Determination of Tocotrienols and Tocophenrols Using HPLC-ECD

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Key Words
Vitamin E, Tocotrienols, Tocopherols, Antioxidants, HPLC-ECD, Palm Oil

Summary
This application describes a sensitive and selective gradient HPLC-ECD method capable of simultaneous determination of multiple tocopherol and tocotrienol isomers.

Introduction
There are eight vitamers of vitamin E (alpha-, beta-, gamma-, and delta-tocopherols and the corresponding tocotrienols) (Figure 1), differing in their biological activity and distribution in foods. Most often vitamin E's role is usually associated with its antioxidant activity. For example, α-tocopherol is a well-defined chain-breaking antioxidant that protects against lipid peroxidation in biological membranes; similarly, α-tocopherol protects biological membranes by preferentially destroying pro-oxidant reactive nitrogen species. Recent research has suggested that tocotrienols are better antioxidants *in vitro*. However, whether they play an important role in antioxidant defenses *in vivo* is questionable as tocotrienols have lower bioavailability than tocopherols following oral ingestion.

Tocotrienols possess a variety of potentially important biological activities. They show novel hypocholesterolemic effects (by down-regulating 3-hydroxyl-3-methylglutaryl-coenzyme A reductase, a key enzyme of the mevalonate pathway) together with an ability to reduce the athero-genic apolipoprotein B and lipoprotein(a) plasma levels. In addition, tocotrienols show anti-thrombotic activity, anti-tumor effects and may act as neuroprotectants. Taken together, tocotrienols may be important in the future for the prevention and/or treatment of cardiovascular disease, cancer, and neuronal degeneration.[1]

Up until now a major issue for active research into the biological effects of these compounds is the lack of a sensitive and selective analytical technique. Presented here is a gradient HPLC-Electrochemical Detection (ECD) method capable of determination of retinol, retinyl acetate, α-, β-, δ-, and γ-tocotrienol, α-, δ-, and γ-tocopherol, β-carotene and coenzyme Q10 in a single analysis.

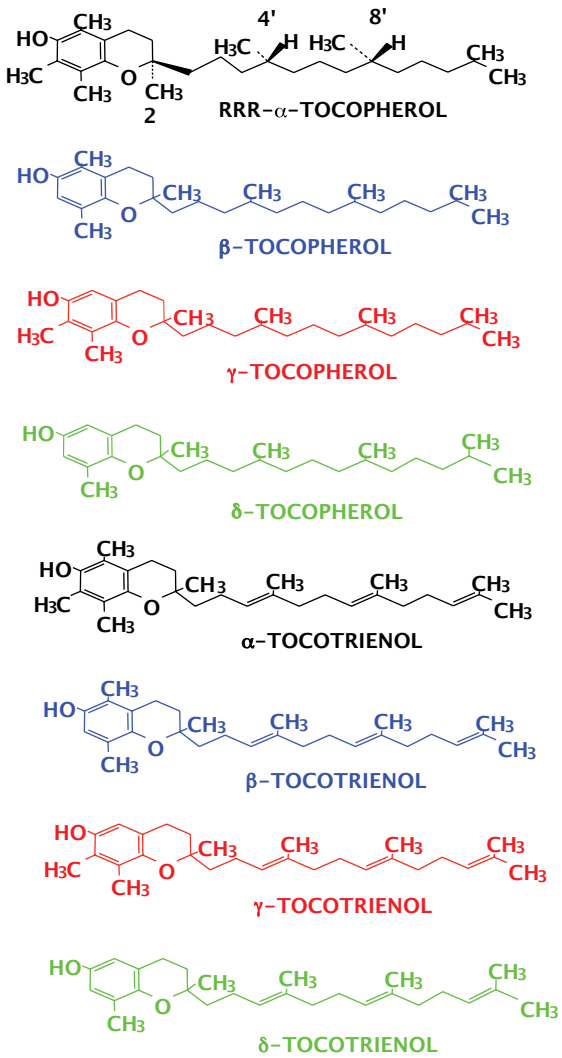

Figure 1. Structures of the various tocopherol and tocotrienol vitamers.

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Materials and Methods

The gradient HPLC system consisted of two pumps, a dynamic mixer, a refrigerated autosampler, a thermostatic organizer, and an eight channel Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector.

