methods

A rapid method for the extraction and determination of vitamin E metabolites in human urine

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Abstract A method for the direct extraction and routine analysis of the vitamin E metabolites γ- and α-carboxyethyl hydroxychroman (γ- and α-CEHC) from human urine has been developed. A relatively small sample volume (5 ml) can be used and, after enzymatic hydrolysis of the conjugated forms and acidification, the metabolites are extracted with diethyl ether. Recovery of α- and γ-CEHC was compared to that of trolox, used as an internal standard, added to 24-h urine collections from vitamin E-unsupplemented volunteers. Various solvent conditions were initially tested; acidification and ether extraction gave the highest recovery. It was found that after addition and extraction from urine, trolox, α- and γ-CEHC are recovered to a similar extent, hence trolox is viable as an internal standard. The samples were analyzed by both GC and HPLC with electrochemical detection (ECD). HPLC-ECD was found to give higher selectivity and higher sensitivity compared to GC or HPLC with UV detection at 290 nm. The HPLC-ECD detection limit was 10 fmol, linearity ($r^2 > 0.98$) was achieved in the range of 40 to 200 fmol, which was found to be optimal for 24-h urines from unsupplemented subjects. Inter-sample variability was typically 2–5%. This greater sensitivity and selectivity means that vitamin E metabolites can be analyzed even in unsupplemented subjects. It is also possible to measure unconjugated forms of the metabolites. Typically these were found to represent ~10% of the total α- and γ-CEHC. This method can be used routinely for the determination of vitamin E metabolites in urine. The new extraction and detection methods described are relatively quick, less laborious, and more cost-effective than previously available methods.—Lodge, J. K., M. G. Traber, A. Elsner, and R. Brigelius-Flohé. **A rapid method for the extraction and determination of vitamin E metabolites in human urine.** ***J. Lipid Res.*** **2000.** 41: **148–154.**

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Vitamin E is a mixture of α-, β-, δ-, and γ- tocopherol and tocotrienols, differing in the methyl substitutions on the chromanoxyl ring, and the saturation of the phytyl tail (1). α-Tocopherol is the most studied because it has the highest biological activity and possesses potent antioxidant activity (2). Although absorption of tocopherol is poorly understood, the release of tocopherol from the liver into the plasma via the lipoproteins is under the control of the hepatic α-tocopherol transfer protein (α-TTP) (3), which selects only naturally occurring *RRR*-α-tocopherol for secretion into the circulation. Plasma α-tocopherol is constantly turned over, the new from the diet replacing the old (4, 5); however, no matter how high the dose of α-tocopherol given, plasma α-tocopherol levels cannot be raised more than 3-fold (5). Hence, there is an upper limit of endogenous α-tocopherol levels which is achievable by supplementation. Thus a regulatory mechanism must exist to what the body can utilize.

Less is known regarding vitamin E metabolism. A number of compounds have been detected in human urine including 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) (6) and 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (discovered as LLU-α (7) or γ-CEHC) which are metabolites of α- and γ-tocopherol, respectively. A similar metabolite of δ-tocopherol has also been found in the urine of rats (8). Interestingly the metabolite of γ-tocopherol (γ-CEHC or LLU-α) was discovered as a natriuretic factor in human urine (7, 9, 10).

These compounds are derived from oxidation of the phytyl side chain so the chromanol structure remains intact. Earlier studies showed metabolites with an open ring system (11, 12). These "Simon" metabolites were thought to be a consequence of metabolic forms of oxidized α-tocopherol (11, 12), as an initial consequence of oxidative stress, but with the discovery of α-CEHC it is likely that the Simon metabolites are a consequence of oxidation during sample preparation and extraction (6). Nevertheless, as α-tocopherol quinone is present in vivo, it is possible that

Abbreviations: CEHC, carboxyethyl hydroxychroman; ECD, electrochemical detection; GC/MS, gas chromatography-mass spectroscopy; TCA, trichloroacetic acid; α-TTP, α-tocopherol transfer protein.

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Simon metabolites are also formed and excreted. Further work is necessary to elucidate this.

The extent of vitamin E metabolism is unknown, although from limited studies so far it appears that α-CEHC in urine comprises less than 5% of administered α-tocopherol (6, 13) while most of γ-tocopherol is metabolized into γ-CEHC and excreted (14). Interestingly, when subjects were given both *RRR*-α-tocopherol and *all-rac*-α-tocopherol, the metabolite of the latter was preferentially metabolized and excreted in the urine (13). This is further evidence that the body selects and retains 2*R*- forms, whilst removing (by metabolism) the other racemic forms (13).

