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Simultaneous quantification of vitamin E and vitamin E metabolites in equine plasma and serum using LC-MS/MS

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Abstract. Vitamin E deficiencies can impact normal growth and development in humans and animals, and assessment of circulating levels of vitamin E and its metabolites may be an important endpoint for evaluation. Development of a sensitive method to detect and quantify low concentrations of vitamin E and metabolites in biological specimens allows for a proper diagnosis for patients and animals that are deficient. We developed a method to simultaneously extract, detect, and quantify the vitamin E compounds alpha-tocopherol (α-TP), gamma-tocopherol (γ-TP), alpha-tocotrienol (α-TT), and gamma-tocotrienol (γ-TT), and the corresponding metabolites formed after β-oxidation of α-TP and γ-TP, alpha-carboxymethylbutyl hydroxychroman (α-CMBHC) and alpha- or gamma-carboxyethyl hydroxychroman (α- or γ-CEHC), respectively, from equine plasma and serum. Quantification was achieved through liquid chromatography–tandem mass spectrometry. We applied a 96-well high-throughput format using a Phenomenex Phree plate to analyze plasma and serum. Compounds were separated by using a Waters ACQUITY UPLC BEH C18 column with a reverse-phase gradient. The limits of detection for the metabolites and vitamin E compounds were 8–330 pg/mL. To validate the method, intra-day and inter-day accuracy and precision were evaluated along with limits of detection and quantification. The method was then applied to determine concentrations of these analytes in plasma and serum of horses. Alpha-TP levels were 3–6 μg/mL of matrix; the metabolites were found at much lower levels, 0.2–1.0 ng/mL of matrix.

Key words: α/γ-tocopherol; alpha-carboxymethylbutyl hydroxychroman; gamma-carboxyethyl hydroxychroman; liquid chromatography–mass spectrometry; vitamin E.

Introduction

Vitamin E is a well-known antioxidant that protects against oxidative stress, specifically the propagation of lipid peroxidation.[3] Vitamin E is a collective term used to describe a group of 8 potent and lipophilic vitamin E isoforms; alpha-, beta-, gamma-, and delta-tocopherol (α-TP, β-TP, γ-TP, δ-TP, respectively) as well as alpha-, beta-, gamma-, and delta-tocotrienol (α-TT, β-TT, γ-TT, δ-TT, respectively), which have a large number of beneficial attributes.[1,7,13,26,30–32] Following ingestion, vitamin E isoforms are metabolized via P450-mediated ω-oxidation to longer chain metabolites, such as carboxymethylbutyl hydroxychroman (CMBHC), followed by β-oxidation to shorter chain metabolites, such as carboxyethyl hydroxychromans (CEHCs). In addition to the TP and TT isoforms being beneficial, several metabolites including γ- and α-CEHC demonstrate similar properties.[11,13,38] The precursor metabolite to α-CEHC, alpha-5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (α-CMBHC), is a minor vitamin E metabolite formed from α-TP and has been investigated as a biomarker of excess/adequate vitamin E.[28] By monitoring vitamin E and vitamin E metabolite levels in biological matrices, it may be possible to determine the level at which vitamin E can protect tissues against oxidative injury.[41]

Vitamin E deficiencies and resulting neurologic diseases affect both humans and animals.[1,2,7,13,26,30,31] In particular, horses receive their dietary intake of vitamin E from grazing pasture, and genetically susceptible foals who do not have access to enough fresh pasture during the first year of their lives may develop equine neuroaxonal dystrophy (eNAD). eNAD is a disease characterized by axonal degeneration within the brainstem and spinal cord, leading to general

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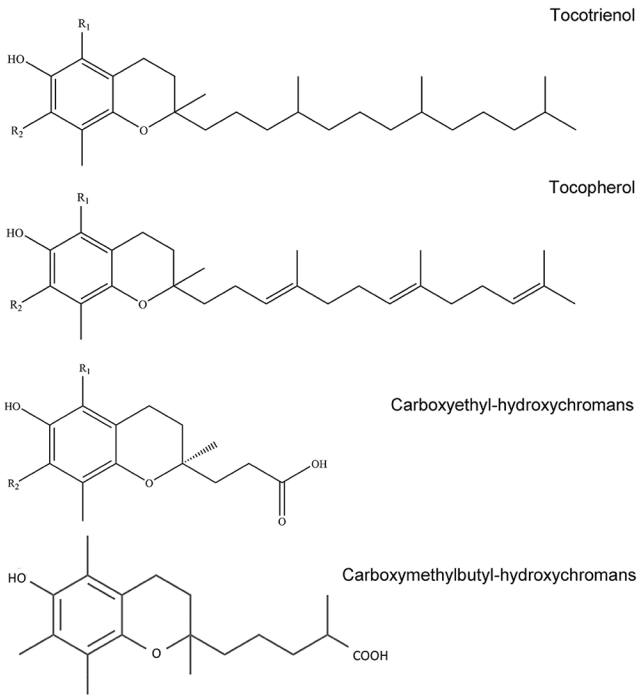

