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Short communication

Quantitative determination of α-, β-, γ- and δ-tocopherols in human serum by high-performance liquid chromatography and gas chromatography–mass spectrometry as trimethylsilyl derivatives with a two-step sample preparation

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Abstract

Using a two-step sample preparation with Extrelut and silica gel extraction in Pasteur pipettes it is possible to quantify all tocopherols in human serum samples by means of normal-phase HPLC with fluorescence detection (λ_{ex} 295 nm, λ_{em} 330 nm) or by GC–MS of their trimethylsilyl (TMS) derivatives. The method has been used in pharmacoepidemiological studies concerning the exposition with vitamin E-containing drugs in Germany. The recovery for all tocopherols is 98% and the limit of detection is 50 pg for α-tocopherol in the HPLC and 40 pg for all TMS-tocopherols in the GC–MS method using the selected ion monitoring mode with a well-tuned GCQ system. Linearity of calibration is excellent for both methods over the full physiological relevant range. Due to the low sample amount needed, the method is suitable for epidemiological and paediatric research.

Keywords: Sample preparation; Tocopherols; Vitamins

1. Introduction

The reliable quantitative determination of all tocopherols in human serum becomes more and more a prerequisite in research concerning their physiological effects in clinical and epidemiological studies. Especially the widespread use of natural, semi-synthetic or purely synthetic α-tocopherol in vitamin or multi-vitamin preparations as well as in foods, animal feed, common goods (e.g. body deodorants, cosmetics or even toilet paper) or in drugs (e.g. contraceptives, analgesics, dermatological and proctological products) due to its antioxidant capacity calls for valid methodology in quantitative determination of the different tocopherols.

Due to massive advertising and the presumed efficiency of α-tocopherol use in cardiovascular diseases or cancer prevention a widespread use of tocopherols and multi-vitamin preparations by the study participants of our pharmacoepidemiological studies during the German Health Surveys could be observed [1,2]. So we decided to measure the distribution of all tocopherols in serum samples of drug users and control persons.

2. Study population

During national and regional health surveys done from 1984–1999 in Germany, consumption data for

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all drugs used by study participants (ca. 25 000 persons) during the last seven days before the examination were collected by use of a standardized drug-usage questionnaire. The groups examined are representative for the national or regional German resident population aged 25–69 years [1]. In all serum samples of tocopherol-drug users and in control groups of persons with no drug usage the concentrations of α-, β-, γ- and δ-tocopherols were measured by the procedures shown below.

3. Methods

3.1. Chemicals

If not otherwise specified, all used chemicals (analytical grade), reference tocopherols (Article No. 15496) and HPLC-solvents were from Merck, Darmstadt, Germany.

3.2. Sample preparation

Extraction of 100 μl serum is performed on Pasteur pipettes filled (filling height 4 cm) with Extrelut (Merck) with 5 ml *n*-hexane 5 min after denaturation of the protein–lipid bonds with 200 μl methanol. This yields a lipid extract containing the total lipids of the serum sample with the exception of some extremely polar lipid classes like phospholipids [3,4]. After evaporation of *n*-hexane this lipid extract is then further purified by chromatography on silica gel (Kieselgel 60 F_{254}, Article No. 10757.1000; Merck) minicolumns made from Pasteur pipettes (filling height 4 cm) by applying all of the formerly mentioned lipid extract with 2×100 μl of *n*-hexane onto the column. After waiting 4 min the column is eluted using 4 ml *n*-hexane–diethyl ether (5:1, v/v) as eluent. This last extract is evaporated to dryness and the remainder can be dissolved in 100 μl of HPLC mobile phase for normal-phase HPLC determination or it can be used for trimethylsilyl(TMS) derivatization of the different tocopherols for GC–MS. Derivatization is done by heating the extract with 100 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey & Nagel, Düren, Germany) for 20 min at 60°C. All evaporations are done with a Vortex-Evaporator (Buchler Instruments, Labconco, Kansas City, MO, USA)

3.3. HPLC conditions

HPLC conditions are as follows: HPLC system HP 1090 (Hewlett-Packard, Waldbronn, Germany); column: LiChrosorb Si 60 (Merck) 7 μm (250 mm×4.6 mm); mobile phase: isooctane–isopropanol (99.5:0.5; v/v); eluent flow: 1.25 ml/min; fluorescence detector: HP 1046A (Hewlett-Packard), λ_{ex}=295 nm, λ_{em}=330 nm; injection volume: 20 μl.

