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Methods for efficient analysis of tocopherols, tocotrienols and their metabolites in animal samples with HPLC-EC

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ABSTRACT

Tocopherols and tocotrienols, collectively known as vitamin E, have received a great deal of attention because of their interesting biological activities. In the present study, we reexamined and improved previous methods of sample preparation and the conditions of high-performance liquid chromatography for more accurate quantification of tocopherols, tocotrienols and their major chain-degradation metabolites. For the analysis of serum tocopherols/tocotrienols, we reconfirmed our method of mixing serum with ethanol followed by hexane extraction. For the analysis of tissue samples, we improved our methods by extracting tocopherols/tocotrienols directly from tissue homogenate with hexane. For the analysis of total amounts (conjugated and unconjugated forms) of side-chain degradation metabolites, the samples need to be deconjugated by incubating with β-glucuronidase and sulfatase; serum samples can be directly used for the incubation, whereas for tissue homogenates a pre-deproteination step is needed. The present methods are sensitive, convenient and are suitable for the determination of different forms of vitamin E and their metabolites in animal and human studies. Results from the analysis of serum, liver, kidney, lung and urine samples from mice that had been treated with mixtures of tocotrienols and tocopherols are presented as examples.

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Abbreviations: α-T, β-T, γ-T and δ-T, α-, β-, γ- and δ-tocopherol, respectively; CEHC, carboxyethyl hydroxychroman; CMBHC, carboxymethylbutyl hydroxychroman; CMHHC, carboxymethylhexyl hydroxychroman; CDMOHC, carboxydimethyloctyl hydroxychroman; -CEHenHC, -carboxymethylhexenyl hydroxychroman; CDMDHC, carboxydimethyldecyl hydroxychroman; HTMTdHC, hydroxytrimethyltridecyl hydroxychroman; CTMDodHC, carboxytrimethyldodecyl hydroxychroman; HPLC, high-performance liquid chromatography; CEAS, Coulochem Electrode Array System; MS, mass spectroscopy.

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1. Introduction

Tocopherols and tocotrienols are synthesized by plants to serve as free radical scavengers (i.e., chain-breaking antioxidants) and are an important group of dietary antioxidants, known as vitamin E [1]. These two groups of compounds have the common structural feature of containing a chromanol ring and a hydrophobic side-chain of 16 carbons. Depending on the pattern of methylation of the chromanol ring, these compounds exist as α-, β-, γ- and δ-tocopherols (α-, β-, γ- and δ-T) each with a saturated side chain, or as α-, β-, γ- and δ-tocotrienols (α-, β-, γ- and δ-T3) each with a side chain containing three double bonds in the side chain. Tocopherols are by far the most important vitamin E forms in terms of abundance in the human diet, tissue levels and antioxidant activities. α-T is the major form of vitamin E found in blood and tissues and is commonly considered as "the vitamin E", even though γ-T is the most abundant tocopherol form found in the major sources of tocopherols in our diet – oils from corn, soybean, canola and other vegetables and nuts [1,2].

All vitamin E forms are absorbed together with other types of lipids as chylomicrons into the lymphatic system and then taken up by the liver. In the liver, α-T is preferably transferred via α-tocopherol transfer protein (α-TTP) to very low density lipoproteins, which enters into blood and then nonhepatic tissues. γ-T and δ-T are not efficiently transported to blood because α-TTP has a low affinity for γ-T and an even lower affinity for δ-T. Tocotrienols, especially γ-T3 and δ-T3 are also not efficiently transferred from the liver to the blood. As a consequence, most of the absorbed γ-T, δ-T, tocotrienols and some of α-T are metabolized via a side-chain degradation pathway. The degradation is initiated with ω-oxidation followed by cycles of β-oxidation of the side chain, to generate a series of carboxychromanol metabolites, such as the terminal metabolites carboxyethyl hydroxychromons (CEHCs) and their precursors, carboxymethylbutyl hydroxychromons (CMBHCs) [1–7]. Using γ-T and γ-T3 as examples, the structures of these compounds and their common metabolic pathways are shown in Fig. 1. Although tocotrienols have three double bonds in the side chain, they can be metabolized to produce the same common short-chain metabolites as tocopherols. The conversion to CMBHC from its precursor, γ-carboxymethylhexenyl hydroxychroman (CMHenHC), has been shown to be catalyzed by the auxiliary enzymes 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase [8].

Metabolites of tocopherols and tocotrienols can undergo glucuronidation and sulfation; both free and conjugated forms have been found in tissues and body fluids [6–13]. For example, the sulfate- and glucuronide-conjugates of γ- and δ-tocopherol side-chain degradation metabolites were found at high concentrations in the plasma and tissues of rats fed diet containing γ-T or γ-T3 [6,9]. It has been suggested that the urinary excretion of tocopherol side-chain degradation metabolites in individuals can be an indicator of adequate or excess tocopherol intake [14]. Recent results have demonstrated the anti-inflammatory activities of long-chain metabolites of γ-T [2]. Blood and tissue levels of different forms of vitamin E and their metabolites are essential data for understanding the biological fates and activities of these compounds. This is especially true for the γ- and δ-forms of tocopherols and tocotrienols, which have recently received a great deal of attention for their beneficial health effects [15–24]. Therefore, it is important to establish sensitive and convenient methods for quantifying different forms of tocopherols, tocotrienols and their metabolites in tissues and body fluids of animals and humans.

Previously, we used a sensitive high performance liquid chromatography (HPLC) method, coupled with a Coulochem Electrode Array System (CEAS) or mass spectrometry (MS), to measure different forms of vitamin E and their metabolites in mouse and human samples and identified 18 tocopherol-derived and 24 tocotrienol-derived side-chain degradation metabolites in mouse fecal samples [25]. Short-chain degradation metabolites, such as γ- and δ-forms of CEHCs and CMBHCs were measured in urine, serum and liver samples after mild enzyme hydrolysis of the conjugated metabolites with glucuronidase and sulfatase [20,22,25]. The reported extraction method and HPLC conditions have the advantage of detecting multiple compounds simultaneously. However, the different polarities of tocopherols/tocotrienols and their metabolites of varied chain lengths resulted in different extraction efficiencies and affected the accuracy of quantification. The different methods of sample preparation before the hydrolysis of the conjugated metabolites also affected the results.

In this study, we examined the different experimental conditions for sample preparation as well as the conditions for HPLC analysis and improved the methods. The improved methods for the analysis of the α-, γ- and δ-forms of tocotrienols, tocopherols and their metabolites are reported herein.

