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Journal of Chromatography A, 976 (2002) 215–220

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of tocopherols, tocopherolquinones and tocopherolhydroquinones by gas chromatography–mass spectrometry and preseparation with lipophilic gel chromatography

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Abstract

Lipophilic gel chromatography using Sephadex LH-20 helps in separating α-, β-+γ-, and δ-tocopherol and also in separation of their oxidation products e.g. the tocopherolquinones or other oxidation products. This preseparation can help to overcome analytical problems due to the complexity of synthesis mixtures of tocopherol oxidation procedures as well as in separation of complex physiological matrices. Determination of the preseparated tocopherols, tocopherolquinones and tocopherolhydroquinones can then be achieved by means of GC–MS measurement of the free substances or their trimethylsilyl derivatives.

Keywords: Sample handling; Tocopherols; Tocopherolquinones; Tocopherolhydroquinones

1. Introduction

The widespread use of α-tocopherol (Vitamin E) in multivitamin drugs as well as additive in the food industry, in feeding livestock, in many drugs as antioxidant [1] and in cosmetics has led to dramatically increased exposure of the whole population in Germany and in most industrialised countries [2]. Due to massive advertising and the presumed efficiency of α-tocopherol use in cardiovascular diseases or cancer prevention a widespread use of tocopherols and multivitamin preparations by the study participants of our pharmacoepidemiological studies during the German Health Surveys could be observed [3,4]. Many of the claimed health benefits of vitamin E usage are not yet proven. In contrary some studies have cast some doubt about the pure benefit of consuming more and more α-tocopherol [5,6]. Despite those doubts and despite the fact that consumption of α-tocopherol tends to rise further, the knowledge concerning the tocopherol metabolism in humans is relatively sparse. Some groups dealing with in vitro oxidation of the tocopherols have published results which show that, in vivo the tocopherolquinones could play an important role during metabolic processes [7–10]. So we decided to develop a GC–MS method that could help to determine the concentration of tocopherolquinones and tocopherolhydroquinones in biological specimens, particularly in human serum samples. Since sample preparation procedures like simple liquid–liquid extraction or the preparation of the unsaponifiable matter are associated with either a lot of matrix interferences in GC–MS and reduced column life-

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00941-X

time or increased risk of losses of analytes, we chose to test the combination of GC–MS and preseparation with lipophilic gel chromatography on Sephadex LH-20.

2. Experimental

2.1. Chemicals

α-Tocopherol was from Sigma (Deisenhofen, Germany); α-tocopherolquinone was from Acros Organics (KMF Laborchemie, Sankt Augustin, Germany). If not otherwise specified, all other used chemicals were of analytical grade and HPLC solvents and α-, β-, γ- and δ-tocopherols (tocopherol-kit, article no. 15496) were purchased from Merck (Darmstadt, Germany). Sephadex LH-20 was from Amersham Biosciences (Freiburg, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and *N*-trimethylsilyl-imidazole (TSIM) were purchased from Macherey–Nagel (Düren, Germany). The quinones of β-, γ- and δ-tocopherol were synthesised from the tocopherols by oxidation with $FeCl_3$ [11] and the tocopherolhydroquinones were synthesised from the tocopherolquinones by reduction with $NaBH_4$ [12].

2.2. Sephadex LH-20 chromatography

The solvent resistant column was from Amersham Biosciences, Freiburg, Germany (Type SR 25/45) with a gel-bed volume of 130 ml and was used with chloroform, LiChrosolv grade as eluent with a flow of ~60 ml/h. The eluting substances were monitored with a LKB Uvicord 4700 (LKB, Bromma, Sweden) at 254 nm and by TLC on precoated silica-gel plates (Type: Kieselgel 60 F 254, article no. 1.05715, Merck) using the following solvent system: light petroleum (b.p. 40–60 °C)–diethylether (1/1, v/v). Detection of the separated substances can be done under UV light or by spraying the TLC plates with phosphomolybdic acid–$HClO_4$ reagent and heating to 120 °C for 15 min.

2.3. GC–MS conditions

GC–MS conditions were as follows: GCQ System (ThermoFinnigan, Egelsbach, Germany); XCALIBUR software version 1.2.2; GC conditions: column: 30 m×0.25 mm I.D., df=0.25 μm Rtx-5MS (Restek, Bad Homburg, Germany); oven: 220 °C for 1 min, 5 °C/min to 290 °C, final temperature for 10 min; injection port: 275 °C, splitless injection; transfer line: 290 °C; He (4.6) flow: 30 cm/s, constant velocity; MS conditions: source temperature: 170 °C; 0.5 s/scan; electron energy: 70 eV, multiplier: 1500 V; acquisition start time: 3 min. For quantification of the different tocopherols, their quinones or hydroquinones full-scan-, selected ion monitoring (SIM)- or MS–MS mode, can be used.