3.4. GC–MS conditions

GC–MS conditions are as follows: GCQ-System (ThermoQuest, Egelsbach, Germany); GC conditions: column: 30 m×0.25 mm I.D., d_f=0.25 μm Rtx-5 ms (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: 280°C; He flow: 30 cm/s. constant velocity; MS conditions: source temperature: 170°C; 0.5 s/scan; multiplier: 1400 V; acquisition start time: 3 min. For quantification of the different tocopherols full-scan, selected ion monitoring (SIM) or MS–MS mode can be used. The ions that can be used for identification and quantification are as follows: for TMS-α-tocopherol: *m/z* 236, *m/z* 237, *m/z* 277 and *m/z* 502; for TMS-β-and γ-tocopherols: *m/z* 222, *m/z* 223, *m/z* 263 and *m/z* 488; and for TMS-δ-tocopherol: *m/z* 208, *m/z* 249 and *m/z* 474.

4. Results and discussion

Fig. 1 shows the HPLC chromatograms of a standard mixture of all tocopherols and Fig. 2 shows a chromatogram of a serum extract. The recovery rate for all tocopherols measured by this method is 98% and the day-to-day precision is ±5% for amounts of ≥1 mg tocopherol/l serum. The limit of detection for all tocopherols is 50 pg. The linearity of the HPLC method was checked and found to be excellent for the physiological relevant range with sample-loop injections of 0.2 ng/20 μl up to 500 ng/20 μl for all tocopherols.