The metabolites of vitamin E may be useful biomarkers for excess vitamin E (6), and may provide an extra mechanism for regulation. This is a relatively new and important area of vitamin E research.

Previous methods to extract and measure these metabolites have involved long and tedious steps (10, 15), with the use of costly equipment such as GC/MS (6, 15) which also involves derivatization and, in some circumstances, the use of deuterated internal standards which are not commercially available (14). We report here simplified methods which use a direct extraction from a few mls of urine and detection by HPLC incorporating electrochemical detection. Such methods are ideal for large numbers of samples and are more routine than GC/MS, and more sensitive than GC with flame ionization detection.

MATERIALS AND MATERIALS

Reagents

α-CEHC and γ-CEHC (LLU-α) were kind gifts from W. J. Wechter. Trolox was obtained from Aldrich, β-glucuronidase (type H-1, contains minimum 300,000 U/g β-glucuronidase activity and minimum 10,000 U/g sulfatase activity) from Sigma, and dl-α-tocopherol acetate from Sanavitan S Swiss Caps, Lohnherstellungs GmgH, Bad Aibling, Germany. Diethyl ether was from Roth GmbH, Karlsruhe, Germany or Mallinckrodt Baker Inc, Paris, KY. Only ethers with low peroxide levels should be used. The GC derivatization compounds BSA (N,O-bis-(trimethylsilyl)-acetamide) was from Aldrich, TMCS (trimethylchlorosilane) was from Merck, and BSTFA (N,O-bis-(trimethylsilyl)-trifluoracetamide) was from Fluka. HPLC solvents were all HPLC grade, and all other chemicals and reagents were highest purity available.

Human subjects protocol

All procedures with human subjects were approved by the institutional review boards of the German Institute for Human Nutrition, UC Davis medical centers, and at Oregon State University. Urine was collected over a 24-h period from either healthy unsupplemented volunteers or from volunteers after supplementation with *all rac*-α-tocopherol (400 mg for 5 days) into large flasks, and frozen immediately. Aliquots were taken when necessary after thawing. No diet restrictions were undertaken.

Extraction procedures

From a 24 h urine collection from normal unsupplemented subjects, 5 ml was taken and the internal standard was added (30 μg trolox final for GC, 10 μg for HPLC-ECD, added in ethanol). The conjugates were then hydrolyzed by the addition of 400 μl enzyme solution (4 mg β-glucuronidase in 450 μl 0.1 m sodium acetate buffer, pH 4.5), and incubation for 4 h at 37°C. After cooling on ice, the samples were acidified by the addition of 50 μl 6 m HCl. Initially, extraction of the metabolites was performed with diethyl ether (4 × 5 ml). Mixing was done by inversion; care was taken not to shake vigorously otherwise foaming could occur. After each step a short centrifuge run (2000 rpm for 1 min) was necessary to separate the layers. This extraction procedure was used for all GC analyses. A one-step ether extraction was later validated, using 15 ml of diethyl ether. In this case the samples could be mixed thoroughly with a vortex mixer for 30 secs. This latter procedure was performed with all HPLC analyses. An aliquot of the top ether fraction was collected and dried down under nitrogen. The following step is dependent upon the analysis technique to be used. For GC, to remove adventitious water, the extract was resuspended twice in 500 μl ethanol and dried under nitrogen. These dried samples were resuspended in 500 μl of derivatization solution (200 μl hexane, 250 μl BSTFA, 40 μl BSA, and 10 μl TMCS) and incubated at 50°C for 2 h with constant shaking. For HPLC, dried samples were resuspended into 200 μl of ethanol and diluted 1:10 with mobile phase or ethanol for analysis.

To measure unconjugated forms of the metabolites, the urine aliquots were acidified (50 μl 6 m HCl, incubated for 5 min on ice) then directly extracted with diethyl ether (1 × 15 ml) as described above. No enzyme solution was added.