2. Materials and methods

2.1. Materials

All tocopherols were purified to >99.5% purity using a flash chromatography (Teledyne Technologies, CA, USA) from commercial sources: α-T and δ-T from Sigma–Aldrich Co. (St. Louis, MO, USA) and a γ-T-rich mixture of tocopherols (γ-TmT) from Cognis Co. (Kankakee, IL, USA). Standards of α-, γ-, δ-CEHCs and α-CMBHC of >95.0% purity were purchased from Cayman Co. (Ann Arbor, MI, USA). α-, γ- and δ-tocotrienol standards of >95.0% purity were generously provided by Davos Life Science (Synapse, Singapore). Lithium acetate, acetic acid, trifluoroacetic acid and sulfatase (S9626, containing 30 U glucuronidase activity per unit of sulfatase) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Acetonitrile, hexane, ethyl acetate, ethanol and methanol were purchased from VWR (Philadelphia, PA, USA). All aqueous solutions were made with nanopure water. Mobile phases were filtered through 0.22 μm nylon membrane and degassed under vacuum prior to use.

2.2. Animal and dietary treatment

All animal experiments were conducted under the protocol no. 02-027 approved by Rutgers University Institutional

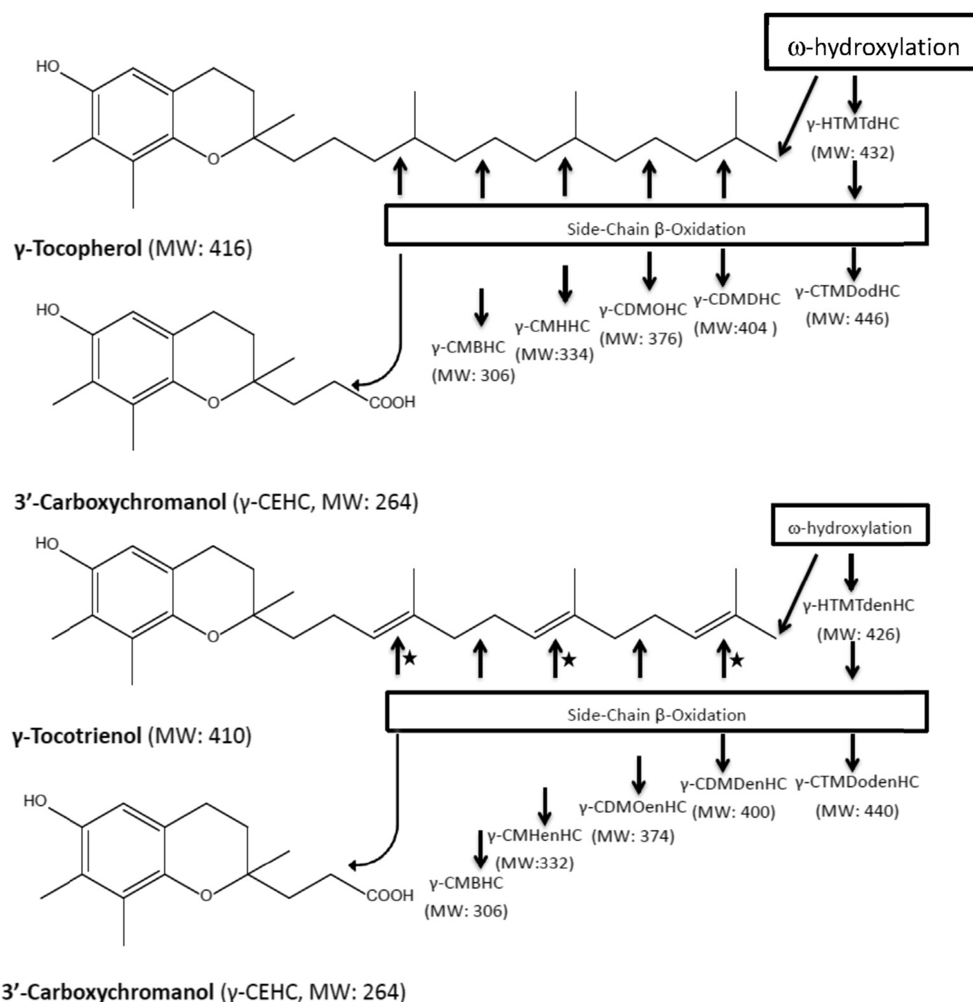

Fig. 1 – Structures of γ-tocopherol and γ-tocotrienol and their side-chain degradation pathways. The side-chain degradation is initiated by ω-oxidation and followed by cycles of β-oxidation, each reducing the chain length by two carbons. The conversion of γ-CMHenHC to CMBHC has been shown to be catalyzed by the presence of auxiliary enzymes 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase [8]. The structures and degradation of other forms of tocopherols and tocotrienols are similar, as the chromanol ring is trimethylated at the 5-, 7- and 8-positions in α-T/T3, and methylated at the 8-position in δ-T/T3.

Animal Care and Use Committee. The animal room was maintained at 20 ± 2 °C, 50 ± 10% humidity and a 12 h light/dark cycle. Animals had free access to food (AIN93M diet) and water. To study tocotrienols, a mixture of tocotrienol (m-T3) solution was prepared by combining 200 mg each of α-, γ- and δ-tocotrienols in 9.4 mL stripped corn oil. The mice used were of C57BL/6J background carrying humanized CYP1A, originally obtained from Jackson laboratories (Bar Harbon, ME, USA), from a breeding colony in our department [26]. This colony was established for our cancer prevention studies using 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) as the carcinogen [20,22–24,27]. We do not think the transgene would affect the disposition of tocopherols and tocotrienols. At 8 weeks of age, 6 mice were given *i.g.* a daily dose of m-T3 solution at 240 mg/kg body weight for 4 day at 0, 24, 48 and 72 h. Urine and blood samples were collected immediately before each dosing at 24, 48 and 72 h, as well as at 3, 7, 27, 51 and 75 h, after the first administration of m-T3 (at 0 h). Urine samples were collected into small beakers by holding the mouse in hand and applying a gentle pressure to induce urination. Blood samples were taken via submandibular puncture at all collection times except for 75 h, which was taken by cardiac puncture. Urine and serum samples were transferred into 2 mL microfuge tubes and immediately frozen on dry ice. All 6 mice were euthanized by ether asphyxiation 75 h after the first administration of m-T3. Liver, kidney, lung and other organs were removed, rinsed in ice-cold saline, patted dry and then frozen on dry ice. All samples were stored at −80 °C until analysis.

