

Free Radical Biology and Medicine: The Use of HPLC with Electrochemical Detection for the Measurement of Pro-Oxidants, Damage Markers, and Protective Mechanisms

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INTRODUCTION

It is an interesting paradox that the very molecule essential for aerobic life, oxygen, is itself highly toxic and gives rise to a variety of reactive oxygen species. These, along with other pro-oxidants (e.g., peroxy-nitrous acid and hypochlorous acid), can cause major damage to essential macromolecules such as DNA, proteins, and membrane lipids. Fortunately, the body is equipped with a variety of protective mechanisms—metal binding proteins, enzymes, and low molecular weight antioxidants—designed to control the formation of pro-oxidants, or to repair macromolecular damage. An imbalance between the production of pro-oxidants and protective mechanisms, sometimes referred to as oxidative stress, is thought to underlie many diseases and to be the cause of aging.

Redox reactions are central to pro-oxidant formation, damage, and protection. For example, in the mitochondrion, oxygen is reduced in a series of single electron transfer reactions resulting in the formation of superoxide, hydrogen peroxide, and finally the hydroxyl free radical. Conversely, α -tocopherol, a chain-breaking antioxidant, prevents lipid peroxidation from taking place by reducing the highly reactive lipid peroxyl radical to the less aggressive lipid hydroperoxide. It should come as no surprise therefore, that electrochemical detection, an analytical approach that uses current flow to quantify an analyte as it undergoes a redox reaction, is uniquely suited to measuring compounds of interest to those studying free radical biology and medicine. Presented here are a number of HPLC-ECD applications including the measurement of: hydroxyl free radical production; 8-hydroxy 2'-deoxyguanosine as a marker of DNA damage; nitro- and chlorotyrosine as markers of peroxy-nitrous acid and hypochlorous acid formation, respectively; and protective fat-soluble vitamins and antioxidants.

PRO-OXIDANTS

Formation

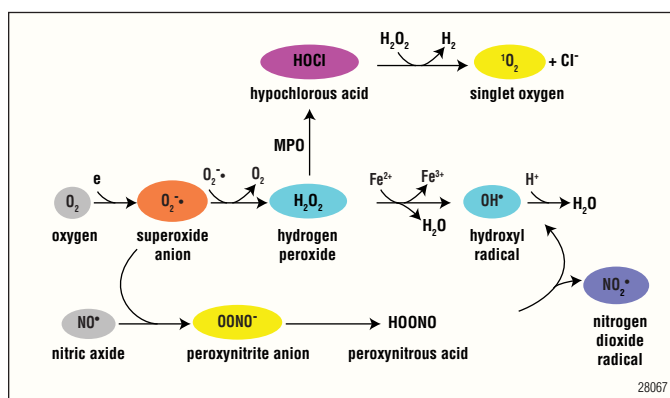
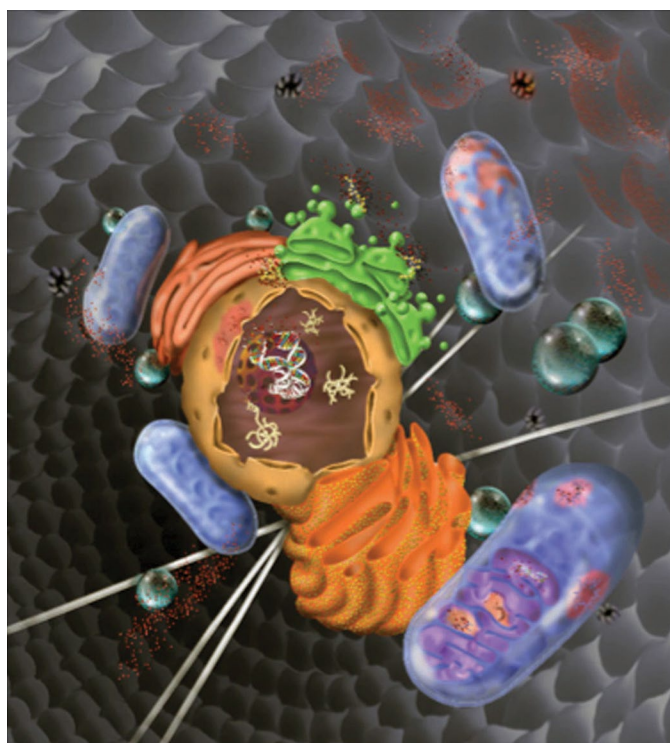


