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Qualitative and Quantitative Analysis of Tocopherols in Toothpastes and Gingival Tissue Employing HPLC NMR and HPLC MS Coupling

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Gingival samples treated with toothpastes containing tocopherols (vitamin E) were investigated employing HPLC chromatography. The aim was to verify that vitamin E is actually enriched in the tissue, which could have beneficial effects on oral health. After determination of the tocopherols available in the toothpastes, control samples from healthy test persons and subjects suffering from gingivitis were analyzed. Subsequently, gingival tissues from diseased test persons who treated their teeth with the toothpastes containing tocopherols using various kinds of concentrations or applications were investigated. The first step of the analysis was a fast and careful extraction employing matrix solid-phase dispersion (MSPD). Afterward, the separation of the different tocopherol homologues existing was performed by HPLC chromatography on highly selective C_{30} RP phases. The identification of the tocopherol homologues was performed using the on-line coupling of HPLC with NMR spectroscopy and mass spectrometry.

In the past years, the importance of tocopherols (vitamin E) has strongly increased in the pharmaceutical and food industries[1]. This is due to their antioxidative properties, that is, the inhibition of lipid oxidation in food and biological systems and the quenching of reactive singlet oxygen.[2−4] This capacity, which is enhanced by synergistic effects of vitamin C and carotenes,[5] results in positive effects on human health. Epidemiological studies indicate beneficial effects against atherosclerosis, cardiovascular disease, certain types of cancer, Alzheimer's and Parkinson's disease, as well as the aging process in general.[6−14]

Lately, it is being discussed, whether a supplementation of vitamin E in toothpastes would improve oral hygiene. This appears to be necessary, because epidemiological studies revealed that 95% of all people are suffering from gingivitis.[15] The main reason for caries and gingivitis is dental plaque that is deposited on the teeth. The plaque bacteria are fed by sugars from food and the glycoproteins in the saliva. When their metabolic products, the endotoxins, migrate into the gingiva, they cause destruction that leads to gingivitis. The initial state of gingivitis is painless and characterized by red and swollen gingiva. Subsequently, the gingivitis becomes painful, showing inflammation, bleeding, and gingival atrophy. The final stage, marked by degradation of the periodontium and loss of teeth, is called periodontitis.[16−18]

The best prophylaxis against caries and gingivitis is a careful plaque removal. This is performed by brushing, especially with toothpastes, because they improve oral hygiene as a result of several additives in combination with the use of dental rinses and floss. For gingival protection, antiinflammatory ingredients from chamomile, aloe, calendula, rosemary, or sage are added. Recently, a toothpaste that contains vitamin E was introduced to the market. Its antioxidative properties might protect the gingival cells against the attack by bacteria and cellular toxins and therefore either prevent the formation or enhance the cure of gingivitis.

To develop their beneficial actions, the tocopherols first need to be enriched in the gingival tissue. The aim of this investigation was to analyze whether the vitamin E from the toothpaste actually migrates into its place of action, the gingiva. A further objective was to determine whether differences between gingival samples from healthy and diseased test persons can be observed. In addition to the two control groups, one with healthy gingivae and one suffering from gingivitis, gingival samples from test persons

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applying (brushing or using a dental tray) toothpastes with different concentrations of tocopherols were analyzed.

The investigation of many biomedical tasks, such as the analysis of tocopherols in gingival samples presented here, needs to be performed using sophisticated analytical methods. Especially, substances that are highly sensitive to environmental influences require an optimized combination of analytical extraction, separation, and detection techniques.

The extraction of the tocopherols from the gingival samples was performed using matrix solid-phase dispersion (MSPD), a new and delicate extraction method that is especially suitable for biological and tissue samples.[19−20] The solid sample was ground with sorbens material, and the mixture was loaded into an SPE cartridge. After a conditioning step, the tocopherols were eluted with methanol.

Vitamin E does not consist of a single substance, but of a mixture of four tocopherol homologues, called α-, *β*-, *γ*-, and *δ*-tocopherol.[21−22] Since all homologues possess different bioavailabilities, it is extremely important to know the exact composition of a given supplement. In addition to the four naturally occurring tocopherols, α-tocopherol acetate is used in industry because of its higher stability. Therefore, it is necessary to perform an HPLC separation. The method established in our group is the HPLC analysis employing C_{30} RP phases, because it combines the advantages of a reversed-phase separation with the improved shape selectivity of the C_{30} alkyl chains, as compared to C_{18} phase separations.[23−25] Now even the separation of the structural isomers *β*- and *γ*-tocopherol can be achieved.