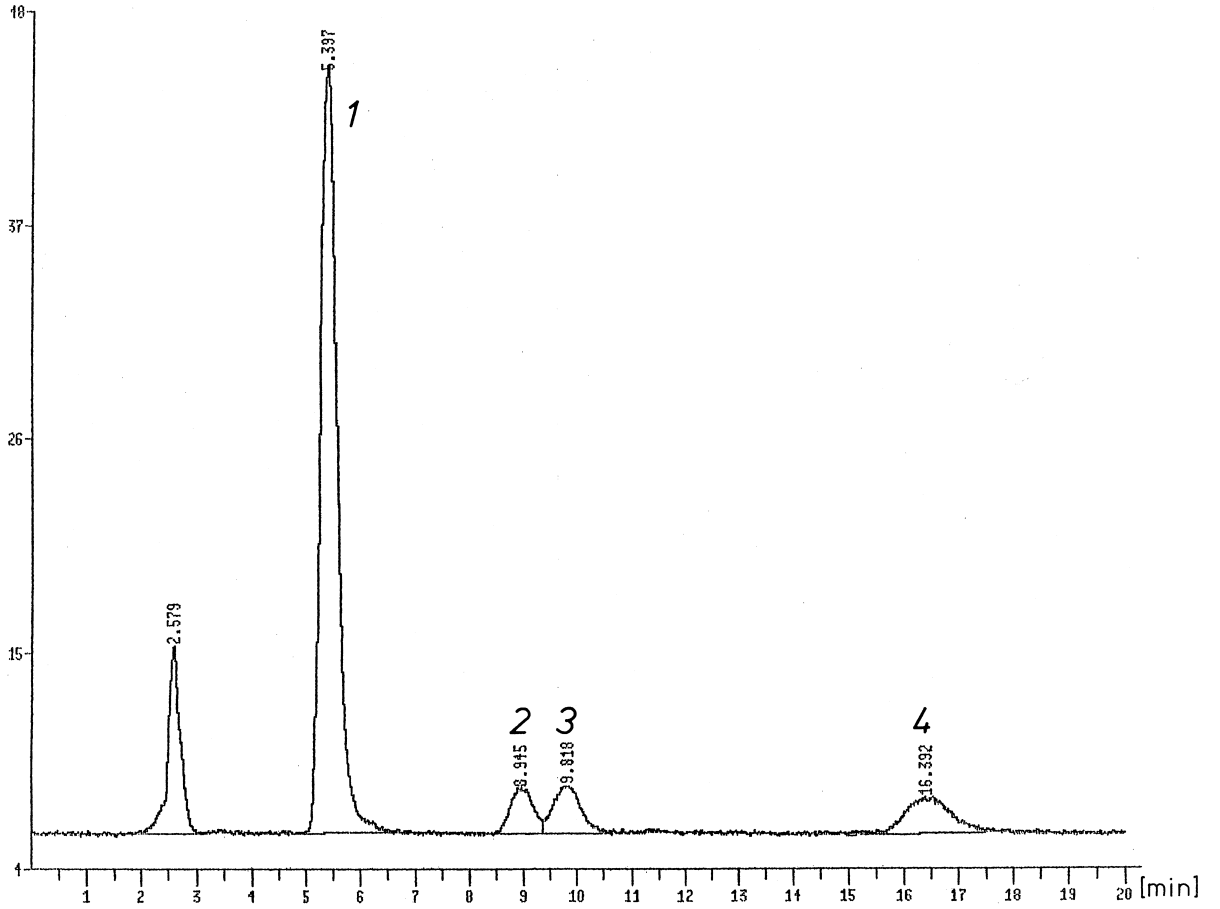

Fig. 1. HPLC chromatogram of a reference-mixture of 170 ng α-tocopherol (1), 13 ng β-tocopherol (2), 15 ng γ-tocopherol (3) and 14 ng δ-tocopherol (4).

Fig. 3 shows a segment of a GC separation of a mixture of the TMS derivatives of δ-, γ-, and α-tocopherol run in full-scan-mode with inserted mass-spectra of TMS-δ- and TMS-γ-tocopherol. The limit of detection for all TMS-tocopherol derivatives we could observe for the above mentioned GC–MS conditions is 40 pg in the SIM mode and the day-to-day precision is ±5% for amounts of ≥1 mg tocopherol/l serum and ±10% for amounts of ≤1 mg tocopherol/l serum.The linearity of the GC–MS method was checked and found to be excellent for the physiological relevant range with injections of 0.05 ng/μl up to 100.00 ng/μl for all tocopherols. Optimal tuning of the GCQ system is essential for measurement of serum samples. Fig. 4 shows the GC–MS separation of the TMS derivatives of α-, β-, γ- and δ-tocopherols of a tocopherol-rich human serum sample containing all four tocopherols. The fact that δ-tocopherol is seen may depend on high consumption of soy-based foodstuff because soy-beans contain high amounts of δ-tocopherol. If it is necessary to separate TMS-β- and γ-tocopherol derivatives totally in using the full-scan mode, another more polar GC column should be used because the Rtx-5ms column separates these sub-stances only partially.

Even in no-drug users a steady increase of the α-tocopherol concentration over the years could be observed in Germany. The mean serum concen-tration rose from 7.50±2.59 mg/l in 1985 to 12.54±3.24 mg/l in 1995. In vitamin-E drug users the mean serum concentration rose from 11.92±4.28