Figure 1. Pathways for reactive oxygen, nitrogen, and halogen species (*mpo* = myeloperoxidase).



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Table 1. Brief Summary of Some of the Different Analytical Methods Used to Determine Reactive Oxygen, Nitrogen, and Halogen Species

ROS/RNS	Measurement	Techniques	Reference
Superoxide	Reaction with Hydroethidine	HPLC-ECD, FLD	Zielonka et al., (2009). <i>Free Radic. Biol. Med.</i> , 46, 329–338.
Hydrogen Peroxide	Technique dependent—direct or indirect	HPLC-ECD, FLD Enzymatic Electrochemical Sensors	Afanasev, I. (2009). <i>Front Biosci.</i> , 1, 153–160. Whiteman et al., (2009). <i>Methods Mol. Biol.</i> , 47, 28–49. Yue et al., (2009). <i>Int. J. Pharm.</i> 375, 33–40.
Hydroxyl Free Radical	Use of scavengers (e.g., salicylic acid, 4-hydroxybenzoic acid, D-phenylalanine) Potential biomarkers (e.g., 8-OH-2'dG; dityrosine)	HPLC-ECD, FLD	Acworth, I.N. (2003). <i>The Handbook of Redox Biochemistry</i> , p/n70-6090 (ESA) and references therein; Acworth et al., (1999). <i>Methods Enzymol.</i> , 300, 297–313. Biondi et al., (2006). <i>Cardiovasc. Res.</i> , 71, 322–330. McCabe et al., (1997). <i>J Chromatogr. B</i> , 691, 23–32.
Nitric Oxide	Indirect - Nitrite/nitrate Direct	HPLC-ECD, UV, FLD; IC-Conductivity Electrochemical sensors	Acworth, I.N. (2003). <i>The Handbook of Redox Biochemistry</i> , p/n70-6090 (ESA) and references therein; Tsikas, D. (2005). <i>Free Radic. Res.</i> , 39, 797–815. Barbosa et al., (2008). <i>Methods Enzymol.</i> , 441, 351–367. Davies and Zhang (2008). <i>Methods Enzymol.</i> , 436, 63–95.
Peroxynitrous Acid	Indirect. Specificity? Possible biomarkers include 3-nitrotyrosine (free and protein-bound), 6-nitrocatecholamines, and 5-nitro- γ -tocopherol	HPLC-ECD, UV; LC-MS(n)	Acworth, I.N. (2003). <i>The Handbook of Redox Biochemistry</i> , p/n70-6090 (ESA) and references therein.
Hypochlorous Acid	Indirect. Specificity? Possible biomarkers include 3-chlorotyrosine and 8-chloro-2'dG	HPLC-ECD, UV; LC-MS(n)	Acworth, I.N. (2003). <i>The Handbook of Redox Biochemistry</i> , p/n70-6090 (ESA) and references therein; Asahi, T., et al., (2010). <i>J. Bio. Chem.</i> , 285, 9282–9291.