Because of their antioxidative properties, the tocopherol homologues are easily oxidizable in the presence of light and oxygen. Subsequentially, the structural assignment of the different homologues to their corresponding peaks in the HPLC chromatogram needs to be performed by on-line coupling with NMR spectroscopy (LC NMR) or mass spectrometry (LC/MS), because they allow the exclusion of light and air in a time-saving manner and, therefore, prevent the analytes from degradation.[26−27] Hereby, NMR spectroscopy is the only method yielding stereochemical information that enables the identification of isomers, whereas the high sensitivity of the mass spectrometry is well-suited for limited amounts of sample.

EXPERIMENTAL SECTION

Materials. The extraction of the tocopherols was performed with MSPD sorbens material isolute C_{18} from Separtis (Grenzach-Wyhlen, Germany). Methanol (LiChrosolv, gradient grade) as well as methanol-d_4 (Uvasol) was obtained from Merck (Darmstadt, Germany). The tocopherol homologues and α-tocopherol acetate were purchased from Calbiochem (San Diego, CA).

Two toothpastes containing 0.2 or 1.0% of tocopherols, respectively, were provided by Block Drug Inc. (Jersey City, NJ).

Study Design. Gingival samples were taken from subjects who had brushed their teeth with a toothpaste containing tocopherols for two weeks before the gingival biopsy. In addition to the two control groups, one with healthy gingivae and one suffering from gingivitis, there were three groups of test persons suffering from gingivitis who used toothpastes containing tocopherols: One group of subjects brushed their teeth twice daily for 60 to 90 s with 2 to 3 cm of a toothpaste containing 0.2% tocopherols, and another group treated their teeth with a toothpaste containing 1.0% tocopherols. A third group brushed their teeth analogously to the second group with the toothpaste containing 1.0% tocopherols. Additionally, they used an elastomer tray for the jaw filled with a thin strip of toothpaste (1.0%), which they wore for 3 min.

Informed consent was obtained from all volunteers and approved with the study design protocol by the Ethics Committee of the Ärztekammer des Saarlandes.

Sample Preparation. *Preparation of the Tocopherol Standard.* Tocopherol standards were prepared by dissolving α-, *β*-, *γ*-, and *δ*-tocopherol and α-tocopherol acetate in methanol until the desired concentrations between 40 and 0.8 μg/mL were reached. For the HPLC NMR coupling, a standard with a concentration of 6.67 mg/mL of each tocopherol was used.

Extraction of the Toothpaste. A 500-mg portion of the toothpaste containing 0.2% of tocopherols and 2.5 mL of methanol were stirred well until the binding agent coagulated. The mixture was then ultrasonicated for 15 min. Afterward, the undissolved substances were removed using a 45-μm filter to prevent clogging of the HPLC column.

Extraction of the Gingival Samples. The gingival samples were taken from the subjects 30 min after they brushed their teeth with the toothpaste containing the tocopherols. The samples were sent to our department under cooled conditions and stored at −30 °C.

The gingival samples were ground with 500 mg of C_{18}(EC)-MSPD-material. The mixture was loaded onto an SPE column and pressed between two frits. The column was conditioned with 5 mL of bidistilled water, and the extraction was performed with 6 mL of methanol. The solvent was evaporated, and the extract dissolved in 500 μL of methanol afterward.

HPLC. The chromatographic separations were performed on an HP1100 HPLC system (Agilent Technologies, Waldbronn, Germany) using a UV detector at 280 nm. The separation was performed employing a highly selective C_{30} RP column (ProntoSil 120-3-C30, Bischoff Chromatography, Leonberg, Germany) with a particle size of 3 μm and a pore width of 120 Å. The column dimensions were 250 × 4.6 mm. The tocopherols were separated using a flow rate of 1 mL/min and pure methanol as mobile phase. The amounts of samples injected were 20−50 μL.