Gas chromatography

GC was performed on a Varian STAR 3400 CX (Varian Instruments, Walnut Creek, CA) containing a Varian 8200 CX autosampler and a GC Star CX series integrator/workstation as previously described (15). Briefly, flame ionization detection was performed after separation on a fused silica DB-5MS capillary column (30 m × 0.25 mm i.d. and 0.25 μm thickness; J & W Scientific, Folsom, CA), after a splitless injection. The heating gradient was as follows: initial temperature of 180°C for 2 min, then an increase of 10°/min to 280°C and 280°C for 10 min. The injector temperature was 260°C and the detector temperature was 300°C. Nitrogen was used as a carrier gas. Typically 1 μl of the derivatized sample solution was injected. Retention times for trolox and α-CEHC were 8.3 and 11.4 min, respectively. Quantification was performed by comparing peak areas of the α-CEHC peak to that of trolox.

HPLC

The HPLC system used was either from Kontron Instruments (Neufahrn, Germany) consisting of two 422S pumps, 365 autosampler, 440 diode array detector, and a Waters 465 electrochemical detector, or from Shimadzu (Pleasanton, CA) consisting of a SCL-10A system controller, two LC-10AD pumps, a SPD-10A UV detector, and a SIL-10AD autosampler. The electrochemical detector was from Bioanalytical Systems (West Lafayette, IN). The former system was used at the German Institute for Human Nutrition while the latter was used at the Linus Pauling Institute.

Separation was performed with an Ultrasep ES100, RP 8 norm, 5 μm column (Sepserv, Berlin) with a gradient system of (A) acetonitrile and (B) McIlvaine buffer (0.01 m citric acid, 0.02 diammonium hydrogen phosphate, pH 4.15) both containing 1% lithium perchlorate, as follows: 80% B for 6.5 min, change to 60% B in 1 min, 60% B for 12 min, then change to 5% B in 4 min, 5% B for 7 min, back to 80% B in 1 min, and 80% B for 5 min. The flow rate was 0.4 ml/min. Detection and quantification of the metabolites were carried out with electrochemical detection operating at an applied voltage of 0.55 V, which was found to be optimal (described later in text). Identification was done by comparing peak retention times of standards, and by spiking with known standard compounds. Usual retention times were 19.5, 23.5, and

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26 min for trolox, γ-CEHC, and α-CEHC, respectively. To quantify amounts in samples, areas were compared to that from the internal standard trolox peak. A factor was also included because of the difference in response at the applied potential between α-CEHC, γ-CEHC, and trolox. Thus a number of standards must be analyzed at the same time as the samples (see below).

RESULTS

A new simplified extraction protocol for vitamin E metabolites was devised to decrease sample preparation time, and to make routine analyses from large clinical studies practical. The foundation for this new method was that of Schultz et. al. (15).

Extraction

At first it was necessary to test various solvent and extraction conditions. Urine (5 ml) from a vitamin E-supplemented subject was used and trolox was added (30 μg) as an internal standard (13, 15). Analysis was performed by GC since this method has been previously employed (15). To test for recovery, parallel urine samples underwent extraction under the various conditions. Trolox was added either directly to the urine before extraction (pre) or immediately prior to derivatization (post). This latter protocol yielded a trolox peak that was then taken as 100%, any deviations from this value represents a difference in recovery. **Table 1** shows the various extraction methods tested, the obtained peak areas, and the calculated recovery of trolox for each solvent and condition. The method that yielded optimal recovery of trolox was acidification followed by ether extraction (4 × 5 ml).

A problem with the ether extraction occurred when the samples were mixed too vigorously, then foaming resulted with loss of metabolite recovery. This was overcome by using a 1 × 15 ml ether extraction, a strategy that also has the benefit of reducing sample handling time and effort.

TABLE 1. Recovery of trolox added to human urine from α-tocopherol-supplemented volunteers under varying conditions

Extraction Method	n	Peak Area Trolox	% Recovery	% CV
Acid/hexane	3	4181	16	20
TCA/hexane	1	4370	21	n.d
Ether	3	14874	60	5
TCA/ether	1	16392	44	n.d
Salt/ether	2	27508	73	6
SDS/ether	2	20813	55	17
Acid/ether	6	39088	104	10
Acid/SDS/ether	4	14205	38	16
Acid/ethanol/ether	4	35326	94	1
Published (ref. 15)	4	28926	77	4
Lyoph/ethanol	2	8530	23	n.d
Lyoph/ether	4	5530	15	n.d
Lyoph/ethanol/ether	2	30565	81	n.d

Analysis by GC. Recovery based on peak area (arbitary units) for trolox added either pre- or post-extraction (described in text). Average peak area for trolox added post-extraction (100%) was 37589 ± 2394 (n = 6); acid, 50 μl 6 m HCl; hexane, 4 × 5 ml; TCA, trichloroacetic acid1% final; ether, 4 × 5 ml; salt, 50 mg Na_2SO_4; SDS, 1% final; ethanol, 1 ml; lyoph, lyophilization after acidification.

