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Analysis Of Vitamin E Metabolites By Liquid Chromatography-Tandem Mass Spectrometry

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ANALYSIS OF VITAMIN E METABOLITES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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LIST OF ABBREVIATIONS

αT, βT, γT, δT: α-, β-, γ-, δ-Tocopherol

αTE, βTE, γTE, δTE: α-, β-, γ-, δ-Tocotrienol

CEHC: 2-(β-carboxyethyl)-6-hydroxychroman

CMBHC: Carboxymethylbutyl Hydroxychroman

9', 11', 13': 9'-, 11'- and 13'-Carboxychromanol

13'-OH: 13'-Hydroxychromanol

9'S, 11'S, 13'S: Sulfated 9'-, 11'- and 13'-Carboxychromanol

5-LOX: 5-lipoxygenase

COX-2: Cyclooxygenase-2

PGE_2: Prostaglandin E_2

SR-B1: Scavenger Receptor Class B Type 1

NPC1L1: Niemann-Pick type C1-Like 1

ABCA1: ATP-Binding Cassette A1

α-TTP: α-Tocopherol Transport Protein

BHT: Butylated Hydroxytoluene

DMEM: Dulbecco's Modified Eagle's Medium

FBS: Fetal Bovine Serum

HPLC-FL: High Performance Liquid Chromatography-Fluorescence

HPLC-UV: High-Performance Liquid Chromatography-Ultraviolet

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry

ESI: Electrospray Ionization

MRM: Multiple-Reaction Monitoring

M/Z: Mass-to-Charge Ratio

QQQ: Triple-Quadrupole

AOM: Azoxymethan

DSS: Dextran sodium sulfate

B.W.: Body Weight

ABSTRACT

Xu, Tianlin. M.S., Purdue University, December 2014. Analysis of Vitamin E Metabolites by Liquid Chromatography-Tandem Mass Spectrometry. Major Professor: Qing Jiang.

Naturally occurring forms of Vitamin E are metabolized to various carboxychromanols and conjugated carboxychromanols. Recent studies showed that vitamin E metabolites, especially the long-chain carboxychromanols are more bioactive than unmetabolized vitamin E forms. It is necessary to quantify vitamin E metabolites in biological environment. Here a simple and effective extraction method was developed to achieve extraction efficacy of more than 90% of various forms of vitamin E and metabolites with less than 10% inter- or intra-day variation. An LC-MS/MS assay was developed and optimized to acquire best sensitivity for the detection of vitamin E and metabolites. This method allows simultaneous detection of all carboxychromanols and sulfated carboxychromanols. Using the optimized extraction and LC-MS/MS analysis conditions, vitamin E metabolites in plasma or feces of animals fed with γT, γTE, δT or δTE-13' were analyzed. Results showed that, the major metabolites in the blood were conjugated γ-CEHC and sulfated long chain carboxychromanols.

CHAPTER 1. LITERATURE REVIEW

1.1 Overview of Vitamin E

Vitamin E is a required nutrient that is synthesized by photosynthetic plants and provides various health benefits to human (1). Vitamin E was identified as a fertility factor in 1922 by Evans and Bishop since vitamin E deficiency leads to fatal resorption. Therefore, vitamin E was named after the Greek word *tokos*, which stands for childbirth (2). There are eight naturally occurring isoforms in the vitamin E family, four tocopherols (α, β, γ and δT) and four tocotrienols (α, β, γ and δTE) (3). These isoforms differ in the methylation on their chromanol ring structures. Tocotrienols differ from tocopherols in the 3 double bonds on their 13-carbon side chain.

Vitamin E is widely consumed in North American diet especially in plant seeds and oil. Dietary sources of vitamin E include olive oil, sunflower oil, almonds, peanuts, etc. Among all the vitamin E isoforms, γT is the major form being consumed in US diet (4). Once absorbed, vitamin E is metabolized in the liver and generates various conjugated and non-conjugated carboxychromanols. The terminal metabolite, 3'-carboxychromanol, carboxyethyl-hydroxychroman (CEHC) and its conjugated counterpart were found

excreted in urine while the unconjugated long chain metabolites were found excreted in feces. Unmetabolized vitamin E is also excreted in feces. αT is more extensively accumulated in body tissue than any of the other vitamin E forms (5,6,7). Thus between 1960 and 1990, researchers focused their attention on αT.

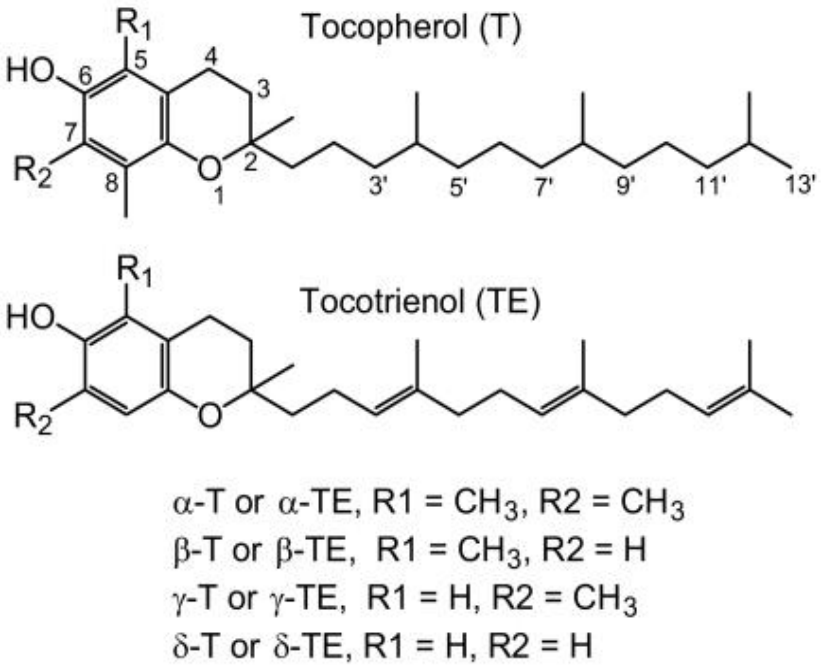

Figure 1.1. Structures of Naturally Occurring Vitamin E

Tocopherols and tocotrienols have a chromanol ring and a 13-carbon side chain. Tocotrienols have 3 double bonds on the side chain while tocopherols have fully saturated side chain. Substitution on R_1 and R_2 positions on the chromanol ring structure determines the subtype α, β, γ and δ of tocopherols and tocotrienols (8).

The first investigation, also the most well-known biological activity of vitamin E was as an antioxidant. αT has been found to be an important lipophilic antioxidant that terminates the chain reaction of lipid peroxidation initiated by free radicals. In the early 1980s, Burton *et al* studied αT's antioxidant property both *in vitro* and *in vivo*. α-T was first found to inhibit lipid peroxidation induced by free radicals in rat liver microsomes by converting the free radicals into harmless hydroperoxide products thus removing the free radicals from the destructive propagative cycle of lipid peroxidation (9). In fact, compared with the commercial antioxidant, butylated hydroxytoluene (BHT), the antioxidant activity of α-T was proved to be approximately 200 times greater with a peroxyl radical. This suggests that αT is a very effective antioxidant.

Since 1990, other biological functions of vitamin E have also been studied. For example, αT was found to dose dependently potentiate basal prostacyclin release, a potent vasodilator and platelet aggregation inhibitor, in rat heart and human umbilical vein endothelial cells (10,11,12). This study demonstrated that in addition to the antioxidant property, αT also exhibits non-antioxidant activities. Besides, α-T has been found to inhibit smooth muscle cell proliferation by inhibiting protein kinase C activity (13).

In late 1990s, researchers attention was drawn to γT, the major dietary form of vitamin E. Studies found that the urinary excreted metabolite of γT, γ-CEHC, is a natriuretic factor. Interestingly, γ-CEHC also inhibits cyclooxygenase-2 (COX-2) catalyzed synthesis of pro-inflammatory prostaglandin E_2 (PGE_2) in macrophages and epithelial cells thus

revealing the bioactivity of vitamin E metabolites (14). This finding led to the investigation of vitamin E metabolism to understand how it becomes the bioactive metabolite.

1.2 Vitamin E Metabolism

The metabolism of vitamin E has been studied ever since 1960s. However, the clear picture of the metabolic pathway weren't characterized until recently. The absorption and distribution of vitamin E has been studied since 1980s using deuterium-labeled αT (15). Pharmacokinetic samples from rats after uptake of deuterium-labeled αT were analyzed by GC-MS to elucidate the transport and distribution of vitamin E. The discovery of urinary excreted metabolites of δT, δ-CEHC enlighted the investigation of catabolism pathway of vitamin E (5). Studies revealed that human liver HepG2 cells actively metabolize γT into γ-CEHC and a 5'-carboxychromanol γT metabolite, 2,7,8-trimethyl-2-(delta-carboxymethylbutyl)-6-hydroxychroman (γ-CMBHC), as identified by GC-MS/MS analysis of the extracted HepG2 cells cultured medium incubated with 50 μM of γT for 48 h (16). It has also been reported that dietary intake of sesame seed or sesamin, a potent inhibitor of cytochrome P450 enzyme, elevated the concentration of γT in plasma, liver and lung of rats supplemented with high level of γT in the diet (17). Based on these evidences, a study by Parker *et al* in 2000 proved that the γT catabolism pathway is cytochrome P450 enzyme dependent and is inhibited by sesamin using HepG2 as the experiment model (18). The hepatic catabolism pathway of truncating the side chain of γT was elucidated by the same group in 2002. Intermediate metabolites of

γT, 7'-, 9'-, 11'- and 13'-carboxychromanols were detected and identified by GC-MS analysis of HepG2 cell cultured media extracts. It was also elucidated that the discrimination of αT metabolism by γT is partially due to their different enzyme specific activity of CYP4F2, the isoform of cytochrome P450 enzyme responsible for the metabolism of vitamin E. This finding brought a new insight to the study of vitamin E metabolism. In 2007, Jiang *et al*. discovered a novel metabolism pathway of γT and δT in human lung epithelial A549 cells by HPLC-FL assay, electrospray and inductively coupled plasma mass spectrometry(19). These novel vitamin E metabolites were identified as sulfated long chain carboxychromanols. This finding provided evidence that sulfation occurs as part of the intracellular metabolism of vitamin E. Vitamin E metabolism is described in more detail in the sections below.

1.2.1 Overview of Intestinal Absorption, Transport and Tissue Distribution of Vitamin E

The absorption of vitamin E was studied in Caco-2 cells and in transgenic mice models. Dietary vitamin E is esterified by fatty acids and incorporated into micelles and hydrolyzed by pancreatic lipase. Once vitamin E is located in the GI lumen, it is absorbed in the middle of gastric intestinal tract, in the distal jejunum (20). Intestinal uptake of vitamin E is initiated by two types of apical membrane bound protein, scavenger receptor class B type 1 (SR-B1) and Niemann-Pick type C1-like 1 (NPC1L1). The major secretion route of intestinal absorbed vitamin E is through chylomicron whereas a smaller fraction is secreted via ATP-binding cassette A1 (ABCA1), both on the basolateralside. Vitamin E secreted from the intestine in chylomicron then transport via

the lymphatic system to the peripheral tissues. The resulting chylomicron remnants are taken up by the liver where vitamin E is metabolized and excreted into the circulation (21).

In hepatocytes, α-tocopherol transport protein (α-TTP) and ABCA1 preferentially binds with α-T and incorporates it into lipoproteins which facilitates its transport into peripheral tissues via the circulation (22,23). Other unbound vitamin E forms are catabolized in hepatocytes. Conjugated and unconjugated vitamin E metabolites generated in the intracellular catabolism are excreted through urine and bile, respectively (5,16). Vitamin E not utilized or metabolized in cell is also discarded via bile by the ABC transporter MDR2 P-glycoprotein (24).

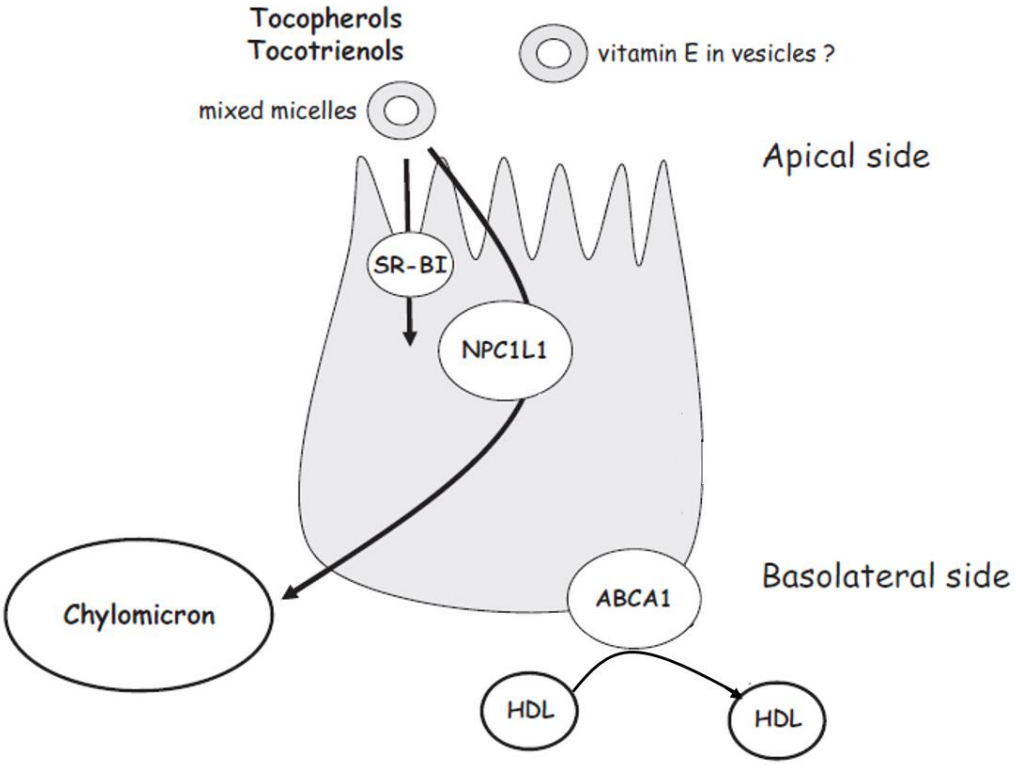

Figure 1.2. Intestinal Absorption of Vitamin E.

Vitamin E uptake occurs at the apical membrane of the intestinal cells by the proteins NPC1L1 and SR-BI. After absorption, a small fraction of vitamin E is secreted by ABCA1 on the basolateral side via an apolipoprotein AI pathway. The main fraction is secreted in chylomicrons via the apolipoprotein B pathway (25).(Adapted from the original figure)

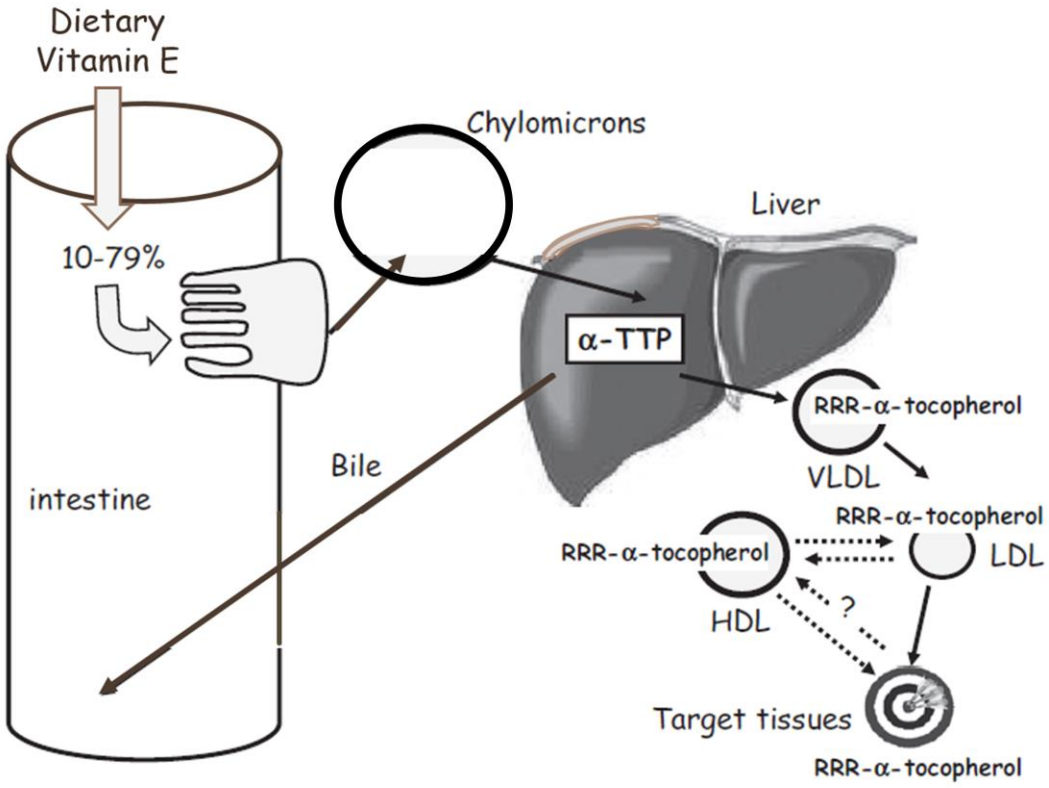

Figure 1.3. Transport and Tissue Distribution of αT

Vitamin E secreted from intestine is packaged in chylomicron and taken up by liver. α-TTP preferentially binds with αT and, with the assistance of ABCA1, incorporates them into lipoprotein which then transports αT into the circulation and distribute to peripheral tissue. Except for αT, large portions of other forms of vitamin E, such as γT, γTE and δT, are catabolized in hepatocytes through cytochrome P450 mediated intracellular catabolism pathway (25).(Adapted from the original figure)

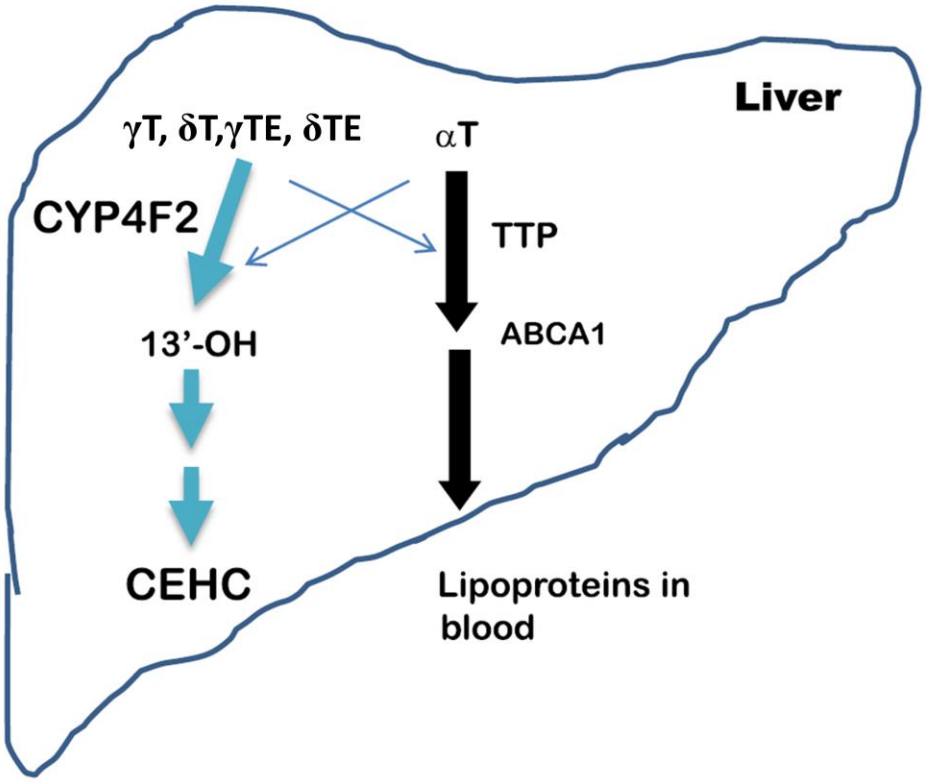

Figure 1.4 Transport of γT, δT and TEs

Except for αT, a majority of the vitamin E forms are metabolized by the cytochrome P450 enzyme mediated intracellular catabolism pathway in the liver. The metabolites generated from the metabolism pathway enters the circulation and the end product, CEHCs are excreted in urine and bile. Small portions of the unmetabolized non-α-forms of vitamin E are transported by α-TTP and incorporated in lipoproteins into the circulation and distributed in peripheral tissue(26).(Adapted from the original figure)

1.2.2 Intracellular Catabolism Pathway of Vitamin E

Vitamin E has been found to be extensively metabolized in human lung epithelial cells and human hepatocytes (16,27). After vitamin E is taken up by the liver and transported into hepatocytes, it is translocated into endoplasmic reticulum and metabolized by cytochrome P450 enzyme mediated ω-hydroxylation, which forms 13'-hydroxychromanol (13'-OH). This water-soluble product is then located into mitochondria and metabolized by 5 cycles of step-wise β-oxidation, which truncates vitamin E side-chain by 2-carbon moiety (18,28). Sulfation, parallel to β-oxidation, has been found to occur to the intermediate and terminal vitamin E metabolites by the effect of cytosolic sulfotransferase(19,29). These sulfated long chain metabolites of vitamin E were found in both cultured A549 human lung epithelial cells and plasma of rats gavaged with γT.