In a second experiment, C57BL/6J mice (from Jackson Laboratories) were given 0.3% γ-TmT enriched AIN-93M diet for 18 days. γ-TmT is a γ-T-rich mixture of tocopherols containing (per g) 130 mg α-T, 15 mg β-T, 568 mg γ-T and 243 mg δ-T. Blood, liver and lung samples were collected and stored as described above for the analysis of tocopherols and their metabolites.

2.3. Serum sample preparation procedures

2.3.1. For analysis of tocopherols and tocotrienols

The serum sample (20 μl) was vortex mixed with 80 μl of 0.1% ascorbic acid and 100 μl of ethanol. The mixture was extracted twice with 1 mL of hexane. The combined hexane extract was dried in a Savant Speedvac SC110 centrifugal vacuum concentrator (Thermo Scientific, Waltham, MA, USA). The residue was redissolved in 100 μl of methanol for HPLC analysis.

2.3.2. For simultaneous analysis of tocopherols, tocotrienols and their long-chain metabolites

In this procedure, long-chain metabolites can be analyzed together with tocopherols/tocotrienols. For this analysis, the conjugated metabolites are converted to the unconjugated forms and the results are the sum of the free and conjugated forms. The serum sample (20 μl) was mixed with 80 μl of 0.1% ascorbic acid, 100 μl of 0.1 M sodium acetate (pH 5.0) and 20 μl of a mixture containing sulfatase (20 U) and β-glucuronidase (600 U). After incubation at 37 °C overnight, to convert the conjugated metabolites to free metabolites, the solution was mixed with 200 μl of ethanol and then extracted twice with 1 mL of hexane. The dried hexane extract was redissolved in 100 μl of methanol for HPLC analysis.

2.3.3. For analysis of short-chain metabolites

Serum (20 μl) was mixed with 80 μl of 0.1% ascorbic acid, 100 μl of 0.1 M sodium acetate (pH 5.0) and 20 μl of a mixture of sulfatase (20 U) and β-glucuronidase (600 U). After incubation at 37 °C overnight, samples were extracted twice with 1 mL of ethyl acetate. The dried ethyl acetate extract was re-dissolved in 100 μl of 40% aqueous methanol for HPLC analysis.

2.4. Tissue sample preparation procedures

2.4.1. For analysis of tocopherols and tocotrienols

The tissue sample (50 mg) was homogenized in 200 μl of 0.1% ascorbic acid and 250 μl of ethanol using an Omni bead ruptor homogenizer (Omni Co., Kennesaw, GA, USA). For the analysis of tocopherols and tocotrienols, the homogenate (200 μl) was extracted twice with 1 mL of hexane. The hexane layer was dried and dissolved in 100 μl of methanol for HPLC analysis.

2.4.2. For analysis of tocopherols, tocotrienols and long-chain metabolites

Tissue homogenate (200 μl) as prepared above was vortex mixed with 1.0 mL of methanol. After centrifugation, the supernatant was transferred to a new tube and dried. Then 100 μl of 0.1% ascorbic acid, 100 μl of 0.1 M sodium acetate (pH 5.0) and 20 μl of a mixture containing sulfatase (20 U) and β-glucuronidase (600 U) were added. The mixture was incubated at 37 °C overnight and was analyzed the same way as that for the serum tocopherols, tocotrienols and long-chain metabolites.

2.4.3. For analysis of short-chain metabolites

Tissue homogenate (200 μl) as prepared above was vortex mixed with 1.0 mL of methanol and 0.5 mL of hexane. After centrifugation, the aqueous supernatant (1.0 mL) was dried and redissolved in a mixture of 100 μl of 0.1% ascorbic acid, 100 μl of 0.1 M sodium acetate (pH 5.0), and 20 μl of sulfatase (20 U)- β-glucuronidase (600 U). The solution was then incubated at 37 °C overnight and analyzed the same way as for serum short-chain metabolites.

2.5. Urine sample preparation procedures

For urine (10 μl), 90 μl of 0.1% ascorbic acid and 100 μl of 0.1 M sodium acetate (pH 5.0), and 20 μl of a mixture of sulfatase (20 U) and β-glucuronidase (600 U) were mixed and incubated at 37 °C overnight. Samples were extracted by ethyl acetate and analyzed the same way as serum short-chain metabolites.

2.6. HPLC conditions

Samples were analyzed on an HPLC system consisting of an ESA Model 542 refrigerated autosampler, two ESA Model 582 dual-piston pumps and an ESA 5600A Coulochem Electrode Array System (CEAS). A Supelcosil C18 reversed-phase column (150 mm × 4.6 mm i.d.; particle size, 5 μm) was used. The column and CEAS detector were housed in a temperature-regulated compartment maintained at 35 ± 0.1 °C and the auto-sampler was maintained at 6 °C. System control, data acquisition and analysis were performed with the CEAS software.

2.6.1. For tocopherols and tocotrienols

The mobile phase was an isocratic aqueous solution containing 52% acetonitrile, 39% ethanol and 15 mM of lithium acetate, pH 4.0. The system was run at 1 mL/min for 30 min.

2.6.2. For tocopherols, tocotrienols and long-chain metabolites

The solvent mixtures, Solvent A (28% acetonitrile and 4% methanol) and Solvent B (84% acetonitrile and 13% methanol), were prepared in nanopure water and vacuum filtered before use. Both Solvents A and B containing 15 mM of lithium acetate at pH 4.0. The mobile phase consisted of an initial 40% A and 60% B at a flow rate of 1.0 mL/min. The linear gradient was changed progressively by increasing to 70% B at 5 min, to 75% B at 10 min, to 76% B at 18 min, to 79% B at 32 min and to 100% B at 33 min. The mobile phase of 100% B was run at 1.25 mL/min until 55 min and then switched to 60% B and a flow rate of 1.0 mL/min, in preparation for the next run.

2.6.3. For short-chain metabolites

The mobile phase consisted of an initial 91% A and 9% B at a flow rate of 0.8 mL/min. The linear gradient was changed progressively by increasing to 45% B at 22 min and to 79% B at 26 min. Afterwards, the mobile phase was rapidly switched to 100% B and run at a flow rate of 1.25 mL/min until 36 min and then switched to 9% B and a flow rate of 0.8 mL/min, in preparation for the next run.

