous formation of its most important oxidation products (5-Fγ T3, 7-Fβ T3, α-T3Q, α-T3Q-4a,5-E, and α-T3Q-7,8-E) in lipid matrices by means of HPLC-DAD-F. For that purpose, lard and depleted wheat germ oil were spiked with 900 μg/g α-T3 (minor impurities of other tocochromanols < 5%) and thermally oxidized under Rancimat conditions at 130 °C (spiked lard) or 90 °C (spiked wheat germ oil) for 1, 3, 4, and 6 h (lard) or for 3, 6, and 10 h (spiked wheat germ oil). Tocochromanols and α-T3 oxidation products were extracted by molecular distillation and analyzed by HPLC. As shown in Figure 2, 5-Fγ T3, 7-Fβ T3, α-T3Q, α-T3Q-4a,5-E, and α-T3Q-7,8-E were detectable in α-T3-spiked lard and wheat germ oil after thermal oxidation under Rancimat conditions and

extraction of analytes by molecular distillation. The α-T3 oxidation products were identified by comparing retention times and UV−vis and EIMS spectra with those of known standards.[16] Figures 3 and 4 show the decrease in α-T3 and the formation of 5-FγT3, 7-FβT3, α-T3Q, α-T3Q-4a,5-E, and α-T3Q-7,8-E in spiked lard (initial concentration = 909 ± 50.0 μg/g) and spiked wheat germ oil (initial concentration = 814 ± 40.5 μg/g) during heating for 6 h (130 °C) and 10 h (90 °C), respectively.

Degradation of α-Tocotrienol. In both lipid matrices, α-T3 showed a rapid exponential degradation (Figure 3). After 3 h, only 29.5 ± 3.31% (lard) or 23.5 ± 0.51% (wheat germ oil) of the initial α-T3 contents remained in the heated samples. After 6 h (lard) or 10 h (wheat germ oil), only traces (<5%) of the initial α-T3 remained in the heated samples. In a further experiment (data not shown), spiked wheat germ oil was heated to 130 °C. After 1 h, α-T3 was no longer detectable in the lipid matrix. Under the given conditions, α-T3 decreased more rapidly in wheat germ oil, which is rich in linoleic acid (C18:2), than in lard, which is rich in oleic acid (C18:1). These findings on α-T3 are in accordance with those of Márquez-Ruiz et al.,[39] who correlated the stability of α-T3 under Rancimat oxidation conditions (100−130 °C) with the saturation degree of the lipid matrix. Contradictory findings have been reported under frying conditions (180 °C),[40] when tocopherols were lost more rapidly in the less unsaturated oils. It seems that the presence of oxygen is an important factor in addition to temperature, presence of antioxidants, and degree of unsaturation of the fatty acids.

Total Amounts of α-T3 Oxidation Products. There were significant differences in quantities and patterns of the α-T3 oxidation products formed in lard and wheat germ oil (Figure 4). The total amount of α-T3 oxidation products significantly increased in lard from 0 to 3 h (5.71 ± 0.929 μg/g) and until 6 h (18.4 ± 2.02 μg/g). In comparison, the total amount of α-T3 oxidation products significantly increased in wheat germ oil from 0 to 3 h (34.1 ± 1.07 μg/g) and until 6 h (46.3 ± 3.03 μg/g). However, after 10 h of heating in wheat germ oil, the total amount of α-T3 oxidation products reached a plateau (44.5 ± 1.39 μg/g). Remarkably, very low amounts of α-T3 oxidation products were formed. In lard and wheat germ oil, only 2.02 ± 0.219 and 5.69 ± 0.372% of the initial α-T3 content converted during the 6 h into α-T3 oxidation products. However, other authors have found significantly higher total amounts of T oxidation products formed during frying experiments: 7.2−78% (α-TQ-4a,5-E, α-TQ-7,8-E, and α-TQ).[17,18,20] An explanation for this could be the intense exposure to oxygen under Rancimat oxidation conditions, which accelerated the oxidation process of tocochromanols. It seems that the α-T3 oxidation products initially formed were not stable in the presence of oxygen and prolonged heating and underwent further oxidation and/or degradation, leading to products not identifiable with the method described here.