HPLC NMR. For the HPLC and NMR coupling, an HP1100 HPLC system was connected to the 120-μL flow probe of a Bruker AMX 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) by a steel capillary. To stabilize the lock, 5% deuterated methanol was added to the HPLC eluent. A 40-μL portion of the tocopherol standard solved in deuterated methanol (6.67 mg/mL) was injected and separated on a C_{30} column (250 × 4.6 mm) at a flow rate of 0.4 mL/min.

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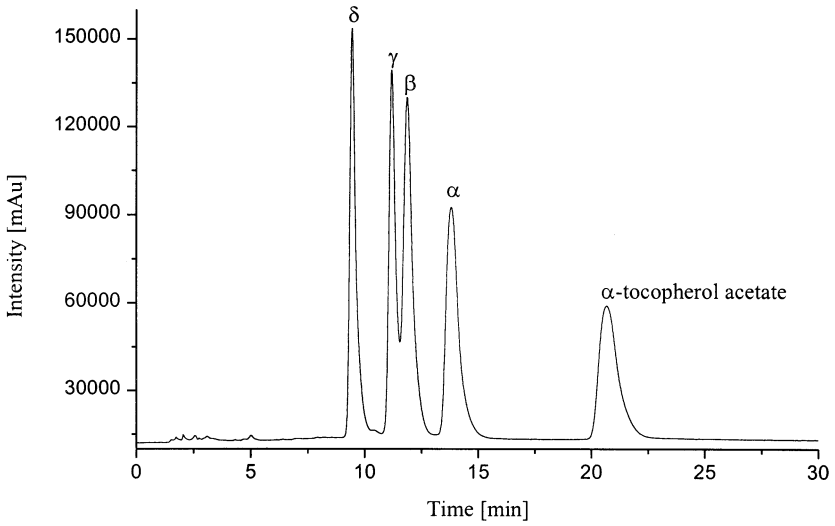

Figure 1. Chromatogram of a standard of α-, *β*-, *γ*-, and *δ*-tocopherol and α-tocopherol acetate in methanol (1000 ng each).

The continuous-flow HPLC NMR measurement was recorded at 295 K with the puls program lc2pnps, which is a continuous-flow experiment with a solvent presaturation using shaped pulses. A total of 128 rows were measured in the F1 dimension, each row having 32 transients and 4k data points over a sweep width of 8621 Hz. Before the Fourier transformation was performed, both dimensions were multiplied by a sine function.

HPLC/MS. Mass spectrometry was performed at the Bruker Esquire-LC Ion Trap LC/$MS^{(n)}$-System (Bruker Daltonik, Bremen, Germany) with an APCI interface and an ion trap. The HPLC/MS coupling was accomplished using an HP1100 system and the software Bruker Data Analysis Esquire-LC 4.0.

To adapt the tocopherol separation for the HPLC/MS coupling, a C_{30} column with a diameter of 2 mm (ProntoSil 120-3-C30, Bischoff Chromatography, Leonberg, Germany) with a particle size of 3 μm and a pore width of 120 Å was used at a flow rate of 0.4 mL/min. Injection size was 10-μL.

The mass spectra were recorded in a mass region of 50−550 *m*/*z*. The detection was performed using atmospheric pressure chemical ionization (APCI) in the positive ion mode. The voltage of the Corona needle was optimized, resulting in a current of 5−7 μA. The drying and carrier gas was nitrogen at a temperature of 300 °C. The temperature of the ionization chamber also was 300 °C.

RESULTS AND DISCUSSION

Calculation of Calibration Curves for the Quantification of the Tocopherols. In the following measurements, the tocopherols were quantified by the method of the external standard. Therefore, it was necessary to calculate a calibration curve for each one of the tocopherols. To accomplish this, a tocopherol standard of a known concentration (40 μg/mL) was prepared from solutions of α-, *β*-, *γ*-, and *δ*-tocopherol as well as α-tocopherol acetate in methanol. This standard and dilutions from it (20, 8, 4, 2, and 0.8 μg/mL) were separated on a highly selective C_{30} HPLC column using pure methanol as an eluent. Figure 1 shows one of the chromatograms obtained.

The peak areas of each tocopherol were plotted against the used amount, and the calibration curve was calculated by linear regression. To improve the correctness, all measurements were performed three times. Each tocopherol has its own calibration curve, because the UV absorptions of the different tocopherols vary at a given wavelength (in this example 280 nm), and therefore, the peak areas vary as well. The slope of each calibration curve then allows calculation of the concentration of unknown amounts of this particular tocopherol, provided the peak area was known. Table 1 displays the functions of the calibration curves and their standard deviations, which were obtained from the series of dilutions of the tocopherol-standard.