Therefore this procedure was used in all following extractions. The recovery of trolox was found to be the same with a 4 × 5 ml versus the 1 × 15 ml extraction (described in the next section).

HPLC-ECD

A previous HPLC method for measurement of α-CEHC (6, 15) was modified to utilize electrochemical detection (ECD). ECD has enormous advantages over UV detection in being more sensitive and more selective, and has been used many times in the past for the measurement of tocopherols and quinones (16, 17).

First the optimum applied potential for both α-CEHC, γ-CEHC, and trolox was ascertained. **Figure 1** shows a hydrodynamic voltammogram from one such experiment performed on a urine extract containing added trolox, α-, and γ-CEHC (10 μg). All three compounds gave different response curves, the maximum for α-CEHC was at 0.5 V and that for trolox and γ-CEHC was 0.65 V. A potential of 0.55 V was chosen for these studies as this gave the optimal peak height versus noise in urine extracts. When a higher potential was used, more interfering peaks were present. The maximum response for each compound was also different. Thus a response factor of ~0.9 for γ-CEHC and ~0.7 for α-CEHC (found to be constant over a concentration range) must be taken into account in analysis using the internal standard trolox at 0.55 V. This assessment must be performed on each day of analysis. A similar voltammogram performed with standards dissolved in ethanol showed similar response curves (data not shown).

Typical calibration curves are linear from 40 fmol to 200 fmol (**Fig. 2**), which was found to be optimal for analysis of the metabolites. The detection limit was ~10 fmol, although the ideal range for quantification should be 5-fold higher.

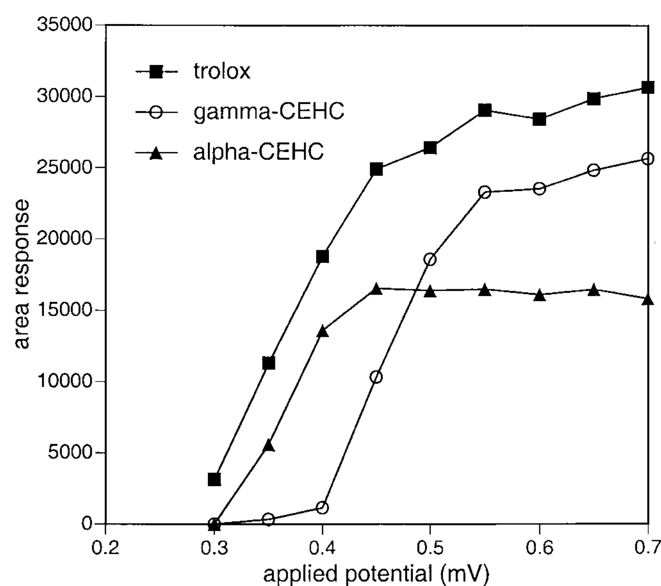

Fig. 1. Hydrodynamic voltammograms for trolox, γ-CEHC, and α-CEHC recovered from urine as described (50 pg injected). The response (function of peak area) for each peak is shown for each applied potential tested. A potential of 0.55V was found to be optimal.

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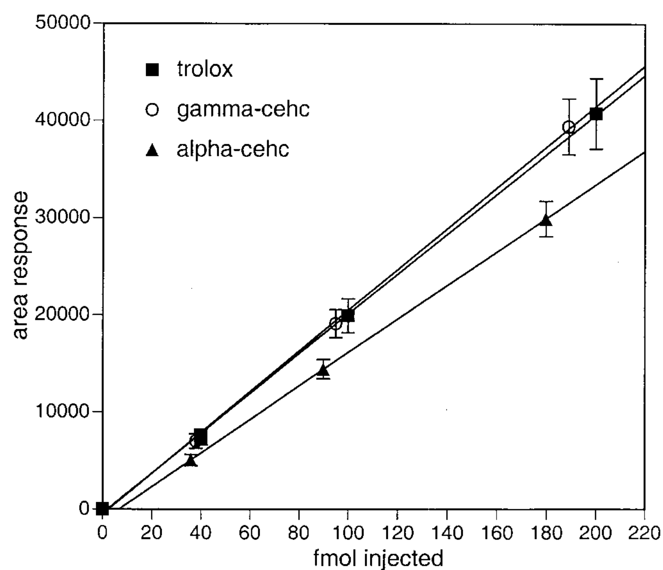

Fig. 2. Standard calibration curves of trolox, γ-CEHC, and α-CEHC showing linearity in the range typically found in human 24-h urine collections. Shown are mean for 6 individual curves recorded separately ($r^2 > 0.98 \pm 0.02$).