2.4. Derivatization procedures

Derivatization of the different tocopherols to form trimethylsilyl (TMS) derivatives for GC–MS is done by heating the substances with MSTFA for 20 min at 60 °C. Tocopherolquinones and tocopherolhydroquinones are derivatized by reaction with TSIM for 30 min at room temperature.

2.5. Quality characteristics

2.5.1. Reproducibility of retention times

The GC–MS conditions mentioned above result in high reproducibility of retention times for all measured substances. Even those substances with higher retention times are eluted with very narrow retention time (t_R) windows. The within-day measured values ($n=10$) for α-tocoquinone and α-tocoquinone-TMS for example are $x_{tR} = 17.14 \pm 0.01$ min and $x_{tR} = 16.73 \pm 0.01$ min, respectively. So the daily t_R variation always was below 0.5% and the day-to-day retention time variation never exceeded 2.0% during a working period of more than 6 months.

2.5.2. Limit of detection (LOD) and limit of quantification (LOQ)

The GC–MS LOD ($S/N>3$ for the base peak) for the TMS derivatives of all tocopherols was 40 pg and the LOQ ($S/N>10$ for the base peak) was 80 pg using the SIM mode. These values were measured with the pure substances and with splitless injection of 1 μl solutions in isooctane. The calibration curves

for the measurement of the TMS tocopherols in serum samples have been shown previously to be linear up to an amount of 15 ng in the SIM mode and recovery data also have been described to be >95% [16,2].

The GC–MS LOD for α-tocoquinone and α-tocoquinone TMS either isolated from the own synthesis mixtures or of purchased reference substance (only available for α-tocoquinone) was determined with 1 ng and the LOQ for the base peak was found to be 10 ng by using full-scan mass spectra and splitless injection of 1 μl solutions in isooctane. Single ion mode detection and quantitation can lower these values by a factor of 10. Using GC–MS suitable capillary columns with a low background noise level is essential to achieve low levels of detection and quantification.

To avoid losses of oxidation-prone analytes during the gel chromatographic preseparation procedure it is necessary to use a closed chromatographic system with sampling valves to avoid contact with ambient oxygen. Under those conditions the recovery of applied lipophilic substances—even of oxidation-prone free tocopherols—from the Sephadex LH-20 gel typically is determined to be 98% [13,14].

3. Results and discussion

Fig. 1 shows the elution diagram of a synthetic mixture of some lipophilic substances on Sephadex LH-20. As can be seen from the elution diagram, clear separations can be achieved for α-, β-+γ- and δ-tocopherols. The relatively strong retardation of the tocopherols on Sephadex LH-20 is due to the fact that the phenolic OH group at the chromane-system interacts with the Sephadex gel matrix when chloroform as an OH-group-free solvent is used as eluent. This effect is even more pronounced for the tocopherolhydroquinones, where the second phenolic OH group introduced into the molecule leads to nearly irreversible retardation of those compounds on the LH-20 gel matrix when using pure $CHCl_3$ as eluent. In contrast to the above mentioned retardation, the loss of the phenolic OH group during the oxidation to the tocopherolquinones leads to shorter elution times compared with the tocopherols and separation of the different tocopherolquinones is not observed. Other lipophilic substances like triglycerides or cholesterolesters elute relatively early from the LH-20 gel column due to their high molecular mass and due to no interaction with the