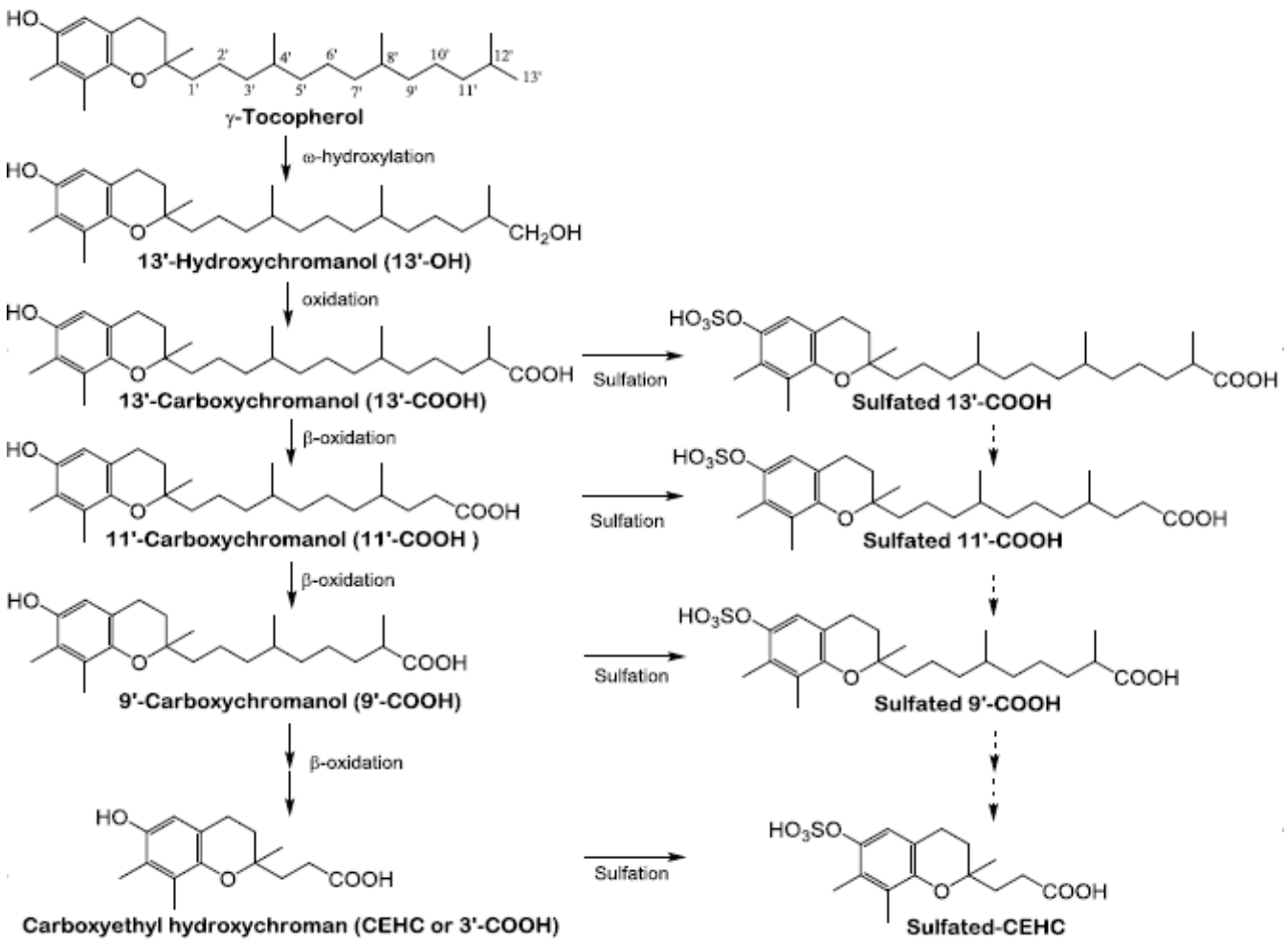

Figure 1.5. Intracellular Catabolism of Vitamin E (Representatively Shown by γT).

Vitamin E metabolism is initiated by CYP4F2 mediated ω-hydroxylation and ω-oxidation in endoplasmic reticulum then followed by 5 cycles of β-oxidation in the mitochondria and peroxisome. Parallel to the β-oxidation, the carboxychromanols are sulfated by cytosolic sulfotransferase and generating sulfated metabolites. Whether the sulfated vitamin E metabolites can also be β-oxidized is not clear yet (19,26). (Adapted from the original figure)

1.2.3 Regulation of Vitamin E Metabolism

It's well known that there's difference of bioavailability among vitamin E forms. However the reason was not revealed until lately. Although being consumed in US diet in a much higher content compared to αT, the tissue retention of γT is relatively low(4).Another evidence of regulation of vitamin E metabolism is that γTE, the counterpart of γT with unsaturated side-chain, are much more extensively metabolized than γT in human liver HepG2 cells and lung epithelial A549 cells (30,31). This evidence suggests that vitamin E metabolism is tightly regulated.

Up to now, only 1 study suggested different intestinal absorption between tocotrienols and tocopherols. The study by Abuasol *et al* in 2012 (32) compared the NPC1L1 mediated intestinal uptake of γTE and αT in rats hepatoma cells. The NPC1L1 kinetic studies demonstrated higher V_{max} value for αT compared with γTE. These findings suggest that intestinal absorption mediated by NPC1L1 favors αT compared to γTE.

Vitamin E metabolism is mainly regulated by the intracellular metabolism and the hepatic excretion. So far, there are three important regulators that have been identified to exhibit different biological activity or affinity towards different forms of vitamin E.

Firstly, α-TTP, the protein that is responsible for the hepatic secretion of vitamin E to the circulation and peripheral tissue, has different binding affinity towards vitamin E isomers. Liposomal membranes were incubated with ^{3}H labeled vitamin E isoforms to

investigate the structural characteristics of vitamin E analogs required for recognition by α-TTP. This study found that αT exhibits the highest ligand binding affinity (100%) and calculated the affinity of other vitamin E forms base on competition: βT, 38%; γT, 9%; δT, 2%; αTE, 12% (33,34). This finding is in line with their tissue concentrations. Interestingly, in patients with deficiency of α-TTP, urinary excreted α-CEHC is at significantly higher level compared to healthy participants (35). The increase of αT degradation in α-TTP deficient patients suggests that α-TTP plays an important role in regulating the metabolism of αT.

Secondly, the cytochrome P450 enzyme mediating the ω-hydroxylation in intracellular catabolism, CYP4F2, exhibits different activity to γT and γTE(36). In human hepatocyte HepG2 cells and human liver microsomes expressing recombinant cytochrome P450 enzyme (isoform CYP4F2) incubated with vitamin E isoforms, both systems have shown to exhibit substrate preference for γTE over γT. Enzyme kinetic study further proved that CYP4F2, the cytochrome P450 enzyme involved in ω-hydroxylation of the methyl group on the phytyl chain, has higher binding affinity and enzyme specific activity towards γTE than γT. The preferential ligand binding and enzyme specific activity of CYP4F2 towards tocotrienols results in faster and more extensive hydroxylation of γTE than γT. This allows the faster metabolism of γTE than γT (36,37).

The third regulator of intracellular catabolism is the cytosolic sulfotransferase. Following the ω-hydroxylation, 13'-OH' side chain is degraded via β-oxidation. These water soluble

carboxychromanols are sulfated in the cytosol. SULT1, a family of human cytosolic sulfotransferases, are so far the only known enzyme mediating the sulfation reaction of tocopherols and their metabolites. Researchers found that in human lung epithelial cells incubated with vitamin E isoforms and ^{32}S labeled sulfate, more ^{32}S-Sulfate was generated in cells incubated with γ-T than α-T (29). This is confirmed by the enzyme kinetic study where SULT1 has 4 fold higher substrate preference and less than 3 fold higher enzyme specific activity for γ-T than α-T. However, SULT1's binding affinity for tocotrienols has not been investigated yet. But it's speculated that SULT1 has higher binding affinity for tocotrienols than tocopherols due to the fact that more sulfated metabolites are generated by γTE than γT(31). Combining the effect of these regulators in intracellular metabolism of vitamin E, among all the tocopherols, α-T is most preferentially bound by α-TTP and is mostly retained in body tissue rather than being metabolized while other forms of vitamin E are preferentially metabolized. Among these unbound forms of vitamin E, γTE is preferentially hydroxylated by CYP4F2 and then β-oxidized and sulfated and excreted via urine or bile.

1.3 Analysis Methods of Vitamin E Metabolites

Quantitative analysis of vitamin E enhances our knowledge about the bioavailability and bioactivity of vitamin E. Since 1940s, researchers investigated analytical methods to quantify vitamin E. The earliest quantification method of vitamin E was by Quaife in 1948 (38). Biological samples, including liver, heart and kidney were homogenized and extracted with organic solvents. Then the extracted samples were reacted with the diazotized *o*-dianisidine to couple with tocopherols and form colored compounds. Samples are then step-wise diluted and measured by the absorbance at 520 nm. He reported > 90% recovery rate of all the biological samples spiked with same concentration of tocopherols. This method had been the most accurate method up to this time. GLC and TLC had also been used to separate tocopherols, however the method has been reported with poor reproducibility(39). In 1970s, researchers used high speed liquid chromatography (HSLC) with reversed phase column to separate tocopherols and used a UV spectrometer to detect signal of tocopherols. This method proved to recover almost 100% of different forms of vitamin E.

Gas chromatography-mass spectrometry (GC-MS) was another popular quantification method of vitamin E. However, GC is not capable of quantifying sulfated vitamin E metabolites due to the inertness of the conjugated phenolic group to chemical derivatization. HPLC-FL detection allowed detection of sulfated metabolites and has good sensitivity to the chromanol ring structure regardless of the length of side chain. However, in terms of samples from animals or human fed with various forms of vitamin

E, metabolites could not be separated due to their similarity in polarity. LC-MS/MS becomes the optimum detection method for vitamin E and metabolites, especially in biological samples due to its ability to eliminate back ground interference and specificity upon each individual compound. The instrumentation of LC-MS/MS will be explained in the following section.

1.4 Instrumentation of LC-ESI-MS

MS/MS analysis specifies the triple quadrupole (QQQ) instrument to monitor specific precursor ion and product ion mass-to-charge ratio thus eliminates the background noise interference and creates a brighter signal for the analytes. Eluents from liquid chromatography are nebulized and the sample droplets are emitted into the ionization zone. Among different ionization methods, electrospray ionization have been shown to exhibit highest sensitivity for vitamin E metabolites. In the ESI zone, sample droplets are charged with a high voltage to electrode charges to ensure formation of ions. These ions then enter the mass spectrometer for analysis. QQQ consist of 3 sets of parallel rods. The first quadrupole (Q1) filters the m/z value of all the ions in the sample and allows selective ions to pass through and enter the second quadrupole (Q2). Q2 is a collision cell in which selective precursor ions are applied with designated collision energy and fragmented by collision induced dissociation. These fragmented ions then enter the third quadrupole (Q3) for a second stage of filtering.

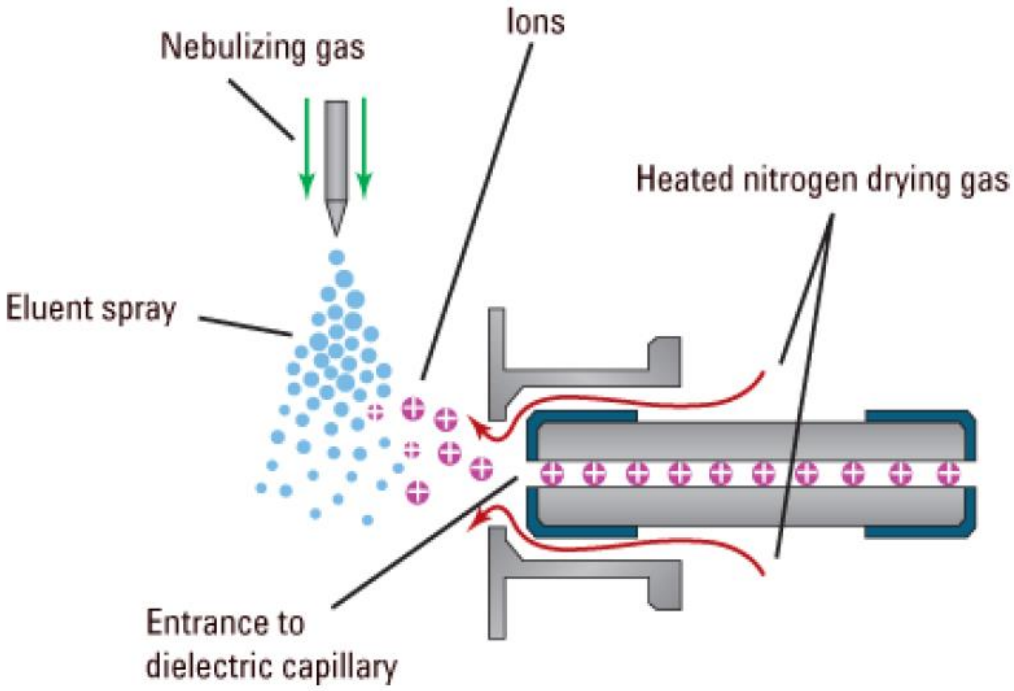

Figure 1.6 Orthogonal Introduction and Electrospray Ionization

Sample is ionized by the nebulizing spray with the addition of super heated nitrogen gas, which significantly improves the signal-to-noise ratio by hastening the drying of the ions (40). Both positive and negative ionization can be achieved (41). (Adapted from the original figure)

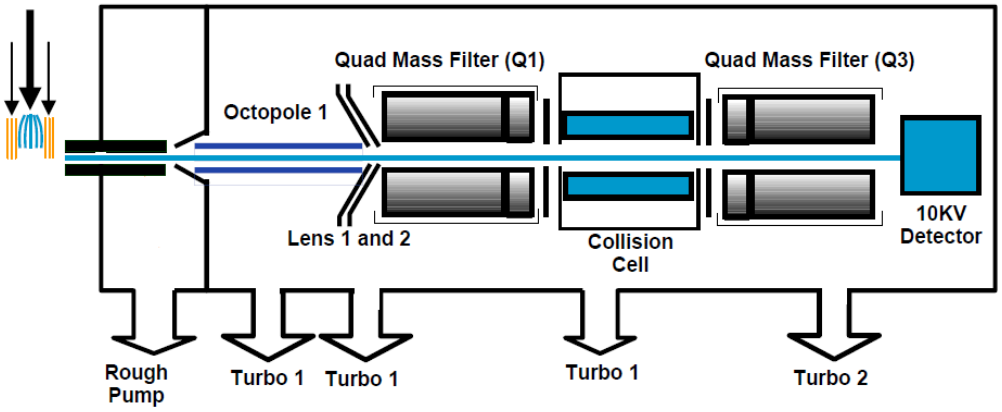

Figure 1.7 Triple Quadrupole MS

After ionization, ions are guided by the octopole to the triple-quadrupole where they are analyzed. Each quadrupole consists of four parallel rods. Ions arrived at the first quadrupole (Q1) are filtered and only selected ions are allowed to pass through. In the Q2, which is a collision cell, precursor ions are fragmented. The fragment ions formed in the collision cell are then sent to the Q3 for a second filtering stage. The triple quadrupole allows monitoring of multiple precursor to product ion transitions (40). (Adapted from the original figure)

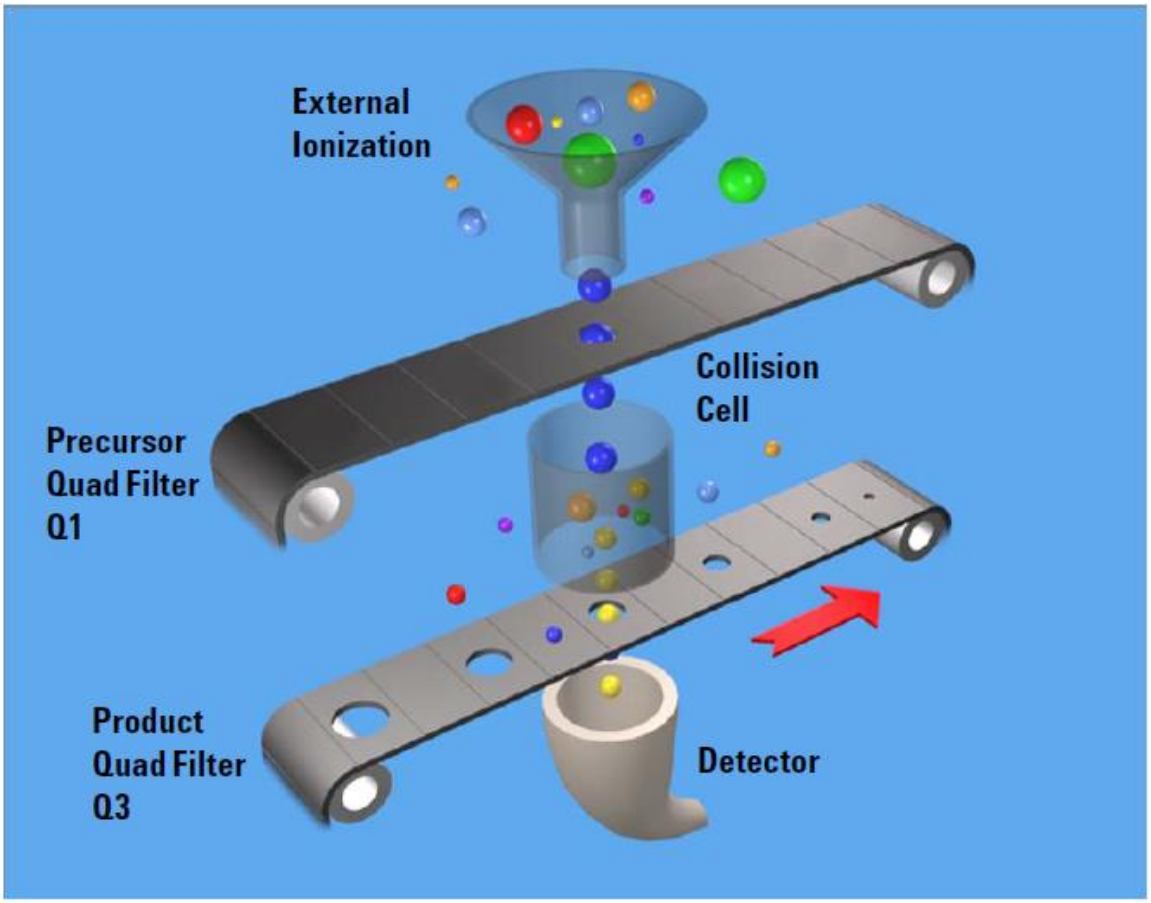

Figure 1.8 Mass Spectrometer

Ions generated by the ionization source are collected into the funnel (Q1). Ions with different mass-to-charge (m/z) ratio are analyzed on a moving belt where ions with designated precursor ion m/z value are allowed to pass through the opening on the belt and travel to the collision cell (Q2). Collision cell is actually a hexapole filled with nitrogen gas. Specified voltage is applied to the ions in the collision cell for fragmentation. The fragment ions are then scanned through the third quadrupole, all product ions (product ion scan MS/MS) or selective product ions (MRM) are passed through the belt and collected into the detector (40). (Adapted from the original figure)

QQQ instrument allows 3 types of sample analysis, total ion scan, product ion scan and multiple reaction monitoring (MRM) analysis.

In the case of a total ion scan, also known as a full scan, only 1 quadrupole will be used to detect all the ions. In this case the sensitivity would be compromised compared to product ion scan or MRM since the filter will scan through all the ions instead of selected ions.

In terms of product ion scan, Q1 and Q2 can be fixed to up to 4 precursor ion m/z and 4 specific collision energy levels in total. Q3 will scan through all the fragment ions and result in a product ion scan MS/MS chromatogram. This process identifies fingerprint of the compounds and is commonly used to optimize the acquisition method that yields highest intensity of the product ion scan.

The most sensitive and most commonly used analysis method of QQQ is MRM mode. In this case, both belts are fixed to allow specific precursor ions and product ions to pass through with designated collision energy. In order to quantify vitamin E metabolites in biological samples, the acquisition methods have been optimized by product ion scan analysis using vitamin E standards.

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CHAPTER 2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Reagents used in cell culture, like trypsin, and fetal bovine serum (FBS), were purchased from Invitrogen (Gland Island, NY). Cell medium, including McCoy's 5A modified medium and Dulbecco's modified eagle medium (DMEM) were purchased from American Type Culture Collection (ATCC company, Manassas, VA). Different forms of vitamin E, including γ-tocopherol and δ-tocopherol, 3-(4, 5-dimethylthiazol-2-yl)-2, DMSO and all other chemicals were purchased from Fluka and Sigma (St. Louis, MO). γ-tocotrienol was a gift from Klaus Kramer (BASF, Ludwigshafen, Germany).

2.2 Cell Lines and Treatment

Human lung epithelial A549cells (purchased from ATCC company, Manassas, VA), were used in this study(19). A549 cells were routinely grown in 75 cm^2 culture flask containing fresh McCoy's 5A medium supplemented with 10% FBS. The flasks were placed in 37^oC, 95% air and 5% CO2 humidified incubator, and the caps were loosened if not vented During experiments, cells were seeded in McCoy's 5A medium containing 10% FBS in each well of the 6-well plates at a density of 1×10^6 cells/well. Cells became attached after overnight incubation prior to treatment. The next day, medium was replaced with.

fresh DMEM containing 1% FBS and γTE 20 μM and incubated for 72h or δTE-13' at 10 μM and incubated for 4, 8, 12, 16 or 24h. Medium was collected to analyze the metabolites.

2.3 Preparation of Vitamin E Standard Solutions

γ-Tocopherol, δ-tocopherol were dissolved in 100% ethanol as stock solutions at the concentration of 500 μM and stored at -20^{o}C.γ-CEHC, α-CEHC, δTE-13' and δT-13' were dissolved in 100% ethanol as stock solutions at the concentration of 2.5mM and stored at -20^{o}C. The stock solution was further diluted into 100μM with 100% ethanol. At the time of experiment, all standard solutions were protected from light by aluminum foil.

2.4 Optimization and Validation of Extraction Method

Various vitamin E standards, namely γ-CEHC, α-CEHC, α-CMBHC, δTE-13', δT-13', δT and γT were spiked into Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) to mimic cell cultured medium samples or plasma samples, respectively, or 100% ethanol as the standard solution, to the final concentrations of 2 μM, 10 μM, 2 μM, 1 μM, 0.4 μM,0.4 μM and 0.4 μM, respectively, to achieve proper detection by HPLC-Fluorescence (HPLC-FL) assay. These vitamin E standards were extracted by the method described in section 2.5 and 2.6 and resuspended and analyzed by HPLC-FL. The recovery rate of vitamin from DMEM and FBS were calculated by comparing their fluorescence intensity with that from the standard solution to calculate the extraction efficiency.

2.5 Extraction of Vitamin E and Metabolites in Cell Cultured Medium

The vitamin E metabolites, tocopherols and tocotrienols were extracted from the cell cultured media by mixing 100ul of cell culture media with 600ul of working methanol solution (contains 0.2mg/ml ascorbic acid) and 500ul hexane. After mixing and a brief centrifugation (10,000 rpm for 2 minutes), the hexane layer which contains tocopherols and tocotrienols and methanol layer that contains the vitamin E metabolites were collected separately. Butylated hydroxytoluene (BHT) solution (66mg/ml) was added to the hexane layer and mixed as an antioxidant of the tocopherols and tocotrienols. Another 400ul of working methanol was added to the protein residue to extract any remaining vitamin E metabolites. After vortex and centrifugation, the methanol layer was combined with the previous one. All organic layers were dried under N_2 and stored in -20^{o}C until use.

2.6 Extraction of Vitamin E and Metabolites in Fetal Bovine Serum and Animal Plasma Samples

The vitamin E metabolites, tocopherols and tocotrienols were extracted from the Fetal Bovine Serum (FBS) or animal plasma samples by mixing 100ul of FBS sample or animal plasma samples with 600ul of working methanol solution (contains 0.2mg/ml ascorbic acid) and 1.2ml of hexane. After mixing and a brief centrifugation (13,200 rpm for 2 minutes), the hexane layer which contains tocopherols and tocotrienols and methanol layer that contains the vitamin E metabolites were collected separately. BHT solution (66mg/ml) was added to the hexane layer and mixed as an antioxidant for the

tocopherols and tocotrienols. Another 400ul of working methanol was added to the protein residue to extract any remaining vitamin E metabolites. After vortex and centrifugation, the methanol layer was combined with the previous one. All organic layers were dried under N_2 and stored in -20^{o}C until use.