Figure 1. Chemical structures of targeted analytes. R_1 = methyl and R_2 = methyl for α-TP, α-TT, and α-CEHC. R_1 = H and R_2 = methyl for γ-TP, γ-TT, and γ-CEHC. The structure for α-CMBHC is shown without substitutions.

proprioceptive ataxia.[6,7] In addition to the lack of vitamin E in foals, adult horses that have been vitamin E deficient for 18 mo can develop lower motor neuron weaknesses, termed equine motor neuron disease (EMND).[5]

Vitamin E and its metabolites have previously been analyzed using high-performance liquid chromatography–ultraviolet visible spectroscopy (HPLC-UV-VIS),[8,29] gas chromatography–mass spectrometry (GC-MS),[21] and liquid chromatography–mass spectrometry (LC-MS).[16,25] As the underlying etiologies for both eNAD and EMND remain unknown, quantification of vitamin E metabolites in diseased horses may provide further insight into the pathophysiology of these conditions. We aimed to develop and validate a sensitive method to measure vitamin E and the vitamin E metabolites (Fig. 1) at low concentrations using liquid chromatography–tandem MS (LC-MS/MS), allowing for the establishment of reference intervals (RIs) in healthy horses maintained on irrigated grass pasture. Additionally, we sought to determine if there were significant differences in vitamin E and vitamin E metabolites in matched serum versus plasma samples.

Materials and methods

Chemicals and materials

DL-α-TP (> 99%), γ-TP (> 96.3%), α-TT (> 97%), γ-TT (> 97%), L-ascorbic acid (pharmaceutical secondary standard, > 99%), and chlorpropamide were purchased from Sigma-Aldrich. The vitamin E metabolites (α-CEHC > 98%, γ-CEHC > 98%, α-CMBHC > 98%) and (12-((cyclohexylamino)carbonyl)amino)-dodecanoic acid (CUDA) were purchased from Cayman Chemical. α-TP-D6 (5-methyl-D3, 7-methyl-D3, > 98%) was purchased from Cambridge Isotope Laboratories. The antioxidant butylated hydroxytoluene (BHT) was purchased from MP Biomedicals. Glacial acetic acid (> 99.7%, analytical grade), methanol (analytical grade), and acetonitrile (ACN; analytical grade) were purchased from Fisher Scientific. Isopropyl alcohol (IPA; analytical grade) was purchased from Millipore Sigma. The 96-well Phree plates and deep-well plates (96/2,000 µL) were purchased from Phenomenex. The vacuum manifold for plate processing was purchased from Waters Corp. Ultrapure water (18.2 mΩ) was obtained using a Milli-Q system (Millipore).

Preparation of stock standard solutions and antioxidant solutions

Stock standard solutions of target analytes and internal standards were prepared in methanol at various concentrations. Working solutions containing all 7 target analytes were prepared at 12.5, 1,250, and 4,000 ng/mL. Additionally, methanolic solutions of α-TP-D6 (1,000 ng/mL), chlorpropamide (50 ng/mL), BHT (10 mg/mL), and CUDA (2 ng/mL) were prepared. Ascorbic acid was prepared fresh daily at 10 mg/mL in water.

Biological sample collection

Matched serum and plasma samples were collected from 16 healthy Thoroughbred horses (2–24 y old, 9 mares and 7 geldings) to establish baseline vitamin E and metabolite levels. These horses were maintained on irrigated pasture for 24 h/d for ≥3 mo prior to sampling, with no supplemental feed provided. Light-protected plasma and serum samples were obtained with signed owner consent under a protocol approved by the University of California's Institutional Animal Care and Use Committee. Plasma samples were collected in lavender-top tubes containing K_2EDTA as the anticoagulant. Serum samples were collected in either empty red-top tubes or serum separator tubes without anticoagulant. Hemolysis was assessed at collection, and no hemolyzed samples were included in the analysis. All samples were centrifuged at 4°C within 4 h of collection and frozen at −80°C until analyzed. Charcoal-stripped negative control serum was prepared in-house using dextran-coated activated charcoal.[17]

Sample extraction from plasma and serum samples

To extract vitamin E and vitamin E metabolites, frozen plasma and serum samples were placed in a refrigerator at 4°C until thawed. Once thawed, samples were inverted and

Table 1. Tandem mass spectrometry parameters and multiple reaction monitoring (MRM) transitions in positive and negative electrospray ionization mode.

Compound	Retention time (min)	MRM transition (*m/z*)	Collision energy (eV)	Ionization mode
Chlorpropamide	5.6	275.7 → **191.0**, 127.1, 80.2	12, 22, 35	Negative
γ-CEHC	5.9	263.3 → **136.1**, 149.1	23, 17	Negative
α-CEHC	6.2	277.3 → **233.1**, 163.1, 150.1	10, 19, 15	Negative
α-CMBHC	7.5	319.4 → **150.1**, 163.1, 275.1	15, 20, 14	Negative
CUDA	7.8	339.0 → **214.1**, 240.1	22, 14	Negative
γ-TT	10.9	411.0 → **151.0**, 191.0, 205.1	18, 13, 11	Positive
α-TP	12.6	431.7 → **165.0**, 137.1, 111.2	18, 36, 14	Positive
α-TT	11.2	425.7 → **165.0**, 205.1, 137.1	16, 12, 33	Positive
γ-TP	12.2	417.7 → **151.0**, 123.1, 97.2	15, 37, 15	Positive
α-TP-D6	12.6	437.4 → **171.1**, 143.1, 97.2	18, 37, 17	Positive

