A Versatile Detector for the Sensitive and Selective Measurement of Numerous Fat-Soluble Vitamins and Antioxidants in Human Plasma and Plant Extracts

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Introduction

Fat-soluble vitamins (FSVs) play essential roles in a wide spectrum of biochemical and physiological processes. One FSV, Vitamin E (tocopherol), along with a suite of other fat-soluble antioxidants (FSAs) (e.g., carotenoids, CoQ10) are purported to help mitigate the potentially disastrous effects of oxidative stress that has been linked to numerous diseases including cancer, atherosclerosis, and neurodegenerative diseases. These compounds are thought to exert their beneficial effects by acting as chain-breaking antioxidants—inhibiting lipid peroxidation of polyunsaturated fatty acids contained within biological membranes, thereby preventing the formation of potentially cytotoxic and highly reactive aldehydes (malondialdehyde and 4-hydroxynonenal).

Although a number of FSVs and FSAs have been measured using HPLC-UV, this approach typically lacks the sensitivity and selectivity required to measure these compounds in biological samples. Electrochemical detection, however, is both sensitive and selective and makes use of the inherent redox activity of these compounds. The Thermo Fisher Dionex CoulArray™ Coulometric Array Detector—the only HPLC electrochemical detector that is fully gradient compatible—uses an array of flow-through, highly efficient electrochemical sensors to generate qualitative voltammetric data to help identify analytes and resolve coeluting compounds. The versatility of this detector is illustrated using a variety of examples including: a global gradient method for determination of FSVs and FSAs in plasma; a gradient method for the analysis of carotenoid isomers in carrots and human plasma; an isocratic method for the measurement of reactive nitrogen species damage to biomembranes measuring 5-nitro-γ-tocopherol in rat astrocytes; a gradient method for the measurement of tocopherol and tocotrienol isomers in palm oil; and an isocratic method for the determination of reduced and oxidized CoQ9 and CoQ10 in human plasma.

Global FSV and FSA Method

The gradient analytical system consisted of two pumps, an autosampler, and a Dionex CoulArray detector.

Column:	MD150, 150 × 3 mm, 3 µM C18
Mobile Phase A:	Methanol: 0.2 M ammonium acetate, pH 4.4, (90:10) (v/v)
Mobile Phase B:	Methanol: 1-propanol: 1.0 M ammonium acetate, pH 4.4, (78:20:2) (v/v/v)
Gradient Conditions:	Isocratic 0% B from 0 to 4 min. Linear increase of phase B from 0 to 80% from 4 to 15 min. Linear increase of phase B from 80 to 100% from 15 to 25 min. Isocratic 100% phase B from 25 to 32 min. Linear decrease of phase B from 100 to 0% from 32 to 35 min.
Flow Rate:	0.8 mL/min
Temperature:	37 °C
Potentials:	200, 400, 500, 700, 800, -1000, 200, 500 mV vs Pd

Sample Preparation

1. Serum or standard (0.2 mL) + diluent (0.2 mL) [10 mg/L butylated hydroxyanisole (BHA) in ethanol]
2. Add 10 µL of 10 µg/mL retinyl acetate (internal standard)
3. Vortex for 1 min
4. Add 1 mL hexane
5. Vortex 10 min and centrifuge (4000 × g, 10 min)
6. Withdraw 0.8 mL of supernatant and evaporate to dryness by vacuum centrifugation
7. Dissolve residue in 0.2 mL EtOH/BHA diluent by vortex-mixing for 5 min
8. Inject 10 µL

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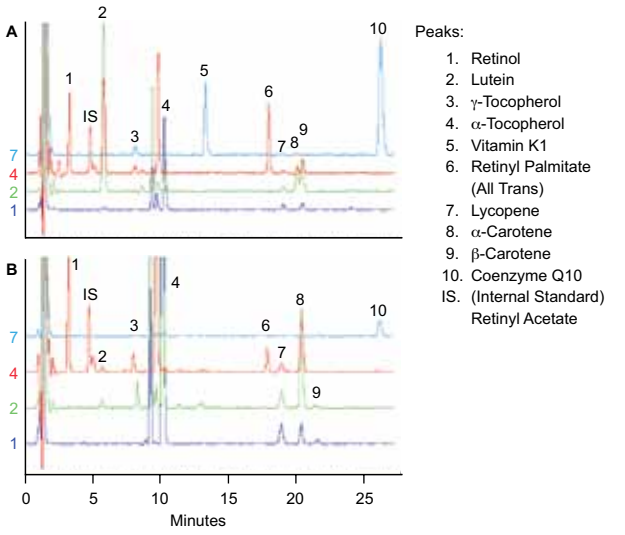
FIGURE 1. Analysis of: A) extracted standard; B) National Institute of Standards and Techology (NIST) medium level serum.