Table 1. Calibration Curves of the Tocopherol Standard and Their Standard Deviations

substance	calibration curve	std dev
α-tocopherol	$y = 0.3117x$	±0.002 12
β-tocopherol	$y = 0.35x$	±0.003 75
γ-tocopherol	$y = 0.2606x$	±0.001 22
δ-tocopherol	$y = 0.2783x$	±0.002 35
α-tocopherol acetate	$y = 0.276x$	±0.001 92

Extraction of Tocopherols from Toothpaste. The measurements show the investigation of the toothpaste that contains 0.2% tocopherols. The first step of the analysis was a careful and, as closely as possible, quantitative extraction of the tocopherols, which was performed with methanol. Sample size for injection on a C_{30} HPLC column was 20 μL. Figure 2 shows the chromatogram that was obtained.

The tocopherol content of the toothpaste could be calculated from the peak areas of the different tocopherols. The results are presented in Table 2. Although only α-tocopherol acetate was added to the toothpaste, because it cannot be oxidized as easily as the other tocopherols, α-, *β*-, *γ*-, and *δ*-tocopherol could also be detected in small amounts. This was due to impurities of the α-tocopherol acetate, which had a purity grade of ∼98% (manufacturer communication).

Identification of the Tocopherol Homologues by HPLC NMR Coupling. After a sufficient chromatographic separation of α-, *β*-, *γ*-, and *δ*-tocopherol, as well as α-tocopherol acetate using a C_{30} HPLC column and pure methanol as the mobile phase had been achieved, the peak assignment was validated by HPLC NMR

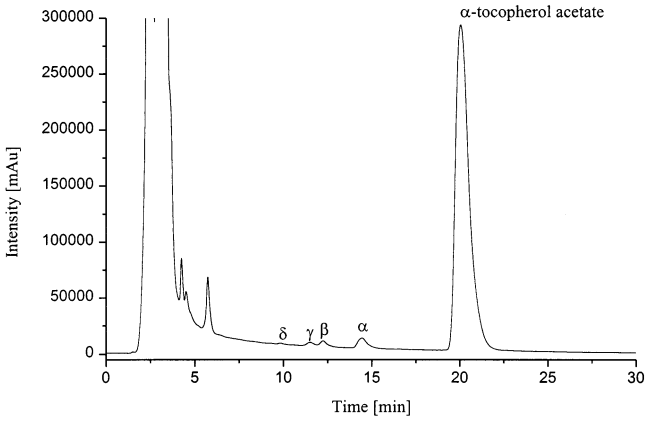

Figure 2. Chromatogram of an extract made from toothpaste containing 0.2% tocopherols in methanol.

Table 2. Composition of the Tocopherols in the Toothpaste

name	% in toothpaste
α-tocopherol	1.8
β-tocopherol	0.7
γ-tocopherol	0.7
δ-tocopherol	0.1
α-tocopherol acetate	96.7

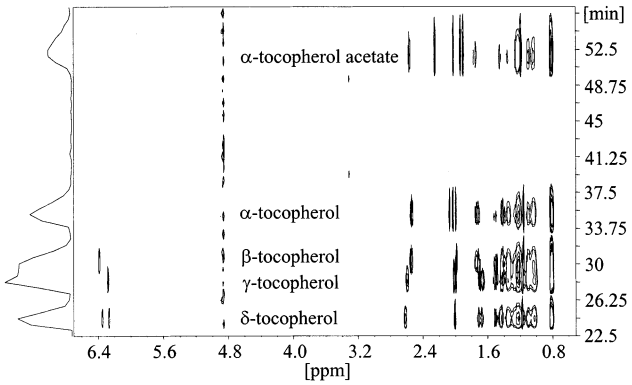

Figure 3. Contour plot of the continuous-flow HPLC NMR separation of a tocopherol mixture.

coupling. To accomplish this, the continuous-flow mode, which allows the simultaneous separation and detection of the sample components, was used. The chromatography was adapted to the NMR spectroscopy by reducing the flow rate to 0.4 mL/min in order to reach a longer residence time of the analytes in the NMR detection cell. Moreover, a higher amount of the tocopherol mixture was injected (266.8 μg of each tocopherol) to compensate for the lower sensitivity of the NMR, as compared to the UV detection.