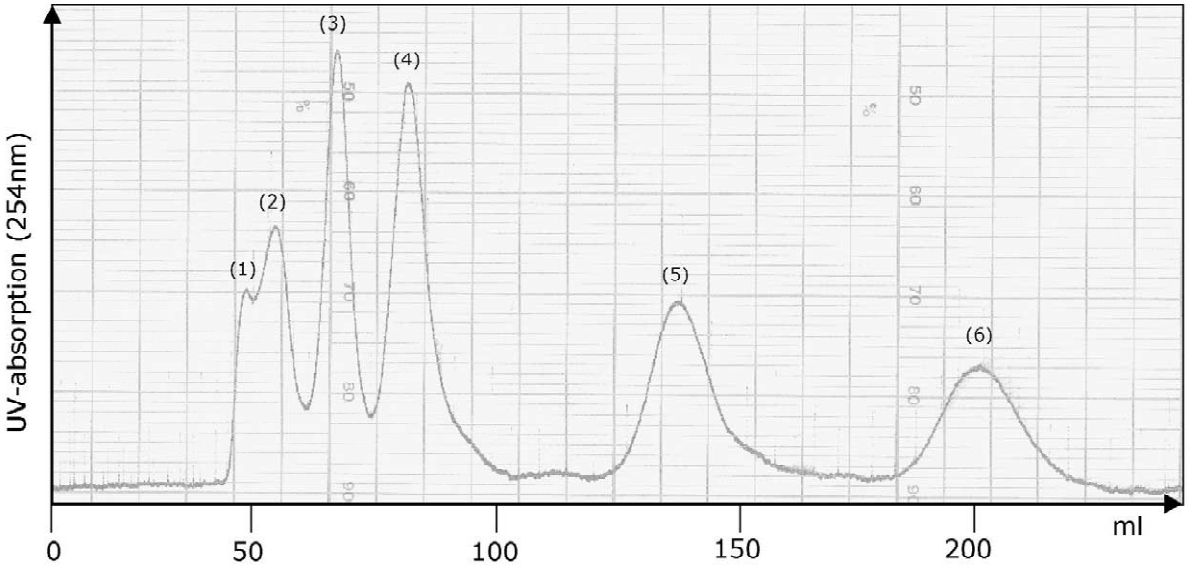

Fig. 1. Sephadex LH-20 elution diagram of a reference mixture of 6.5 mg triolein (1), 5.7 mg cholesterolpalmitate (2), 80 μg α-tocopherolquinone (3), 400 μg α-tocopherol (4), 1 mg β-tocopherol (5) and 1 mg δ-tocopherol (6); gel-bed 130 ml, eluent: chloroform.

Table 1
Elution volumes of lipophilic substances on Sephadex LH-20 [vol. of the gel bed: 130 ml; eluent: $CHCl_3$; flow-rate: ~60 ml/min]

Substance	M_r	Elution volume (ml)
Triolein	885.5	45–60
Cholesterolpalmitate	625.1	50–65
α-Tocopherolquinone	446.7	61–76
β-+γ-Tocopherolquinone	432.7	61–76
δ-Tocopherolquinone	418.7	61–76
α-Tocopherol	430.7	73–95
Cholesterol	386.7	85–110
β-+γ-Tocopherol	416.7	120–155
δ-Tocopherol	402.7	185–230

gel matrix. Here the molecular sieving effect is determining the elution sequence observed. Table 1 shows the elution volumes of the substances tested with our system.

GC–MS measurement of the free or derivatized substances can be performed on relatively nonpolar stationary phases like Rtx-5MS. For the derivatization of the tocopherols MSTFA can be used as described earlier [2] and for the quinones and hydroquinones TSIM should be used. Fig. 2 shows an example for the separation of underivatized tocopherolquinones and Fig. 3 shows the elution order of TMS derivatives of the tocopherolhydroquinones. In contrast to the tocopherols and tocopherolquinones, which can be measured in free as well as in derivatized form, the tocopherolhydroquinones can be separated using the Rtx-5MS GC column only as TMS derivatives. Good separations are observed for the free tocopherolquinones as well as for the TMS derivatives of tocopherolquinones and hydroquinones. For the tocopherols and their TMS derivatives the β- and γ-forms are only partially separated. The main ions