Product ions used for quantitation are shown in bold.

mixed to ensure homogeneity. Six hundred μL of ACN was added to each well of a Phree plate. Ten μL of ascorbic acid (10 mg/mL) and BHT (10 mg/mL) as antioxidants, along with 10 μL of α-TP-D6 (1 μg/mL in methanol) and chlorpropamide (50 ng/mL in methanol) as internal standards, were added to each tube. Select wells were then spiked with 10 μL of working solutions containing all 7 analytes at various concentrations for calibrators and quality control (QC) samples, or 10 μL of methanol for samples. Then, 150 μL of plasma/serum sample or water for controls and calibration curve was added to each well and mixed by pipette. A gentle vacuum of 5–10 mm Hg was then applied to collect the flow-through containing the metabolites. After the wells appeared to be dry, 800 μL of an ACN/IPA mix (9:1) was added to the wells to extract the tocopherol and tocotrienol compounds, and eluent was collected. The contents were transferred to a 2-mL microcentrifuge tube and evaporated to dryness (45°C) in vacuo (VacuFuge; Eppendorf). Each residue was then reconstituted in 50 μL of methanol containing CUDA (2 ng/mL) and vortexed, sonicated (2 min), vortexed, and centrifuged (8 min) at 14,000 × *g*. Approximately 40 μL of supernatant was transferred into HPLC vials for analysis.

Instrumental analysis

The analysis of vitamin E and metabolites was accomplished using a LC-MS/MS system, which consisted of an Advance HPLC system coupled with an EVOQ Elite triple-quadrupole mass spectrometer (Bruker). The chromatography system was fitted with a 130 Å, 1.7 μm, 2.1 mm × 100 mm ACQUITY BEH C18 column with a corresponding Van Guard pre-column (Waters). Mobile phases consisted of ultrapure water (18.2 mΩ) with 0.1% acetic acid (channel A) and methanol with 0.1% acetic acid (channel B) delivered over a reverse-phase gradient. Initially, 5% B was increased to 45% B over 2 min, which was then increased to 98% B over 6 min, held for 8 min, and returned to 5% B immediately after 16 min, and then held for 6 min for re-equilibration to initial conditions. The column was maintained at 50°C, and the flow rate was set at 250 μL/min. Analytes were monitored by multiple reaction monitoring (MRM) in positive mode electrospray ionization for the tocopherols and tocotrienols, and negative mode electrospray ionization for the metabolites (Table 1, Figs. 2, 3). Nitrogen gas was used as the nebulizer gas, and argon was used as the collision gas. Other source parameters were as follows: nebulizer gas flow 45 arbitrary units (a.u.), probe gas flow 35 a.u, temperature of the heated probe 300°C, cone gas flow 15 a.u., cone temperature 300°C, and the capillary voltage 4,000 V. The injection volume per sample was 5 μL. MSWS 8.1 software (Bruker) was used for all data analysis and processing.

Method validation

To determine the performance of the method, intra- and inter-day accuracy and precision were evaluated. An external calibration curve in water was utilized because a truly blank matrix (serum or plasma) was unavailable given the endogenous nature of the targeted analytes. Nine calibration standards of 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1,000 ng/mL for all 7 analytes were used, plus additions of 200 ng/mL of internal standard α-TP-D6, 10 ng/mL of internal standard chlorpropamide, and 2 ng/mL of the instrument internal standard CUDA. An additional 3 calibration standards were prepared at 2,000 ng/mL, 5,000 ng/mL, and 10,000 ng/mL, only containing α-TP and the internal standards to account for high levels of α-TP expected in serum and plasma of healthy horses. Three QC samples at low, medium, and high levels were prepared at final concentrations of 2.5, 250, and 800 ng/mL in water, respectively (n = 5/level). The recovery of the methodology was assessed by comparing the response of neat standards to extracted samples in water. The matrix effects of the method were assessed by calculating the ratio of peak area response from a charcoal-stripped serum sample