2.7. Identification and quantification

A standard serum solution with predetermined quantities of different forms of tocopherols, tocotrienols and their metabolites were processed in parallel with the samples and run for

every 6 samples on HPLC to serve as a standard and quality control sample for the HPLC. The eluent was monitored using the CEAS with potential settings at 100, 200, 300 and 400 mV. The elution time and identity of these compounds were established in our previous work using HPLC-MS [25]. The analyte was identified by the elution time (the ratios of the peak heights among the four voltage channels were used for confirmation, if needed) and quantified by comparing the peak height with that of the serum standard.

2.8. Statistical analyses

Statistical analyses were conducted using GraphPad Prism software. One-way ANOVA with Turkey's post hoc test was

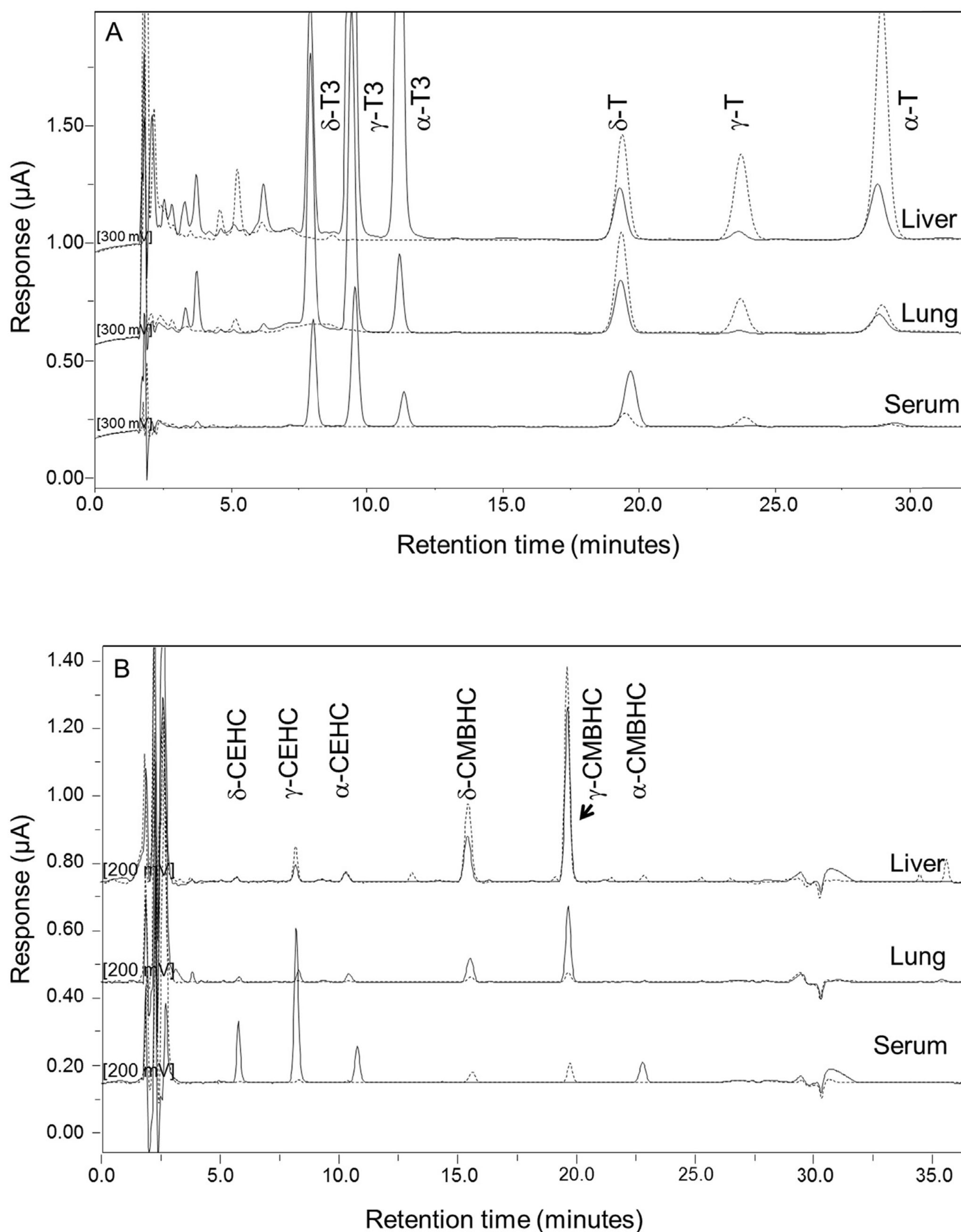

Fig. 2 – HPLC chromatograms of tocopherols and tocotrienols (A) and CEHCs and CMBHCs (B) in mouse serum, lung, and liver. All samples were collected at 3 h after the fourth oral dose of tocotrienol mixture. Solid lines, samples from mice treated with 4 daily doses of m-T3; dotted lines, samples from mice treated with γ-TmT-enriched diet for 18 days.