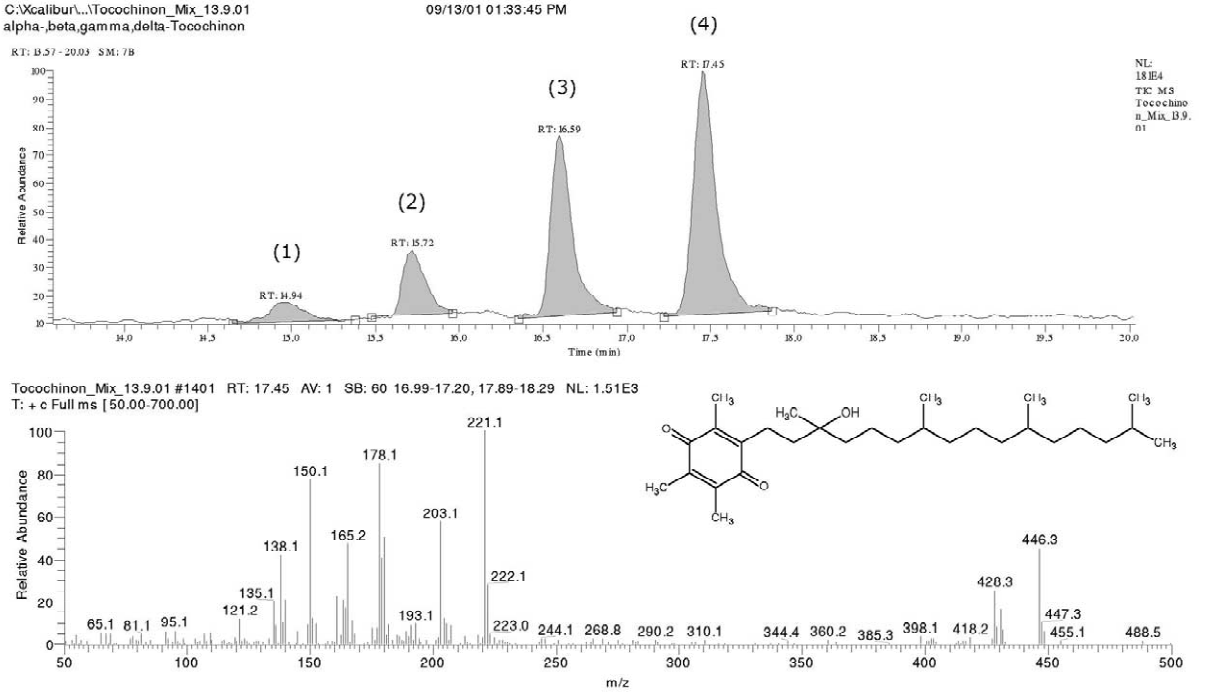

Fig. 2. Segment of the GC–MS chromatogram (upper trace) of underivatized tocopherolquinones with δ-tocopherolquinone (1), β-tocopherolquinone (2), γ-tocopherolquinone (3), α-tocopherolquinone (4) and the mass spectrum (lower trace) of α-tocopherolquinone; experimental conditions see 2.3.

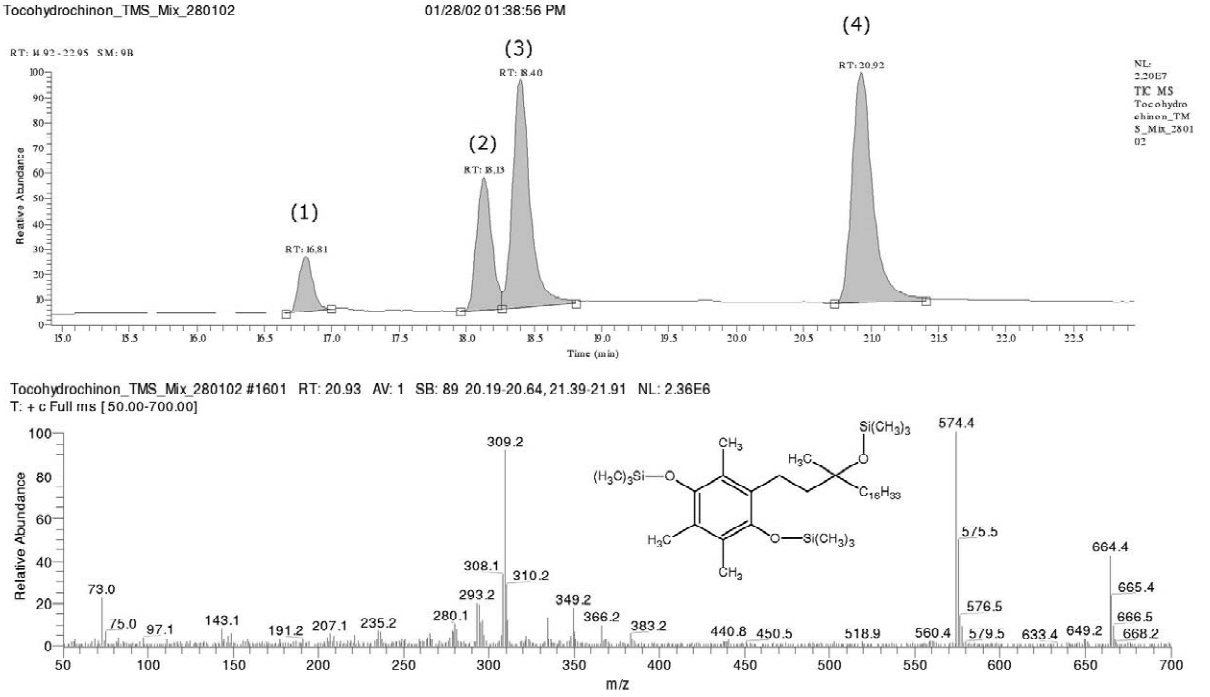

Fig. 3. Segment of the GC–MS chromatogram (upper trace) of the TMS derivatives of tocopherolhydroquinones with δ-tocopherolhydroquinone-triTMS (1), β-tocopherolhydroquinone-triTMS (2), γ-tocopherolhydroquinone-triTMS (3), α-tocopherolhydroquinone-triTMS (4) and the mass spectrum (lower trace) of α-tocopherolhydroquinone-triTMS; experimental conditions see 2.3.