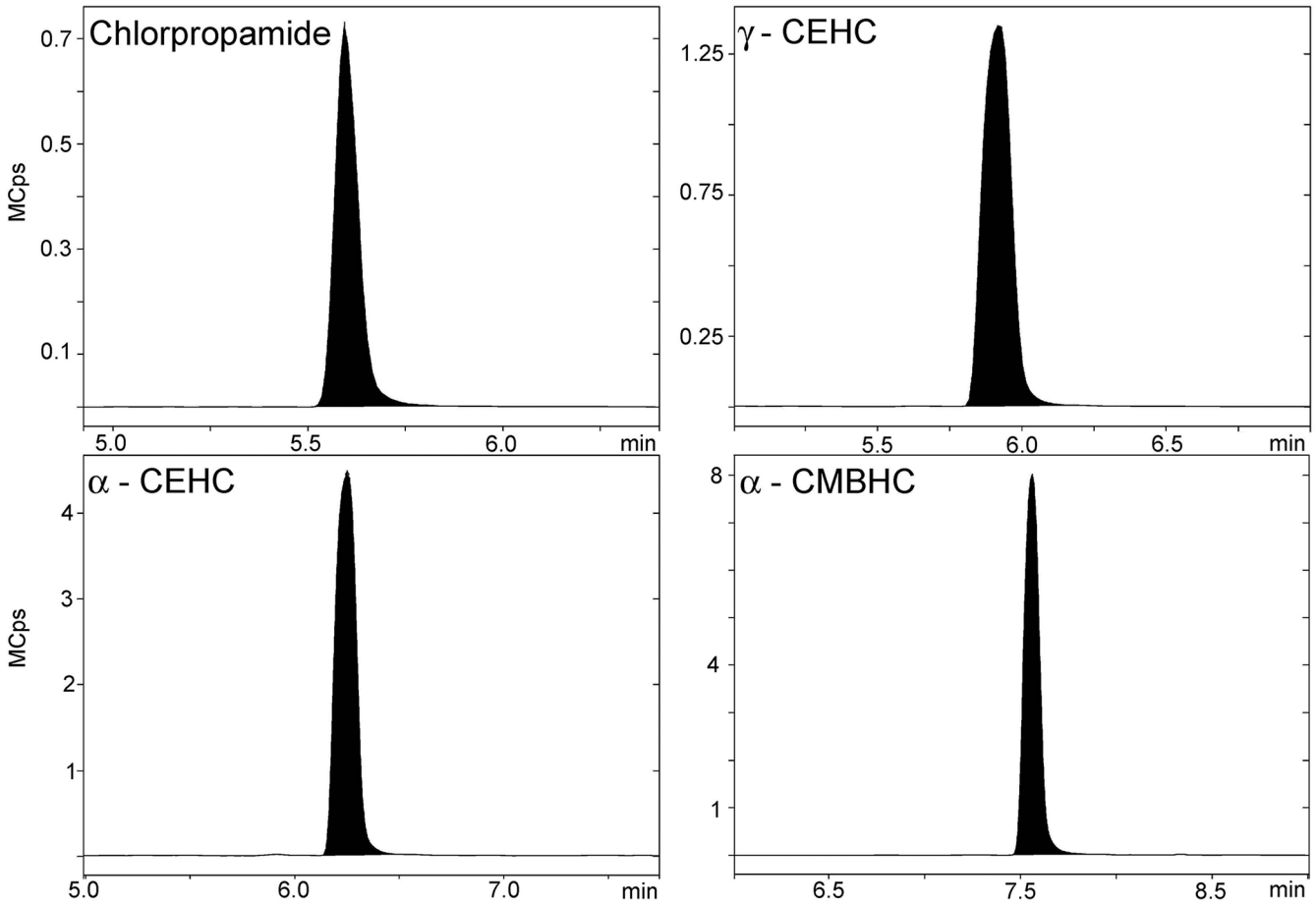

Figure 2. Representative LC-MS/MS chromatograms for chlorpropamide, γ-CEHC, α-CEHC, and α-CMBHC in a 250 ng/mL quality control sample.

to an extracted standard. In addition to the positive control samples, a water negative control was run each day to monitor contamination or significant carry-over.

Data analysis

Statistical analyses were performed with Prism v.7.0 (GraphPad). Normality was evaluated using a Shapiro–Wilk test and evaluation of residuals using a Q-Q plot. Correlation between matched plasma and serum samples was assessed using a Pearson coefficient for normally distributed data, and a Spearman coefficient for non-normally distributed data. An unpaired *t*-test with a Welch correction was used to assess sex differences for all analytes in both plasma and serum samples. Statistical significance was set at $p \leq 0.05$.

Results

The spray voltage (in both positive and negative mode), cone temperature, cone gas flow, heated probe temperature, probe gas flow, and nebulizer gas flow were optimized following either the infusion of standards (10 ng/μL) or injection of an extracted sample to obtain the greatest instrument sensitivity. Source and MRM scan parameters were evaluated for vitamin E and vitamin E metabolites in both positive and negative mode using 3 different acidic modifiers (0.1% formic acid, 0.1% acetic acid, or 1.0% acetic acid) in MeOH/H_2O 50:50. Based on signal intensities, the metabolites ionized with greater efficiency with 0.1% acetic acid as the modifier. Decreasing the spray voltage below 4,000 V resulted in decreased peak areas for all analytes. The peak areas were not affected when switching between cone temperatures of 200, 250, and 300°C. Heated probe temperature was tested at 250, 300, and 350°C, with 300°C having the largest response for most of the compounds. Cone gas flow was evaluated at 15 and 20 a.u. with a nebulizer gas flow at 40 and 50 a.u., respectively, with the lower flows producing better results.