Figure 3 displays the contour plot of the HPLC NMR separation, which can be correlated to the chromatogram of the HPLC separation of the tocopherol mixture (see Figure 1). On the *x* axis, the chemical shift is plotted, and the *y* axis shows the time scale of the separation. The chromatogram plotted next to the *y* axis was obtained by summarizing the intensities of the proton signals, although due to the time resolution of about 38 s, the resolution of the chromatogram is certainly much worse than the resolution of a corresponding UV chromatogram (resolution 0.5 s).

The classical interpretation of the ^{1}H NMR spectra cannot easily be applied when the continuous-flow measurement is graphically shown as a contour plot. In this case, the analysis of single rows is more suitable. In Figure 4, the ^{1}H NMR spectra of the different tocopherols taken at the corresponding peak maximums are plotted. The assignment of the structures of the different tocopherols to their ^{1}H NMR spectra[28] can be performed via the interpretation of the signals of the aromatic protons (between 6.2 and 6.3 ppm) or via the interpretation of the protons of the methyl groups substituted on the aromatic ring (between 2.0 and 2.2 ppm). Furthermore, the methyl group of the acetate group of the α-tocopherol acetate can easily be detected as an additional signal at 2.3 ppm.

Analysis of Gingival Samples Regarding the Presence of Tocopherols. The main target of the investigations was the analysis of gingival samples that were taken from test persons who had brushed their teeth for two weeks before the biopsy with a toothpaste containing tocopherols. The aim was to analyze whether the tocopherols were enriched in the gingiva, because only in this case may the tocopherols have a positive effect on the gingiva and, therefore, on oral health.

In addition to healthy and diseased control groups, three groups of subjects suffering from gingivitis used different types of pretreatment before the gingival biopsy. The first group of subjects brushed their teeth for two weeks before the biopsy twice daily for 60 to 90 s with 2 to 3 cm of a toothpaste containing 0.2% tocopherols, and the second group treated their teeth with a toothpaste containing 1.0% tocopherols. The third group of test persons brushed their teeth analogously to the second group with a toothpaste containing 1.0% tocopherols. Additionally, they used an elastomer tray for the jaw filled with a thin strip of toothpaste (1.0%), which they wore for 3 min. The last application of the toothpaste containing tocopherols was 30 min before the gingival biopsy.

The extraction of the tocopherols from the gingival samples was performed using MSPD. The samples were ground with C_{18} sorbens material, the mixture was loaded onto an SPE cartridge, and pressed to yield a stable column bed. After a conditioning step, the tocopherols were extracted with methanol.

A 50-μL portion of the extract was injected for the chromatographic separation performed on the C_{30} column with pure methanol. Figure 5 shows a chromatogram obtained from a gingival sample that was pretreated with the toothpaste containing 0.2% tocopherols. It is remarkable that even though strong matrix effects were predominant, a small peak at ∼20 min could be observed that indicates the presence of α-tocopherol acetate. HPLC/MS investigations could verify this presumption.

The amounts of α-tocopherol acetate found in the gingival samples are presented in Table 3. It can clearly be seen that the amount of tocopherols available in the gingivae of subjects from the healthy control group is higher than the value of untreated test persons suffering from gingivitis. Nevertheless, both control groups show very low values, because the α-tocopherol acetate found may result only from the regular tocopherol intake from food or supplements.

When the gingival tissue is brushed with a toothpaste containing tocopherols, a significant enrichment ($p < 0.05$, outliers not