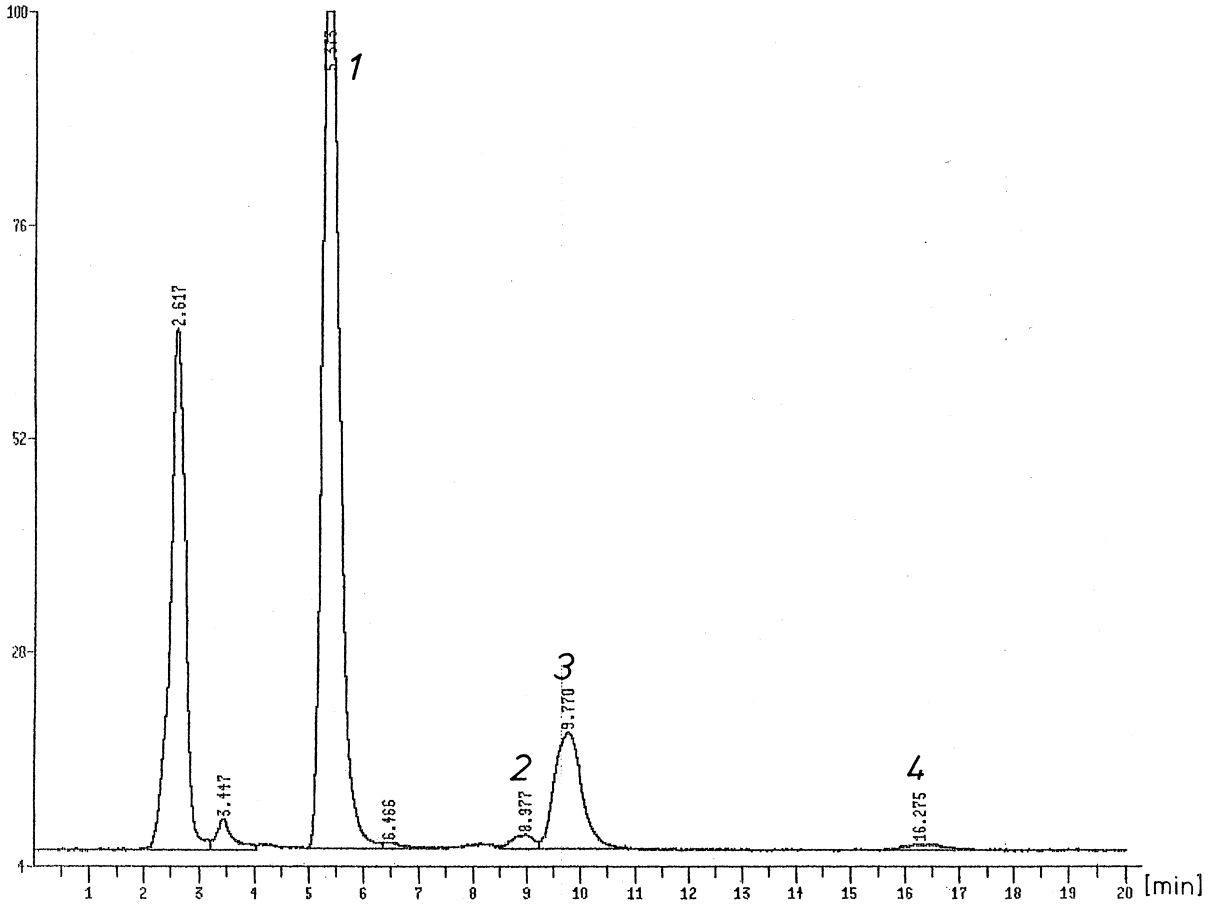

Fig. 2. HPLC chromatogram of the tocopherols extracted from a human serum sample; 448.5 ng α-tocopherol (1), 8.2 ng β-tocopherol (2), 79.8 ng γ-tocopherol (3) and 5.1 ng δ-tocopherol (4), injected sample volume 20 μl.

mg/l in 1985 to 15.83±4.88 mg/l in 1995. In the eastern part of Germany (the former GDR) the mean serum concentration of α-tocopherol in 1992 was found to be 7.57±3.20 mg/l for no-drug-users and 8.22±3.43 mg/l in users of vitamin E-containing drugs. Those study participants consuming α-tocopherol-containing drugs had clearly reduced levels of β- and γ-tocopherols in their serum samples. It could be shown that this effect was dose-dependent. δ-Tocopherol could be found only in 1% of all serum samples examined, its concentration reached levels of 0.01–0.50 mg/l and seemed also to be diminished by consumption of α-tocopherol [5]. Measurements of serum samples from the latest Federal Health Survey 1998–1999 are in progress and the results will be presented [6].

5. Conclusions

Normal-phase HPLC using LiChrosorb Si 60 as well as GC–MS measurements with adequate sample preparation are appropriate tools for the quantitative determination of all different tocopherols in serum samples. The described HPLC method easily achieves the separation of all tocopherols. This is in contrast to RP-HPLC described in other publications [7,8] where β- and γ-tocopherols are not separated. The two-step prepurification yields extracts which are largely free of polar serum-components. By this sample pre-treatment, the life of the HPLC column is extended and the necessity of frequent calibrations due to changing the column can be minimized. So the analysis of >1000 serum samples with one