Calibration curves were generated by calculating relative responses (analyte peak area/internal standard peak area) for each point on the standard curve. The calibration was fitted with a linear regression for vitamin E compounds and a quadratic regression for the metabolites (Table 2). Method accuracy, precision, recovery, and matrix effects were evaluated

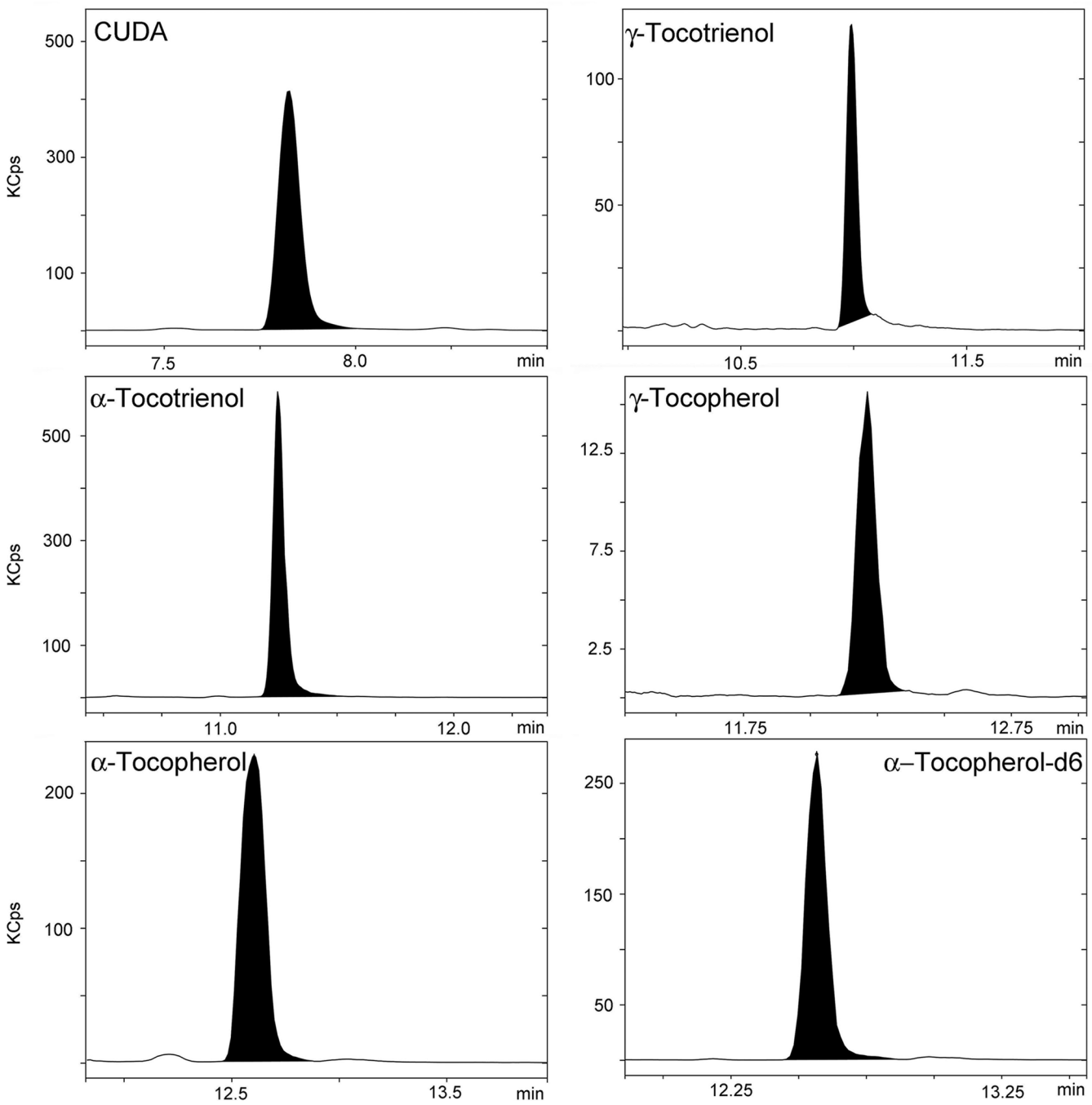

Figure 3. Representative LC-MS/MS chromatograms for CUDA, γ-TT, α-TT, γ-TP, α-TP, and α-TP-D6 in a 250-ng/mL quality control sample.

during method validation. The limits of detection and limits of quantification were determined by diluting a standard containing all 7 analytes in methanol until signal-to-noise ratios of 3:1 and 10:1 were obtained, respectively (Table 2). The linear/quadratic regression for all compounds was 0.1–1,000 ng/mL (α-TP regression extended to 10,000 ng/mL) with coefficients of determination (R^2) > 0.99 for each day.

The accuracy and precision of all analytes were evaluated by determining intra-day and inter-day statistics (Table 3) using QC samples spiked with low, medium, and high concentrations. Intra-day accuracies were 73.9–121%, depending on the target analyte. Assay precision was evaluated, and the coefficient of variation (% CV) was calculated for each concentration level. For intra-day precision, % CVs were

Table 2. Limits of detection (LOD), limits of quantification (LOQ), upper limits of quantification (ULOQ), percent recovery, matrix effect ratio, type of regression used for the calibration curve, and representative regression equations of targeted compounds.