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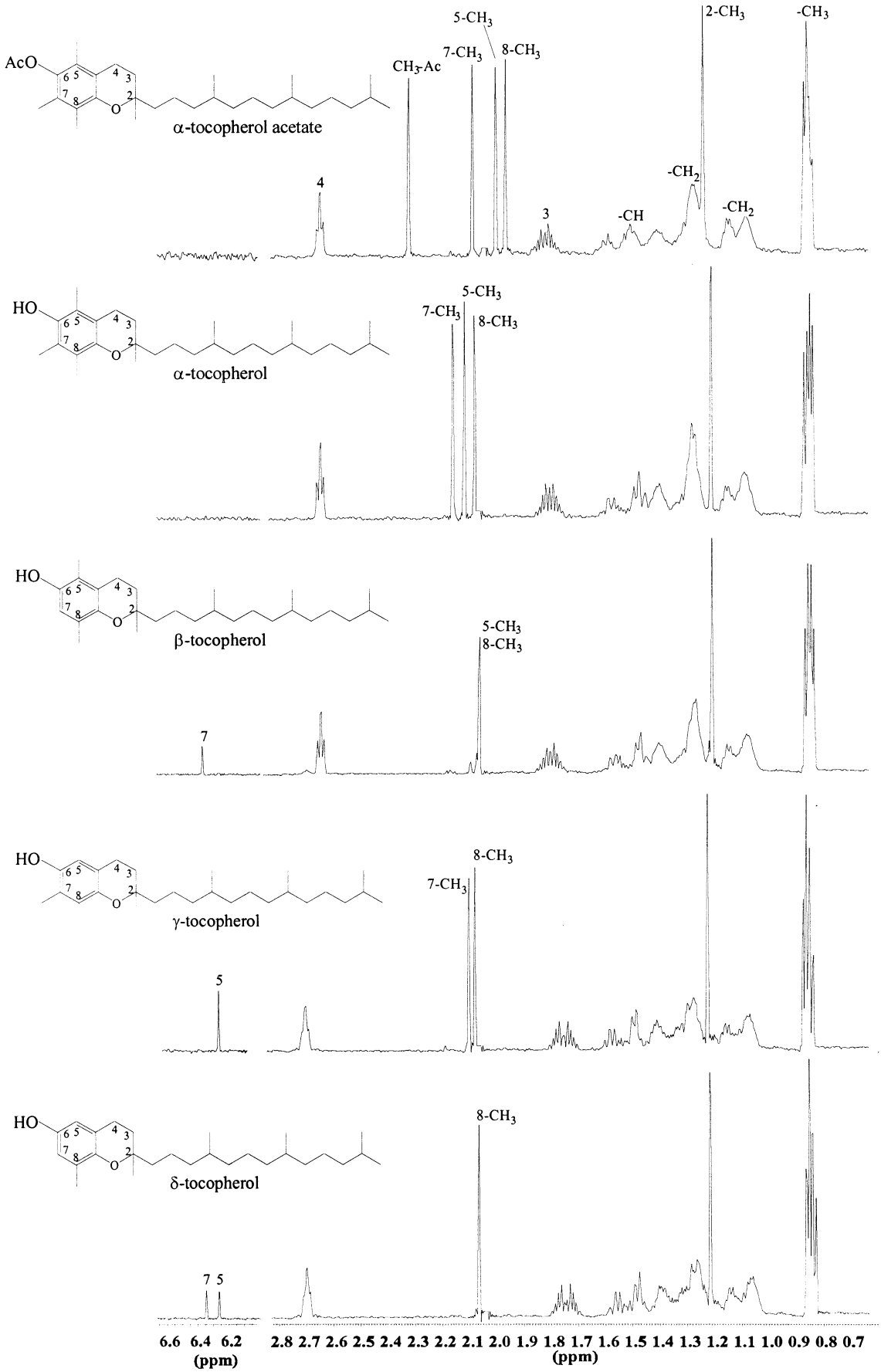

Figure 4. ^{1}H NMR spectra of the tocopherols, extracted from the contour plot of the continuous-flow measurement (Figure 3) at the corresponding peak maximums.

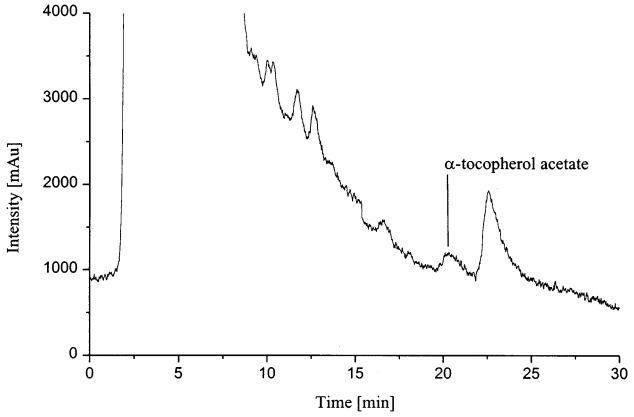

Figure 5. Chromatogram of an extract made from gingiva treated with a toothpaste containing tocopherols.