Only some channels (Ch) are shown for clarity. Ch 1 = 200 mV; Ch 2 = 400 mV; Ch 4 = 700 mV; Ch 7 reoxidation at 200 mV following reduction on Ch 6 at -1000 mV.

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Carotenoid Isomers

The gradient analytical system consisted of two pumps, an autosampler, and a Dionex CoulArray detector.

Column:	C30, 4.6 × 250, 5 µm
Mobile Phase:	1 M ammonium acetate, pH 4.0: methyl-*tert*-butyl ether: methanol, 2:35:63 (v/v/v)
Flow Rate:	1 mL/min
Temperature:	Ambient
Potentials:	+100 to +520 mV (vs Pd) in 60 mV increments

Sample Preparation

Sample was mixed with ethanol/BHA extracted with hexane, dried down, and reconstituted in-phase.

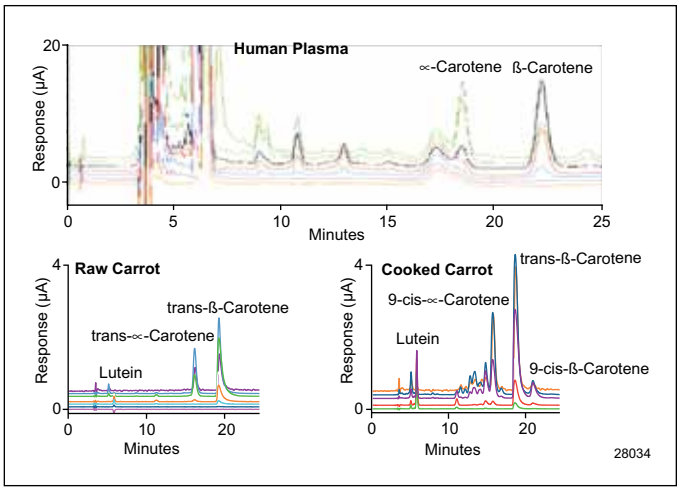
Table 1. Observed vs Expected Values Obtained from NIST[a] Sera

	Concentration					
	Low			Medium		
Name	**Obs.**	**Exp.**	**Diff. %**	**Obs.**	**Exp.**	**Diff. %**
Retinol	292	296	-1.35	498	514	-3.11
Lutein	71.5	62	15.3	74.0	62.9	17.6
γ-Tocopherol	1660	1700	-2.35	2230	2300	-3.00
α-Tocopherol	7490	7070	5.94	10,500	10,100	3.96
Retinly Palmitate	82.4	99	-16.8	193	178	8.4
Lycopene	163	200	-18.5	263	300	-12.3
α-Carotene	24.5	20	22.5	33.7	30	12.3
β-Carotene	222	224	-0.89	576	596	1.23
Coenzyme Q10	59.8	NA	NA	95.7	NA	NA
Vitamin K1	<0.98	NA	NA	< 0.98	NA	NA

NA-value not available in control.

[a] Control sera obtained from NIST Standard Reference Material™ 968b.

FIGURE 2. Analysis of human plasma and raw and cooked carrot samples.



5-Nitro-γ-Tocopherol (An Indicator of Membrane Damage)

The isocratic analytical system consisted of one pump, an autosampler, and a PDA preceding a 12-channel Dionex CoulArray detector.

Column:	TosoHaas, ODS-80TM C18, 4.6 × 250 mm, 5 µm
Mobile Phase:	30 mM Lithium acetate: acetonitrile: methanol: and acetic acid (5:83:12:0.2) (v/v/v/v)
Flow Rate:	2.0 mL/min
Temperature:	Ambient
Inj. Volume:	60 µL
Potentials:	+200, +300, +400, +525, +600, +625, +650, +675, +700, +750, +825, +900 mV vs Pd

Sample Preparation

Rat brain astrocytes were prepared according to Hensley, K.; Williamson, K. S.; Floyd, R. A. Measurement of 3-Nitrotyrosine and 5-Nitro-γ-Tocopherol by High-Performance Liquid Chromatography with Electrochemical Detection. *Free Radical Biol. Med.* **2000**, *28* (4), 520–528 and Williamson, K. S. et al. The Nitration Product 5-Nitro-γ-tocopherol is Increased in the Alzheimer Brain. *Nitric Oxide* **2002**, *6* (2), 221–227.

FIGURE 3. Analysis of standards; 500 pM each on column (reproduced with permission of Dr. Kenneth Hensley, Oklahoma Medical Res. Fdn.).