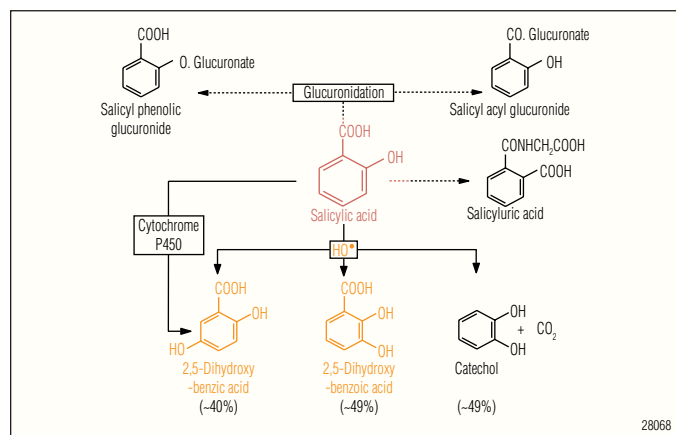


Figure 2. Hydroxyl free radical production can be determined by scavenging with salicylic acid (SA) and measuring the dihydroxybenzoic acid (DHBA) isomers thus formed using HPLC-ECD.

Conditions

Column:	ESA DHBA-250, 250 × 3.0 mm, 5 μ m
Mobile Phase:	50 mM Sodium acetate, 50 mM citric acid, 8% (v/v) methanol, 2.0% (v/v) isopropanol, pH 2.5 with phosphoric acid
Flow Rate:	0.5 mL/min
Temp:	Ambient
Detection:	Model 5300, Coulochem® III
Guard Cell:	Model 5020 EGC = +775 mV
Analytical Cell:	Model 5010 E1 = +250 mV; E2 = +750 mV

Experimental Protocol

Male Sprague Dawley rats received 180–200 g either saline or salicylic acid (SA) (100 mg/kg) i.p. Animals were decapitated after 30 min and tissue samples (brain, liver, kidney, and blood) were removed and kept on ice. The brain was dissected into striata, hippocampi, and cortex. Solid tissues were ultrasonicated in ice-cold 0.2 M perchloric acid (containing 100 μ M EDTA and 100 μ M sodium metabisulfite; 1:5 or 1:10 w/v). Following centrifugation (12.5 krpm, 5 min, 4 °C), the supernatant was passed through a 0.2 μ m filter prior to analysis. Blood was centrifuged (as above), the plasma was removed, deproteinized (as above, but 1:5 v/v) and filtered (as above). In a second experiment, animals received either saline or SA as above followed by either saline or amphetamine (5 mg/kg i.p.) 30 min later. Following an additional 30 min period the animals were sacrificed, their brains removed, and processed as above.

RESULTS

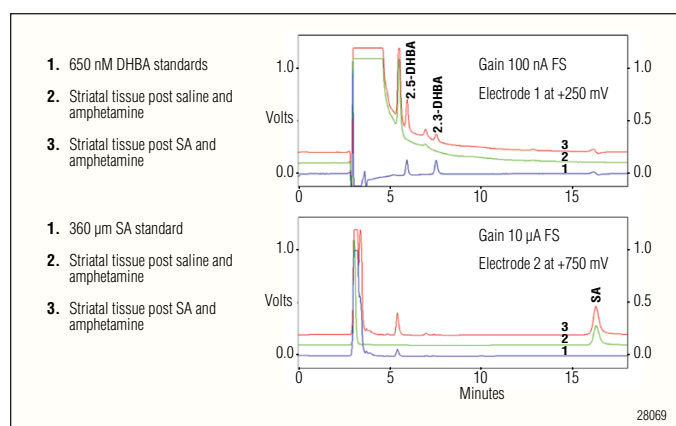


Figure 3. Chromatograms showing resolution of DHBA isomers (upper) and SA (lower) in amphetamine treated rats. (Acworth et al., *Methods Enzymol.* **1999**, 300, 297–313).