Table 3. Results of the Investigation of the Gingival Samples

sample no.	amt of α-toco acetate/1 mg of gingival sample [ng/mg]
Healthy Subjects, No Treatment	
271	3.1
276	8.1
281	28.0
287	1.2
av	10.1
without 281	4.1
Diseased Subjects, No Treatment	
460	8.2
462	*a*
464	*a*
467	*a*
469	*a*
475	*a*
477	*a*
478	1.1
482	*a*
av	1.0
Diseased Subjects, Brushing 0.2% Ac	
455	50.7
459	4.0
465	25.4
470	9.1
476	27.2
av	23.3
Diseased Subjects, Brushing, 1.0% Ac	
453	58.0
461	*a*
468	70.7
471	339.7
479	14.5
483	9.1
av	82.0
without 471	30.5
Diseased Subjects, Brushing and Tray, 1.0% Ac	
454	52.2
463	12.7
466	1.5
481	1.9
av	17.1

[a] Not detectable.

considered) of α-tocopherol acetate in the gingiva can be observed, which indicates that the antioxidant effect of the vitamin E can be exerted. Nevertheless, neither the concentration of tocopherols in the toothpaste nor the type of application seems to have a great influence on the enrichment of the vitamin E in the gingival tissue, because the differences using various concentrations or applications are quite small. It appears that the maximum enrichment is already reached using the toothpaste containing 0.2% α-tocopherol acetate.

One could also expect to observe the presence of α-tocopherol in the gingival samples, because α-tocopherol acetate is usually hydrolyzed to α-tocopherol in an aqueous environment. In this context, the formation of α-tocopherol cannot be seen yet in the HPLC chromatogram, because the last treatment occurred only 30 min before the gingival biopsy, so that the hydrolysis could not take place rapidly enough.

Mass Spectrometric Analysis of the Gingival Samples. The chromatographic separation of gingival samples treated with toothpastes containing tocopherols showed a peak at a retention time of ~20 min that indicated the presence of α-tocopherol acetate. This assumption had to be validated by a mass spectrometric analysis (MS). For this purpose, the HPLC-MS coupling was optimized using atmospheric pressure chemical ionization (APCI), because it is a straightforward (as opposed to the use of postcolumn agentation[29]) and well suitable method for the ionization of the nonpolar tocopherols. A narrow-bore HPLC column (2 mm instead of 4.6 mm) and, therefore, a lower flow rate adapted the chromatography to the mass spectrometry.

The investigation reveals that, even though the spectrum is dominated by the matrix components (e.g., base peak chromatograms (BPC) of m/z 338), which is impressively displayed in Figure 6 a), a peak of m/z 473 can be observed in the BPC, which can be correlated to the peak of the protonated molecule mass of α-tocopherol acetate, at 8.2 min (the use of a 2.0-mm HPLC column yields shorter retention times). This is shown in the extension of Figure 6.

Because of the careful ionization via APCI, the mass spectrum between 8.0 and 8.3 min is dominated by the protonated molecule mass of α-tocopherol acetate, whereas the MS/MS analysis of the peak reveals a fragmentation pattern characteristic for α-tocopherol acetate (Figure 6c,d). It can either undergo a retro-Diels–Alder reaction splitting off the alkyl chain to m/z 207 or it can exchange the acetate group for a hydroxyl group, forming α-tocopherol (m/z 431). α-Tocopherol can also undergo a similar retro-Diels–Alder reaction to a fragment with m/z 165.

CONCLUSION

The optimization and combined use of the extraction technique MSPD with the chromatographic separation method HPLC enabled the analysis of tocopherols in gingival samples. Hereby, the identification of the light and air-sensitive tocopherol homologues was accomplished by employing HPLC NMR and HPLC/APCI-MS coupling.

It could be verified that an enrichment of vitamin E in the gingiva occurs when the gingival tissue is treated with toothpastes containing α-tocopherol acetate. This is an important milestone on the way of showing that the supplementation of toothpastes with tocopherols may prevent the formation or enhance the cure of gingivitis, a dental disease present in 95% of all people. The next step will be a clinical study to examine the effects of the supplementation.