α-Tocotrienol Quinone Epoxids. In the present study, α-T3QEs were the major identifiable α-T3 oxidation products formed in spiked lard and spiked wheat germ oil (Figure 4). This is in accordance with previous studies that detected α-TQEs as the major α-T oxidation products in spiked maize germ oil[17] and spiked model fats.[20] The total amount of α-T3QEs increased significantly in spiked lard from 0 to 3 h (2.34 ± 0.341 μg/g) and until 6 h (10.3 ± 0.777 μg/g) and in spiked wheat germ oil from 0 to 3 h (24.7 ± 0.945 μg/g) and until 6 h (34.1 ± 2.67 μg/g). In spiked wheat germ oil the total amount reached a plateau (35.7 ± 1.02 μg/g) after 10 h. Interestingly, less α-T3Q-4a,5-E was formed than α-T3Q-7,8-E in spiked lard (130 °C), whereas this situation was reversed in spiked wheat germ oil (90 °C) (Figures 2 and 4). The levels of α-T3Q-4a,5-E and α-T3Q-7,8-E in spiked lard increased from 0 to 3 h (0.949 ± 0.066 μg/g) and until 6 h (2.96 ± 0.592 μg/g) and from 0 to 3 h (1.39 ± 0.275 μg/g) and until 6 h (7.35 ± 0.185 μg/g), respectively. On the other hand, levels of α-T3Q-4a,5-E and α-T3Q-7,8-E in spiked wheat germ oil increased from 0 to 3 h (16.0 ± 0.819 μg/g) and until 6 h (22.4 ± 1.75 μg/g) and from 0 to 3 h (8.63 ± 0.126 μg/g) and until 6 h (11.7 ± 0.924 μg/g), respectively. Murkovic and co-workers[17] identified both α-TQ-4a,5-E and α-TQ-7,8-E in α-T-spiked maize germ oil at a low temperature (90 °C), whereas they found that only α-TQ-7,8-E is formed at high temperatures (180 or 220 °C). These results are in accordance with our findings.

In contrast, Verleyen et al.[20] found that more α-TQ-4a,5-E was formed than α-TQ-7,8-E in all experiments. Those experiments were performed at frying conditions between 175 and 225 °C with different model triacylglcerols with different degrees of unsaturation. It is especially noteworthy that the differences in the amounts of α-TQ-4a,5-E and α-TQ-7,8-E formed were lower in the more highly saturated triacylglcerols. The higher amount of α-T3Q-4a,5-E than α-T3Q-7,8-E in wheat germ oil at low temperature as well as in the model system[16] may be explained by the preferred reaction at position 5 compared to position 7.[41]

α-Tocotrienol Quinone. There were also significant differences in the amounts of α-T3Q formed in spiked lard and wheat germ oil (Figure 4). After 3 and 6 h, significantly more α-T3Q was formed in wheat germ oil than in lard. The level of α-T3Q increased continuously in spiked lard from 0 to 3 h (2.11 ± 0.509 μg/g) and until 6 h (4.44 ± 0.656 μg/g). In comparison, the amount of α-T3Q significantly increased in wheat germ oil from 0 to 3 h (8.30 ± 0.076 μg/mL) and until 6 h (9.82 ± 0.116 μg/mL), but after 10 h, the amount of α-T3Q fell to 5.05 ± 0.284 μg/g (Figure 4). Remarkably, α-T3Q was formed in the heated samples as long as α-T3 was still present. Verleyen et al.[20] and Murkovic et al.[17] consistently found that only a smaller percentage of the α-T converted into α-TQ (<10% relative to the initial α-T level) than to α-TQEs. Murkovic et al.[17] assumed that α-TQEs are formed to a greater extent than α-TQ because the resonance stability of the tocopherylepoxide radical is lower than that of the tocopheryl radical and that the tocopherylepoxide radical thus has a greater tendency to add peroxy radicals ($ROO^{\bullet}$) at C-8. Interestingly, Rennick and Wagner[18] found that the percentage of the α-T converting into α-TQ increased in nonspiked samples with a concentration of α-T below 200 μg/g. This phenomenon may be due to the fact that at high temperature and high concentration and in the presence of oxygen, α-tocochromanols can easily act as prooxidants by undergoing side reactions leading to the loss of antioxidant efficiency.[2] In our study, both α-T3 and α-T3 oxidation products, especially α-T3Q, underwent further oxidation and/or degradation leading to products that were not identifiable with the method described here.