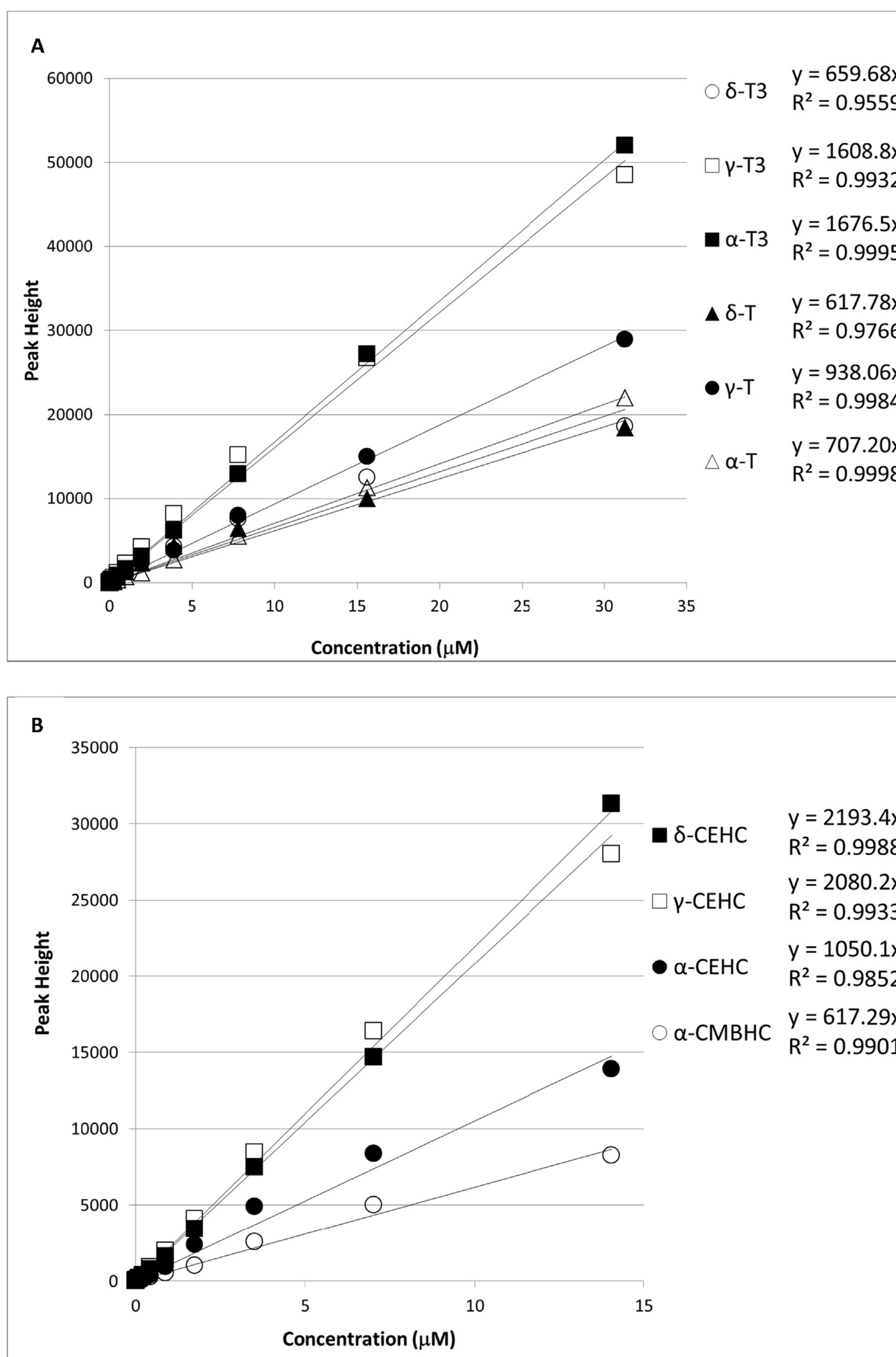

Fig. 3 – Calibration curves for tocopherols and tocotrienols (A) and CEHCs and CMBHCs (B). The injection volume was 50 μl. Each data point represents the average of two measurements.

used for statistical analysis. All data are presented as a mean ± SEM. Significance was assigned at $P < 0.05$.

3. Results and discussion

3.1. HPLC analysis

In our previous analysis, dried samples prepared for tocopherols and short-chain metabolites were re-dissolved in 70% methanol before injection onto the HPLC and a run time of 75 min was required for one sample. The advantage of this previous method is that both groups of compounds can be analyzed in one run. The disadvantage is the incomplete solvation of both tocopherols and short-chain metabolites in this solvent, which affects the accuracy of the quantification. To avoid this problem, we now analyze tocopherols/tocotrienols and their short-chain metabolites separately. For the analysis of tocotrienols and tocopherols, samples are dissolved in 100% methanol and analyzed with an isocratic solvent elution system in a 30 min run. For short-chain metabolites, samples were dissolved in 40% aqueous methanol and analyzed with a gradient elution system with a run time of 45 min. Fig. 2A shows a chromatogram of tocopherols and tocotrienols in serum, lung and liver samples, with α-, γ- and δ-forms of tocopherols and tocotrienols clearly separated without interfering peaks. Fig. 2B shows a chromatogram of short-chain metabolites in serum, lung and liver with α-, γ- and δ-CEHCs and CMBHCs clearly separated.

The analytes were identified and quantified by comparing their peak heights with those of the serum standard. The latter were determined by spiking known amounts of α, γ or δ forms of tocopherols, tocotrienols or CEHCs, as well as α-CMBHC to the serum sample and then measuring the increment amounts of these compounds to make a standard curve, which was used to determine the quantities of each analyte in the un-spiked serum sample. These analytes have been identified by HPLC-MS in parallel with HPLC-CEAS in our previous studies [25], and the elution time of a specific analyte did not vary in different samples. Tocopherols and tocotrienols were linear in the range of 1 nM–30 μM and CEHCs and α-CMBHC were linear in the range of 1 nM–14 μM (Fig. 3). The detection limit (a signal to noise ratio of 3:1) for α-, γ- and δ-tocopherols and tocotrienols was 0.2 pmol, and that for α-, γ- and δ-CEHCs and α-CMBHCs was 0.1 pmol. Because we did not have pure standards for γ- and δ-CMBHCs, the corresponding γ- and δ-CEHCs were used as surrogate standards (to obtain estimated values).

Table 1 – Intraday and interday variations of analysis of plasma samples.[a]

	Intraday variations		Interday variations	
	Concentration (μM)	CV[b] (%)	Concentration (μM)	CV (%)
δ-T	0.26 ± 0.11	4.26	0.27 ± 0.02	5.33
γ-T	4.00 ± 0.18	4.52	4.14 ± 0.10	4.06
α-T	20.80 ± 0.96	4.64	21.54 ± 1.16	4.51
δ-T3	10.40 ± 0.31	2.95	10.51 ± 0.18	2.77
γ-T3	9.20 ± 0.15	1.59	9.31 ± 0.36	2.60
α-T3	9.00 ± 0.31	3.41	9.25 ± 0.22	3.17
δ-CEHC	105.20 ± 2.39	2.27	104.70 ± 2.17	2.07
γ-CEHC	478.10 ± 8.99	1.88	481.30 ± 7.03	1.46
α-CEHC	30.70 ± 1.53	4.97	31.70 ± 1.48	4.68
δ-CMBHC	18.00 ± 0.63	3.50	18.20 ± 0.49	2.70
γ-CMBHC	22.50 ± 0.65	2.89	22.60 ± 0.60	2.67
α-CMBHC	4.60 ± 0.17	3.69	4.70 ± 0.13	2.69

[a] The data are expressed as means ± SD and coefficient of variance for the analysis of mouse serum samples in six intraday determinations for 10 days. The averages from each day were used to calculate interday variations.
[b] CV, coefficient of variation.