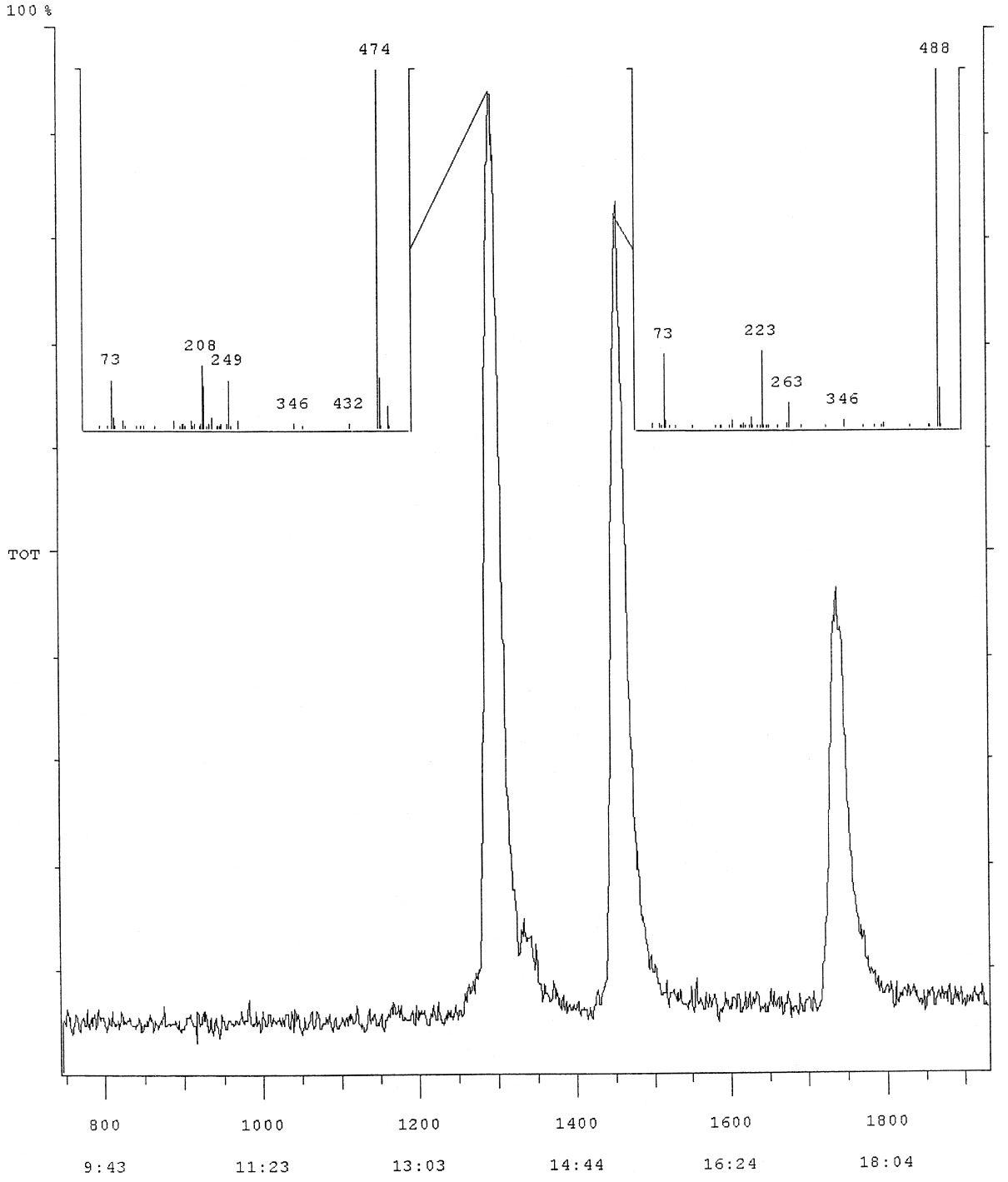

Fig. 3. Segment of the GC–MS chromatogram (total ion current trace) of a standard-mixture of δ-, γ-, and α-tocopherols as TMS derivatives with inserted mass spectra of TMS-δ-tocopherol (first eluting peak) and TMS-γ-tocopherol (second eluting peak) derivatives. Absolute amount for each substance 70 ng.