To test for recovery of vitamin E metabolites, known amounts of α-CEHC (10, 1, and 0.2 μg) and γ-CEHC (10, 1, and 0.2 μg) were added with trolox (10 μg) to 5-ml aliquots of 24-h urine collections from unsupplemented subjects (four different urines tested), and the samples were extracted as described above and analyzed by HPLC-ECD. Recovery of α- and γ-CEHC was ascertained by comparing peak areas for the standards added either directly to urine and extracted as described (pre), or added to urine extracts immediately prior to analysis (post). Peak areas for the latter represent 100%. Typical recoveries and peak area ratios for trolox, α-CEHC, and γ-CEHC are shown in **Table 2**. Recoveries were typically different from individual urines, ranging from 73 to 100% (n = 2–6). However, within each urine, the recovery of trolox, α- and γ-CEHC were similar. This is better represented by comparing the peak area ratio of α- and γ-CEHC:trolox after pre- and post-addition of the standards. Generally a difference in only ±3% was observed. Thus although recovery is not always 100%, trolox, γ- and α-CEHC are extracted together to a similar degree, hence trolox is viable as an internal standard.

TABLE 2. Recovery of trolox, α- and γ-CEHC from human urine analyzed by HPLC-ECD

Standard Added	% Recovery			α-CEHC: Trolox		γ-CEHC: Trolox	
	Trolox	α-CEHC	γ-CEHC	Pre	Post	Pre	Post
μg							
10	88	89	92	0.75	0.75	1.05	1.00
	80	81	76	0.80	0.81	1.07	1.02
	73	74	84	0.71	0.70	1.19	1.03
	78	81		0.73	0.70		
	91	89		0.64	0.66		
	78	76		0.83	0.82		
1	97	95	93	0.71	0.70	0.96	0.92
	105	104	101	0.83	0.82	1.18	1.13
0.2	77	75	60	0.67	0.65	0.87	0.65
	98	100	104	1.02	1.04	1.53	1.63

Shown are percentage recoveries after addition of various amounts of the standards to 24-h urine collections from unsupplemented individuals. Each row represents a different subject (urine). The peak area ratio from pre- and post-addition of standards is a measure of viability; if the extraction is viable, then pre- and post-ratios should be similar. Each value represents the average of two individual extractions on the same sample.

Typical chromatograms of urine extracts from an unsupplemented subject are shown in **Fig. 3**. The peaks for trolox, γ-CEHC, and α-CEHC are detectable and easily resolved. Thus the method is effective for samples containing low levels of metabolites. When UV and ECD detection are compared, then the superior sensitivity afforded by ECD is seen (compare Fig. 3A with 3C and 3B with 3D). The UV 290 nm detection shows broad unresolved peaks, which are potentially difficult to integrate. The same samples were also analyzed by GC (data not shown). Although peaks for trolox and α-CEHC are resolved by GC, the number of interfering peaks is higher than with HPLC-ECD, and the signal-to-noise ratio is lower. Thus HPLC-ECD is more sensitive than GC in this particular system, and also removes the need for the preparation of silyl derivatives.

The coefficient of variation (CV) was measured to test reproducibility of the method. Two 24-h urine collections were analyzed six times separately, and the vitamin E metabolites were analyzed using either the internal standard method or with an external calibration curve. The latter curve was obtained by injecting known volumes of a solution containing trolox, γ- and α-CEHC in ethanol, over a range from 10 to 50 pg. The results are shown in **Table 3**. The CV ranged from 2 to 5% when determined by the internal standard, and these increased to 6 to 13% using the external calibration curve. The estimated concentrations from both methods were similar; however, using the internal standard corrects for any loss of recovery and thus has the lower CV, and is more reliable.

Typical values for γ- and α-CEHC excretion in 24-h urine collections from unsupplemented volunteers are shown in **Table 4**. Values ranged from ~100 to 800 μg/day for γ-CEHC and 60 to 400 μg/day for α-CEHC (n = 6). γ-CEHC excretion was either equal to or up to 2.5-fold greater than α-CEHC within each individual urine.