5-Formyl-γ-tocotrienol and 7-Formyl-β-tocotrienol. In both lipid matrices, the aldehydic oxidation products 5-FγT3 and 7-FβT3 were formed steadily to a small extent, whereby 5-FγT3 was more likely to be formed than 7-FβT3 (Figure 4). These findings are in accordance with the results of Rosenau et al.,[41] who found that in α-tocochromanols position 5a is more likely to oxidize than position 7a. Compared with wheat germ

oil, the amount of 5-FγT3 formed was significantly higher in lard. The levels of 5-FγT3 and 7-FβT3 in spiked lard increased from 0 to 3 h (1.06 ± 0.056 μg/g) and until 6 h (3.26 ± 0.518 μg/g) and from 0 to 3 h (0.199 ± 0.023 μg/g) and until 6 h (0.387 ± 0.065 μg/g), respectively. On the other hand, the levels of 5-FγT3 and 7-FβT3 in spiked wheat germ oil increased from 0 to 3 h (0.782 ± 0.023 μg/g) and until 6 h (1.71 ± 0.125 μg/g) and from 0 to 3 h (0.382 ± 0.022 μg/g) and until 6 h (0.620 ± 0.062 μg/g), respectively (Figure 4). Pirisi et al.[19] investigated the photolysis of α-T-spiked trioleine and showed that 5-FγT was formed in small concentrations (<5%). Other authors have reported the formation of 5-FγT and 7-FβT by reaction with triethylamin at 180 °C in liquid paraffin[42] or in polyolefins during a melt process.[43] All of these studies are in accordance with our observation that the formation of 5-FγT3 is more likely than that of 7-FβT3. To the best of our knowledge, the present study is the first to identify aldehydic tocochromanol oxidation products in natural lipid matrices.

Dimer or Trimer Oxidation Products. We observed in a pretest that dimer or trimer oxidation products of α-T3 do not evaporate during the molecular distillation process, obviously due to their high molecular weights (data not shown). Consequently, these oxidation products were not detected in the distillate of the α-T3-spiked oxidized lipid matrices (Figure 2). Direct HPLC-DAD-F analysis (without prior molecular distillation) of the oxidized lipid matrices gave no positive indication of dimer and trimer oxidation products (data not shown), although in the model system *n*-hexane autoxidation of α-T3 was proven to generate dimer and trimer oxidation products.[16] However, data from the literature are contradictory for the formation of tocochromanol dimers and trimers in oxidized lipid matrices. Some previous studies do indicate the formation of tocochromanol dimers and trimers in lipid matrices,[21,24,44] but these results are not in agreement with those of Yamauchi and coauthors,[45] who showed that α-T suppresses the formation of methyl linoleate hydroperoxides in methyl linoleate and that the main oxidation products of α-T are epoxy-8a-hydroperoxy-α-tocopherones and 8a-alkylperoxy-α-tocopherones, which convert to α-TQE and α-TQ. The α-T dimers and trimers are formed only in a reaction mixture containing large amounts of α-T. The polymerization reaction is very slow, and therefore polymerization of α-T$^\bullet$ is less likely in the presence of other radicals. However, dimers and trimers in lipid matrices may also be artifacts of too harsh enrichment conditions such as saponification.[44]

Finally, it can be concluded that the oxidative degradation of α-T3 and the formation of α-T3 oxidation products 5-FγT3, 7-FβT3, α-T3Q, α-T3Q-4a,5-E, and α-T3Q-7,8-E are influenced by temperature, oxygen supply, heating time, and lipid matrix, especially the fatty acid composition.

Proposed Reaction Pathway. Figure 1 shows the proposed reaction pathway leading to the formation of the most important α-T3 oxidation products. In the lipid matrix, unsaturated fatty acids undergo autoxidation with formation of alkyl (R$^\bullet$), alkoxy (RO$^\bullet$), and peroxy radicals (ROO$^\bullet$). The lipids RO$^\bullet$ and ROO$^\bullet$ react with α-T3 to form the corresponding nonradical lipid products and the resonance-stabilized α-tocotrienyl radical. The α-tocotrienyl radical reacts further with additional ROO$^\bullet$ or RO$^\bullet$ to form the corresponding 8a-peroxy-α-tocotrienone or 8a-alkoxy-α-tocotrienone or via epoxidation to epoxy-8a-peroxytocotrienone or epoxy-8a-alkoxytocotrienone. These intermediate products react easily by hydrolysis to form α-T3Q and α-T3QEs.[46] Formation of the aldehydic α-T3 oxidation products 5-FγT3 and 7-FβT3 seems to be a competitive reaction mechanism alongside the formation of α-T3Q or α-T3QEs. A possible formation mechanism for 5-FγT3 and 7-FβT3 proceeds via oxidation of α-tocotrienolquinone methides, which can be formed from the α-tocotrienyl radical by one-electron oxidation or from 8a-peroxy-α-tocotrienone by 1,4-elimination (the latter reaction step is not shown in Figure 1). Nagata et al.[35] and Al-Malaika et al.[47] assumed a prooxidative reaction mechanism for the formation of 5-FγT and 7-FβT alongside the formation of α-TQ. An explanation for the increased incidence of 5-FγT3 and 7-FβT3 in lard in comparison to wheat germ oil could be that the slower lipid peroxidation (due to the lower unsaturation degree in lard) and the concomitant slow consumption of α-T3 lead to the oxidation of α-T3 to 5-FγT3 and 7-FβT3 in the presence of sufficient oxygen.