Compound	LOD (ng/mL)	LOQ (ng/mL)	ULOQ (ng/mL)	% Recovery	Matrix effect ratio	Calibration curve regression utilized	Representative regression equation
α-TP	0.008	0.025	10,000	44	0.42	Linear	$0.0038x + 0.0084$
γ-TP	0.333	1.11	1,000	41	0.22	Linear	$1.37e^{-4}x + 2.87e^{-5}$
α-TT	0.029	0.095	1,000	44	0.36	Linear	$0.0047x + 4.4e^{-5}$
γ-TT	0.125	0.417	1,000	42	0.35	Linear	$0.002x - 4.21e^{-4}$
α-CMBHC	0.010	0.032	1,000	66	0.42	Quadratic	$-2.1e^{-5}x^2 + 0.069x + 0.270$
α-CEHC	0.022	0.074	1,000	93	0.54	Quadratic	$-1.2e^{-5}x^2 + 0.054x + 0.130$
γ-CEHC	0.026	0.087	1,000	96	0.53	Quadratic	$-3.9e^{-6}x^2 + 0.016x + 0.056$

Table 3. Intra-batch accuracy and precision, and inter-batch accuracy and precision using liquid chromatography–mass spectrometry.

Analyte	Nominal concentration (ng/mL)	Day 1		Day 2		Day 3		Inter-day	
		Accuracy* (%)	Precision† (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy‡ (%)	Precision§ (%)
α-TP	2.50	114	6.58	119	2.50	109	5.00	114	5.83
	250	99.1	5.23	115	3.63	101	6.14	105	7.96
	800	103	4.95	108	2.23	102	4.44	104	4.55
γ-TP	2.50	102	17.5	81.4	9.63	103	5.67	95.6	15.8
	250	113	7.52	76.9	4.53	82.6	6.99	93.7	19.6
	800	101	8.57	79.3	21.5	84.0	7.80	88.2	16.5
α-TT	2.50	84.3	5.10	80.4	9.97	92.6	7.95	85.8	9.53
	250	94.6	7.98	109	4.39	109	7.12	104	9.11
	800	91.9	14.9	107	2.03	109	1.58	103	10.6
γ-TT	2.50	115	8.06	93.1	18.7	106	5.21	104	13.7
	250	75.7	6.63	76.5	22.1	73.9	11.5	75.3	14.0
	800	78.0	15.3	87.7	11.4	77.2	3.29	80.9	12.0
α-CMBHC	2.50	99.5	7.11	116	2.86	98.8	24.4	105	8.88
	250	98.3	5.60	116	0.96	98.9	11.8	105	10.6
	800	101	12.5	109	3.14	107	2.87	106	7.57
α-CEHC	2.50	114	5.65	118	2.88	110	4.12	114	10.2
	250	108	5.46	118	2.33	116	4.18	114	5.48
	800	101	11.5	110	1.96	112	2.81	108	7.46
γ-CEHC	2.50	114	2.87	124	3.27	115	5.48	118	5.29
	250	117	1.88	121	3.55	113	6.80	117	5.07
	800	109	6.02	108	2.47	118	6.09	111	6.20

* Intra-day accuracy was measured as the amount of target analytes added to a blank matrix, carried throughout the extraction procedure, and then analyzed. The % accuracy was based on the mean of 5 samples at low, medium, and high concentration levels.
† Inter-day precision was measured as the amount of target analytes added to a blank matrix, carried throughout the extraction procedure, and then analyzed. The inter-day % precision is determined by the average % recoveries obtained in days 1–3.
‡ Intra-day accuracy was measured by the amounts obtained from the coefficients of variation (% CV). The % accuracy is based on the averages of the % CVs of 5 samples at low, medium, and high concentration levels.
§ Inter-day precision was measured by the amounts obtained from the coefficients of variation (% CV). The inter-day precision % is determined by the average % precisions obtained in days 1–3.

0.96–24.4%. The inter-day accuracy was based on the average obtained over 3 d, at each concentration level. Inter-day accuracies were 85.8–117%, 75.3–117%, and 80.9–111%, for the low, medium, and high concentration levels, respectively. The inter-day precision was determined by the average obtained in days 1–3. The inter-day precisions were 5.29–15.8%, 5.07–19.6%, and 4.55–16.5%, for the low, medium, and high concentration levels, respectively. The matrix effects (matrix effect ratio) and recovery were evaluated by comparing peak area responses from neat standard to either a charcoal-stripped serum sample or extracted standard at equivalent concentrations (Table 2).