It is also possible to measure free or unconjugated vitamin E metabolites in the urine samples with this method. A comparison of unconjugated versus conjugated α- and γ-CEHC in 24-h urine collections from unsupplemented subjects is shown in **Fig. 4**. Typically, for both α- and γ-CEHC, the unconjugated form comprises from 5 to 25% of the total metabolites.

DISCUSSION

We describe here a relatively simple and rapid method for the extraction and analysis of urinary tocopherol me-

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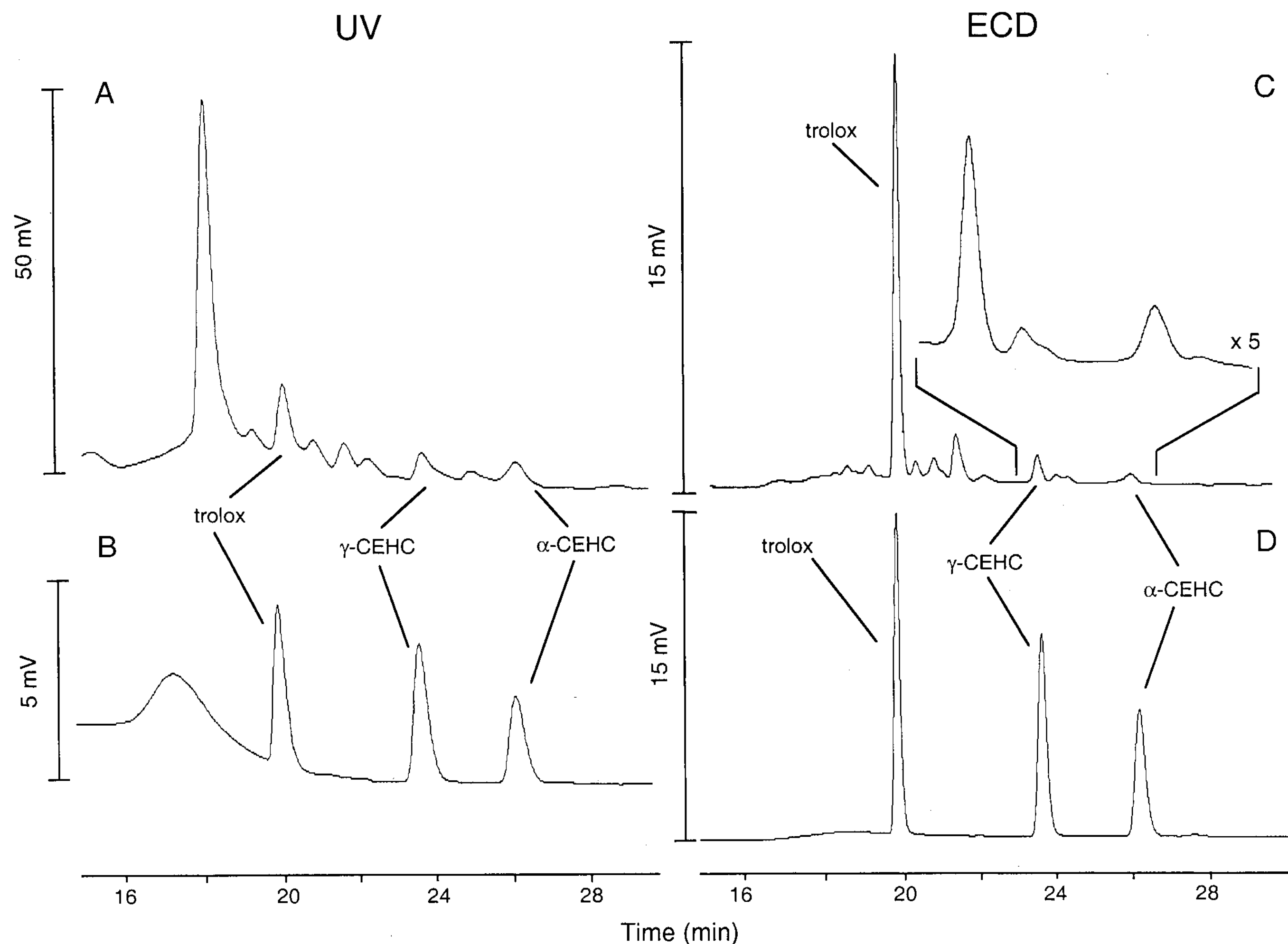