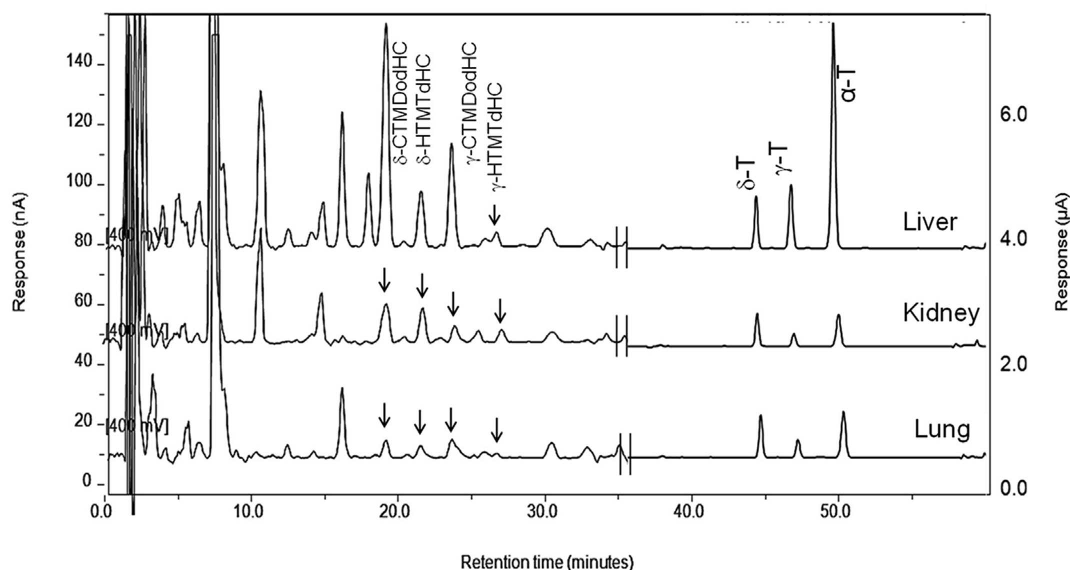

Fig. 4 – Long-chain metabolites determined in mouse tissues together with their parent tocopherols. The tissue samples were from mice treated with a γ-TmT-supplemented diet for 18 days. For the kidney and lung samples, "↓" was added to identify the peaks.

This pre-extraction spiking method also allowed us to determine the "process efficiency", which is the combined effect of extraction efficiency and matrix effect, by comparing the increment peak height with that from direct injection (neat standard). Under the conditions described in "Materials and Methods", the "process efficiencies" of the analytes in our analysis were over 80% for serum, liver, kidney, lung and urine samples. The differences in "process efficiency" of an analyte between serum and other samples were within 10%. Since the analytes were extracted, dried and then dissolved in methanol before injection onto HPLC, the matrix effect of the analysis was low and the "process efficiency" was determined mainly by extraction efficiency (or recovery). The HPLC analysis had good precision. The difference between duplicated runs were below 5%, therefore duplicated samples were not needed for most samples. With proper procedures for sample preparation, the results were highly reproducible. Interday and intraday variabilities (CVs) were below 5%, with the exception of δ-T (interday CV, 5.33%) (Table 1).

Using our present conditions, the column life-time was approximately 1000 injections and the life-time of the detector (CEAS) was approximately one year. With a new detector, the analyte peaks showed mostly in the lower voltage channels (100 and 200 mV) and maximal peak heights of different analytes were observed in different channels. With time of usage, the peaks became more prominent in higher voltage channels (300 and 400 mV). However, such changes are corrected by the standard serum samples run simultaneously with the samples to be analyzed.

By modifying the conditions of Jiang et al. [6,9], we adapted a procedure to measure α-, γ- and δ-tocopherols together with their long-chain metabolites in the same HPLC run. The procedure of Jiang et al. [6,9] measured mainly conjugated long-chain tocopherol metabolites. We modified the ethyl acetate extraction procedure to an ethanol/hexane extraction, which afforded better recovery of long-chain metabolites. A chromatogram of liver, kidney and lung samples from γ-TmT supplemented mice is shown in Fig. 4. The γ- and δ-forms of carboxytrimethyldodecyl hydroxychromon (CTMDodHC) and hydroxytrimethyltridecyl hydroxychroman (HTMTdHC) were clearly separated in a method that also analyzed α-, γ- and δ-T. These peaks were not quantified for lacking of standard compounds. The α-form of these metabolites was not detectable. This method should also be able to measure α-, γ- and δ-tocotrienols when present, eluted between γ-HTMTdHC and δ-T.

Table 2 – Comparison of mouse tissue tocopherol/tocotrienol levels analyzed using tissue homogenates versus tissues supernatants (supernatant values in parenthesis).[a]

δ-T3	γ-T3	α-T3	δ-T	γ-T	α-T
Concentration (μM)					
11.75 (1.17)	45.70 (2.46)	97.94 (2.24)	0.11 (0.17)	1.56 (0.14)	65.95 (1.12)
11.45 (1.54)	59.76 (4.75)	115.93 (3.60)	0.13 (0.09)	2.25 (0.10)	60.88 (1.32)
8.81 (1.03)	35.16 (2.11)	51.37 (1.68)	0.11 (0.09)	1.04 (0.07)	60.88 (1.32)
5.87 (0.53)	27.42 (1.23)	99.94 (2.60)	0.26 (0.09)	3.46 (0.07)	71.02 (1.62)
8.81 (1.09)	47.46 (3.16)	117.93 (3.20)	0.11 (0.10)	2.77 (0.07)	76.10 (1.72)
6.46 (0.73)	22.85 (0.88)	29.98 (0.60)	0.11 (0.00)	1.04 (0.00)	86.24 (1.01)
16.15 (2.23)	22.85 (1.76)	31.98 (1.60)	0.09 (0.00)	0.97 (0.10)	31.45 (0.61)
19.09 (2.13)	28.12 (1.76)	31.98 (1.20)	0.13 (0.00)	1.11 (0.07)	30.44 (0.56)
22.02 (2.35)	39.55 (3.16)	59.96 (3.00)	0.15 (0.01)	1.31 (0.10)	35.51 (0.51)
18.79 (1.91)	42.19 (2.64)	35.98 (2.00)	0.14 (0.01)	3.74 (0.17)	27.40 (0.71)
13.21 (1.91)	19.34 (1.58)	23.99 (1.20)	0.07 (0.01)	0.55 (0.07)	30.44 (0.51)
8.81 (2.03)	10.55 (2.55)	9.99 (2.40)	0.09 (0.01)	0.42 (0.21)	25.37 (1.42)

[a] Mice were treated with 4 daily doses of m-T3 (240 mg/kg), and samples collected at 75 h after the initial dose as described in Materials and Methods. Values for 6 liver samples (upper panel) and 6 lung samples (lower panel) are shown.