Serum and plasma α-TP, γ-TP, α-CMBHC, α-CEHC, and γ-CEHC concentrations were significantly positively correlated (Fig. 4A, 4B, 4D, 4E, respectively). Serum α-TP, γ-TP, and γ-CEHC concentrations were higher than matched

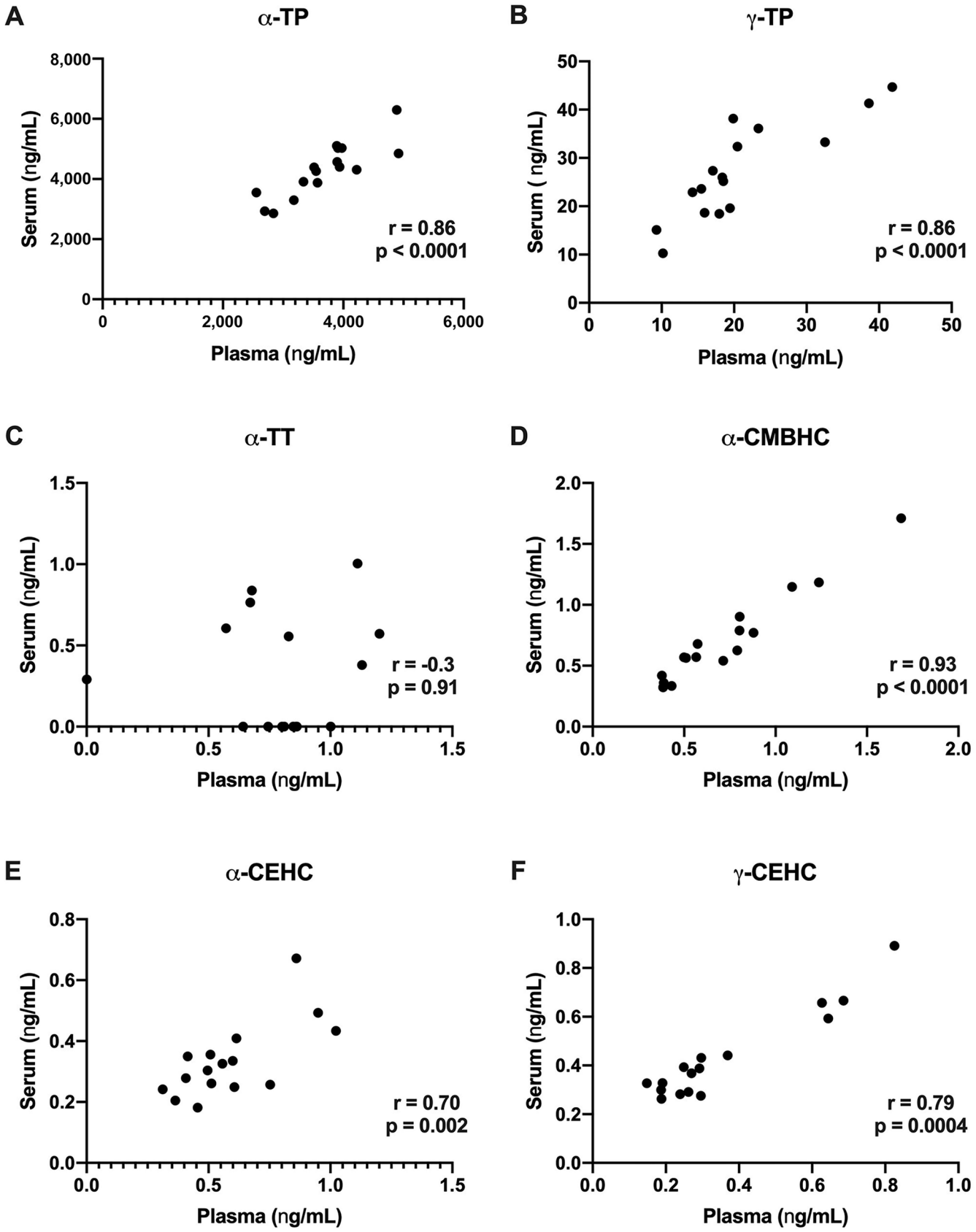

Figure 4. Correlations of matched plasma and serum: **A.** α-TP, **B.** γ-TP, **C.** α-TT, **D.** α-CMBHC, **E.** α-CEHC, and **F.** γ-CEHC. Significant positive correlations were identified for all analytes except α-TT.

plasma samples, whereas plasma α-CEHC was higher than matched serum samples. Serum and plasma values of α-TT were not significantly correlated (Fig. 4C), with higher values in plasma versus serum. Serum and plasma γ-TT concentrations from healthy horses maintained on pasture were below the reporting limit for most data points and therefore excluded from further analysis.

Discussion

We developed and ultimately applied our LC-MS/MS method to analyze plasma and serum from 16 healthy Thoroughbred horses maintained on pasture to determine endogenous levels of vitamin E and vitamin E metabolites. Our data generated are similar to the findings of a previous study,[7] in which α-TP levels in healthy Thoroughbred horses were 3–5 μg/mL in plasma or serum. Our data support this claim; our α-TP serum and plasma concentrations were 4.42 ± 1.02 μg/mL and 3.72 ± 0.68 μg/mL, respectively. Results were higher than previously reported α-TP plasma (2.8 ± 0.9 μg/mL) levels of healthy Thoroughbreds, Quarter Horses, and other breeds maintained on hay and alfalfa pellets.[33] This is likely the result of different diets, with our Thoroughbred population maintained on irrigated pasture 24 h/d. With regards to sex, α-TP was the only analyte that showed significant differences, with mares demonstrating higher concentrations of α-TP in both plasma and serum ($p = 0.007$ and 0.01, respectively).