Application of the Method to Wholemeal Rye Bread and Dried Brewer's Spent Grain. To assess the applicability and importance of the method, two different food matrices were analyzed for the presence of α-T3 oxidation products: commercially available wholemeal rye bread and dried brewer's spent grain. For this, α-T3 oxidation products were extracted along with tocochromanols from the food matrices and determined by HPLC-DAD-F. As shown in Figure 5, 5-FγT3, α-T3Q, α-TQ, α-T3Q-4a,5-E, and α-T3Q-7,8-E were detectable in wholemeal rye bread and dried brewer's spent grain after *n*-hexane extraction and further extraction by molecular distillation. The α-T3 oxidation products and tocochromanols determined in the food matrices with the newly developed method are given in Table 5. α-T3Q was the main α-T3 oxidation product determined in wholemeal rye bread and dried brewer's spent grain. Furthermore, α-TQ was determined in wholemeal rye bread and dried brewer's spent grain, presumably due to the presence of its precursor α-T in high concentration. However, compared with the thermal oxidation of α-T3 in spiked lipid matrices, comparatively high concentrations of α-T3Q and α-TQ were formed in wholemeal rye bread and dried brewer's spent grain. Furthermore, 5-FγT3 and α-T3Q-4a,5-E, as well as α-T3Q-7,8-E, were also detected in trace amounts. The tocochromanol pattern and contents are comparable to those described in the literature.[10,12] Although the presence of oxidation products showed that oxidation processes had taken place, no conclusions can be made about the original tocochromanol contents on the basis of the available data.

In conclusion, with the aid of the newly developed molecular distillation procedure it was possible to efficiently extract the most important oxidation products of α-T3 (α-T3Q, α-T3Q-4a,5-E, α-T3Q-7,8-E, and 5-FγT3, as well as 7-FβT3) from lipid matrices (heat-treated lard and wheat germ oil) and from α-T3-rich food matrices (wholemeal rye bread and dried brewer's spent grain). Furthermore, these five oxidation products were identified and quantitated in these matrices for the first time. The newly developed molecular distillation procedure makes it possible to investigate oxidation products of α-T3 in small quantities (<0.78 μg/g) in lipid matrices, which is the prerequisite to tracing and understanding the formation kinetics of these oxidation products in fat, oils, and other T3-rich foods. Another advantage of this method is that α-T3 oxidation products can now be enriched to obtain sufficient amounts for conducting physiological studies with these potentially bioactive products.

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Notes

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ABBREVIATIONS USED

5-FγT, 5-formyl-γ-tocopherol; 5-FγT3, 5-formyl-γ-tocotrienol; 7-FβT, 7-formyl-β-tocopherol; 7-FβT3, 7-formyl-β-tocotrienol; DAD, diode array detection; EIMS, electron impact mass spectroscopy; F, fluorescence; HPLC, high-performance liquid chromatography; IDL, instrument limit of detection; IDC, instrument detection capability; IQL, instrument limit of quantitation; MDL, method limit of detection; MQL, method limit of quantitation; NMR, nuclear magnetic resonance spectroscopy; PBI, particle beam interface; TBME, *tert*-butyl methyl ether; UV−vis, ultraviolet−visible spectroscopy; α-, β-, γ-, δ-T, α-, β-, γ-, δ-tocopherol; α-, β-, γ-, δ-T3, α-, β-, γ-, δ-tocotrienol; α-T3Q, α-tocotrienolquinone; α-T3Q-4,a5-E, α-tocotrienolquinone-4a,5-epoxide; α-T3Q-7,8-E, α-tocotrienolquinone-7,8-epoxide; α-T3QM, α-tocotrienolquinone methide; α-TQ, α-tocopherolquinone; α-TQ-4a,5-E, α-tocopherolquinone-4a,5-epoxide; α-TQ-7,8-E, α-tocopherolquinone-7,8-epoxide; α-TQM, α-tocopherolquinone methide; $R^{\bullet}$, alkyl radical; $RO^{\bullet}$, alkoxy radical; $ROO^{\bullet}$, peroxy radical.

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