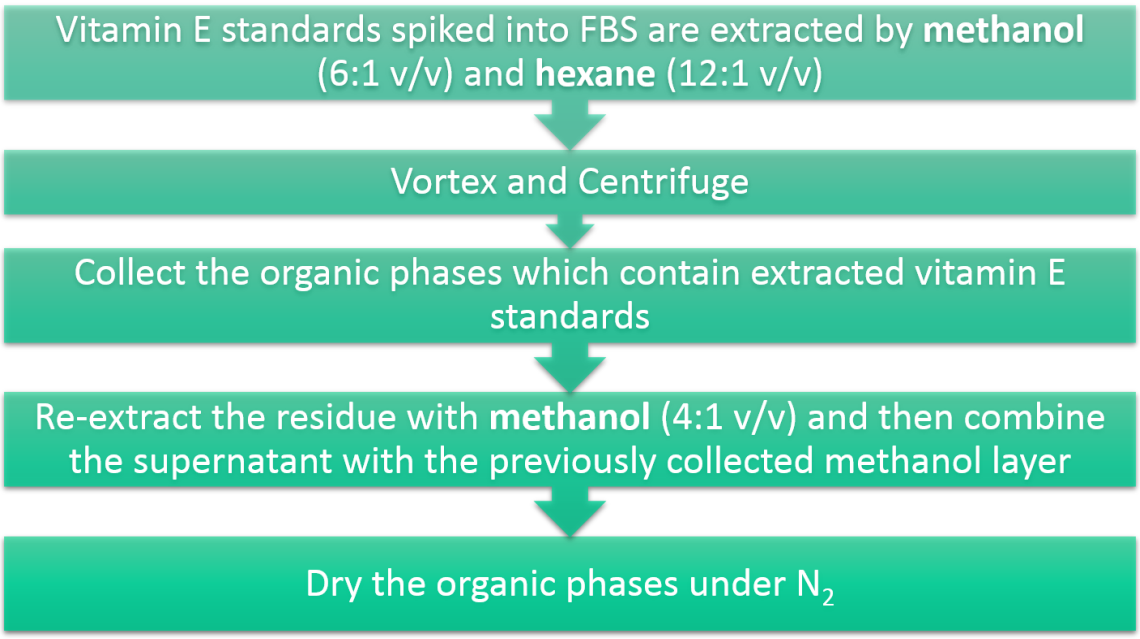


Figure 2.1 Method of Extraction of Vitamin E Standards in FBS

Vitamin E standards spiked into FBS are extracted by methanol (6:1 v/v) and hexane (12:1 v/v). After 1 min of vigorous vortex and 2 min of centrifugation at 13,000 rpm, organic phases which contain vitamin E standards are collected. The residue was re-extracted with methanol (4:1 v/v). The supernatant was combined with the previously collected methanol layer. All the organic phases were dried under N_2.

2.7 Extraction of Vitamin E Metabolites in Animal Diet

The vitamin E metabolites were extracted from the diet pellets fed to animals by dissolving around 150 mg of chopped animal diet in 1ml of working methanol solution (contains 0.2mg/ml ascorbic acid). After mixing, the diet sample is further homogenized with homogenizer and pestle for around 30 strokes. Put the sample on ice for 2 min and then isolate 350ul of supernatant to a new tube. Dry under N2 and store at -20 ^{o}C until analysis.

2.8 Extraction of Vitamin E Metabolites in Animal Feces

The vitamin E metabolites, tocopherols and tocotrienols were extracted from the animal feces by dispersing around 25 mg of animal feces in 200ul of working methanol solution (contains 0.2mg/ml ascorbic acid). The feces pellet was broken down to small pieces and then another 1.8 ml working methanol solution was added. The dispersion was homogenized with the pestle and the homogenizer for around 30 strokes until the particles are mostly broken down. After homogenization, put the sample on ice for 2 min and then collect 1.8ml of supernatant to a new tube. Centrifuge at 10,000 rpm for 5 min at 4^{o}C and then transfer 1.4ml supernatant to a new tube. Dry under N_2 and store at -20^{o}C until analysis.

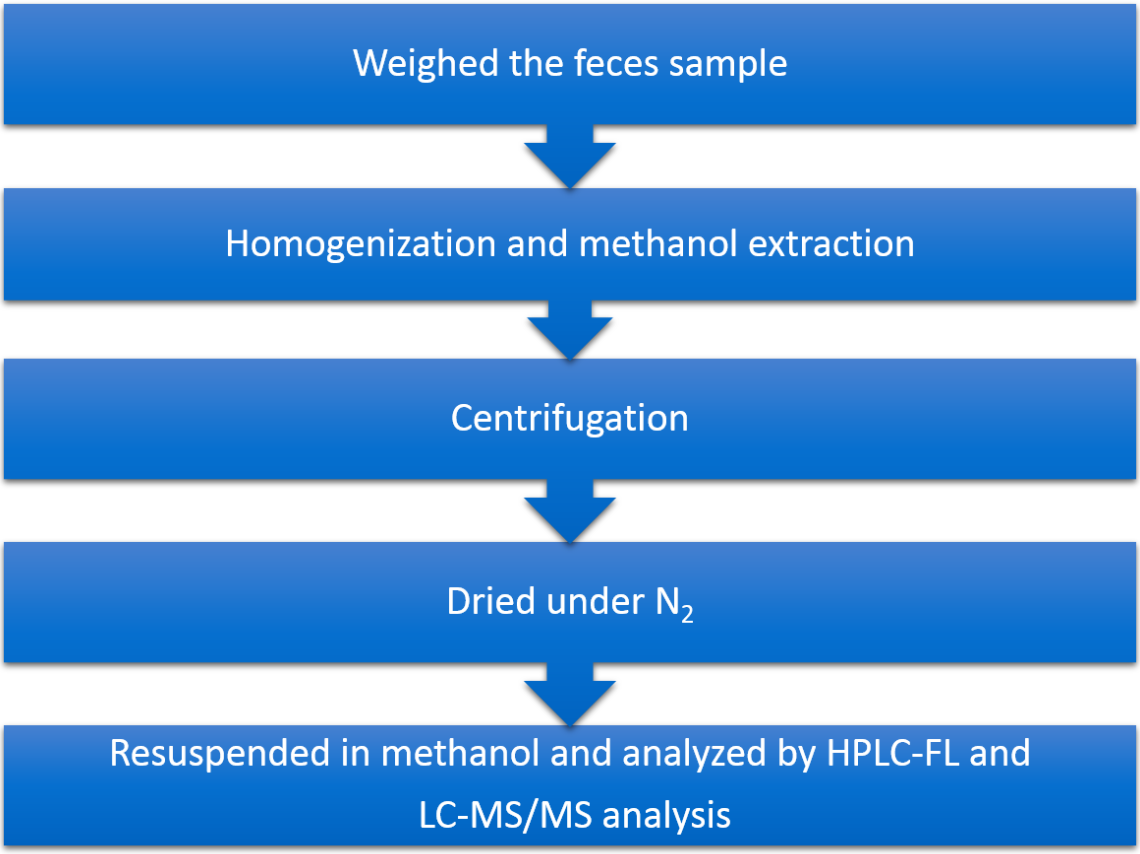


Figure 2.2 Method of Extraction of Vitamin E Metabolites in Animal Fecal Samples

As described in section 2.8, animal fecal samples were weighed and homogenized under a homogenizer. Vitamin E metabolites were extracted by 2 ml of methanol. 1.8 ml of supernatant was collected after sitting on ice for 2 min. The dispersion was centrifuged at 10,000 rpm at 4 ^{o}C for 5 min and 1.4 ml of supernatant was collected to a new tube. The sample was dried under N_2 and resuspended for HPLC-FL and LC-MS/MS analysis.

2.9 Sulfatase Treatment of Vitamin E Metabolites

100 µl of plasma or cell cultured medium sample was extracted by the method described in section 2.5 and 2.4, respectively. Extracted plasma and medium samples were each dissolved in 1 µl of ethanol and reconstituted in the incubation buffer, 0.2 M NaAc solution at pH 5.0. Sulfated vitamin E metabolites were desulfated by sulfatase (sulfatase from *Helix pomatia* S9626, purchased from Sigma) at 30 U/ml (dissolved in 2 mg/ml NaCl solution). The enzyme concentration and buffer used in the sulfatase treatment were based on the recommendations of the manufacturer (Sigma). After 3h or overnight incubation at 37 ^{o}C with vortexing during the initial 3h of incubation, samples were re-extracted twice with working methanol solution (contains 0.2 mg/ml ascorbic acid) and analyzed by HPLC-fluorescence. In the case of quantifying γTE-9'S in isolated γTE-9'S solution and vitamin E metabolites in biological samples, internal standard δTE-13' was spiked in each sample to a final concentration of 0.5 µM to check extraction efficiency and stability of vitamin E metabolites during overnight incubation.

2.10 High-Performance Liquid Chromatography-Fluorescent (HPLC-FL) Assay for Measuring Vitamin E Metabolites

Extracted vitamin E metabolites from cell cultured medium, plasma, feces and animal diets (method described in 2.4, 2.5, 2.7 and 2.6, respectively) were resuspended in working methanol (0.2mg/ml ascorbic acid) followed by mixing and brief centrifugation (10,000 rpm for 2 min). The supernatant was transferred to a vial and injected by Hitachi HPLC auto-sampler. The flow rate was 1.05 ml/min. Metabolites were separated using

HPLC and detected by a Shimadzu RF-10AXL spectrofluorometric detector (Shimadzu, Columbia, MD). The excitation and emission wavelengths were set at292 and 327 nm, respectively. The samples were injected through a Hitachi L-7200 auto-sampler (San Jose, CA). The mobile phases were A (35% acetonitrile and 65% 10 mM ammonium acetate at pH 4.3) and B (96% acetonitrile and 4% 10 mM ammonium acetate at pH 4.3). The metabolites were separated on a 5 mm Supelcosil LC-18-DB column, 4.6 × 3 × 150 mm (Supelco, Bellefonte, PA), using a flow rate of 1.0 ml/min with the following gradient: 100% A for 8 min, linearly increasing to 100% B from 8 to 30 min, 100% B until 55 min, and then back to 100% A at 56 min. Vitamin E and their metabolites were quantified by external standards due to the good linearity between the concentration of vitamin E and vitamin E metabolites and their peak area (19).

2.11 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Extracted samples are analyzed by liquid chromatography-tandem mass spectrometry equipment at the Metabolites Profiling Facility at Bindley Bioscience Center. Vitamin E pellets were dissolved in working methanol (0.2 mg/ml ascorbic acid) followed by mixing and brief centrifugation (10,000 rpm for 2 min). Internal standard, α-CMBHC or δTE-13' were spiked into each sample to the final concentration of 0.5 µM. The supernatant was transferred to a vial and 10 µl from each sample was injected by Agilent auto-sampler (model G1367D). Biological samples, including cell cultured medium, animal feces and plasma samples, were injected through an Agilent G1312B binary pump and separated by an Atlantis dC 18 column, 3 µm, 2.1 ×150 mm from Water's Corporation (Milford,

MA), using a flow rate of 0.3 ml/min and the following gradient: 100% A for 1 min, linearly increasing to 100% B from 1 to 30 min, 100% B until 40 min, and then back to 100% A at 43 min. Mobile phase A consisted of acetonitrile/ethanol/water (165/135/700 v/v/v) and mobile phase B consisted of acetonitrile/ethanol/water (539/441/20 v/v/v)(42).

The LC-MS/MS analysis was done with an Agilent 1200 liquid chromatography system coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer equipped with a jet stream electrospray ionization (ESI) source (Santa Clara, CA).Multiple reaction monitoring (MRM) mode, product ion scanning mode and total ion scanning mode were used to analyze each compound. Negative polarity ESI was used with the following source conditions: gas temperature 325°C, gas flow 10 L/minute, nebulizer pressure 30 psi, sheath gas temperature 250°C, sheath gas flow 7 L/minute, capillary voltage 4000 V, nozzle voltage 1500V, and an electron multiplier voltage of-300V. In total ion scanning mode, the range of precursor ion mass-to-charge ratio was set between 100 and 1000. In product ion scanning mode, the mass-to-charge ratio of the precursor ion was set at 453, 479, 521 or 427 to analyze γTE-9'S, γTE-11'S, γTE-13'S or δTE-13' (2-double-bond form), respectively. The collision energy was set at 15, 25, 35 and 45 V to observe the fragmentation pattern. The condition of gradient and ion source used in total ion scanning mode and product ion scanning mode were the same from those used in multiple reaction monitoring mode. All data was evaluated with Agilent Mass Hunter Qualitative and Quantitative Analysis software, version B.01.04 or B.06.00.

2.12 Optimization of MS/MS Parameters of LC-MS/MS Analysis

MS/MS parameters, also called the acquisition method, specify the conditions by which the Agilent 6460 QQQ mass spectrometer acquired data from the samples. These parameters include precursor ion mass, product ion mass, collision energy, polarity, etc. These parameters were optimized by conducting product ion scanning which allows comparison of the intensity of varied fragmentation conditions. The following table is a final version of the acquisition method after the optimizations were made to the method.

Table 2.1 Optimized MS/MS Parameters

Compound Name	*Precursor Ion*	*Product Ion*	*Fragmentor*	*Collision Energy*
α-CEHC (α-3’-COOH)	277.2	233	120	10
α-CMBHC (α-5’-COOH)	319.2	150	180	20
α-CMHHC (α-7’-COOH)	347.2	163	180	20
αT-9'	389.3	163	120	35
αT-11'	417.3	163	120	35
αT-13'	459.4	163	120	35
αT-13’-OH	445.4	163	120	35
α-Tocopherol	429.4	163	120	20
δ-CEHC (δ-3’-COOH)	249.1	205	120	10
δ-SO3-CEHC	329.1	135	120	45
δ-CMBHC (δ-5’-COOH)	291.2	135	180	20
δ-CMHHC (δ-7’-COOH)	319.2	135	180	20
δT-9'	361.3	135	120	35
δT-11'	389.3	135	120	35
δT-13'-OH	417.3	135	120	35
δT-13'	431.3	135	120	35
δT-9'S	441.2	135	120	45
δT-11'S	469.2	135	120	45
δT-13'S	511.3	135	120	45
δ-Tocopherol	401.4	135	120	35
δTE-5' (1 double bond)	289.2	135	120	35
δTE-7' (1 double bond)	317.2	135	120	35
δTE-9' (1 double bond)	359.2	135	120	35
δTE-9' (2 double bond)	357.2	135	120	35
δTE-11' (2 double bond)	385.3	135	120	35
δTE-11' (1 double bond)	387.3	135	120	35
δTE-13'-OH	411.3	135	120	35
δTE-13' (3 double bond)	425.3	135	120	30
δTE-13'-2 (2 double bond)	427.3	135	120	30
δTE-9'S (1 double bond)	439.2	135	120	45
δTE-9'S (2 double bond)	437.2	135	120	45

Table 2.1 Continued

Compound Name	*Precursor Ion*	*Product Ion*	*Fragmentor*	*Collision Energy*
δTE-11'S (2 double bond)	465.2	135	120	45
δTE-11'S (1 double bond)	467.3	135	120	45
δTE-13'S (3 double bond)	505.2	135	120	45
δTE-13'S (2 double bond)	507.3	135	120	45
δ-tocotrienol	395.3	135	120	35
γ-CEHC (γ-3'-COOH)	263.1	219	120	5
γ-CMBHC (γ-5'-COOH)	305.2	149	180	20
γ-CMHHC (γ-7'-COOH)	333.2	149	180	20
γT-9'	375.3	149	120	40
γT-11'	403.3	149	120	40
γT-13'-OH	431.4	149	120	40
γT-13'	445.3	149	120	40
γT-SO3-CEHC	343.1	149	120	30
γT-9'S	455.2	149	120	45
γT-11'S	483.3	149	120	45
γT-13'S	525.3	149	120	45
γ-Tocopherol	415.4	149	120	40
γTE-5' (1 double bond)	303.2	149	120	25
γTE-7' (1 double bond)	331.2	149	120	25
γTE-9' (2 double bond)	371.2	149	120	25
γTE-9' (1 double bond)	373.3	149	120	25
γTE-11' (2 double bond)	399.3	149	120	25
γTE-11' (1 double bond)	401.3	149	120	25
γTE-13'-OH (3 double bond)	425.3	149	120	35
γTE-13' (3 double bond)	439.3	149	120	35
γTE-13' (2 double bond)	441.3	149	120	35
γTE-9'S (2 double bond)	451.2	149	120	45
γTE-9'S (1 double bond)	453.2	149	120	45
γTE-11'S (2 double bond)	479.2	149	120	45
γTE-11'S (1 double bond)	481.2	149	120	45
γTE-13'S (3 double bond)	519.3	149	120	45
γTE-13'S (2 double bond)	521.3	149	120	45
γ-Tocotrienol	409.3	149	120	35

2.13 Quantitation of Vitamin E and Metabolites by LC-MS/MS Assay

A mixture of 100 μM of each of the vitamin E standards, namely γ-CEHC, α-CEHC, δTE-13', δT-13', δT, γT (for the quantification of animal plasma and fecal samples, also included γTE-9'S and αT standards) was step-wise diluted to 10 μM, 1 μM, 0.5 μM, 0.1 μM and 0.05 μM (for the quantification of animal plasma and fecal samples, diluted the standard mixture to 5 μM, 1 μM and 0.2 μM instead). Standard curve and R^2 value was calculated to examine the linearity of each standard. Usually the response of LC-MS/MS is in linear relationship to each standard's concentration. Concentration of vitamin E metabolites in samples are calculated by the external standard with the most similar structure and its peak area at a similar range as the unknown sample. Usually the peak area of the external standard is averaged by its adjacent points on the standard curve that fall in the linear range.

2.14 Isolation of γTE Metabolites from A549 Cells Cultured Medium

Medium of A549 cells cultured with 20 μM of γTE for 72h was collected and extracted by working methanol (0.2 mg/ml ascorbic acid) and hexane. Extracted medium sample was injected by HPLC auto-sampler and detected by ultraviolet (UV) detector (UVD340U) with excitation wavelength at 292 nm. The column and condition of gradient was the same as those used in HPLC-fluorescent assay. Eluent was collected when a peak appeared on the chromatogram. The components of these isolated γTE metabolite samples were identified by LC-MS/MS assay.

2.15 Quantification of Isolated γTE-9'S by HPLC-Fluorescence-UV Analysis

In order to quantify sulfated vitamin E metabolites, concentration of isolated γTE-9'S was measured by an HPLC-fluorescence-UV (HPLC-FL-UV) assay. Concentrated isolated γTE-9'S solution was incubated with incubation buffer (0.2 M NaAc solution) or sulfatase solution (20 mg/ml dissolved in 2 mg/ml NaCl solution) at 30 U/ml at 37 ^{o}C for 3 h at pH 5.0. Each sample was treated with 0.2 mg/ml ascorbic acid as antioxidant. During the 3 h incubation, each sample was gently vortexed each 15 min. After incubation, internal standard δTE-13' was spiked in each sample to the final concentration of 0.5 μM prior to methanol extraction. Each sample was then dried under N_2 and resuspended in working methanol (contains 0.2 mg/ml ascorbic acid) and injected by HPLC auto-sampler. Eluents were separated by the condition stated in section 2.9 and were detected by the UV detector and the fluorescent detector.

2.16 Animal Study

All animal studies were approved by the Purdue Animal Care and Use Committee. Male Wistar rats (230 - 260 g) (Charles River) were caged separately and routinely consumed a control diet (2018 Teklad Global 18% Protein Rodent Maintenance diet, Harlan Teklan) and tap water. Rats were grouped randomly and gavaged with a single dose of γT at 10, 50 or 100 mg/kg body weight, γTE at 10 or 50 mg/kg body weight or tocopherol-stripped corn oil for rats in the control groups. Rats were euthanized 6 h following the gavage and plasma was collected from the animals.

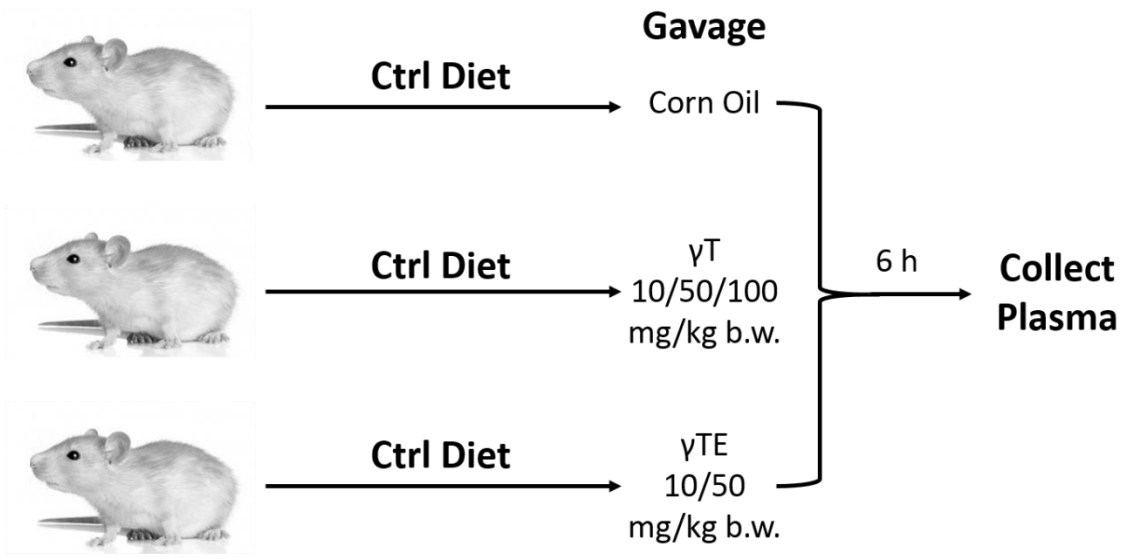


Figure 2.3 Design of Animal Study with Rats

Rats were fed a control diet and gavaged with γT at 10, 50 or 100 mg/kg b.w., γTE at 10 or 50 mg/kg b.w. or corn oil as control group. Their plasma were collected 6 h after being gavaged.

In another study, Male Balb/c mice (5–6weeks) from Harlan (Indianapolis, IN) were single-housed under controlled temperature and were given unrestricted access to diets and water. Mice were randomly grouped into control, γT supplementation and δT supplementation groups. Mice in the control group received control diet (AIN-93G) while the supplementation groups received diet fortified with γT or δT at 0.1% diet. After a week of acclimatization and diet supplementation (for the supplementation group), mice were given an intraperitoneal injection of azoxymethane (AOM) at 10 mg/kg to induce colon cancer. A week following the AOM injection, colitis was induced in all animals by 7 days of administration of 1.5% DSS (dextran sodium sulfate) in drinking water. Animals were observed for another 18 weeks and euthanized on day 140 following the AOM injection. Plasma was collected from these animals. Their food intake was recorded every day and provided in adequate amount throughout the 140-day experiment.