LC Conditions	
Column:	Thermo Scientific™ Hypersil™ BDS C18, 3 µm, 3.0 × 150 mm
Temperature:	32 °C
Mobile Phase A:	Acetonitrile – Water, 90:10 (v/v) containing 20 mM Sodium Perchlorate and 5 mM Perchloric Acid
Mobile Phase B:	Acetonitrile – 1-Propanol, 65:35 (v/v) containing 20 mM Sodium Perchlorate and 10 mM Perchloric Acid
Gradient Conditions:	Initial isocratic condition using 10% B for 4 min, then a linear gradient from 10–100% B for 21 min followed by a 9 min hold at 100% B before returning to initial conditions for 5 min. Total run time was 40 min
Flow Rate:	0.6 mL/min
Injection Volume:	10 µL, tray at 4 °C
Detector Conditions	
Detector:	Model 5600A CoulArray
Applied Potentials:	-700, 0, 75, 150, 225, 300, 375, and 450 mV (vs. Pd)

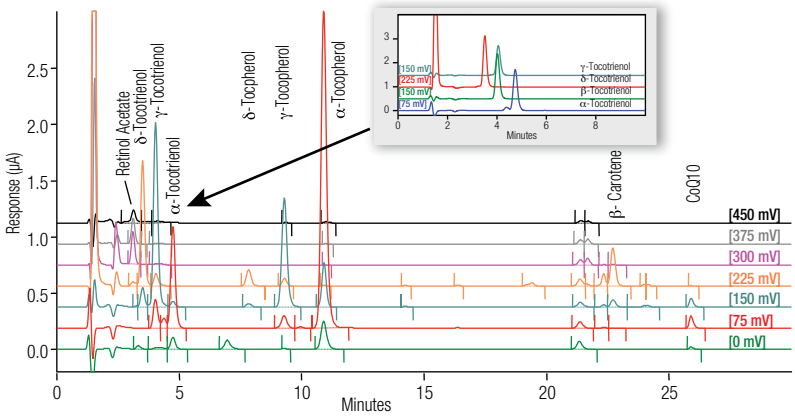

Figure 2. Chromatographic and voltammertric separation of standards. Inset shows the behavior of the tocotrienol isomers.

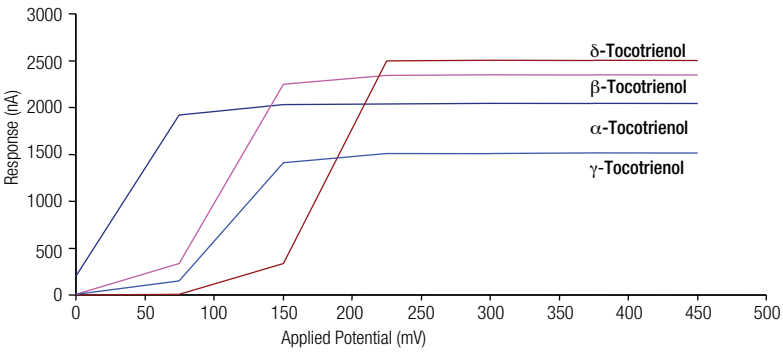

Figure 3. Current-voltage behavior of the tocotrienol isomers.

Standard Preparation

The α-, β-, δ-, and γ-tocotrienols were obtained from Calbiochem and were used without further purification. Retinol, retinyl acetate, α-, δ-, and γ-tocopherols, β-carotene and coenzyme Q 10 were obtained from Sigma Chemical Co., St. Louis, M0. Stock standards were made by dissolving approximately 10 mg of each compound in 10 mL of ethanol with the exception of the carotenoids and Q10. For these more lipophilic compounds, 1.0 mg was dissolved in 5.0 mL of hexane followed by dilution with 15 mL EtOH. Stock solutions were then assayed spectrophotometrically and assigned a concentration value prior to the addition of 10 mg/L butylated hydroxyanisole (BHA) as a preservative. Dilutions were made in ethanol containing 10 mg/L BHA.

Sample Preparation

Palm oil samples obtained were obtained from Drs. Sumit Shah and Paul Sylvester of University of Louisiana, College of Pharmacy. Samples were weighed and diluted with ethanol prior to injection. The samples were placed in a –80 °C freezer for storage prior to analysis.

Results and Discussion

The separation of tocotrienol isomers was achieved using both chromatographic and voltammetric techniques as shown in Figure 2. The current voltage relationship for the various tocotrienol isomers are illustrated in the hydrodynamic voltammogram shown in Figure 3. These figures illustrates that alpha tocotrienol has a lower oxidation potential (75 mV vs. Pd) than the beta and gamma tocotrienol isomers (150 mV vs. Pd), while the delta isomer has the highest oxidation potential (225 mV vs. Pd) of the group. This is similar to the response observed for the alpha, gamma and delta tocopherol isomers shown in Figure 2. The separation method employed was not able to separate the beta and gamma tocotrienol isomers.

Figure 4 illustrates that excellent linear response from 125 fmol to 25 pmol for both delta and gamma isomer and 250 fmol to 25 pmol for the alpha isomer was observed. Recently, this dynamic range was further extended up to 2.5 nmol for these isomers (data not shown). Thus a dynamic range of more than 105 can be achieved using the CoulArray Coulometric Array Detector. The limit of detection for the various tocotrienol isomers is 250, 74 and 10 (fmol) for the alpha, delta and gamma tocotrienols, respectively. The response for the alpha tocotrienol isomer was limited due to auto-oxidation even though preservative antioxidants were included in the stock solutions and all subsequent dilutions. Previous work on the analysis of fat-soluble vitamins at ESA, now part of Thermo Scientific, has shown that a lower limit of detection of less than 20 fmol was achieved for the analysis of alpha tocopherol. The reason for this discrepancy is unknown and will require further investigation.