Fig. 3. HPLC chromatograms comparing UV 290 nm and electrochemical detection in the analysis of urinary vitamin E metabolites. A typical UV trace of a urine extract from an unsupplemented subject is shown in (A) and is compared with a standard mixture of trolox, γ- and α-CEHC (50 pg injected) (B). The same samples analyzed by electrochemical detection are shown in (C) and (D). The inset in (C) shows an expanded region (×5) from 23 to 27 min where γ- and α-CEHC elute.

tabolites. A small amount of sample is needed, and after enzyme hydrolysis, a one-step ether extraction isolates the metabolite plus the internal standard trolox. Analysis confirmed that α-CEHC, γ-CEHC, and trolox are recovered together to a similar degree, hence trolox is viable as an internal standard. Improved routine analytical sensitivity was achieved by incorporating HPLC with electrochemical detection. Both sensitivity and resolution were superior in comparison with GC flame ionization detection or with HPLC with UV detection. This improved method is

TABLE 3. Reproducibility of the assay for α- and γ-CEHC from human urine analyzed by HPLC-ECD

	Internal Standard (Trolox)		External Calibration Curve	
Urine (n = 6)	α-CEHC	γ-CEHC	α-CEHC	γ-CEHC
		μg excreted/day		
A				
Mean ± SE	335.61 ± 10.36	317.76 ± 8.58	367.85 ± 23.11	360.02 ± 28.77
CV%	3.08	2.69	6.28	7.99
B				
Mean ± SE	82.05 ± 3.39	212.76 ± 4.32	89.93 ± 11.43	193.79 ± 14.72
CV%	4.14	2.03	12.71	7.59

Two separate 24-h urine collections from vitamin E-unsupplemented subjects were used (A and B). The extraction and analysis was performed six times and determinations were performed using either the internal standard (trolox) or by an external calibration curve.

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TABLE 4. Typical values for α- and γ-CEHC excretion per day in unsupplemented individuals (n = 6) determined by HPLC-ECD

Subject	γ-CEHC	α-CEHC	γ-CEHC/ α-CEHC
	μg/day	*μg/day*	
1	139	116	1.2
2	149	60	2.5
3	240	106	2.3
4	394	271	1.4
5	407	181	2.2
6	821	404	2.0

quicker, less laborious, and more cost effective than previous GC/MS methods, and thus can be used in a routine basis for the measurement of vitamin E metabolism.

Methods for analysis of both α- and γ-CEHC from human urine have been reported, however both involve the use of GC/MS (14, 15) and the use of deuterated standards which are commercially unavailable (14). Here we report a HPLC method for simultaneous determination of these metabolites in unsupplemented urine, which uses trolox as an internal standard. Our recovery experiments showed that trolox, α- and γ-CEHC are extracted to a similar degree, therefore any losses during the extraction procedure are compensated for. The reproducibility of the extraction and analysis was also good. The coefficient of variation was less than 5%.

The majority of α- and γ-CEHC appear as conjugates in the urine, which necessitates enzymatic hydrolysis prior to extraction and quantification. The actual conjugated species is largely unknown, but based on results of a recent study with γ-CEHC (14), is presumed to be mainly a glucuronide. However, as the enzyme used in this study contained both glucuronidase and sulfatase activity, realistically both forms could have been present.

This is the first report of unconjugated forms of α- and γ-CEHC in human urine. Unconjugated forms were quantitated by simply extracting without enzymatic hydrolysis. Acidification prior to ether extraction was performed, thus it is possible that a small amount of hydrolysis occurred resulting in an overestimate of the total amount. However, under the conditions used, this is not presumed to contribute significantly. The unconjugated forms typically represented approximately 10% of the total, although there did not appear to be any correlation between free and conjugated within individuals. The analysis of unconjugated forms of the metabolites may be useful as the unconjugated form of γ-CEHC has been shown to be a powerful natriuretic factor in human urine (7).