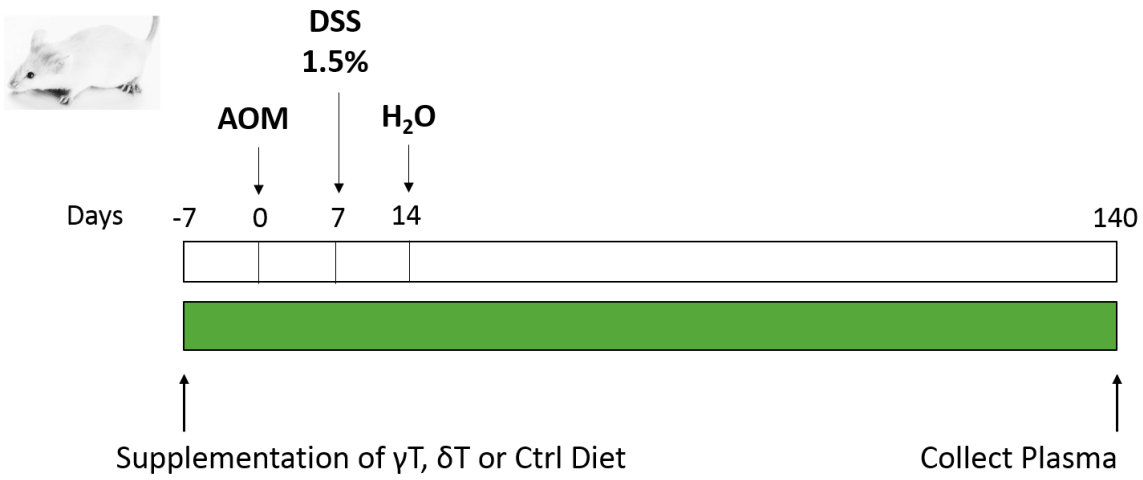


Figure 2.4 Design of Animal Study with Mice

Male mice were supplemented with control diet (AIN-93G), diet fortified with 0.1% of γT or δT. After a week, mice were injected AOM at 10 mg/kg b.w. to induce colon carcinogenesis. Another week later, colitis was induced by administration of 1.5% of DSS in drinking water. Water was changed to normal tap water after a week of DSS administration. Animals were observed for another 18 weeks then euthanized. Plasma and fecal samples were collected from the animals.

CHAPTER 3. RESULTS

3.1 Extraction Efficiency

In order to obtain the optimum ratio of volume of organic solvent versus biological samples, vitamin E standards were spiked in DMEM, FBS and ethanol at the same concentration. These vitamin E standards were then extracted from DMEM and FBS samples, mimicking the biological samples, with varied volumes of hexane and working methanol (0.2 mg/ml ascorbic acid) and injected by HPLC auto-sampler and analyzed by fluorescent assay. The recovered concentration of standards are calculated by the peak area of directly injected ethanol dissolved vitamin E standards. To validate the optimum extraction method, extraction efficiency and inter-day, intra-day variation were calculated based on the recovered concentration of vitamin E standards.

The extraction efficiency result shows that for FBS samples, 6 volumes of working methanol (0.2 mg/ml ascorbic acid) and 12 volumes of hexane with another extraction of 4 volumes of working methanol extracts more than 90% of all the standards spiked into FBS.

Table 3.1 Extraction Efficiency and Intra-Day and Inter-Day Variation of Vitamin E Metabolites Extracted from FBS Samples

	External Standards						
	γ-CEHC	**α-CEHC**	**α-CMBHC**	**δTE-13'**	**δT-13'**	**δT**	**γT**
Average Extraction Efficiency	97%	89%	96%	98%	95%	107%	101%
Inter-Day Variation	3%	11%	6%	10%	5%	11%	8%
Intra-Day Variation	9%	4%	7%	4%	3%	7%	6%

In four parallel experiments, FBS/working methanol/hexane/working methanol (1/6/12/4 v/v/v/v) extraction method yields 97% recovery rate of γ-CEHC, 89% of α-CEHC, 96% of α-CMBHC, 98% of δTE-13', 95% of δT-13', 107% of δT and 101% of γT. The inter-day and intra-day variation of all standards are within 11%.

3.2 Optimization of LC-MS/MS Acquisition Method for Sulfated γTE Metabolites

3.2.1 Identification of Profile of Isolated γTE Metabolites by LC-MS Total Ion Scan

In order to optimize the MS/MS parameter of sulfated γTE metabolites, γTE metabolites were separated from A549 cells culture medium using HPLC-UV assay. These isolated metabolites were first identified by total ion scan LC-MS assay with negative ESI. The range of ion mass was set between 100 - 1000. The chromatogram was then extracted to examine detected ion mass.

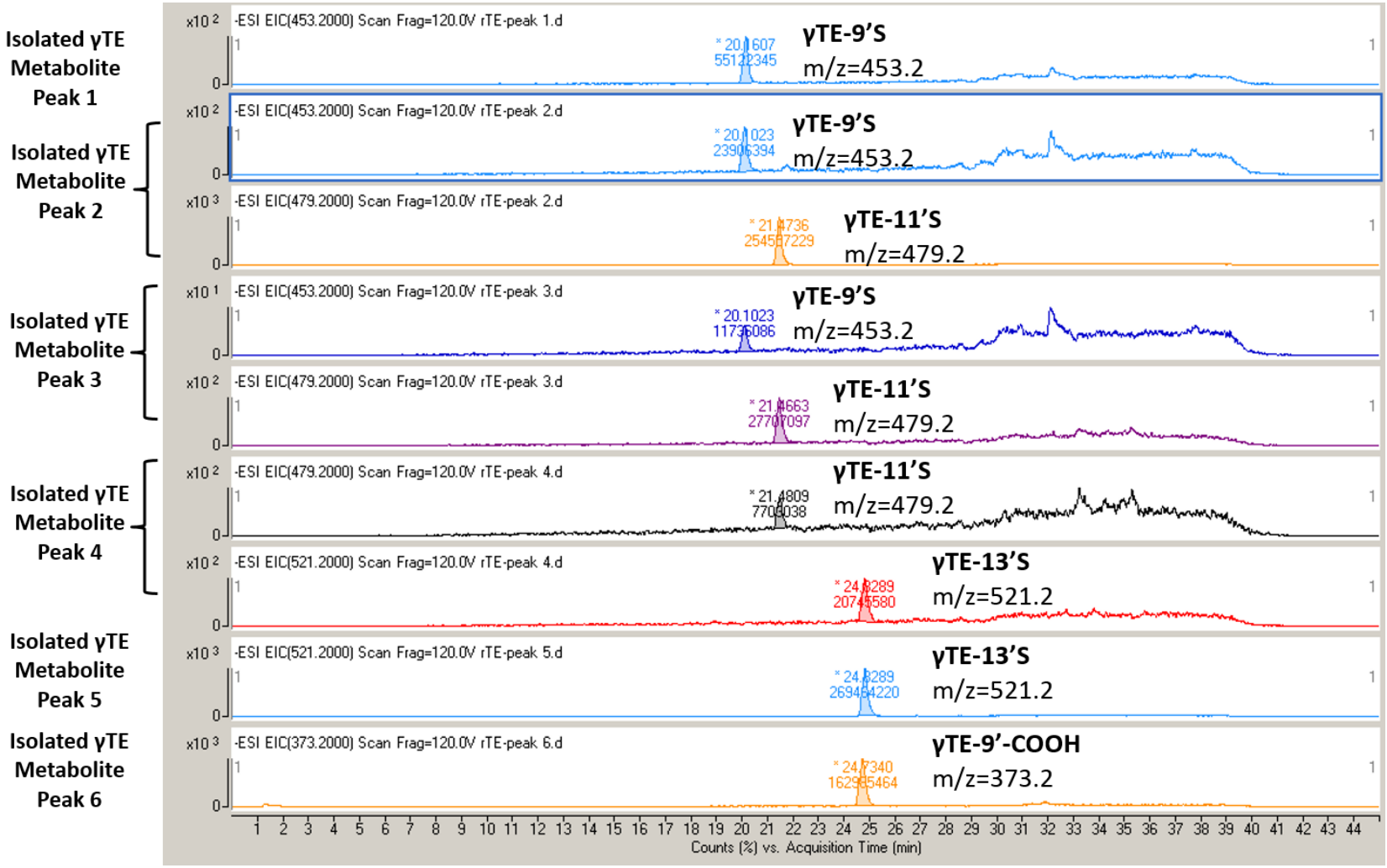


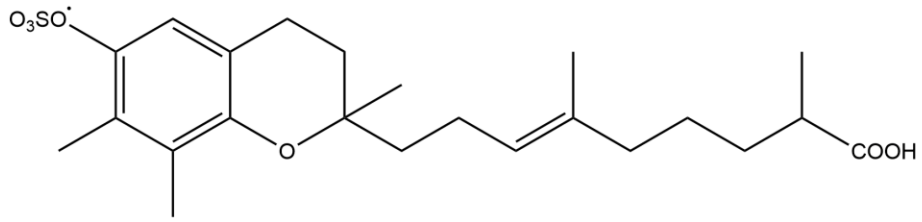
Figure 3.1 Profile of Isolated γTE Metabolites by Total Ion Scan of LC-MS Analysis

As shown in Figure 3.1, the major ion in isolated peak 1 displayed an m/z of 453.2, which is consistent with that of γTE-9'S ($[M-Z]^-$ = 453.2) while m/z of 479.2, 521.2 and 373.2 displayed in other isolated peaks suggest that they might be γTE-11's, γTE-13'S and γTE-9'. Their profile needs to be further confirmed by their fragmentation pattern by performing MS/MS assay.

Table 3.2 Intensity of Ions by Total Ion Scan of LC-MS Analysis

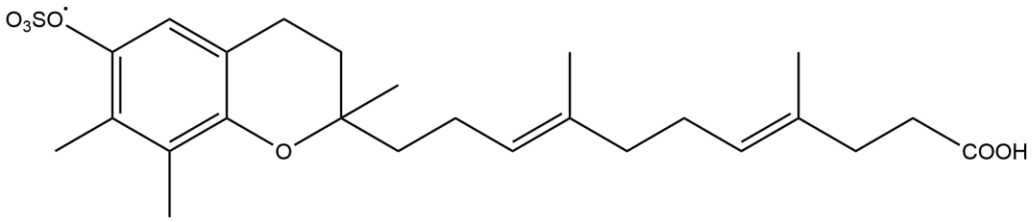
Peak #	m/z Value	Peak Profile	Retention Time (min)	Peak Intensity
1	**453.2**	**γTE-9'S**	**20.16**	**5.5E+07**
2	453.2	γTE-9'S	20.10	2.4E+07
2	**479.2**	**γTE-11'S**	**21.47**	**2.5E+08**
3	453.2	γTE-9'S	20.10	1.2E+07
3	479.2	γTE-11'S	21.46	2.8E+07
4	479.2	γTE-11'S	21.48	7.7E+06
4	521.2	γTE-13'S	24.82	2.1E+07
5	**521.2**	**γTE-13'S**	**24.83**	**2.7E+08**
6	**373.2**	**γTE-9'**	**24.73**	**1.6E+08**

Sample labeled in bold, isolated γTE metabolite peak 1, 2, 5 and 6 are most abundant in γTE-9'S, γTE-11'S, γTE-13'S and γTE-9' respectively, among all the samples. Based on the intensity of the ions detected in isolated γTE metabolites, product ion scan MS/MS mode analysis will be performed on these isolated peaks.



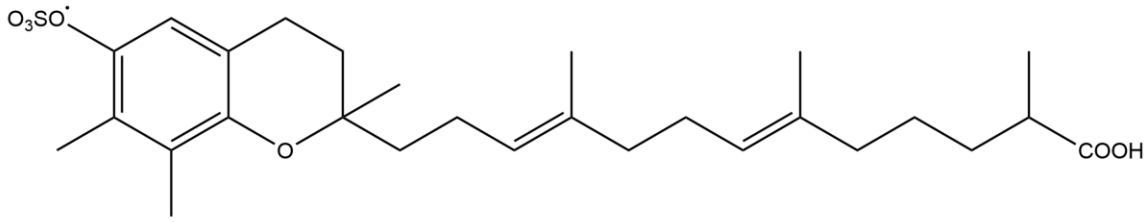
γTE-9'S

Chemical Formula: $C_{23}H_{33}O_7S^{\bullet}$ Exact Mass: 453.19



γTE-11'S

Chemical Formula: $C_{25}H_{35}O_7S^{\bullet}$ Exact Mass: 479.21



γTE-13'S

Chemical Formula: $C_{28}H_{41}O_7S^{\bullet}$ Exact Mass: 521.26

Figure 3.2 Molecular Structure of Ionized Sulfated γTE Long Chain Carboxychromanols

After negative mode ionization, the sulfated γTE long chain carboxychromanols are deprotonated thus loses 1molecular weight. The parental ion mass of γTE-9'S, γTE-11'S and γTE-13'S, would be 453.2, 479.2 and 521.2, respectively. The ion mass detected by total ion scan analysis is consistent with the postulated ion mass.

3.2.2 Identification of Profile of Isolated γTE Sulfated Metabolites by Product Ion Scan LC-MS/MS Analysis

Based on the result of total ion scan LC-MS analysis, peak 1 is the major peak of γTE-9'S, peak 2 and peak 5 are most abundant in γTE-11'S and γTE-13'S, respectively. Product ion scan LC-MS/MS analysis was performed on these isolated metabolites to confirm the profile of these metabolites and optimize the MS/MS parameters to acquire the highest intensity of these sulfated γTE metabolites. The fragmentation pattern of these metabolites in MS/MS mode would suggest the structure of these molecules which allows identification of the metabolites in the samples.

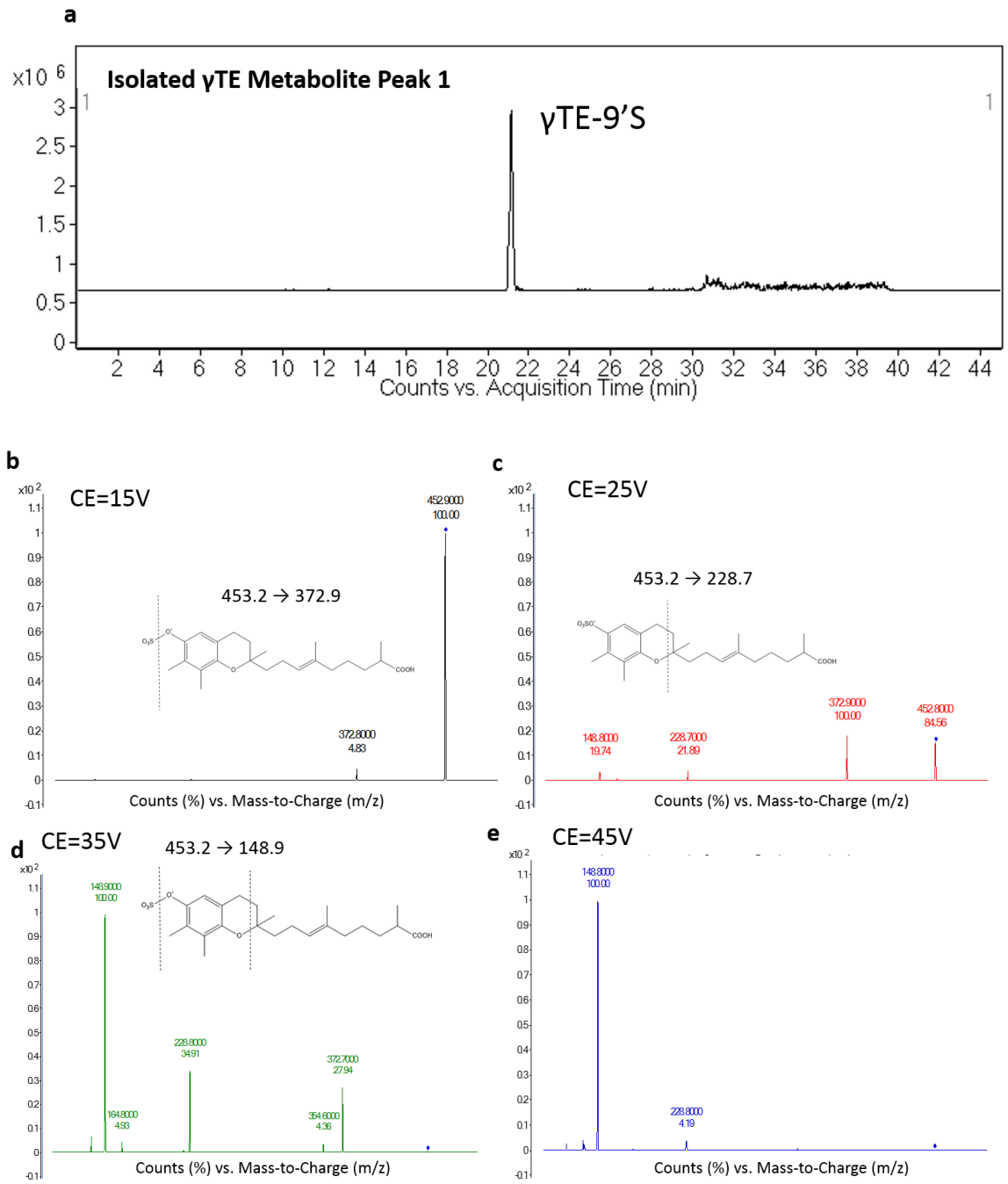


Figure 3.3 Chromatogram and MS/MS Spectrum of Product Ion Scan LC-MS/MS Analysis of Isolated γTE Metabolite Peak 1

Figure 3.3.a is the chromatogram of product ion scan analysis of isolated γTE metabolite peak 1 with MS/MS mode. 3.3.b, c, d, e are MS/MS spectrum of the product ions generated from precursor ion with m/z of 453.2 at collision energy level of 15, 25, 35 and 45 V, respectively. The major product ion in figure 3.3.b with m/z of 373, suggests the presence of SO_3 group in the molecular structure. The major product ion in figure 3.3.c and 3.3.d with m/z of 229 and 149, respectively, suggests retro-Diels-Alder fragmentation of the break in the bond between the O-1 and C-2 position and another bond between the C-3 and C-4 position in the chromanol structure, which is commonly observed with vitamin E metabolites (Figure 3.3). Based on these fragmentation patterns, we are able to confirm the isolated metabolite in peak 1 is γTE-9'S. The product ion with m/z of 149 at collision energy of 45 V showed the highest intensity among all the product ions generated at different collision energy levels.

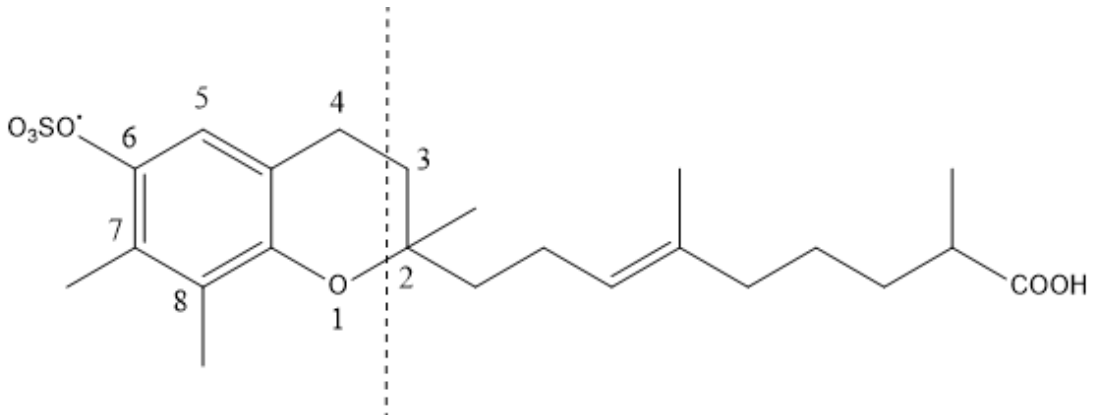


Figure 3.4 Retro-Diels-Alder Fragmentation of γTE-9'S

Retro-Diels-Alder fragmentation pattern has been observed in many chromanol structure molecules. It is also a common fragmentation pattern of vitamin E metabolites (43). In MS/MS mode, at 30 V of collision energy level, the bond between O-1 and C-2 and the bond between C-3 and C4 are broken.

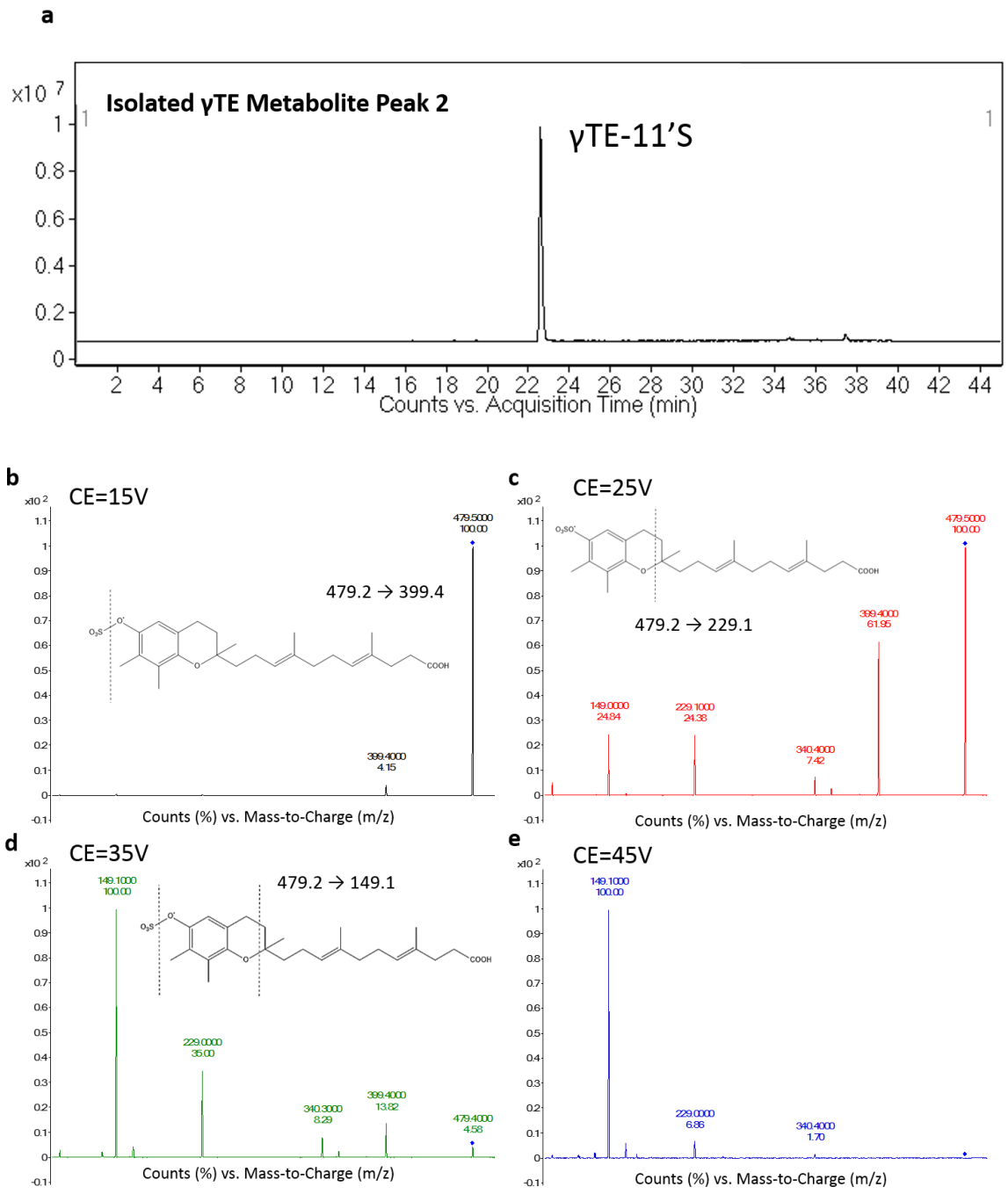


Figure 3.5 Chromatogram and MS/MS Spectrum of Product Ion Scan LC-MS/MS Analysis of Isolated γTE Metabolite Peak 2

Figure 3.5.a is the chromatogram of product ion scan analysis of isolated γTE metabolite peak 2 with MS/MS mode. 3.5.b, c, d, e are MS/MS spectrum of the product ions generated from precursor ion with m/z of 479.2 at collision energy level of 15, 25, 35 and 45 V, respectively. The major product ion in figure 3.5.b with m/z of 399, suggests the presence of SO_3 group in the molecular structure. The major product ion in figure 3.5.c and 3.5.d with m/z of 229 and 149, respectively, suggests retro-Diels-Alder fragmentation of the break in the bond between the O-1 and C-2 position and another bond between the C-3 and C-4 position in the chromanol structure (Figure 3.4). Based on these fragmentation patterns, we are able to confirm the isolated metabolite in peak 2 is γTE-11'S. The product ion with m/z of 149 at collision energy of 45 V showed the highest intensity among all the product ions generated at different collision energy levels.