DISCUSSION

The SA scavenging approach is an effective way to measure the production of hydroxyl free radicals in a number of tissues. With a rate constant of $\sim 5 \times 10^9$ – 10^{10} $M^{-1}sec^{-1}$, it is 2.5 and 22 times more rapid than approaches using D-phenylalanine and 2'-deoxyguanosine, respectively. With an LOD of ~ 1 pg for the DHBA isomers, the sensitivity of the approach enables the use of minimal amounts of SA—a major advantage over similar less sensitive approaches where the need for higher levels of SA can have unwanted physiological effects. Another major benefit to our approach is that it uses two sensitive and selective coulometric electrodes in series: the upstream sensor detects the DHBA isomers while the downstream sensor detects SA. This overcomes the poor sensitivity and issues with chromatographic interferences that typically plague single amperometric electrode methods. Finally, the coulometric sensor has a wide dynamic range (typically pg– μ g) enabling the measurement of low levels of DHBAs and high levels of SA on the same detector. Our approach does not require the use of UV detection for the measurement of SA.

DNA MARKERS

Formation

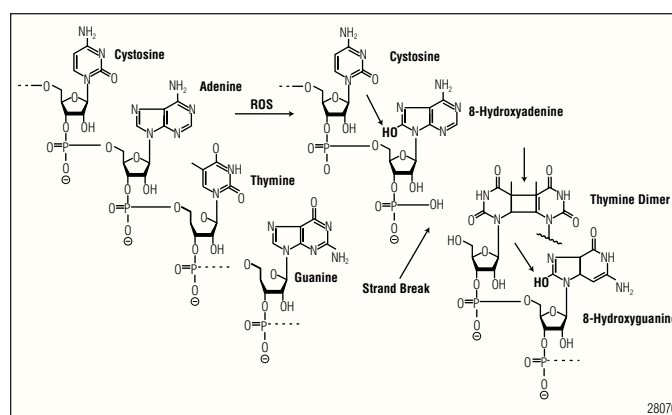


Figure 4. Examples of ROS-induced damage to DNA including adduct formation and strand scission. For the sake of clarity only one of the DNA strands is shown.

Table 2. Tissue Production of DHBA Isomers Following Treatment with Saline (Control) or SA*

Tissue	2,3-DHBA (pmol/g) Control	2,3-DHBA (pmol/g) Post SA	2,5-DHBA (pmol/g) Control	2,5-DHBA (pmol/g) Post SA	SA Control	SA (nmol/g) Post SA	2,3-DHBA/SA ($\times 10^{-3}$)	2,5-DHBA/SA ($\times 10^{-3}$)
Kidney	49	780	190	6,170	—	298	2.6	21
Liver	54	268	—	3,344	—	206	1.3	16
Brain Striatum	—	93	—	48	—	28	3.3	1.7
Brain Cortex	—	114	—	136	—	48	2.4	2.8
Serum (pmol/mL)	36	753	214	10,130	—	935	0.8	11

*Acworth et al., *Methods Enzymol.* **1999**, 300, 297–313.

DNA is relatively fragile and can be damaged in a number of ways (e.g., exposure to ionizing radiation, alkylating agents, and pro-oxidants), resulting in strand breaks and DNA adduct formation. Guanine is particularly susceptible.

The formation of the adduct 8-hydroxy-2'-deoxyguanosine (8-OH-2'dG) in DNA can result in a G→T transversion, and is one of the most mutagenic adducts identified so far.

MEASUREMENT

Adducts liberated from DNA can be measured using a number of approaches, and these have been evaluated by The European Standards Committee on Oxidative DNA Damage (ESCODD). GC-MS has identified numerous adducts, however, care must be exercised to prevent artifact formation. Although LC-MS(n) is selective, it is not always sufficiently sensitive to measure basal adduct levels (Collins et al., *Arch. Biochem. Biophys.* **2004**, 423, 57–65). Furthermore, LC-MS(n) is expensive and can be difficult to use. ESCODD found that HPLC with coulometric electrochemical detection is sensitive, selective, and is capable of measuring dose-induced 8-OH-2'dG formation in test samples (Collins et al., *Arch. Biochem. Biophys.* **2004**, 423, 57–65).