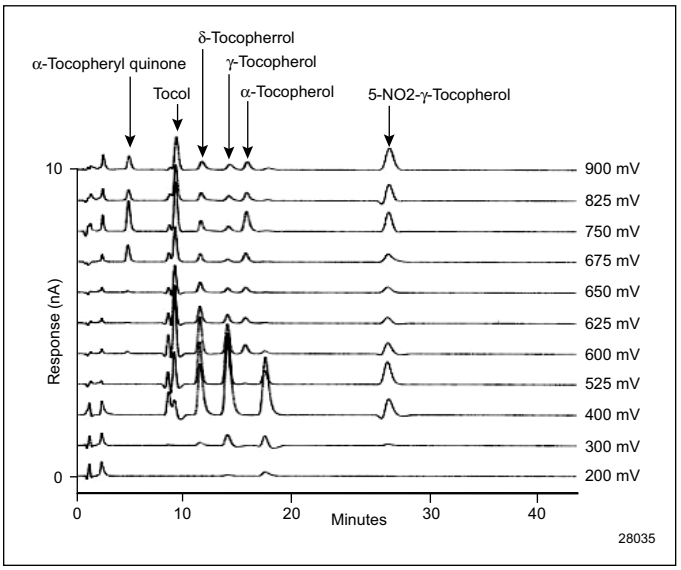
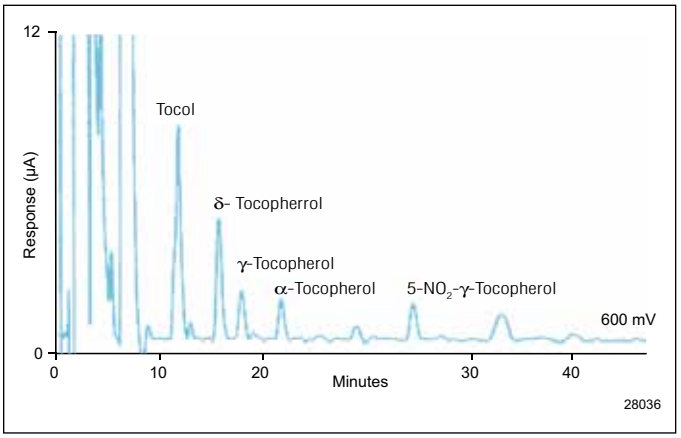


FIGURE 4. Primary rat astrocytes 36 h following stimulation with endotoxin. (The 5-nitro-γ-tocopherol peak is equivalent to 2.5 pmol on column.) Only one channel is shown for clarity. (Reproduced with permission of Dr. Kenneth Hensley, Oklahoma Medical Res. Fdn.)



Tocopherols and Tocotrienols

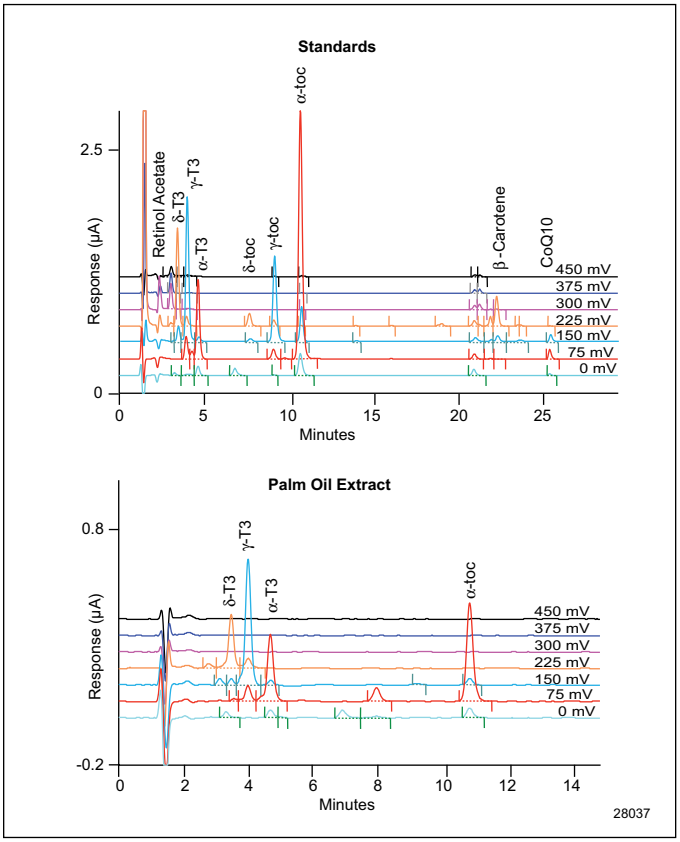
The gradient HPLC-ECD system consisted of two pumps, a refrigerated autosampler, a thermal chamber, and an 8-channel Dionex CoulArray detector.