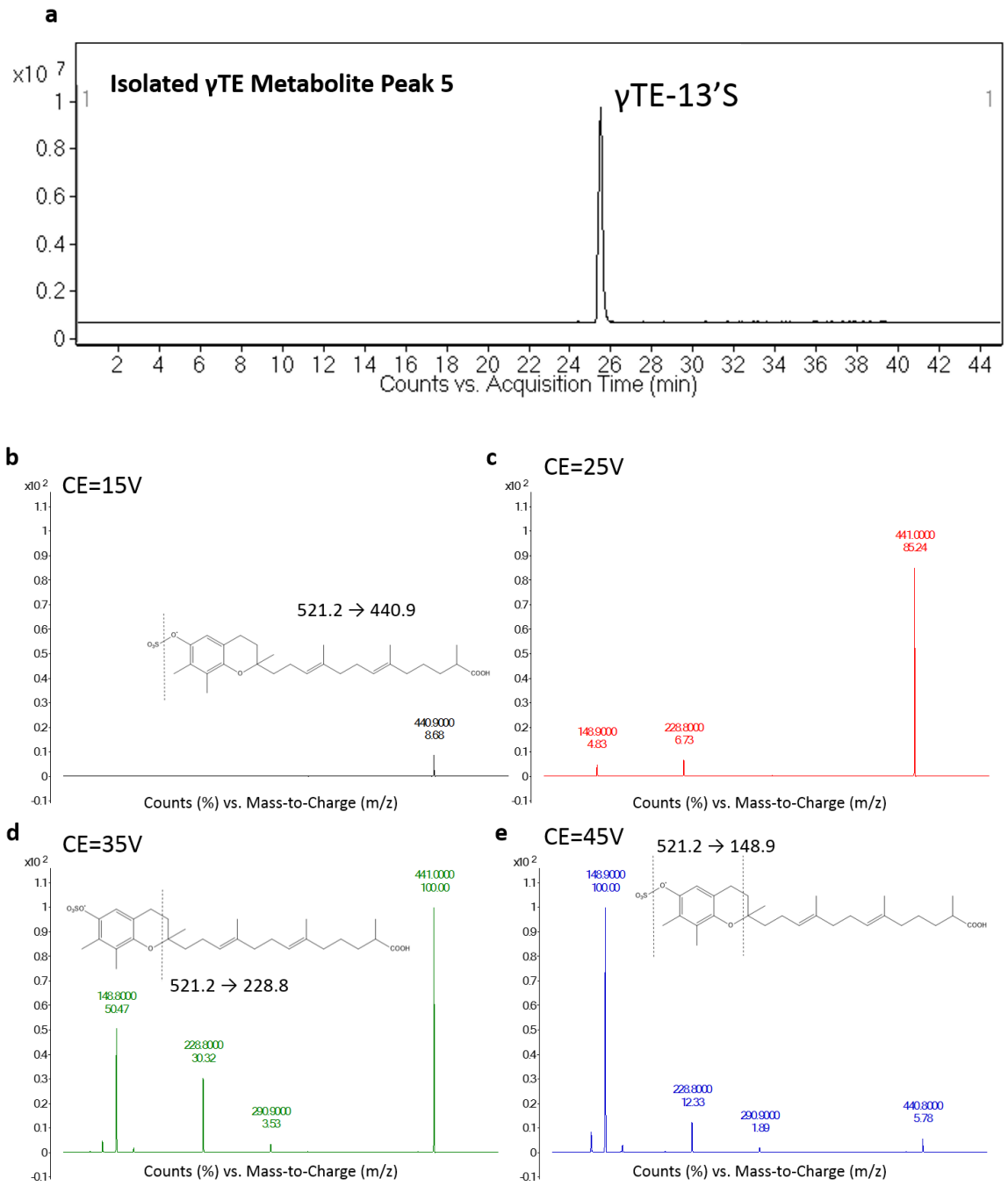


Figure 3.6 Chromatogram and MS/MS Spectrum of Product Ion Scan LC-MS/MS Analysis of Isolated γTE Metabolite Peak 5

Figure 3.6.a is the chromatogram of product ion scan analysis of isolated γTE metabolite peak 5 with MS/MS mode. 3.6.b, c, d, e are MS/MS spectrum of the product ions generated from precursor ion with m/z of 521.2 at collision energy level of 15, 25, 35 and 45 V, respectively. The major product ion in figure 3.6.b with m/z of 441, suggests the presence of SO_3 group in the molecular structure. The major product ion in figure 3.6.c and 3.6.d with m/z of 229 and 149, respectively, suggests retro-Diels-Alder fragmentation of the break in the bond between the O-1 and C-2 position and another bond between the C-3 and C-4 position in the chromanol structure (Figure 3.4). Based on these fragmentation patterns, we are able to confirm the isolated metabolite in peak 5 is γTE-13'S. The product ion with m/z of 441 at collision energy of 25 V showed the highest intensity among all the product ions generated at different collision energy levels.

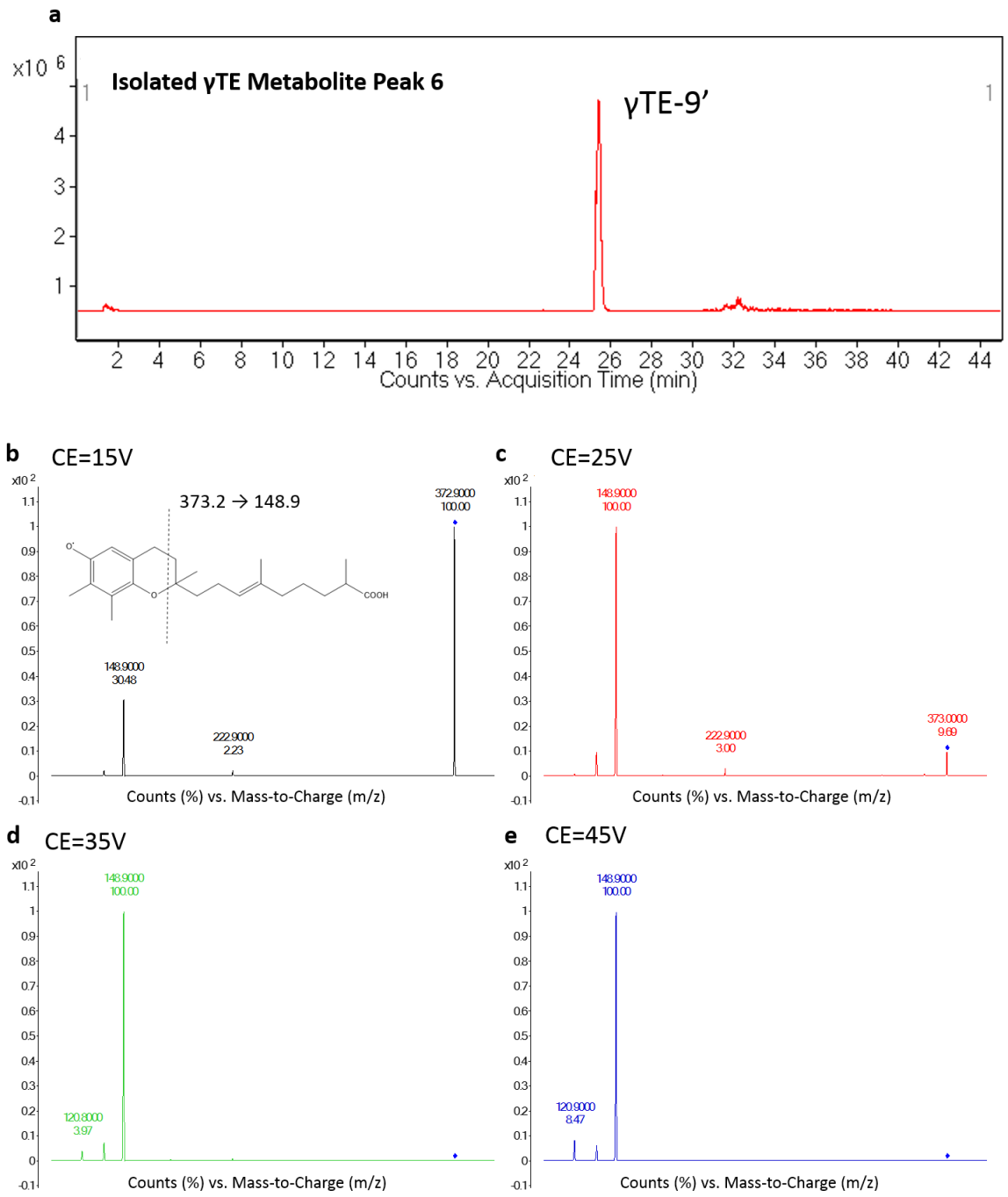


Figure 3.7 Chromatogram and MS/MS Spectrum of Product Ion Scan LC-MS/MS Analysis of Isolated γTE Metabolite Peak 6

Figure 3.7.a is the chromatogram of product ion scan analysis of isolated γTE metabolite peak 6 with MS/MS mode. 3.7.b, c, d, e are MS/MS spectrum of the product ions generated from precursor ion with m/z of 373.2 at collision energy level of 15, 25, 35 and 45 V, respectively. The common major product ion in figure 3.7.c, 3.7.d and 3.7.e with m/z of 149, suggests retro-Diels-Alder fragmentation of the break in the bond between the O-1 and C-2 position and another bond between the C-3 and C-4 position in the chromanol structure (Figure 3.4). Based on its fragmentation pattern, we are able to confirm the isolated metabolite in peak 6 is γTE-9'. The product ion with m/z of 149 at collision energy of 25 V, showed the highest intensity among all the product ions generated at different collision energy levels, although only slightly higher than the intensity of product ion with m/z of 149 at 35 V.

3.2.3 Confirmation of Optimum MS/MS Parameters of Isolated γTE Metabolites by LC-MS/MS Analysis in Multiple-Reaction Monitoring (MRM) Mode

In order to confirm that the intensity of product ion generated in LC-MS/MS product ion scan mode would be consistent with that generated in MRM mode, isolated γTE metabolites were analyzed by multiple reaction monitoring (MRM) mode LC-MS/MS assay. The most abundant product ion generated at each collision energy level was added to the list of MS/MS parameter (Table 3.3) prior to analysis to confirm the most sensitive MS/MS parameter for each sulfated γTE metabolites in MRM mode LC-MS/MS analysis.

Table 3.3 MS/MS Parameters of Isolated Sulfated γTE Metabolites Tested in MRM Mode

Compound Name	**Precursor Ion m/z Ratio**	**Product Ion m/z Ratio**	**Collision Energy (V)**	**Polarity**
γTE-9'S	453.1	373.0	25	Negative
γTE-9'S	453.1	229.1	35	Negative
γTE-9'S	453.1	149.1	45	Negative
γTE-11'S	479.1	399.0	25	Negative
γTE-11'S	479.1	229.1	35	Negative
γTE-11'S	479.1	149.1	45	Negative
γTE-13'S	521.1	441.0	25	Negative
γTE-13'S	521.1	229.1	35	Negative
γTE-13'S	521.1	149.1	45	Negative

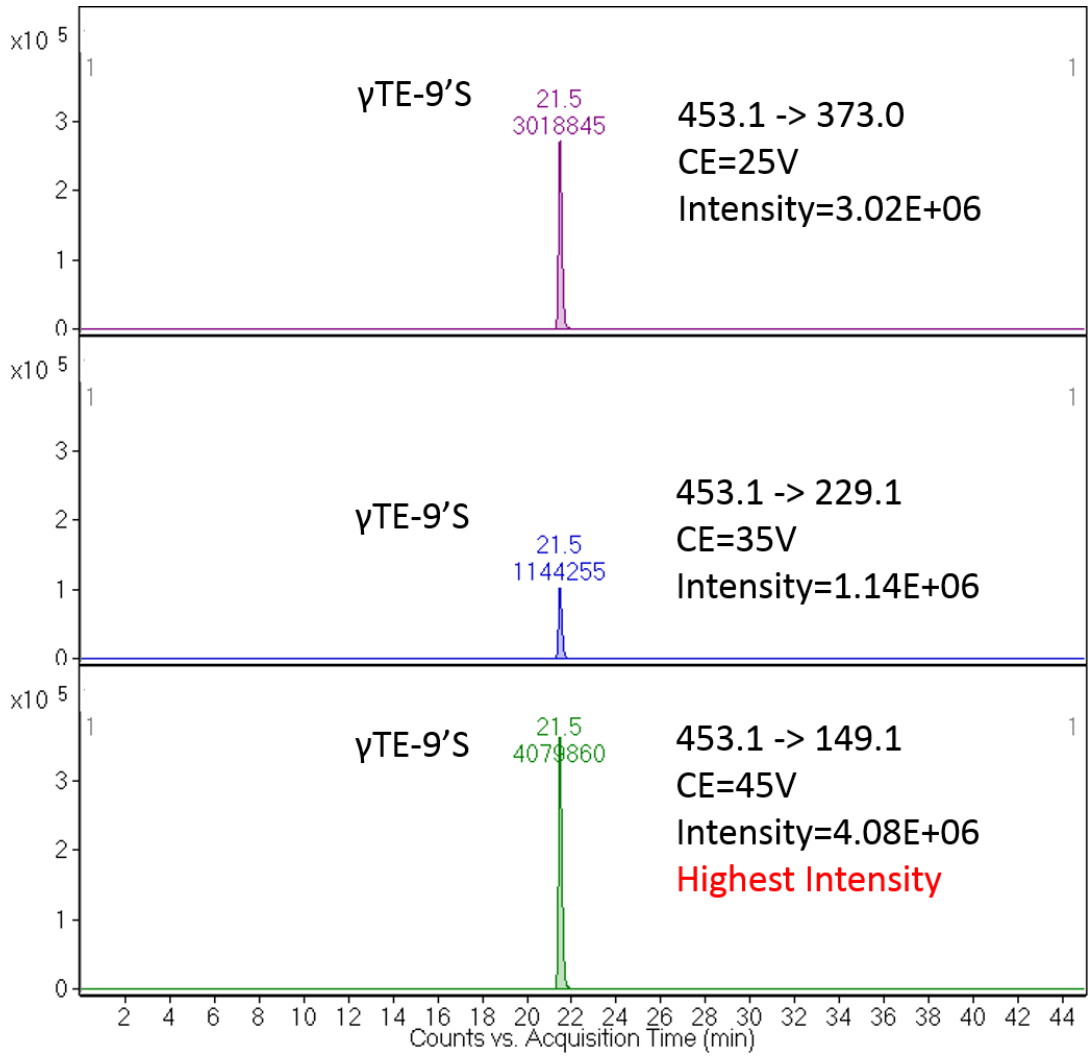


Figure 3.8 Chromatograms of LC-MS/MS Analysis of Isolated γTE-9'S with Different MS/MS Parameters in MRM mode

As shown in Figure 3.8, the sensitivity of MRM mode LC-MS/MS analysis with the transition 453 → 149 at collision energy of 45 V is the highest among all the examined acquisition parameters of γTE-9'S, followed by the transition 453 → 373 at 25 V and 453 → 229 at 35 V. This result is consistent with the result of product ion scan analysis of γTE-9'S. Thus the optimum MS/MS parameters ofγTE-9'S for MRM mode LC-MS/MS analysis are: precursor ion with m/z of 453.2, product ion with m/z of 149.1, collision energy of 45 V.

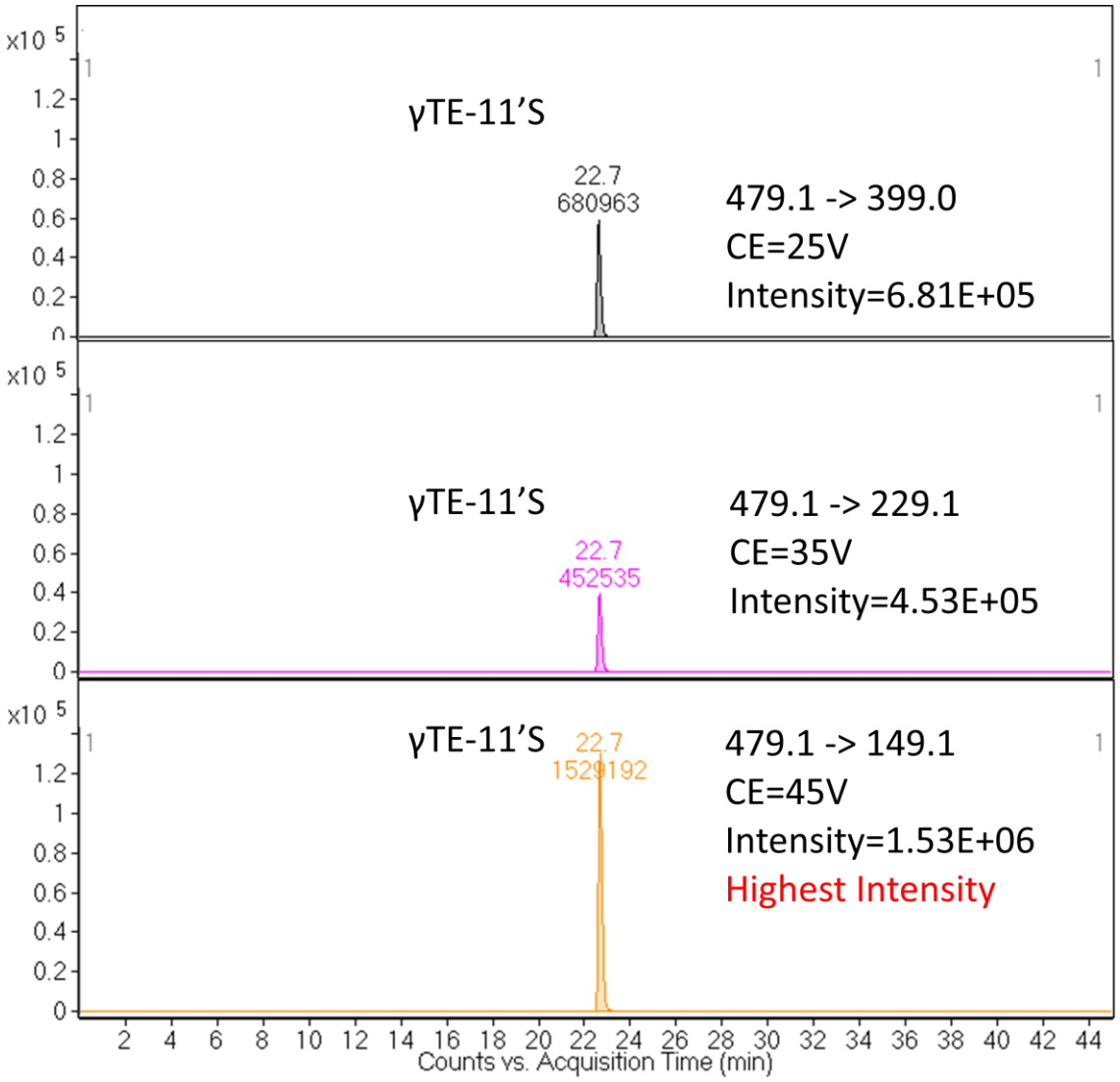


Figure 3.9 Chromatograms of LC-MS/MS Analysis of Isolated γTE-11'S with Different MS/MS Parameters in MRM mode

As shown in Figure 3.9, the sensitivity of MRM mode LC-MS/MS analysis with the transition 479 → 149 at collision energy of 45 V is the highest among all the examined acquisition parameters of γTE-11'S. The intensity of peaks detected by other parameters, namely 479 → 399 at 25 V and 479 → 229 at 35 V, are a lot lower compared to 479 → 149 at 45 V. This is consistent with the result of product ion scan analysis of γTE-11'S. Thus the optimum MS/MS parameters of γTE-11'S for MRM mode LC-MS/MS analysis are: precursor ion with m/z of 479.2, product ion with m/z of 149.1, collision energy of 45 V.