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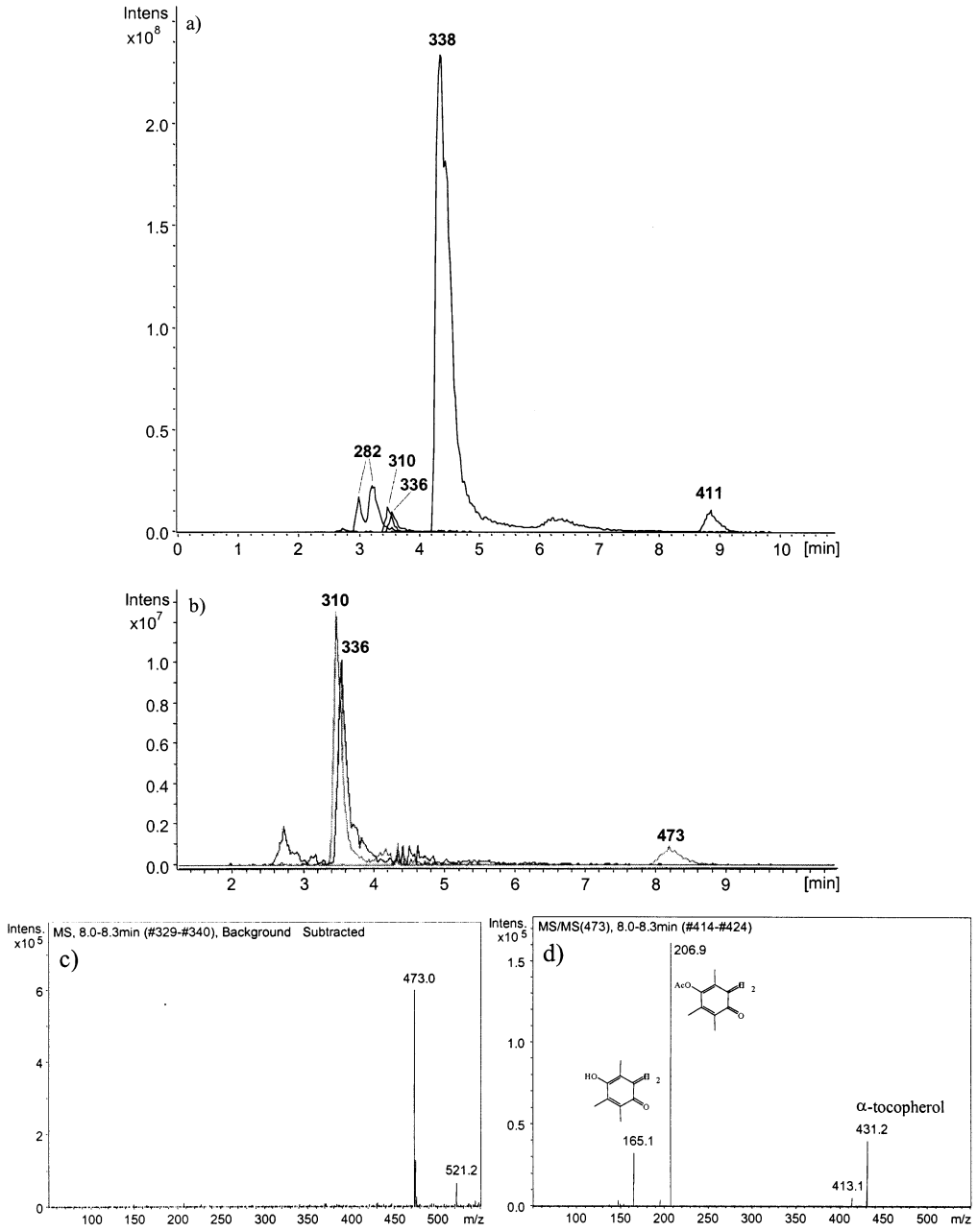

Figure 6. (a) Base peak chromatograms (BPC) of *m*/*z* 282, 310, 336, 338, and 411 (unidentified matrix components); (b) extension of (a) with BPC of *m*/*z* 310, 336, and 473 (α-tocopherol acetate); (c) MS spectrum; and (d) MS/MS spectrum of α-tocopherol acetate from gingival tissue treated with a toothpaste containing tocopherols.

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