Conditions

Column: YMC Basic, 4.6 × 150 mm, 3 μm
Mobile Phase: 100 mM Sodium acetate, 5% methanol (v/v), pH 5.2
Flow Rate: 1.0 mL/min
Temperature: 31 °C
Detection: Coulochem III (5300)
5010 Analytical Cell: E1 = +400 mV; E2 = +800 mV

Experimental Protocol

To show the applicability of the method to measuring DNA damage, DNA was extracted from calf thymus and was subjected to singlet oxygen-induced photosensitization using methylene blue and irradiation (75 W incandescent light source, 30 min). A glass plate was placed between the light source and sample in order to prevent heat-induced damage. The production of the 8-OH-2'dG adduct was then determined in the isolated and hydrolyzed DNA (McCabe et al., *J. Med. Food* **1999**, 2, 209–214).

RESULTS

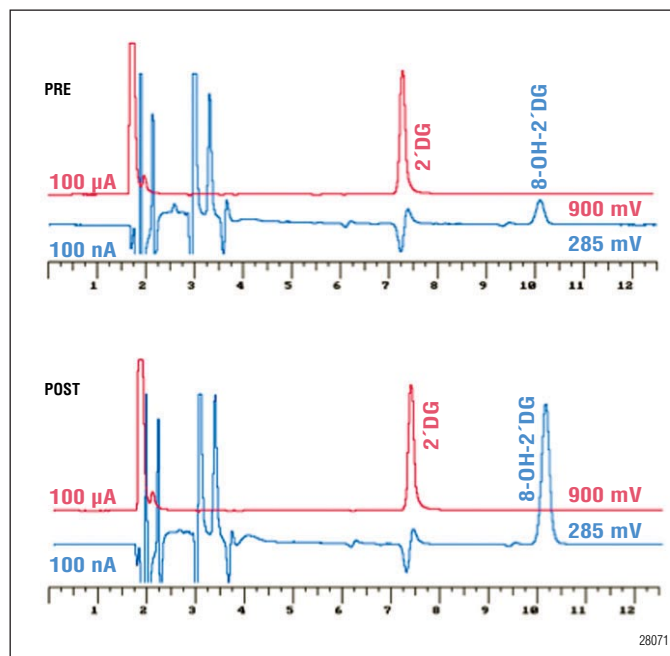


Figure 5. Chromatograms show a significant formation of 8-OH-2'dG adduct following exposure of DNA to singlet oxygen. Note: The downstream 900 mV channel (orange) selective for 2'dG is presented at 1000x less sensitivity than the upstream 285 mV channel selective for 8-OH-2'dG.

DISCUSSION

The HPLC-ECD method is extremely sensitive (LOD ~20 pg on column) and has the dynamic range needed for the simultaneous measurement of low levels of adducts and high levels of unaltered parent base (2'dG). Once again, the advantage of selective and sensitive coulometric electrodes can be seen. The assay was linear ($r^2 > 0.9991$). As little as 40 μg of DNA is needed for the assay. Exposure of DNA to singlet oxygen caused a marked increase in adduct formation and a >300% increase in the 8-OH-2'dG/2'dG ratio compared to controls (exposed to light but not to methylene blue).

The range of bases, nucleosides and adducts can be markedly extended by the use of a gradient HPLC along with a CoulArray® multi-electrode ECD and a post-array UV detector. (McCabe et al., *J. Med. Food* **1999**, 2, 209–214) used this approach to measure 23 DNA relevant analytes.

AMINO ACID DAMAGE MARKERS

Formation

Free amino acids or their residues in peptides and proteins can be covalently modified by reaction with pro-oxidant species. Although the production of protein carbonyls has been used extensively as a possible indicator of oxidative stress, it appears to be nonspecific as carbonylation can take place by reactions not involving attack by pro-oxidant species. Modification of tyrosine residues appears to be a much better indicator of oxidative stress with different products being formed from different pro-oxidants (e.g., 3-nitrotyrosine from RNS; 3-chlorotyrosine from hypochlorous acid).