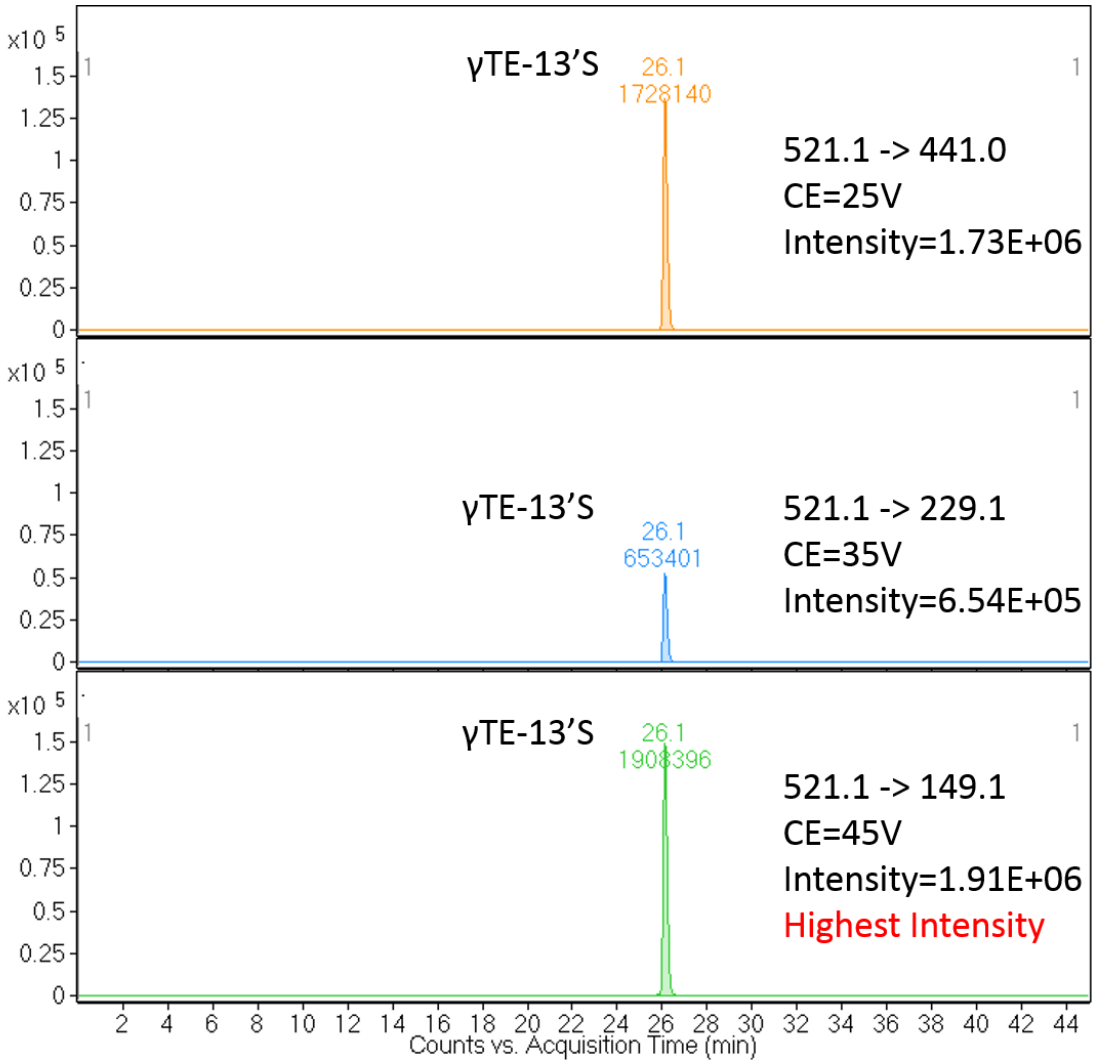


Figure 3.10 Chromatograms of LC-MS/MS Analysis of Isolated γTE-13'S with Different MS/MS Parameters in MRM mode

As shown in Figure 3.10, the sensitivity of MRM mode LC-MS/MS analysis with the transition 521 → 149 at collision energy of 45 V is the highest among all the examined acquisition parameters of γTE-13'S. The intensity of peak detected by the transition 521 → 441 at 25 V is only 10 % smaller while the intensity of peak detected by the transition 521 → 229 at 35 V is only 1/3 compared to the other two transitions. However, in product ion scan LC-MS analysis of γTE-13'S, the intensity of the product ion with m/z of 441 at 25 V was 25% higher than that of m/z of 149 at 45 V. The difference in sensitivity between product ion scan mode and MRM mode might explain the situation. For LC-MS/MS analysis of γTE-13'S in MRM mode, the optimum parameters are: precursor ion with m/z of 521.2, product ion with m/z of 149.1, collision energy of 45 V.

3.3 Quantification of Isolated γTE-9'S Standard Solution by HPLC-FL-UV

In order to quantify γTE-9'Sas an external standard, γTE-9'Swas isolated from cell cultured medium and was quantified by HPLC-UV detection coupled with fluorescent assay. Due to lack of external standards for the sulfated metabolite we were not clear about their linearity and sensitivity in UV and FL detection, quantification of γTE-9'S requires enzyme deconjugation to γTE-9' which can be quantified by δTE-13' by HPLC-UV detection. It has been demonstrated by our previous study (not published data) that UV intensity is not as sensitive as the fluorescence intensity to the chromanol ring configuration and side-chain modification. This makes δTE-13'an excellent external standard for γTE-9'. However, the intensity of the UV signal appears to be around 100 times lower than the intensity of the fluorescence signal. In order for the UV detector to detect γTE-9', we used concentrated γTE-9'S solution and converted around 90% into γTE-9' by the sulfatase. The concentration of γTE-9'S was calculated by adjusting the concentration of γTE-9' quantified by HPLC-UV analysis by the percentage converted γTE-9'S as measured by HPLC-fluorescent assay. Due to the linear relationship between fluorescent intensity and percentage of solvent B, fluorescent intensity is lower for γTE-9'S compared to γTE-9' (it elutes later than γTE-9'S). Thus connecting the fluorescent detector with the UV detector allows simultaneous detection of γTE-9'S (by the fluorescent detector) and γTE-9' (by the UV detector). The recovery rate of internal standard (δTE-13') spiked in each sample was within 85% - 95%. A sample calculation of concentration of γTE-9'S was included in the Appendix.

Table 3.4 Concentration of γTE-9'S Calculated by HPLC-FL-UV Analysis

γTE-9'S Conc. (μM)	**Average Conc. (μM)**	**Std Dev**	**CV%**
1471	1480	44	3%
1445			
1543			
1459			

The experiment was repeated on 4 different days. The average concentration of γTE-9'S in the concentrated isolated sample is 1.48 ± 0.044 mM.

The concentrated γTE-9'S standard solution was diluted 10 times to 148 μM with ethanol and stored at -20 ^{o}C. It was further diluted to 10 μM along with other vitamin E standards (γ-CEHC, α-CEHC, δTE-13', δT-13', δT, γT and αT) by step-wise dilution in order to quantify vitamin E metabolites using LC-MS/MS analysis with MRM mode.

3.4 Analysis of Tocopherol and δTE-13' in Animal Diet Pellets by HPLC-FL

Two forms of animal diets, control (Ctrl) diet (AIN-93G) and 250 mg/kg δTE-13' supplemented diet (purchased from Dyets Inc., Bethlehem, PA), were extracted and analyzed by HPLC-FL. The concentrations of components in different diets were calculated by external standards. The primary purpose for this experiment was to examine the concentration of δTE-13' in the diet to more accurately calculate the δTE-13'treatment given to the animals. Four independent extractions and analyses were conducted using both control diet and treatment diet.

Table 3.5 Concentration of Vitamin E in the Control Diet and δTE-13' Fortified Diet

	AIN-93G Diet	**AIN-93G+δTE-13' Diet**
	Average Conc. (mg/kg)	**Average Conc. (mg/kg)**
γ-CEHC	27.72± 13.58[a]	28.38± 13.33[a]
δTE-13'	n.d.	218.86± 36.30
δT	7.47± 2.56[a]	7.93± 1.08[a]
γT	25.12± 6.56[a]	22.41± 7.55[a]

n=3

Between the control diet and treatment diet, there's no significant difference in concentration of γ-CEHC, δT and γT. There's significantly ($p = 0.005$) higher concentration of δTE-13'in the treatment diet (218.86 ± 36.30 mg/kg). δTE-13' wasn't detected in the control diet. The average concentration is 10% smaller than the concentration suggested by the manufacturer but considering the loss during extraction and variation of fluorescent detection the difference is acceptable. The methanol extraction probably compromised the recovery rate of tocopherols due to the poor hydrophobicity of tocopherols.

3.5 Analysis of Vitamin E and Metabolites in Feces of δTE-13' Supplemented Mice by HPLC-FL Analysis

Feces of mice fed with control diet or δTE-13' supplemented diet were extracted with methanol and analyzed by HPLC-FL assay. In 5 independent experiments, feces of rats fed with control diet or δTE-13' supplemented diet were extracted and analyzed. Vitamin E and metabolites were quantified by external standards.

Table 3.6 Concentration of Vitamin E and Metabolites in Mice Feces

	Ctrl Diet Feces	**δTE-13' Diet Feces**
Peak Profile	**Average Conc. (nmol/g)**	**Average Conc. (nmol/g)**
δTE-13'	n.d.	1475.18± 565.16
δT	14.97± 8.49[a]	14.84± 2.06[a]
γT	92.56± 25.77[a]	91.03± 18.91[a]
αT	296.05± 70.85[a]	310.56± 69.69[a]

n=3 for feces of mice fed with control diet;
n=4 for feces of mice fed with δTE-13' supplemented diet

There's no significant difference in the concentration of tocopherols between feces of mice fed with ctrl diet and δTE-13' supplemented diet. However, there's significantly ($p = 0.002$) higher concentration of δTE-13' in feces of mice fed with δTE-13' supplemented diet (1475.18 ± 565.16 nmol/g) compared to feces of mice fed with the control diet (not detected).

Several peaks which eluted earlier than δTE-13' were detected by the fluorescent detector. However, due to lack of external standard, we couldn't identify the profile of these metabolites. These samples were then analyzed by LC-MS/MS with MRM mode to identify the profile of these metabolites.

3.6 Analysis of Vitamin E and Metabolites in Feces of δTE-13' Supplemented Mice by LC-MS/MS Analysis

The remaining fecal samples after the HPLC-FL analysis were stored at -20 ^{o}C and analyzed by LC-MS/MS with MRM mode. MRM mode LC-MS/MS analysis allows identification of each individual metabolite due to their unique transitions (precursor ion and product ion m/z value) while in HPLC-FL analysis the profile of the metabolites were suggested by the retention time. In the case of lack of external standards or peaks overlapping with each other, the profile of the peaks could not be identified from fluorescent chromatogram. As shown in the result of HPLC-FL analysis of animal diets, animals were given multiple forms of vitamin E. Our previous studies (data not published) with HPLC-FL analyzed A549 cells culture medium incubated with vitamin E suggested that metabolites of different forms of vitamin E would overlap on the fluorescent chromatogram. Thus, for samples that contained multiple forms of vitamin E metabolites, LC-MS/MS MRM mode analysis is necessary to identify profile of the metabolites. LC-MS/MS analysis in MRM mode also eliminates background noise since the triple-quadruple instrument only acquires ions with m/z ratio specified by the MS/MS parameters.

Mixed vitamin E standards at different concentrations were also injected to calculate concentration of metabolites and tocopherols in feces samples. The inter-day variation of internal standards added in each sample was within 15%.

3.6.1 Analysis of Vitamin E and Metabolites in Feces of δTE-13' Supplemented Mice by LC-MS/MS Analysis with Original MRM Acquisition Method

The δTE-13' fortified in the animal diet had 3 double bonds in its side chain (Figure 3.11). However, previous studies using A549 cell cultured medium suggested the structure of γTE 13-carbon side-chain metabolite have 2 double bonds in its side-chain (31). Since our primary goal was to quantify the bioavailability of δTE-13' in biological environment and we were not clear of the metabolism of δTE-13' (3-double-bond) in mice at that point, the MS/MS parameter of 2-double-bond form of δTE-13' (Figure 3.11) was not included in the acquisition method. The MS/MS parameter of 3-double-bond form of δTE-13' (precursor ion $[M-H]^-$ = 425, product ion $[M-H]^-$ = 135, collision energy = 35 V) was postulated from the MS/MS parameter of γTE-13', which has similar molecular structure with δTE-13' except the number of double bonds in the side chain.

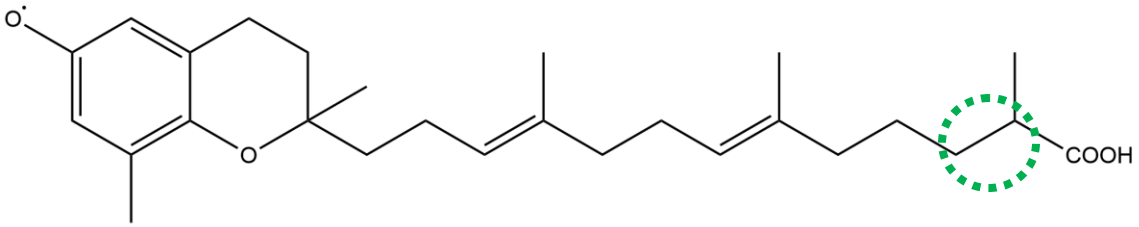
Table 3.7 Concentration of Vitamin E Metabolites in Feces of Mice Fed with Control Diet or δTE-13' Supplemented Diet Analyzed by LC-MS/MS Analysis in MRM Mode with Original MRM Acquisition Method

	Ctrl Diet	**δTE-13' Diet**
Peak Profile	**Average Conc. (nmol/g)**	**Average Conc. (nmol/g)**
δTE-9'	n.d.	14.51 ± 4.78
δTE-11'	n.d.	82.39 ± 27.03
δTE-13'	n.d.	147.30 ± 151.93
δT-13'	5.58 ± 2.76[a]	4.55 ± 1.47[a]
γT-9'	3.08 ± 1.68[a]	2.37 ± 0.84[a]
γT-11'	5.20 ± 2.34[a]	3.27 ± 1.07[a]
γT-13'	17.87 ± 10.47[a]	11.60 ± 3.89[a]
δT-13'-OH	5.81 ± 3.23[a]	4.16 ± 1.35[a]
γT-13'-OH	11.82 ± 7.01[a]	8.05 ± 2.51[a]

n=5

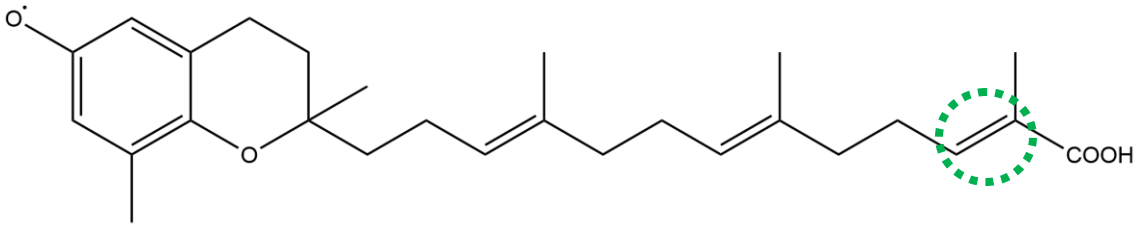
The concentration of δTE-13' (3-double-bond form) calculated from LC-MS/MS analysis (147.3 ± 151.93 nmol/g) is 10X smaller than the concentration calculated from HPLC-FL analysis (1475.18 ± 565.16 nmol/g).

We speculate that this difference might due to under-estimation of δTE-13' by LC-MS/MS analysis with MRM mode. δTE-13' (3-double-bond form) might have been metabolized to 2-double-bond form in mice. In order to find out the form of δTE-13' metabolite in mice fecal samples, total ion scan LC-MS analysis will be performed on these samples.



δTE-13'
2-Double-Bond

Chemical Formula: $C_{27}H_{39}O_4^{\bullet}$
Exact Mass: 427.28



δTE-13'
3 Double Bonds

Chemical Formula: $C_{27}H_{37}O_4^{\bullet}$
Exact Mass: 425.27

Figure 3.11 Molecular Structure of Ionized 2-Double-Bond and 3-Double-Bond Form of δTE-13'

Animal diet fortified with δTE-13' with 3 double bonds was given to the animals. This molecule is possibly metabolized to the 2-double-bond form in mice since previous study showed in A549 cells culture medium incubated with γTE at 20 µM for 72 h, the 13'-carboxychromanol of γTE were identified by LC-MS/MS to be 2-double-bond form. However, the existence of 2-double-bond form of δTE-13' needs to be confirmed by total ion scan and product ion scan analysis of the fecal samples.

3.6.2 Identification and Optimization of MS/MS Parameters of δTE-13' Metabolites in Mice Feces by LC-MS Analysis Total Ion Scan and Product Ion Scan Mode

In order to find out the form of δTE-13' metabolite in mice feces, samples were analyzed by total ion scan LC-MS analysis in the range of 200 - 500 m/z value in which the ion mass of δTE-13' falls into. The chromatogram was further analyzed in the range of 10 m/z value to examine δTE-13' metabolites detected by the instrument.

The fecal samples were further analyzed by product ion scan LC-MS/MS analysis targeting a precursor ion with m/z of 427.1 (2-double-bond form of δTE-13') and 385.2 (δTE-11') with collision energy at 5/15/30/45 V to examine its fragmentation pattern and its optimum MS/MS parameter for MRM analysis.

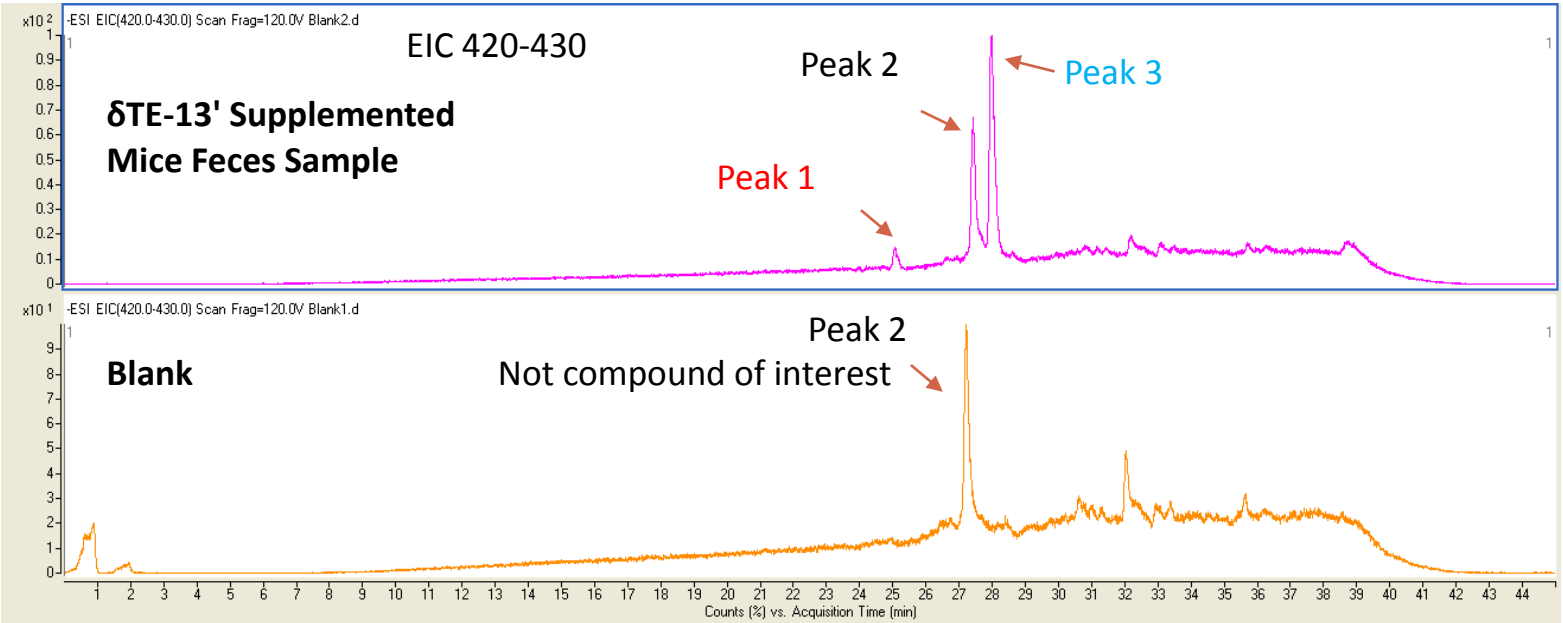


Figure 3.12 Extracted Ion Chromatogram of δTE-13' Supplemented Mice Feces Sample and Blank (methanol) in 420 - 430 m/z

As shown in the chromatogram, there are 3 peaks on the extracted ion chromatogram of δTE-13' supplemented mice feces sample, 1 of them in common with the extracted ion chromatogram of blank sample. Peak 1 and peak 3 might be our compounds of interest.

Peak 1

Peak 3

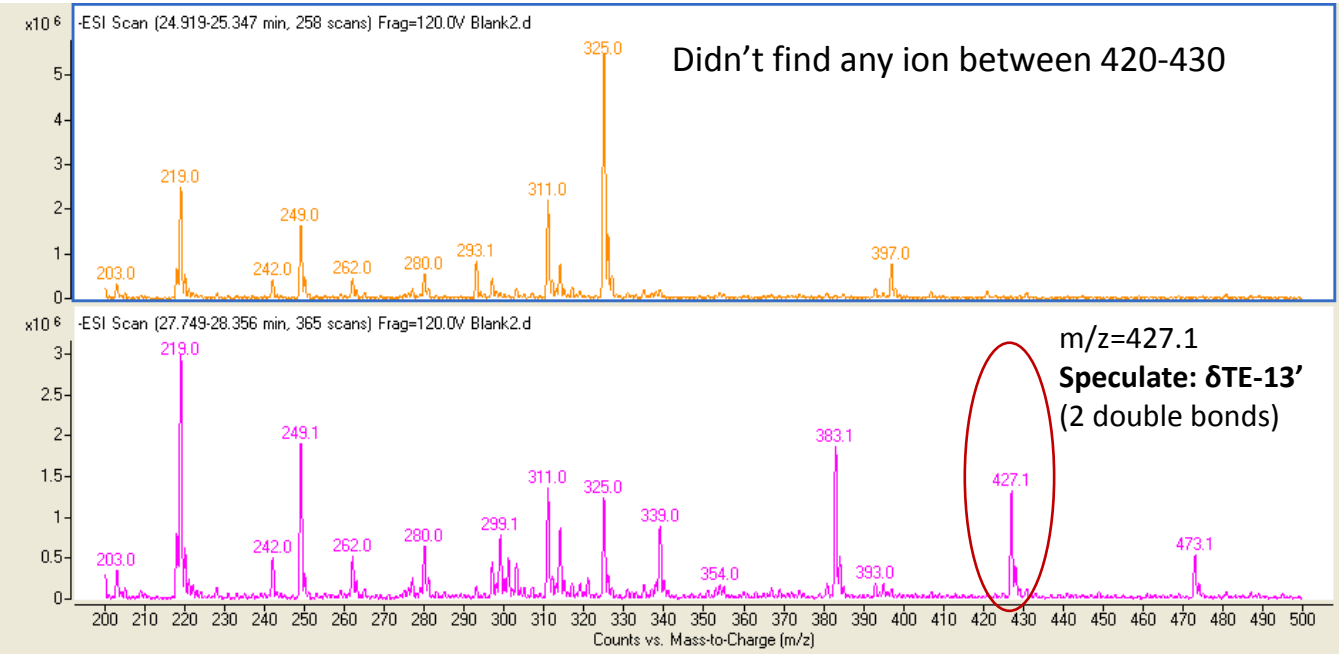


Figure 3.13 MS Spectrum of Peak 1 and 3 in Extracted Ion Chromatogram of δTE-13' Supplemented Mice Fecal Samples in 420 - 430 m/z

In peak 1 spectrum there was no ion detected within 420 to 430 m/z value. But in peak 3 spectrum, ion with m/z of 427.1 was detected. Based on its ion mass, this compound is speculated to be δTE-13' with 2-double-bond. However it needs to be confirmed by its fragmentation pattern with product ion scan LC-MS/MS analysis.