Column:	Hypersil® BDS, 150 × 3.0 mm, 3 µm, C18
Phase A:	Acetonitrile: water, 90:10 (v/v) containing 20 mM sodium perchlorate and 5 mM perchloric acid
Phase B:	Acetonitrile: 1-propanol, 65:35 (v/v) containing 20 mM sodium perchlorate and 10 mM perchloric acid
Gradient:	Isocratic 10% B from 0 to 4 min. Linear increase of phase B from 10 to 100% B for 21 min. Isocratic 100% B for 9 min before returning to initial conditions for 5 min. Total run time was 40 min.
Flow Rate:	0.6 mL/min
Temperature:	32 °C
Inj. Volume:	10 µL
Potentials:	-700, 0, 75, 150, 225, 300, 375, and 450 mV vs Pd

Sample Preparation

Palm oil samples 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.

FIGURE 5. Analysis of tocotrienols (T) and tocopherols (toc) in palm oil extract. Note expanded time scale for palm oil extract chromatogram.



Oxidized and Reduced CoQ9 and CoQ10 (Ubiquinone and Ubiquinol Speciation)

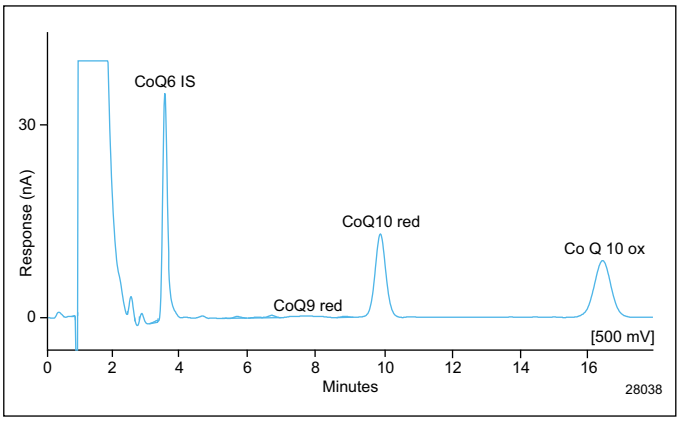
The isocratic HPLC-ECD system consisted of two pumps, a refrigerated autosampler, a thermal chamber, and a 3-channel Dionex CoulArray detector.

Column:	Capcell Pak® C18 MG 100 (4.6 × 50 mm; 3 µ)
Mobile Phase:	Methanol: 1-propanol: 1.0 M ammonium acetate, pH 4.4, (78:20:2) (v/v/v)
Flow Rate:	1 mL/min
Temperature:	32 °C
Inj. Volume:	50 µL (tray at 4 °C)
Applied Potentials:	Model 5021A: +700 mV (vs Pd); Model 5011A: E1 = -700 mV; E2 = +500 mV

Sample Preparation

Blood must be collected in a heparinized Vacutainer® tube. Following centrifugation for 10 min at 4 °C, the plasma was immediately removed and stored at -80 °C until analyzed. A 100 µL volume of plasma was vortexed with 50 µL internal standard and 400 µL (cold) 2-propanol. After centrifugation, the supernatant was transferred to an autosampler vial and analyzed promptly.

FIGURE 6. Analysis of extracted human plasma. For clarity, only the final oxidative analytical channel is shown.



Discussion

- Coulometric array detection is both sensitive (pg) and selective.
- Analytes are identified both by retention time and by voltammetric behavior across the electrode array.
- This versatile detector can be used for either global or targeted analysis of FSVs and FSAs in both mammalian and plant tissues.

Table 2. Analysis of Several NIST Human Plasma Samples Comparing This Data to That Obtained Using Other Analytical Methods

NIST Sample ID	314	315	316	317	318	324	325	326	327	328
TOTAL CoQ10 NIST-Assigned Value (µg/L)	611 ± 54	618 ± 103	618 ± 89	776 ± 135	620 ± 90	770 ± 77	986 ± 99	1190 ± 119	740 ± 74	350 ± 35
ESA CoQ10 Red	150	370	540	585	600	51	522	1042	658	192
ESA CoQ10 Ox	310	220	50	585	600	51	522	1042	658	192
ESA CoQ10 total [ΣRed + Ox] (% red)	460 (32)	590 (63)	590 (92)	745 (79)	705 (85)	612 (8)	903 (58)	1157 (90)	716 (92)	261 (74)
Other CoQ10 Red	nd	195	488	472	452	140	150	830	780	200
Other CoQ10 Ox	49	326	120	263	127	450	700	360	110	150
Other CoQ10 total [ΣRed +Ox] (% red)	49 (-)	521 (80)	608 (80)	735 (64)	579 (78)	590 (24)	850 (17)	1190 (70)	890 (88)	350 (57)

Total CoQ10 amounts were in good agreement to NIST values. Although NIST values do not report levels of reduced and oxidized forms separately, data from our method show considerable oxidation of the NIST samples. When sample preparation and storage were controlled appropriately, levels for normal patient samples (n = 30) were found to be: CoQ10 0x = 48 ± 23 (µg/L); CoQ10 Red = 775 ± 290; CoQ10 Total = 827 ± 303; 94% in reduced form.

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