Values reported here for α- and γ-CEHC in unsupplemented subjects were found to be similar to those reported previously for tocopherol metabolites in baseline values as determined by GC/MS (13). Large inter-individual differences were noticed with the levels of α- and γ-CEHC, however this is to be expected when the diets are not controlled and subjects are taken randomly. Generally, within individual urines, γ-CEHC excretion either equalled or was up to 3 fold higher compared to α-CEHC

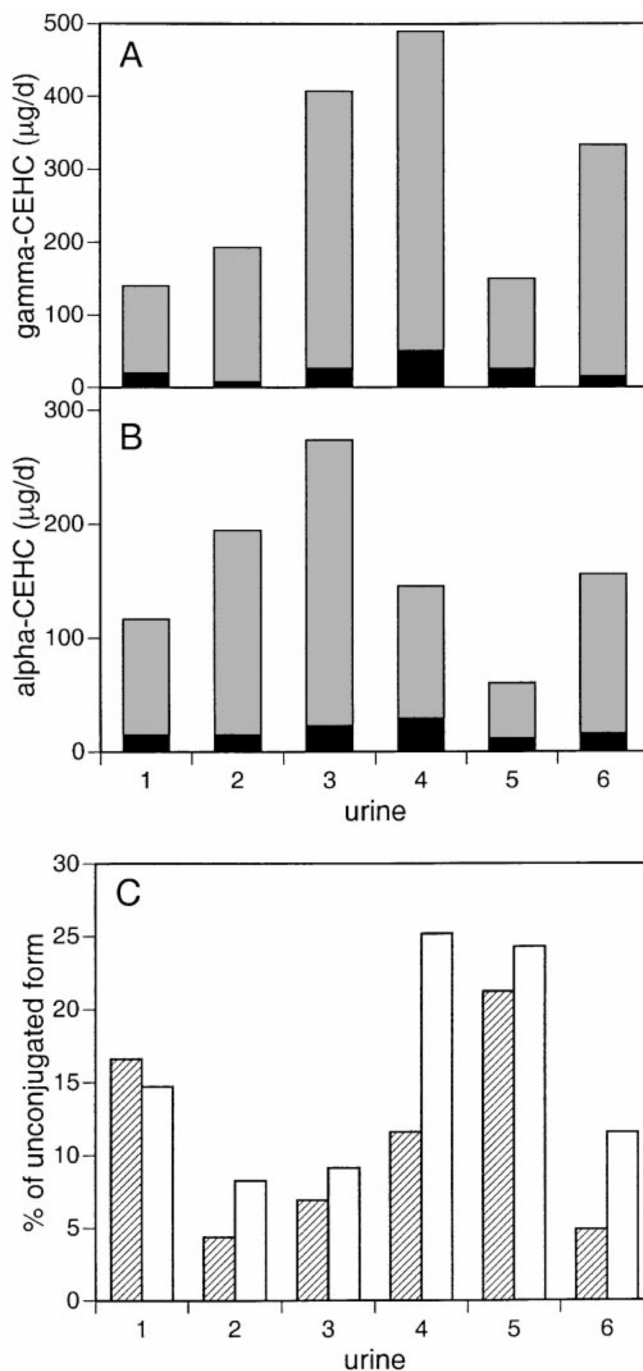

Fig. 4. Comparison of unconjugated (■) and conjugated (⊠) forms of α- and γ-CEHC in 24-h urine collections from unsupplemented subjects. Amounts of γ-CEHC are shown in A, while that of α-CEHC are in shown in B. The percentage of the free unconjugated form of α-CEHC (□) and γ-CEHC (▨) in each urine sample is shown in C.

excretion, again in accordance with previous observations (13). This may be a dietary consequence in that, at least in the US, intake of γ-tocopherol is greater than α-tocopherol, and may not be due to a difference in metabolism. However, there is discrimination and selective retention of α-tocopherol in the liver (5), whilst most of the dietary γ-tocopherol is metabolized to γ-CEHC (14). Moreover, γ-CEHC may have a metabolic role as a natriuretic factor

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(7). Such a 'secondary' function for α-CEHC has not been demonstrated. Thus α- and γ-tocopherol metabolism may be regulated independently, and not solely under the control of the hepatic α-tocopherol transfer protein.

When *RRR*-α- and *all rac*-tocopherol are taken together, the all racemic tocopherol is preferentially metabolized and excreted as α-CEHC (13) in line with similar observations showing a preferential uptake of the *RRR*- form into plasma (5). Because plasma levels of α-tocopherol are not able to be raised more than 3-fold no matter what the level of supplementation (5), this raises the question what happens to the remainder, is it distributed elsewhere or simply metabolized and excreted. Thus measurements of vitamin E metabolites may represent one way of examining a maximal level of endogenous vitamin E obtained from supplementation, as previously proposed (6). It is also likely that metabolism and excretion play a regulatory role.

Therefore it will be of interest to study further the relationship between plasma vitamin E and urinary excretion.

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