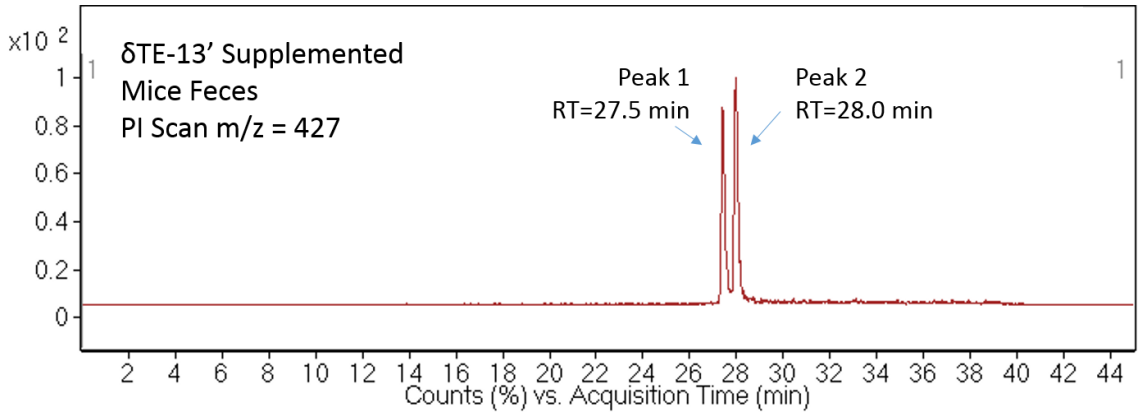


Figure 3.14 Chromatogram of Product Ion Scan LC-MS/MS Analysis of δTE-13' Supplemented Mice Feces at m/z of 427

As shown on figure 3.14, there are 2 separate peaks on the chromatogram of product ion scan LC-MS/MS analysis of δTE-13' supplemented mice feces targeting a precursor ion m/z of 427. The MS/MS spectrum of these 2 peaks at different collision energy levels are shown in the figure below.

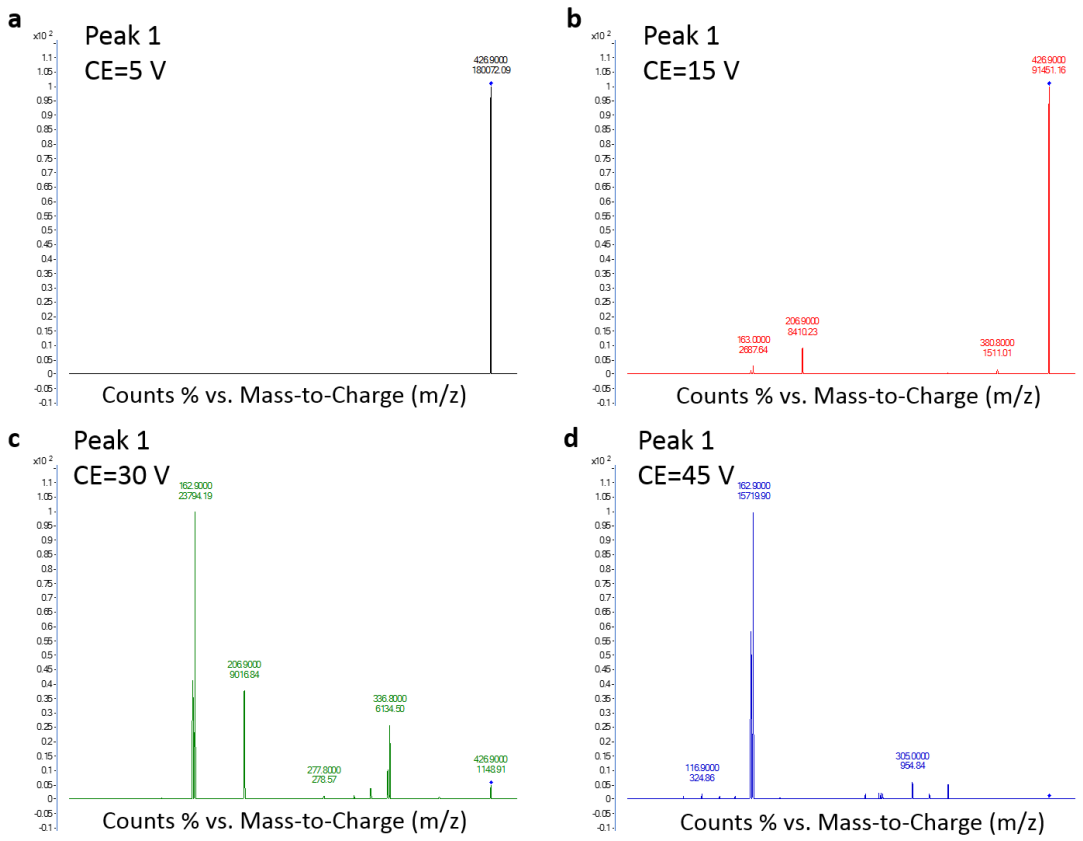


Figure 3.15 MS/MS Spectrum of Peak 1 of Product Ion Scan of δTE-13' Supplemented Mice Feces at m/z of 427 with Various Collision Energy Levels

Figures 3.15.a, b, c, d represent the MS/MS spectrum of peak 1 in the chromatogram of product ion scan of δTE-13' supplemented mice feces at m/z of 427 at collision energy level of 5, 15, 30 and 45 V, respectively. The fragmentation of the compound at collision energy at 5 or 15 V was poor. At 30 V and 45 V of collision energy, the major product ion with m/z of 163 was generated at similar levels but slightly higher at 30 V of collision energy. We did not recognize the fragmentation pattern of this particular ion from its precursor ion, δTE-13'. We've only observed the product ion with m/z of 163 from α-tocopherol and its metabolites, which has 2 additional methyl groups on its chromanol

ring structure. We could not identify the profile of peak 1 on the product ion scan chromatogram although its precursor ion mass matches with the 2-double-bond form of δTE-13'.

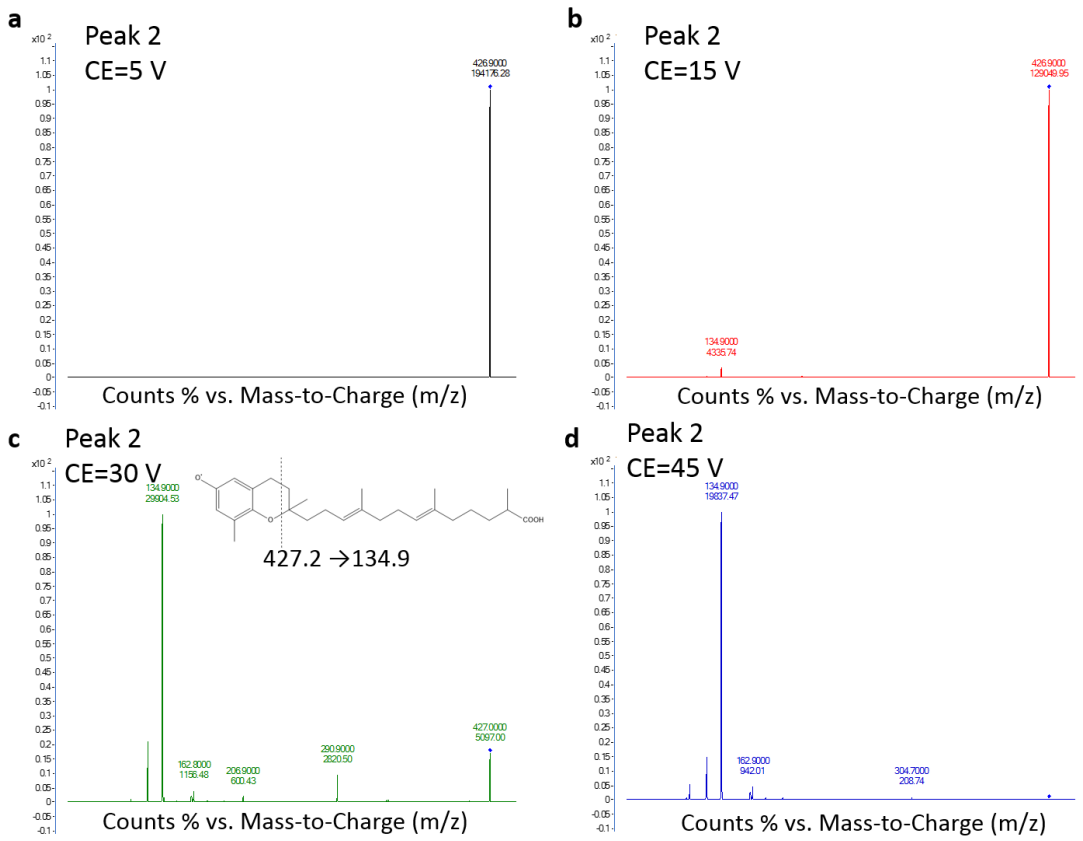


Figure 3.16 MS/MS Spectrum of Peak 2 of Product Ion Scan of δTE-13' Supplemented Mice Feces at m/z of 427 with Various Collision Energy Levels

Figures 3.16.a, b, c, d represent the MS/MS spectrum of peak 2 in the chromatogram of product ion scan of δTE-13' supplemented mice feces at m/z of 427 at collision energy level of 5, 15, 30 and 45 V, respectively. The fragmentation of the compound at collision energy at 5 or 15 V was poor. At 30 V and 45 V of collision energy, the m/z value of the major product ion generated was both 135. The level of the major product ion generated at 30 V of collision energy was higher than that at 45 V. This fragmentation pattern is similar to those observed in other vitamin E metabolites. Based on the product ion scan MS/MS spectrum of peak 2, we confirmed the existence of 2-double-

bond form of δTE-13' and its optimum MS/MS parameters: precursor ion at m/z of 427.2, product ion at m/z of 134.9, collision energy at 30 V. This MS/MS parameter was implemented in the acquisition method of QQQ instrument and feces samples were re-analyzed using the modified MS/MS parameters.

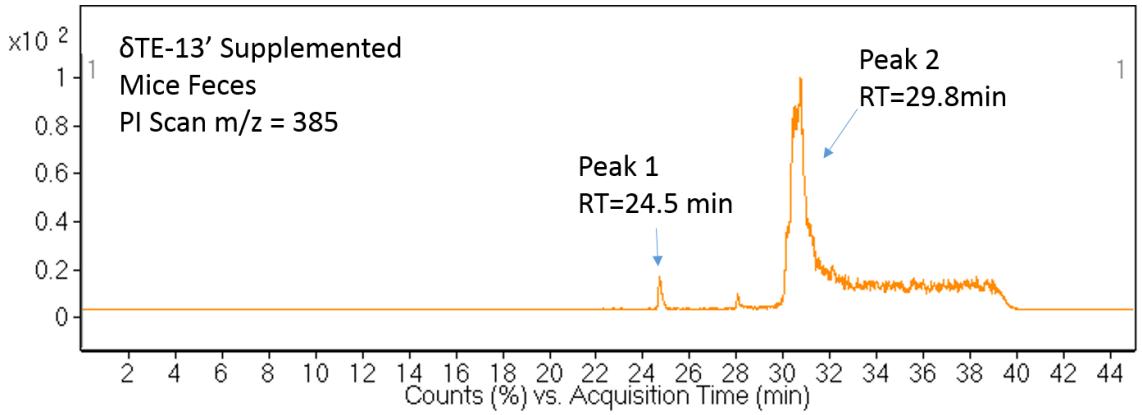


Figure 3.17 Chromatogram of Product Ion Scan LC-MS/MS Analysis of δTE-13' Supplemented Mice Feces at m/z of 385.2

On the chromatogram of product ion scan LC-MS/MS analysis of δTE-13' supplemented mice feces at m/z of 385.2, there are 2 major peaks. The bigger peak (Peak 2) eluting at 29.8 min does not match the retention time of our compound of interest (δTE-11' RT = 24.5 min). Peak 1 eluting at 24.5 min matches the retention time of our interest compound, δTE-11'. The MS/MS spectrum of this peak at various collision energy levels are shown in the figure below.

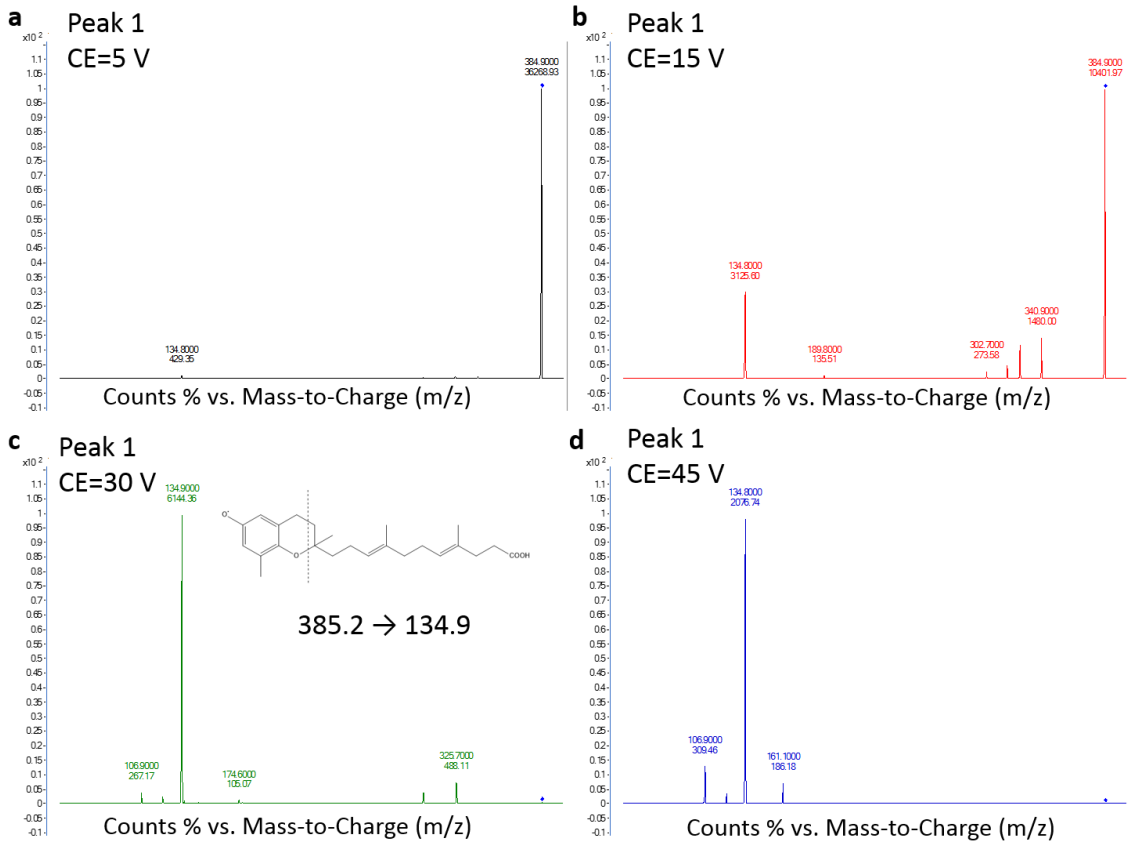


Figure 3.18 MS/MS Spectrum of Peak 1 of Product Ion Scan of δTE-13' Supplemented Mice Feces at m/z of 385.2 with Various Collision Energy Levels

Figures 3.18.a, b, c, d represent the MS/MS spectrum of peak 1 in the chromatogram of product ion scan of δTE-13' supplemented mice feces at m/z of 385.2 at collision energy level of 5, 15, 30 and 45 V, respectively. The fragmentation of the compound at collision energy at 5 or 15 V was poor. At 30 V and 45 V of collision energy, the m/z value of the major product ion generated was both 135. The level of the major product ion generated at 30 V of collision energy was higher than that at 45 V. This fragmentation pattern is consistent with δTE-13' and other vitamin E metabolites. Thus we confirm this

peak as δTE-11' and its optimum MS/MS parameters are: precursor ion m/z = 385.2, product ion m/z = 134.9, collision energy = 30 V. The optimum MS/MS parameter is consistent with the current parameters in the acquisition method.

3.6.3 Analysis of Vitamin E and Metabolites in Feces of δTE-13' Supplemented Mice by LC-MS/MS Analysis with Modified MRM Acquisition Method

After identifying the 2-double-bond form of δTE-13' and its optimum MS/MS parameters, feces samples were re-analyzed by LC-MS/MS MRM mode analysis to calculate concentration of vitamin E metabolites.

Table 3.8 Concentration of Vitamin E Metabolites in Feces from Mice Fed with Control or δTE-13’ Supplemented Diet Analyzed by LC-MS/MS Analysis in MRM Mode with Modified MRM Acquisition Method

	Ctrl Diet	**δTE-13’ Diet**
Peak Profile	**Average Conc. (nmol/g)**	**Average Conc. (nmol/g)**
δTE-9'	n.d.	10.61 ± 5.36
δTE-11'	n.d.	70.48 ± 24.05
δTE-13'	n.d.	150.82 ± 162.27
δTE-13' (2 Double Bond)	n.d.	294.19 ± 85.41
δT-13'	5.58 ± 2.76[a]	4.70 ± 1.76[a]
γT-9'	3.08 ± 1.68	n.d.
γT-11'	5.20 ± 2.34	n.d.
γT-13'	17.87 ± 10.47[a]	10.68 ± 4.42[a]
δT-13'-OH	5.81 ± 3.23[a]	4.43 ± 2.08[a]
γT-13'-OH	11.82 ± 7.01[a]	8.29 ± 3.26[a]

n=5

As shown in the table above, the major metabolite in δTE-13' supplemented mice feces samples is δTE-13' with 2 double bonds (294.19 ± 85.41 nmol/g). The concentration of 3-double-bond form of δTE-13' is 150.82 ± 162.27 nmol/g. δTE-11' and 9' were detected at lower concentration. The major metabolite excreted in feces of mice after δTE-13' supplementation is long chain δTE carboxychromanols. None of these δTE long chain metabolites were detected in feces of mice fed with ctrl diet. The 13'-carboxychromanol and hydroxylated metabolites from δT and γT were detected in both control and supplemented fecal samples. Low levels of 9'- and 11'-carboxychromanol of γT were also detected in control fecal samples but not in supplemented mice fecal samples.

Table 3.9 Comparison of Concentration of δTE-13' in δTE-13' Supplemented Mice Feces Samples Calculated from LC-MS/MS MRM Analysis and HPLC-FL Analysis

	LC-MS/MS Analysis			**HPLC-FL**
Conc. (nmol/g)	**δTE-13' (425.1 → 135)**	**δTE-13'-2 (427.1 → 135)**	**Sum of δTE-13'**	**δTE-13'**
Average	150.82	294.19	**445.01**	**1475.18**
StdDev	162.27	85.41	**199.38**	**565.16**

Although the newly implemented MS/MS parameter for 2-double-bond form of δTE-13' allowed detection of both forms of δTE-13' in the fecal samples, there's still a 3-fold difference between the concentration calculated from HPLC-FL analysis and LC-MS/MS analysis. We speculate that HPLC-FL analysis over-estimated the concentration of δTE-13' due to overlapping of the 2 peaks. Since the 2 compounds are very similar in structure and polarity, the retention time of δTE-13' with 2 double bonds or 3 double bonds are almost the same. Peaks overlapping with each other would influence the shape of the peak thus compromise the accuracy of the intensity of the peak. We conclude that the result from LC-MS/MS analysis with MRM mode is more reliable.

3.7 Optimization of Condition of Complete Deconjugation of Vitamin E Metabolites

In order to quantify metabolites in conjugates in biological samples, complete deconjugation is required to quantify conjugated metabolites more accurately by calculating the increase of unconjugated metabolites. MS/MS parameters for sulfated conjugates have been set up and optimized with an external standard, γTE-9'S. However, we rely on the increase of unconjugated metabolites to calculate other forms of conjugated metabolites, such as glucuronide conjugates. Per information from Sigma Aldrich, the sulfatase from *Helix pomatia* exhibits sulfatase activity at 14.2 U/mg (1 U of sulfatase deconjugates 1 µmole of sulfated p-nitrocatechol at 37 ^{o}C at pH 5.0) and glucuronidase activity at 541.8 U/mg (1 U of glucuronidase deconjugates 1 µg of phenolphthalein glucuronide at 37^{o}C at pH 5.0). We propose that by optimizing the treatment condition, we would be able to achieve complete deconjugation of conjugated metabolites in biological samples.

Our study demonstrated that overnight sulfatase treatment completely deconjugatedγTE-11'S existing in plasma of rats supplemented with γTE at 50 mg/kg after 16 h of sulfatase treatment at 37 ^{o}C at pH 5.0. Our study also demonstrated that 3h incubation desulfated 90% of isolated γTE-9'S. Experiments were conducted using A549 cells culture medium incubated with γTE at 20 µM for 72h which contains large amounts of long chain sulfated metabolites. Different sulfatase treatment conditions were experimented to achieve complete desulfation.

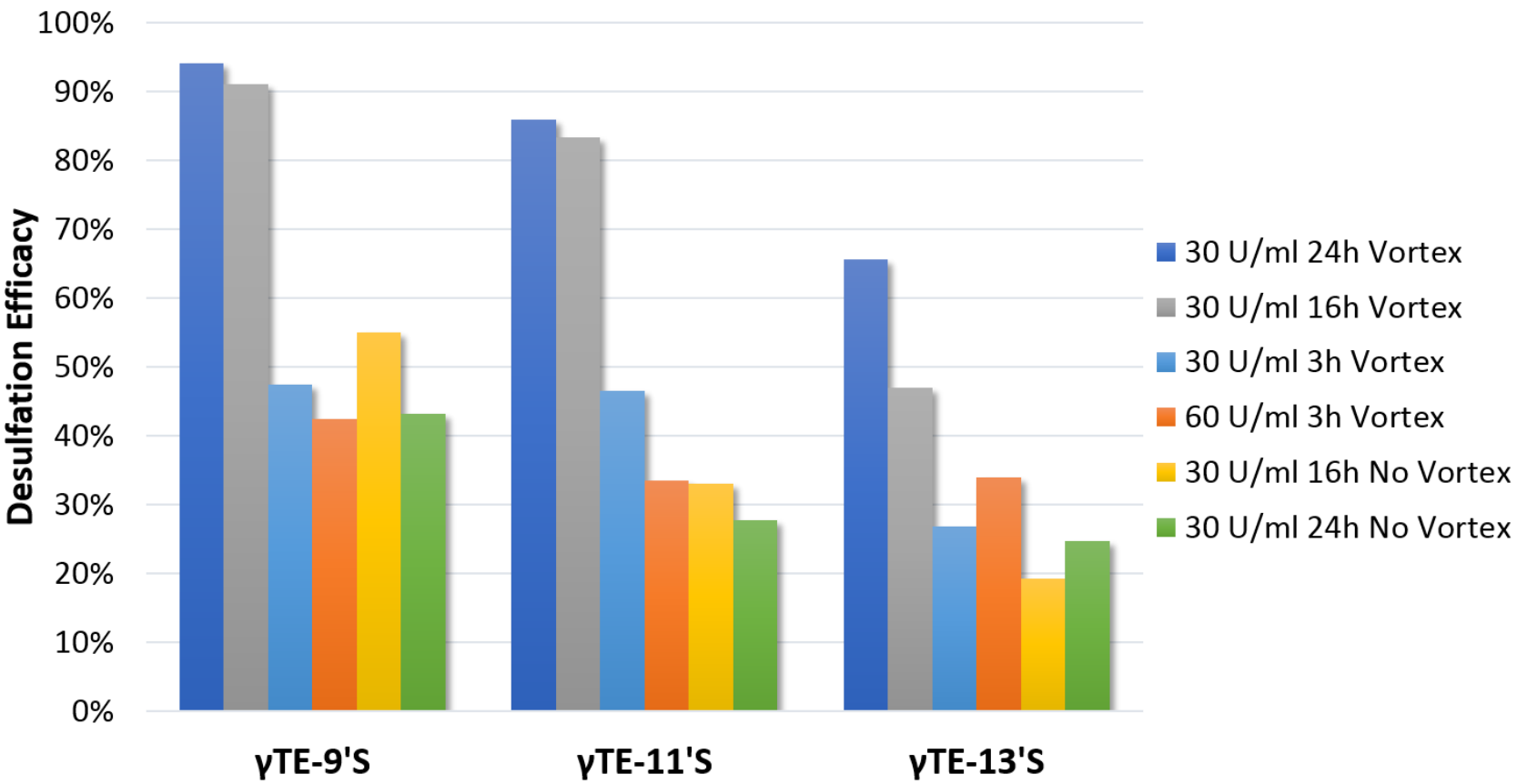


Figure 3.19 Desulfation Efficacy of Long Chain Sulfated γTE Metabolites Extracted from Cell Cultured Medium Analyzed by HPLC-FL Assay

As seen in the figure, although 30 U/ml sulfatase treatment at 3 h desulfated 90% of purified γTE-9'S, in samples with multiple forms of conjugated vitamin E metabolites the same condition only achieved desulfation of 47% of γTE-9'S and 11'S. Even if the amount of sulfatase was doubled, the desulfation efficacy was still around 40% for γTE-9'S and even lower for longer chain sulfates. When incubation was prolonged, 24 h sulfatase treatment at 30 U/ml desulfated 94% of γTE-9'S, 86% of γTE-11'S and 66% of γTE-13'S. 16 h sulfatase treatment at 30 U/ml desulfated a little bit less than 24 h treatment, but still achieved 91% and 83% desulfation of γTE-9'S and 11'S, respectively. The initial vortexing every 15 min during the first 3 h of incubation seemed to be necessary for the complete desulfation as samples without initial vortexing resulted in only 40 - 50% desulfation after overnight incubation. Based on these results, the optimum sulfatase treatment condition for complete deconjugation is sulfatase treatment at 30 U/ml for 24 h at 37 ^{o}C at pH 5.0 with vortexing during the first 3 h of incubation.