The analysis of tocotrienols and tocopherols from palm oil is shown in Figure 5. The amount of tocotrienols and tocopherols observed in palm oil samples were high and has been reported in mg/kg amounts. Since the CoulArray Coulometric Array Detector is capable of detecting fmol levels of tocotrienols it should be possible to determine the levels of these compounds from plasma samples after the consumption of foods, such as palm or rice bran oil, which are rich in tocotrienols. In a recent paper that utilized the CoulArray detector for the analysis of tocotrienols from tissue samples,[2] no tocotrienols could be determined even though the levels of the tocopherols were easily detected in rats fed standard diets supplemented with corn oil. By using higher initial plasma volumes in combination with the analytical approach described in this paper, it should be feasible to work out a suitable methodology for the analysis of plasma tocotrienols. Due to susceptibility of these compounds to undergo auto-oxidation the extraction method must include suitable protective antioxidants. In the current study the antioxidant BHA did not completely prevent the degradation of alpha tocotrienol. Further attempt to stabilize this compound by using 10 times the amount of alpha tocopherol and Coenzyme Q10 improved the situation but did not completely abolish its degradation. It is important to establish a suitable technique for the analysis of tocotrienols from plasma since it is thought that alpha tocotrienol may be 40–60 times more potent in preventing lipid oxidation than alpha tocopherol.[3,4] There is also evidence that they may play an important role in lowering cholesterol and may influence the progression of certain cancers.[5]

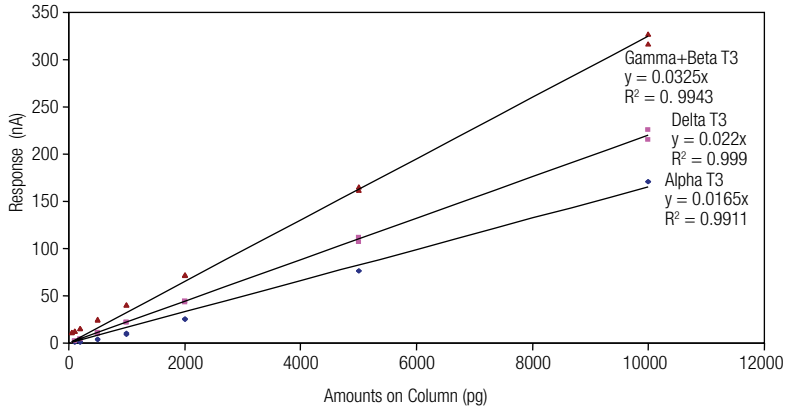

Figure 4. Linearity of the toctrienol standards.

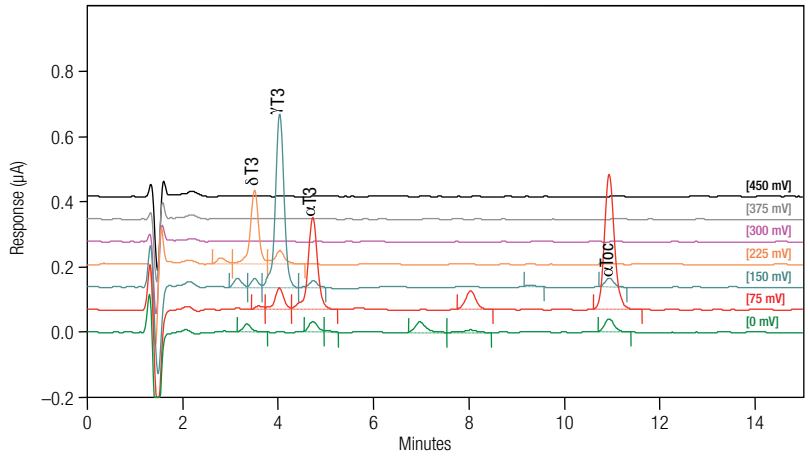

Figure 5. Separation of analytes from palm oil.

Conclusion

Gradient HPLC with multichannel electrochemical detection provides an excellent means of simultaneous detection of tocotrienol and tocopherol isomers. The sensitivity provided with the CoulArray electrochemical technique is a significant improvement over previous methods. This allows for the analysis of these compounds from tissue samples that contain lower levels. Both chromatographic and voltammetric resolution provided should assist in compound identification from biological samples.

Ordering Information

Description	Part Number
5600A CoulArray 8-Ch Detetector Inst 120 Vac	70-4324
CoulArray Thermal Organizer	70-4340T
Accessory Kit, CoulArray Detector to UltiMate 3000 System	70-9191
SR-3000 Solvent Rack Without Degasser	5035.9200
HPG-3400RS Biocompatible Binary Rapid Separation Pump with Two Solvent Selector Valves	5040.0046
WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler	5841.0020

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Other References of Interest

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