Various analytical approaches have been utilized to detect vitamin E and its metabolites[9,10,12,14,16,19–25,27,29,37,38,40] and are summarized in in-depth reviews.[36,37] Given its high endogenous concentration in serum or plasma, α-TP measurement does not require a highly sensitive methodology, but measurement of other vitamin E isoforms and metabolites requires highly sensitive approaches with circulating levels commonly observed at either low ng/mL or sub ng/mL concentrations.[12,19,27] The use of a triple-quadrupole mass spectrometer allows for improvements in detection levels compared to single-quadrupole instruments.[14] Most notably, our approach allows very low level detection of both α- and γ-CEHC at over an order of magnitude lower concentrations compared to other studies. Others have found similar accuracy and precision values in plasma and liver homogenates using GC-MS and LC-MS approaches.[19,25] Although our results showed slightly greater variance in precision, and lower recoveries for γ-TP and γ-TT, we have demonstrated simultaneous detection of vitamin E and vitamin E metabolites using a 22-min method, minimizing sample preparation and run times compared to previous investigations.[19,25] Some studies have compared the differences in detecting vitamin E through LC-MS/MS analysis to GC-MS analysis after derivatization to trimethylsilyl derivatives.[16,21,25] Derivatizing vitamin E samples for GC-MS analysis not only adds a time-consuming and costly step, but also adds the risk of hydrolysis.[16]

The use of internal standards during bioanalytical sample preparation and analysis is a commonly utilized approach to account for losses observed during processing or to correct for matrix effects that are observed during the electrospray ionization process.[34] Given the potential for degradation or oxidation of analytes during sample preparation and to compensate for varied instrument responses, we used 2 internal standards. Alpha-TP-D6 was used to correct for vitamin E responses, and chlorpropamide was used to correct the responses for the metabolites. Alpha-TP-D6 was initially used as an internal standard for all 7 target analytes. Because of the differences in chemical structure and ionizing modes between the metabolites and vitamin E compounds, α-TP-D6 did not properly correct for the metabolites detected in negative mode with observed concentrations ~120–230% of predicted values, indicating that the metabolites required a different internal standard. Initially, Trolox was evaluated as an internal standard based on structure similarities to the metabolites and data from previous studies,[19,22] but resulted in poor recoveries (0.2–22.8%). Chlorpropamide was ultimately evaluated and selected as an internal standard for the metabolites.[15] Our data suggest that the internal standards were helpful in correcting for both recovery and ion suppression or enhancement, although the use of stable isotope–labeled internal standards for all of the targeted analytes that are not commercially available would further strengthen the methodology.

The analysis of vitamin E and its metabolites is challenging for a variety of reasons, and our study has some limitations that should be noted and can be addressed in future work. First, our approach uses only one stable isotope–labeled internal standard (α-TP-D6) given the lack of commercial stable isotope–labeled reference standards for the remaining compounds, which increases the likelihood of uncorrected biases being introduced during sample processing.[34] Second, the endogenous nature of the targeted compounds included in our analysis makes obtaining a "blank" matrix that does not contain the compounds of interest challenging. There are several potential bioanalytical approaches to address these challenges, such as background subtraction, method of standard addition, or use of a surrogate matrix.[35,39] Thus, similar to the work of others, we used a surrogate matrix (water) to prepare calibration curves.[12,14,20] Although this approach is not ideal given the use of non–matrix-matched calibrators potentially resulting in unaccounted-for biases in results, it was suitable as part of our preliminary evaluation of vitamin E and its metabolites in serum or plasma. One alternative surrogate matrix is charcoal-stripped serum/plasma samples, although, based on our data (not shown), charcoal stripping was more effective at reducing the background levels of the metabolites, it was less effective for less polar compounds, such as α-TP, and was not utilized. Further studies would be strengthened using the background subtraction approach in the analysis of vitamin E and its metabolites.[10] Third, instability during sample

processing is also of concern when working with vitamin E isoforms, and thus our approach utilized a combination of BHT and ascorbic acid to attempt to mitigate degradation during sample preparation.[14,36,37] Likewise, the stability of vitamin E and its metabolites in biological fluids after collection and storage is of further interest and, although not addressed specifically in our study, is an area for further investigation. Others have shown that α-TP concentrations are stable when stored refrigerated or frozen for 1–2 wk, although extended storage did result in decreased levels after 6 mo of storage below −10°C.[4,12,23] Additionally, given that several of the metabolites analyzed in our study are excreted as phase II metabolites (glucuronide, sulfate, amino acid conjugates) in urine, our approach focusing on non-conjugated compounds may not assess the full spectrum of vitamin E metabolites present in circulation.[36,37] Last, it should be noted that others have shown that hemolysis of samples can have dramatic impacts on α-TP concentrations and it is likely that other similar compounds will be impacted in the same fashion, and thus care should be taken when collecting and analyzing samples.[4,12,18]

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Declaration of conflicting interests

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