3.8 Analysis of Vitamin E Metabolites in Animal Plasma and Feces Samples

Plasma and fecal samples collected from rats or mice gavaged or supplemented with vitamin E were extracted with methanol and hexane and analyzed by HPLC-FL or LC-MS/MS. Plasma samples from rats gavaged with a single dose of γT or γTE was added with 0.5 μM of δTE-13' before extraction to ensure extraction efficiency and correct for possible loss during the experiment. Plasma samples were treated with or without the sulfatase to calculate glucuronide conjugates in the plasma samples. The hexane fraction and methanol fraction extracted from each sample were combined and treated with an internal standard (0.5 μM of α-CMBHC) before analysis to monitor consistency of injection and ionization of the LC-ESI-MS instrument. MS/MS parameters allowed identification of each metabolite and tocopherol or tocotrienol, simultaneously. Vitamin E metabolites were quantified by external standards with different side chain at 0.2 μM. Tocopherols and tocotrienols were quantified by their external standards at 10 μM. The internal standard spiked in each sample before analysis (α-CMBHC to a final concentration of 0.5 μM) was all within 15% inter-day variation.

Table 3.10 Concentration of Vitamin E Metabolites in Plasma of Rats Collected 6 h after Single Dose Gavage with γT at 10 or 50

100 mg/kg Body Weight or Tocopherol-Stripped Corn Oil

	Ctrl	**γT 10 mg/kg**	**γT 50 mg/kg**	**γT 100 mg/kg**
Peak Profile	**Average Conc. (μM)**	**Average Conc. (μM)**	**Average Conc. (μM)**	**Average Conc. (μM)**
γ-SO3-CEHC	0.033 ± 0.018	0.13 ± 0.07	0.73 ± 0.35	0.94 ± 0.50
γ-CEHC	0.12 ± 0.038	0.14 ± 0.069	0.29 ± 0.10	0.24 ±0.04
"Glucuronidated" γ-CEHC	n.d.	n.d.	n.d.	2.03 ± 0.65
α-CEHC	0.16 ± 0.080	0.17 ± 0.12	0.25 ± 0.070	0.16 ± 0.047
γTE-9'S (1DB)	0.017 ± 0.013	0.011 ± 0.010	n.d.	0.010 ±0.0022
γTE-11'S (2DB)	0.036 ± 0.015	0.051 ± 0.024	0.050 ± 0.026	0.050 ± 0.0086
γTE-11'S (1DB)	n.d.	n.d.	0.038 ± 0.026	n.d.
γT-9'S	n.d.	0.11 ± 0.050	0.44 ± 0.28	0.55 ± 0.10
γT-11'S	0.032 ± 0.038	0.25 ± 0.12	0.96 ± 0.56	0.62 ± 0.26
γT-13'S	n.d.	0.011 ± 0.0022	0.042 ± 0.014	0.070 ± 0.034
αT-13'	n.d.	0.0052 ± 0.00088	0.0043 ± 0.00042	0.0054 ± 0.0019
γT-9'	n.d.	n.d.	n.d.	0.00035 ± 0.00031
γT-11'	n.d.	n.d.	n.d.	0.0015 ± 0.00050
γT-13'	n.d.	n.d.	0.013 ± 0.0059	0.020 ± 0.0082
γT-13'-OH	n.d.	0.017 ± 0.0029	0.081 ± 0.022	0.0630 ± 0.041
αT-13'-OH	0.0043 ± 0.0032	0.0063 ± 0.00077	0.0081 ± 0.00029	0.0050 ± 0.0017
δT	5.48 ± 3.04	3.47 ± 1.91	4.51 ± 1.39	2.48 ± 2.17
γT	n.d.	1.83 ± 1.08	7.45 ± 1.96	9.24 ± 4.96
αT	24.04 ± 12.32	27.68 ± 7.59	27.43 ± 7.26	11.29 ± 2.12

Three or five samples in each treatment group were extracted and analyzed. Result of LC-MS/MS analysis in MRM mode showed that the major metabolite of γT in rats are conjugated γ-CEHC and long chain sulfated carboxychromanols. Among the sulfated long chain carboxychromanols detected in plasma of rats gavaged with γT, 11'S has the highest concentration among 3 different dosages. As the gavaged dosage increases, the amount of γ-CEHC conjugates increased. Tocopherols are also found in rats plasma in all groups in relatively high concentration, especially αT, which is poorly metabolized compared to δT and γT. Concentration of γT in plasma of rats increased as the gavaged dosage of γT was increased.

Table 3.11 Concentration of Vitamin E Metabolites in Plasma of Rats Gavaged with γTE at 10 or 50 mg/kg Body Weight or Tocopherol-Stripped Corn Oil in Ctrls

	Ctrl	**γTE 10 mg/kg**	**γTE 50 mg/kg**
Peak Profile	**Average Conc. (μM)**	**Average Conc. (μM)**	**Average Conc. (μM)**
γ-SO3-CEHC	n.d.	0.46± 0.24[a]	1.60 ± 0.61[b]
"Glucuronidated" γ-CEHC	0.10	1.15± 0.47	N/A
γ-CEHC	0.08	0.22± 0.14[a]	0.25 ± 0.068[a]
α-CEHC	n.d.	0.13±0.064[a]	0.17 ± 0.061[a]
γTE-9'S (1DB)	n.d.	0.27± 0.084[a]	0.41 ± 0.23[a]
γTE-11'S (2DB)	0.017	0.95± 0.34[a]	1.08 ± 0.79[a]
γTE-11'S (1DB)	n.d.	0.062± 0.019[a]	1.02 ± 1.59[a]
γTE-13'S (3DB)	n.d.	0.041± 0.016[a]	0.10 ±0.0156[b]
γT-9'S	n.d.	0.018± 0.0040[a]	0.026 ± 0.0094[a]
γT-11'S	n.d.	0.017± 0.0070	n.d.
γTE-7' (1DB)	n.d.	n.d.	0.0032 ± 0.00080
γTE-9' (1DB)	n.d.	n.d.	0.0051 ± 0.00082
γTE-11' (2DB)	n.d.	n.d.	0.0088 ± 0.0015
γTE-13' (2DB)	n.d.	0.0026± 0.0023	n.d.
γTE-13' (3DB)	n.d.	0.0068± 0.0026[a]	0.022 ±0.0031[b]
αT-13'	0.0049	0.0058± 0.0002[a]	0.0049 ± 0.00035[b]
γTE-13'-OH	n.d.	0.027± 0.0047[a]	0.086 ± 0.0060[b]
γT-13'-OH	n.d.	0.0027± 0.00010[a]	0.0017 ± 0.00020[b]
αT-13'-OH	0.0057	0.0051± 0.0016[a]	0.0063 ± 0.0017[a]
γTE	n.d.	n.d.	1.16 ± 0.08
δT	3.59	3.14± 1.26[a]	4.23 ± 1.84[a]
αT	22.80	26.78± 3.12[a]	21.65 ± 1.31[b]

n=2 for control groups; n=3 for treatment groups

Two plasma samples from the control group and three plasma samples from each treatment group was extracted and analyzed by LC-MS/MS with MRM mode. In plasma of rats gavaged with a single dose of γTE at 10 or 50 mg/kg body weight, the major metabolites are also conjugated γ-CEHC and sulfated long chain carboxychromanols. The amount of conjugated γ-CEHC increased 4-fold when the administration dose increased 5-fold however the amount of γ-CEHC stayed at a similar level. Interestingly, γTE-11'S, the major sulfated long chain metabolite in rats plasma, are found existing with both 1 double bond and, more predominantly, 2-double-bonds. There's approximately 2-fold increase in the amount of γTE-13'S and 11'S (1-double-bond form) when the dosage was increased 5 times, however the amount of 2-double-bond form of 11'S was similar. In all treatment groups, δT and αT were excreted at relatively high concentration and are not influenced by the dose of γTE. In plasma of rats gavaged with 50 mg/kg body weight of γTE, approximately 1 μM of γTE was also detected.

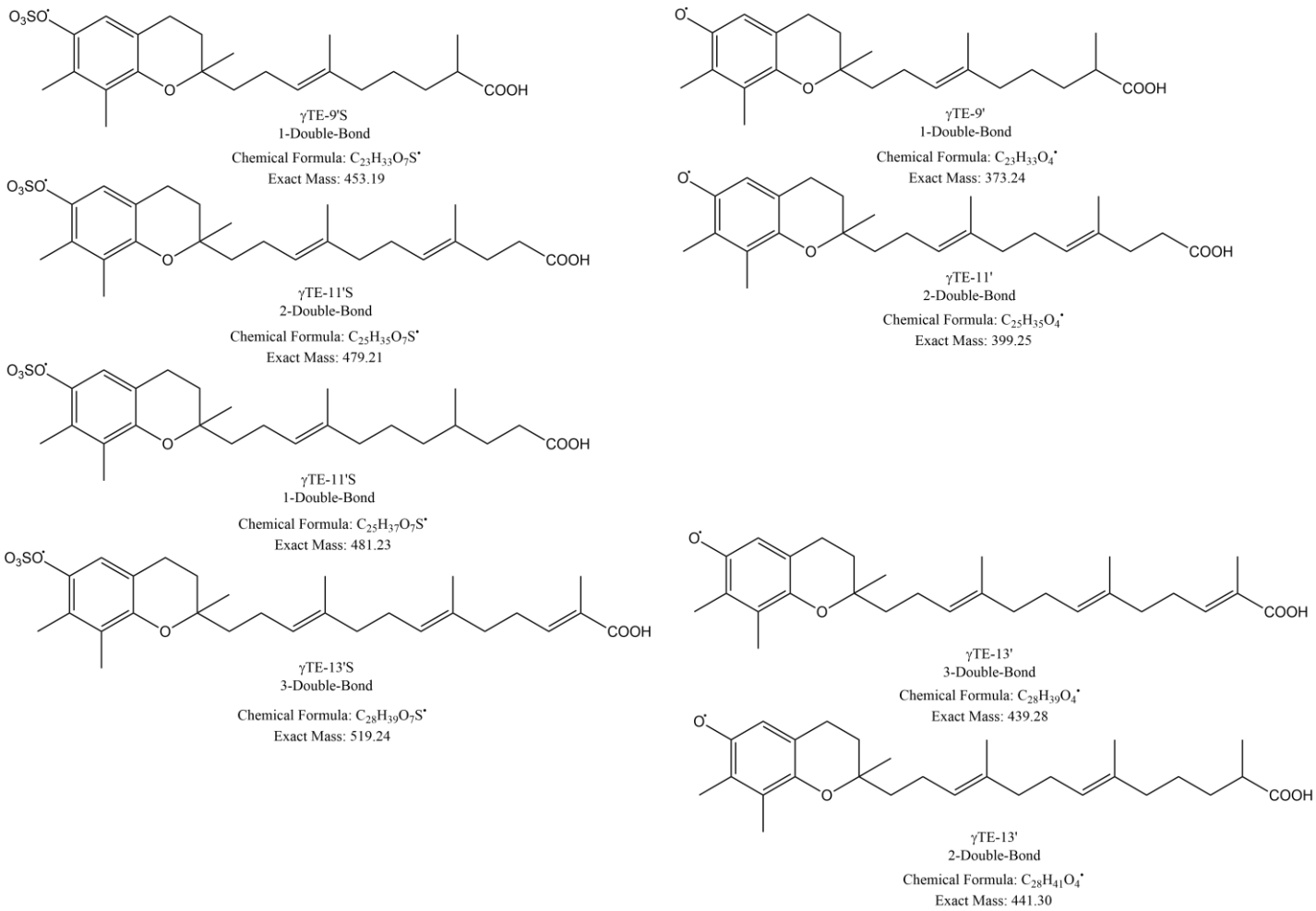


Figure 3.20 Molecular Structure of Ionized Long Chain Metabolites of γTE in Plasma of Rats 6h after Being Gavaged with γTE

As shown in Table 3.11 and Figure 3.20, in the plasma of rats collected 6 h following the gavage of γTE at 10 or 50 mg/kg b.w., γTE-9'S was only detected in 1-double-bond form, the major metabolite γTE-11'S, was detected in both 2-double bond and 1-double-bond form but the 2-double-bond metabolite was the predominant form, γTE-13'S was only detected in 3-double-bond form. However, as for the unconjugated long chain carboxychromanols, γTE-9' was detected only in 1-double-bond form, γTE-11' was only detected in 2-double-bond form, γTE-13' was detected in both 2- and 3-double-bond forms. These unconjugated long chain carboxychromanols were detected at relatively low concentration compared to the conjugated long chain carboxychromanols.

Table 3.12 Concentration of Vitamin E Metabolites in Plasma of AOM-DSS Colon Carcinogenesis Induced Mice Supplemented with γT or δT at 0.1% Diet or Control Diet

Ctrl Diet		**γT Diet**		**δT Diet**	
Peak Profile	**Average Conc. (μM)**	**Peak Profile**	**Average Conc. (μM)**	**Peak Profile**	**Average Conc. (μM)**
α-CEHC	0.034 ± 0.011	γ-CEHC	0.085 ± 0.031	γ-CEHC	0.012 ± 0.0043
		α-CEHC	0.026 ± 0.0067	α-CEHC	0.029 ± 0.0064
		γT-9'S	0.040 ± 0.011	δT-9'S	0.032 ± 0.0056
		γT-11'S	0.032 ± 0.016	δT-11'S	0.045 ± 0.0092
		γT-13'S	0.0085 ± 0.0042	δT-13'S	0.032 ± 0.0073
		γT-11'	0.0014 ± 0.0013	δT-13'	0.010 ± 0.0052
		γT-13'	0.021 ± 0.0021		
		αT-13'	0.0036 ± 0.00072		
		γT	0.59 ± 0.73	δT	0.98 ± 1.07
αT	8.41 ± 0.28	αT	6.07 ± 1.66	αT	5.94 ± 1.10

n=3

Three plasma samples of each treatment group were extracted and analyzed by LC-MS/MS with MRM mode. Mice were injected AOM at 10 mg/kg body weight and fed 1.5% DSS in drinking water. In these mice, the concentration of γT or δT supplemented in their diet were very low. Major components in plasma of γT supplemented mice were γ-CEHC, γT and high amount of αT, which had similar level with the other 2 groups. 9'S and 11'S of γT were detected in relatively low concentration but higher than those of unconjugated long chain carboxychromanols.

In the plasma of rats supplemented with δT, the concentration of metabolites were very low. The major components are sulfated long chain metabolites, αT and δT. Different from the previous study when rats were gavaged with single dose of γT or γTE, these mice were supplemented with γT or δT at 0.1% in their diet. This concentration is a lot lower than the dose gavaged into rats. The metabolism of rats and mice are also different. Another factor to consider is that these mice were induced with colon cancer by AOM-DSS. This might also played a role in the metabolism of vitamin E in mice.

Table 3.13 Concentration of Vitamin E Metabolites in Feces of AOM-DSS Colon Carcinogenesis Induced Mice Fed with δT at 0.1% Diet or Control Diet

	Ctrl	**δT Diet**
Peak Profile	**Average Conc. (nmol/g)**	**Average Conc. (nmol/g)**
γT-9'	2.09	11.80
γT-11'	5.70	24.42
γT-13'	35.50	184.14
γT-13'-OH	21.31	63.26
δT-13'S	n.d.	32.12
δT-9'	n.d.	61.24
δT-11'	1.69	220.31
δT-13'	15.49	1828.63
δT-13'-OH	25.08	1995.49
αT-9'	0.97	4.90
αT-11'	2.98	7.93
αT-13'	15.41	38.86
αT-13'-OH	12.56	24.82
δT	n.d.	3659.67
γT	n.d.	0.32
αT	425.09	433.45

Fecal samples of mice in the control and δT supplemented group of the same study were extracted and analyzed by LC-MS/MS analysis in MRM mode. Two samples from each group were analyzed. The major components excreted in mice fed with δT diet were δT-13' and δT-13'-OH. δT were also excreted at very high concentration. Combined with the result of the metabolites in the plasma, in AOM-DSS colon cancer induced mice, δT is primarily metabolized to δT-13' and δT-13'-OH which were excreted in high levels in bile.

CHAPTER 4. CONCLUSION AND DISCUSSION

We have developed a simple and effective extraction method that recovered more than 90% of different types of vitamin E. This simplified extraction method reduced the volume of samples consumed in extraction and also reduced the preparation time.

The optimization of MS/MS parameters using purified sulfated γTE metabolites and feces of mice supplemented with δTE-13' improved the sensitivity of detecting vitamin E metabolites in biological samples. With the optimized extraction and LC-MS/MS acquisition method, we are able to detect all of the vitamin E metabolites in biological samples simultaneously.

We observed the 2-double-bond form of δTE-13' as the major metabolite excreted in mice fed with the 3-double-bond form of δTE-13. The 2-double-bond metabolite was also formed in A549 cells cultured with 3-double-bond form of δTE-13'. Consistent with the result in our previous study that in A549 cell cultured medium incubated with γTE at 20 μM for 48 h, γTE is metabolized to the 2-double-bond form of 13'-carboxychromanol(31). This suggests that the reduction of the double bond on the C-13 position on the

side chain of tocotrienols might be an important step leading up to the removal of 2-carbon moiety by β-oxidation.

In the plasma of rats collected 6 h after being gavaged with a single dose of γT at 10, 50 or 100 mg/kg b.w. or γTE at 10 or 50 mg/kg b.w, the major metabolites were conjugated γ-CEHC and sulfated long chain carboxychromanols. This is also consistent with our study published in 2009 (31). Interestingly, with the gavaged dose of γT increased from 10 to 50 mg/kg b.w., the increase of plasma concentration of 9'S, 11'S and sulfated γ-CEHC was almost proportional. However when the dose was further increased to 100 mg/kg, only a small increase in plasma concentration of metabolites was observed.

In the plasma of rats collected 6 h after being gavaged with a single dose of γTE at 10 or 50 mg/kg b.w., we found that the major metabolite, γTE-11'S exists in both 2-double-bond form and 1-double bond form while γTE-13' also exist in both 2-double-bond and 3-double-bond form. This result further suggests that LC-MS/MS analysis is a more accurate quantification method compared to the HPLC-FL assay which was unable to separate the γTE metabolite with only 1 double bond difference in the side chain. The discrepancy in quantification of δTE-13' between HPLC-FL assay and LC-MS/MS assay has been shown in our study with feces of mice fed with δTE-13'. The overlapping peaks in fluorescent chromatogram might cause overestimation of the peak area and concentration of the metabolites.

In feces of AOM-DSS induced mice fed with 0.1% δT or γT diet, δT-13' and δT-13'-OH were detected at 1.8 - 2.0 μmol/g in feces of mice supplemented with δT. These results suggest that in AOM-DSS induced mice, dietary supplemented δT or γT was substantially metabolized.

We attempted to treat plasma samples with overnight incubation with sulfatase/ glucuronidase to deconjugate conjugated metabolites in the plasma samples. However, the condition that have been proved to convert most of the sulfated long chain γTE metabolites in cell cultured medium did not achieve as high desulfation efficacy in plasma samples. Previously published study showed that overnight incubation of plasma samples with sulfatase at higher concentration achieved complete deconjugation of conjugated vitamin E metabolites (44). The reason for the decreased desulfation efficacy in plasma sample is not clear at this point. However, by spiking the sulfated long-chain carboxychromanol into FBS and comparing the desulfation efficacy of overnight incubation with sulfatase at 30 U/ml vs. 60 U/ml might help answering the question.

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APPENDIX

APPENDIX

Sample Calculation of Concentration of Isolated γTE-9'S

1) HPLC-UV Quantification of γTE-9'

   UV intensity of converted γTE-9' = 2.62×10^4

   UV intensity of 10 μM (0.2 nmole) of δTE-13' = 2.07×10^4

   Concentration of γTE-9' = $\frac{2.62\times10^4}{2.07\times10^4} \times 10\ \mu\text{M} = 12.64\ \mu\text{M}$

2) HPLC-FL Quantification of Desulfation Efficacy of γTE-9'S

   FL intensity of γTE-9'S in the control sample = 8.22×10^6

   FL intensity of γTE-9'S in the sulfatase treated sample = 1.16×10^6

   Desulfation efficacy of γTE-9'S = $\frac{8.22\times10^6-1.16\times10^6}{8.22\times10^6} = 86\%$

3) Calculation of Concentration of γTE-9'S

$$\frac{\text{Concentration of } \gamma\text{TE} - 9'}{\text{Desulfation Efficacy of } \gamma\text{TE} - 9'\text{S}} = \frac{12.64\ \mu\text{M}}{86\%} = 14.71\ \mu\text{M}$$

   Original concentration is 100 times more concentrated = 14.71 μM × 100 = 1.47 mM