Table 2
MS fragments suitable for identification or quantification for tocopherols, tocoquinones and tocohydroquinones as free substances and TMS derivatives

Substance	Key fragments (m/z)
α-Tocopherol	165, 205, 430
β-+γ-Tocopherol	151, 191, 416
δ-Tocopherol	137, 177, 402
α-Tocopherol-TMS	236, 237, 277, 502
β-+γ-Tocopherol-TMS	222, 223, 263, 488
δ-Tocopherol-TMS	208, 249, 474
α-Tocopherolquinone	178, 221, 428, 446
β-+γ-Tocopherolquinone	164, 189, 414, 432
δ-Tocopherolquinone	150, 175, 418
α-Tocopherolquinone-TMS	263, 293, 341, 503
β-Tocopherolquinone-TMS	249, 279, 341, 489
γ-Tocopherolquinone-TMS	251, 279, 341, 489
δ-Tocopherolquinone-TMS	175, 265, 341
α-Tocopherolhydroquinone-triTMS	309, 574, 664
β-+γ-Tocopherolhydroquinone-triTMS	560, 650
δ-Tocopherolhydroquinone-triTMS	546, 636

that can be used for GC–MS identification and quantification are shown in Table 2.

4. Conclusions

Lipophilic gel chromatography on Sephadex LH-20 is able to help in preseparation steps of tocopherols and their oxidation products in complex synthesis mixtures or in physiological matrices. Of special value is the ability of Sephadex LH-20 to separate substances not only according to their molecular mass but also in dependence of OH groups with phenolic properties. The usage of Sephadex LH-20 for separation covers a variety of substances like antioxidants, lipophilic vitamin and substances in pollution or in drug research [13–15]. This preseparation step can help to get better interpretable

mass spectra and eliminates disturbing background signals which often obscure spectral interpretations. Additionally the problems due to dirty ion volumes in ion trap systems can be diminished and the time the GC–MS system is working properly can be significantly enhanced.

The application of the above described analytical techniques for the detection of tocopherol oxidation products in human serum samples is currently developed and recovery data for the tocoquinones from serum will be communicated in a following publication.

References

[1] Rote Liste, Bundesverband der pharmazeutischen Industrie, Editio Cantor Verlag, Aulendorf (Württ.), Germany, 2001.
[2] H.-U. Melchert, E. Pabel, J. Chromatogr. A 896 (2000) 209.
[3] H.-U. Melchert, B. Görsch, H. Hoffmeister. Nichtstationäre Arzneimittelanwendung und subjektive Arzneimittelverträglichkeit in der bundesdeutschen Wohnbevölkerung der 25-bis 69jährigen. RKI-Schrift 1/95, MMV Medizin Verlag, München 1995.
[4] H. Knopf, H.-U. Melchert, Bundesgesundheitsblatt 41 (No. 11) (1998) 505.
[5] Q. Jiang, S. Christen, M.K. Shigenaga, B.N. Ames, Am. J. Clin. Nutr. 74 (2001) 714.
[6] H.-U. Melchert, H. Knopf, E. Pabel, M. Braemer-Hauth, Y. Du, Int. J. Clin. Pharmacol. Therap. 39 (2001) 488.
[7] L. Gille, K. Staniek, H. Nohl, Free Rad. Biol. Med. 30 (2001) 865.
[8] A.J. Ham, D.C. Liebler, Arch. Biochem. Biophys. 339 (1997) 157.
[9] G.T. Vatassery, Biochim. Biophys. Acta 926 (1987) 160.
[10] G.T. Vatassery, Lipids 24 (1989) 299.
[11] P.P. Nair, I. Sarlos, J. Machiz, Arch. Biochem. Biophys. 114 (1966) 488.
[12] D.C. Liebler, J.A. Burr, L. Philips, A.J. Ham, Anal. Biochem. 236 (1996) 27.
[13] H.-U. Melchert, Chem. Mikrobiol. Technol. Lebensm. 2 (1973) 94.
[14] H.-U. Melchert, Deutsche Lebensmittel Rundschau 71 (1975) 400.
[15] H. Henke, Preparative Gel Chromatography on Sephadex LH-20, Hüthig, Heidelberg, 1994.
[16] http://www.thermo.com (Application Note AN 9158)