Table 3 – Serum tocopherol/tocotrienol metabolite levels when the serum samples were incubated with glucuronidase/sulfatase directly or after precipitation of proteins with methanol.[a]

δ-CEHC	γ-CEHC	α-CEHC	δ-CMBHC	γ-CMBHC	α-CMBHC
Concentration (μM)					
0.41 (0.47)	0.28 (0.23)	0.01 (0.00)	1.85 (2.26)	1.24 (1.24)	0.01 (0.00)
0.70 (1.05)	0.75 (0.80)	0.02 (0.01)	5.35 (6.64)	2.99 (3.28)	0.15 (0.01)
3.40 (3.16)	1.69 (1.31)	0.07 (0.02)	1.60 (1.44)	0.58 (0.40)	0.00 (0.00)
1.64 (1.76)	1.13 (1.13)	0.05 (0.01)	3.50 (3.91)	1.75 (1.75)	0.17 (0.00)
1.70 (1.93)	1.13 (1.08)	0.02 (0.01)	2.57 (2.83)	1.60 (1.60)	0.09 (0.01)
2.34 (2.58)	0.99 (0.94)	0.01 (0.00)	1.34 (1.29)	0.44 (0.29)	0.00 (0.00)

[a] Mice were treated with 4 doses of m-T3 (240 mg/kg) and serum samples were collected at 75 h. The concentrations of 6 serum samples were obtained in a procedure with direct deconjugation are shown versus a procedure with deconjugation after protein precipitation (value in parentheses).

3.2. *Tissue samples preparation: using tissue homogenates vs. supernatants*

For analyzing tissue levels of tocopherol and tocotrienol, we compared the results from using tissue homogenates versus tissue supernatants (Table 2). In both liver and lung samples, the values obtained from analysis of tissue homogenates were much higher than those from the supernatants, and the discrepancy was the largest in the results of α-T and α-T3. It is likely that in the tissue homogenates, most of the tocopherols and tocotrienols are bound to proteins or cell debris and are precipitated upon centrifugation. Therefore, not all the tocopherols/tocotrienols are extracted into the supernatants. This result suggests that in studies when tissue supernatants were used for the analysis, the tissue levels of tocopherols and tocotrienols may have been underestimated.

On the other hand, short-chain metabolites analyzed using tissue supernatants yielded similar results as compared to using the homogenates. When tissue homogenates were used, it required longer times for drying the ethyl acetate

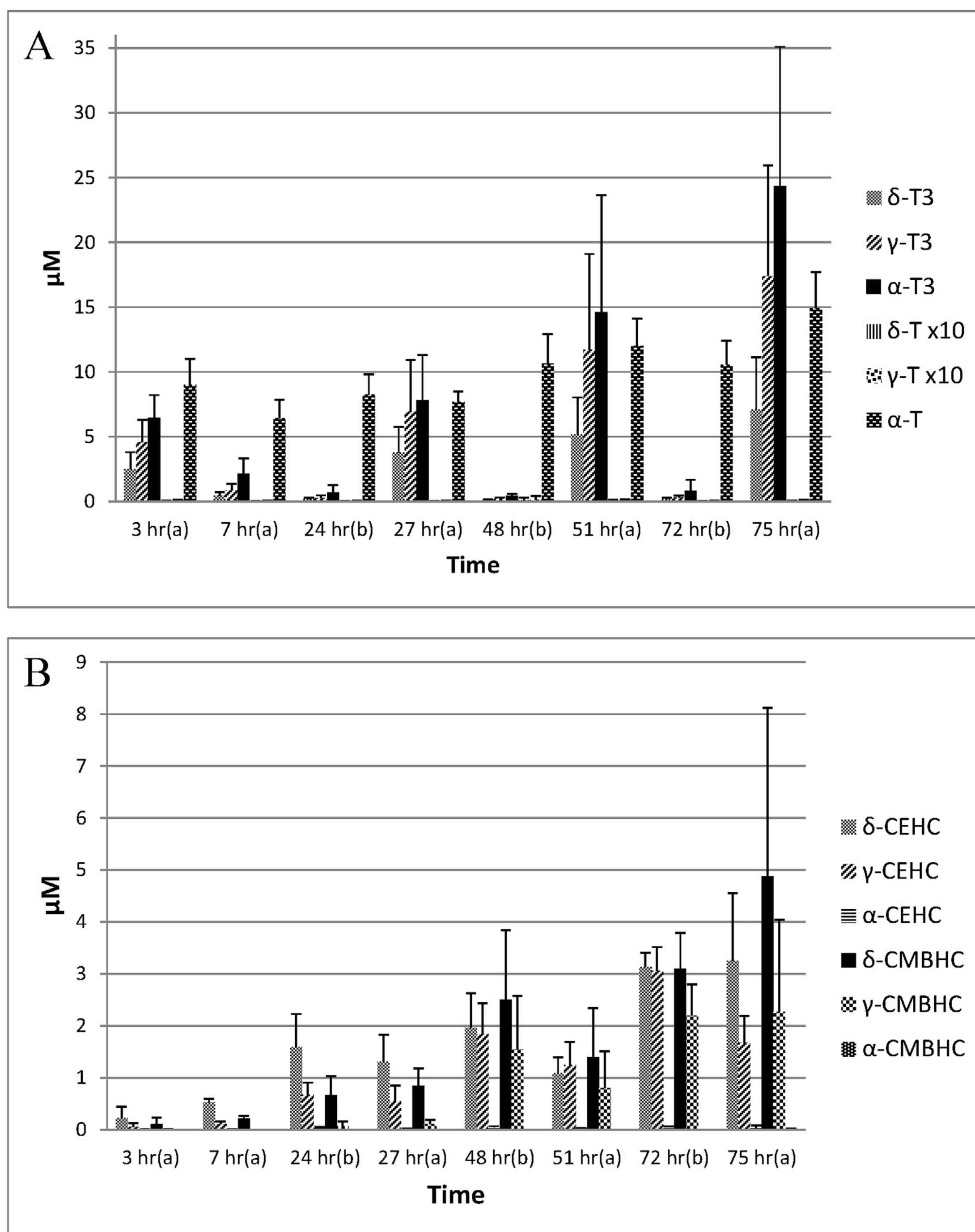

Fig. 5 – Serum levels of tocopherols and tocotrienols (A) and CEHCs and CMBHCs (B): Samples were collected at 3, 7, 24, 27, 48, 51, 72, and 75 h in the experiment with daily oral dose of tocotrienols (m-T3). The data are expressed as mean ± SD (n = 6).