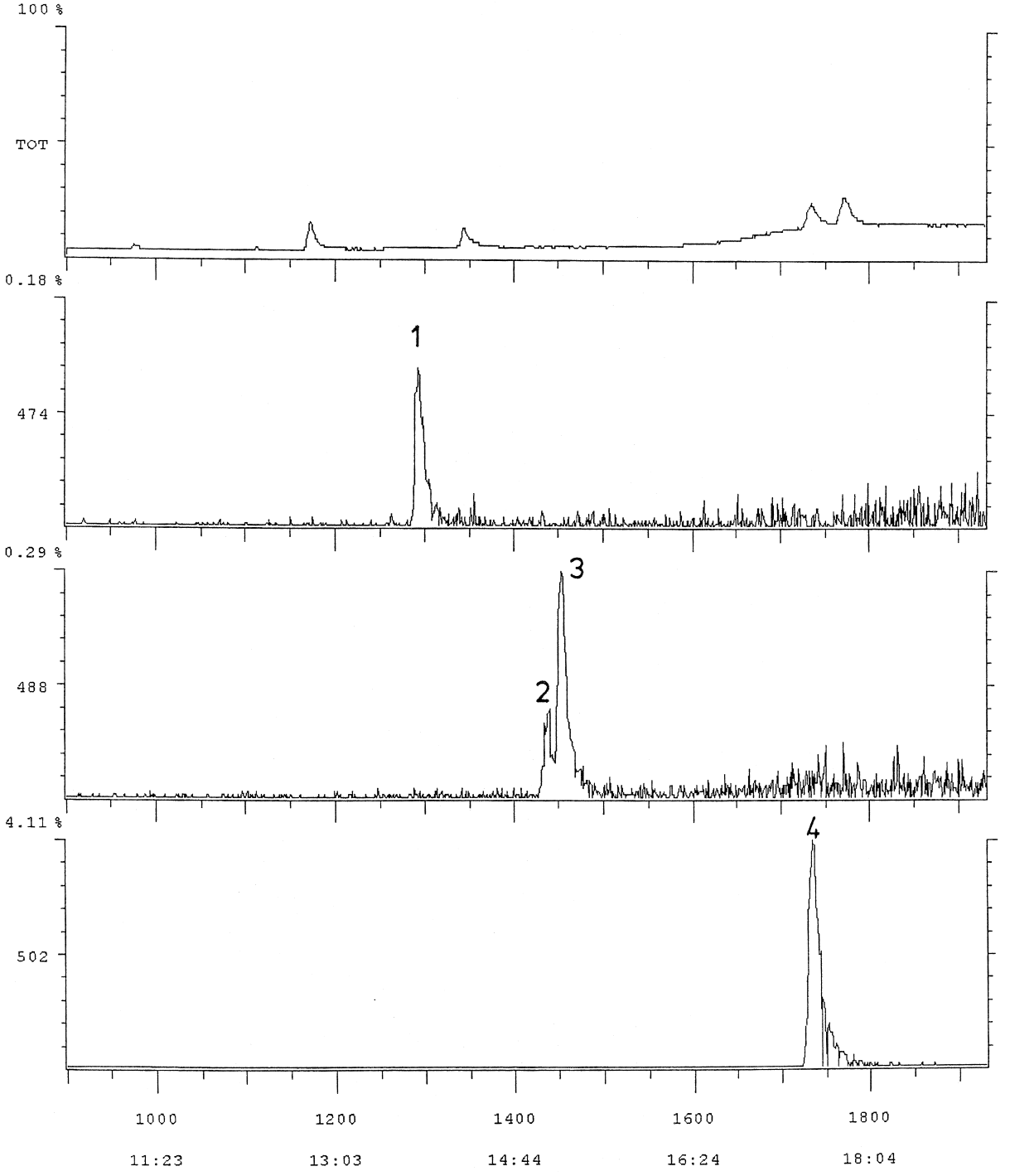

Fig. 4. Segment of the GC–MS chromatogram of the tocopherol TMS derivatives after derivatization of an extract from a tocopherol-rich human serum sample containing 0.7 mg δ-tocopherol (1), 0.1 mg β-tocopherol (2), 1.2 mg γ-tocopherol (3) and 20.1 mg α-tocopherol (4) per litre serum. As can be seen in the 488 m/z trace, the Rtx-5ms column is not able to separate the TMS derivatives of β- and γ-tocopherols completely.

column is possible. The HPLC measurements with fluorescence detection could be used advantageously in paediatric diagnostics where the available amount of blood or serum mostly is small. GC–MS method should be considered when fluorescence measurement in HPLC is disturbed by interfering constituents in serum samples or when the high specificity of the MS measurement must be used for badly prepurifiable samples. Owing to the emerging evidence of the potential physiological importance [9–12] of a balanced presence of all the different tocopherols, use of natural tocopherol mixtures as vitamin supplement should be considered when such medication becomes necessary.

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