extract and often yielded inconsistent results, possibly due to presence of lipids and proteins, which complicate the sample preparation before HPLC analysis. In our present method, the tissue homogenates are mixed with methanol and hexane, which extract the short-chain metabolites and precipitate proteins and cell debris, before deconjugation with β-glucuronidase/sulfatase. In this procedure, we found that acidification (with 2–20 μl acetic acid) of the deconjugated samples did not increase the efficiency of extracting short-chain metabolites into ethyl acetate. Therefore, this step is not included in the procedure.

For the analysis of lung-chain metabolites, the tissue homogenates (200 μl) were mixed with methanol (100 μl). This allowed the long-chain metabolites and tocopherols/tocotrienols to be in the supernatant upon centrifugation. The supernatant were dried and reconstituted for enzyme digestion to hydrolyze the conjugated metabolites. This study had better recovery as compared to digesting the tissue homogenate directly. This procedure demonstrates the feasibility for analyzing long-chain metabolites together with their parent vitamin E forms. However, standard compounds are needed for their qualification and for checking the recoveries from different tissue samples.

3.3. *Serum sample preparation for analysis of short-chain metabolites: using serum versus deproteinated serum*

We compared the results of two sample preparation procedures: 1) serum samples were incubated with β-glucuronidase/sulfatase directly to deconjugate the conjugated metabolites of tocopherols/tocotrienols and 2) serum samples (20 μl) were deproteinated with methanol (1 mL) before deconjugation. These two methods yielded comparable results (Table 3) and therefore the first method is adapted in our procedure.

3.4. *Time-dependent changes of serum tocotrienols/tocopherols and their metabolite levels after oral supplementation with m-T3*

Mice were treated with four oral doses of m-T3 at 0, 24, 48 and 72 h and serum samples were collected at different time points. As shown in Fig. 5A, rather higher serum concentrations of δ-, γ- and α-T3 were observed at 3 h after the m-T3 dose and the levels decreased at 7 h and 24 h. The levels raised at 3 h after each of the subsequent dose, and the level was higher than that produced by the previous doses. By the fourth dose of m-T3, the increments in α- and γ-T3 from 72 h to 75 h were approximately 3-fold higher than those from 24 to 27 h (caused by the second dose). The reason for this progressive increment is unknown and remains to be investigated. The results suggest that in studies like this, it is important to report the time of blood sample collection in reference to the time of agent administration. When the agent is given in the diet, because rodents eat most at night, the levels of tocopherols/tocotrienols in the morning are expected to be higher than in the afternoon. Overnight fasting will yield even lower levels. At all the time points, α-T3 was presented at the highest concentrations, γ-T3 at lower concentrations and δ-T3 even lower.

Serum concentrations of γ- and δ-CEHCs and CMBHCs increased throughout the four days of the experiment in the lower micromolar range, while α-CEHC and CMBHC levels were very low, indicating that α-T and α-T3 are not extensively metabolized (Fig. 5B). Similarly, urinary γ- and δ-CEHCs and CMBHCs levels were increased gradually in the first 2–3 days, reaching levels of 175 μM for γ-CEHC and 260 μM for γ-CMBHC (Table 4). The corresponding levels of δ-CEHC and -CMBHC were approximately half of these values (Table 4). These urine metabolite levels were 30–100 fold higher than those in the serum.

Table 4 – Urine tocotrienol metabolite levels after four daily oral doses of tocotrienol mixture.[a]

		δ-CEHC	γ-CEHC	α-CEHC	δ-CMBHC	γ-CMBHC	α-CMBHC
		Concentration (μM)					
3 h(a)	Mean	10.4	0.0	8.1	8.0	14.2	0.2
	SD	5.7	0.0	6.2	6.0	12.8	0.4
7 h(a)	Mean	18.8	78.2	8.1	18.3	24.7	1.3
	SD	8.3	55.6	6.0	14.3	21.9	2.9
24 h(b)	Mean	44.4	128.5	15.7	47.9	77.3	2.2
	SD	13.1	33.5	10.0	22.9	50.4	2.6
27 h(a)	Mean	40.2	130.2	18.2	56.9	94.7	5.7
	SD	10.8	30.4	9.1	26.5	69.2	4.5
48 h(b)	Mean	79.4	181.5	19.9	101.3	119.0	4.6
	SD	15.6	34.0	16.7	33.4	94.9	5.3
51 h(a)	Mean	67.1	168.7	15.6	105.5	210.4	8.2
	SD	12.9	32.3	9.1	44.9	129.6	8.3
72 h(b)	Mean	72.6	175.2	17.7	123.4	259.5	10.3
	SD	16.1	46.2	9.5	50.8	118.2	9.3
75 h(a)	Mean	63.7	148.7	13.5	121.2	267.6	11.0
	SD	15.9	29.6	8.2	29.0	107.7	6.2

[a] Mice were treated with 4 doses of γ-T3 (240 mg/kg) at 0, 24, 48 and 72 h and urine samples were collected 3 h after (a) or right before (b) dosing.

4. Concluding remarks

We have refined our HPLC/CEAS method for the determination of tocopherols, tocotrienols and their chain-degradation metabolites. Our procedures for sample preparation, using solvent extraction and centrifugation to precipitate protein during phase separation, are more convenient and economical than some of the solid-phase extraction procedures. Our new HPLC conditions provide efficient and clear separation of analytes with minimal interfering peaks. The CEAS detecting system provides high sensitivity, comparable to MS or florescence detection, but is much more sensitive than UV absorption. A limitation of the CEAS is that it does not provide the structural information that can be provided by MS, and electrochemical detection can only detect redox active forms of vitamin E and their metabolites. Overall, the presently described procedure is a very sensitive, convenient and rather inexpensive method for the routine analysis of tocopherols, tocotrienols and their metabolites of large numbers of samples in laboratory and epidemiological studies.

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