MEASUREMENT

A number of different analytical methods are used to measure modified amino acid residues either free or liberated (post hydrolysis of peptides/proteins). These approaches include immunochemical techniques as well as HPLC-UV, HPLC-FLD, GC-MS and LC-MS(n). HPLC-ECD offers a number of advantages in that it is sensitive, selective, easy to operate and as is the case with the CoulArray, provides both quantitative and qualitative data.

Conditions

Column: MD150, 150 × 4.6 mm 3 μm ODS
 Mobile Phase: 75 mM NaH₂PO₄, 1.7 mM OSA, 100 μL/L TEA, 10% (v/v) CH₃CN, pH = 3.0 with H₃PO₄, 100 mM Sodium acetate, 5% methanol (v/v) pH 5.2
 Flow Rate: 0.6 mL/min
 Temperature: Ambient
 Detection: CoulArray (5600)
 Potentials: 0 to +720 mV in 80 mV increments

RESULTS

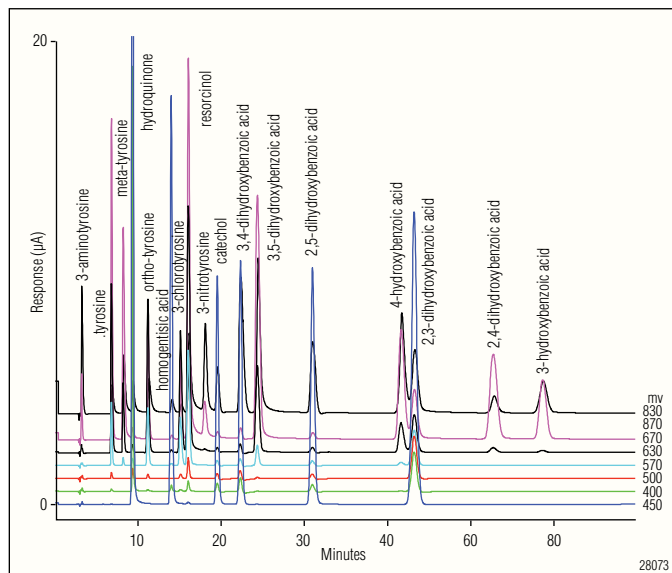


Figure 7. CoulArray chromatogram showing both chromatographic and electrochemical resolution of different modified tyrosine residue standards (5 ng each on column).

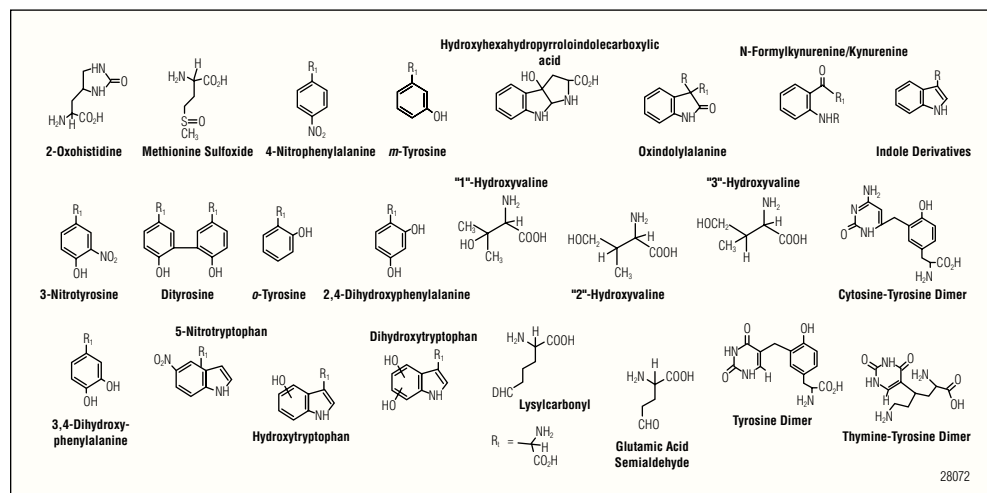


Figure 6. Just a few of the modifications that can be formed when amino acids, either free or as part of proteins, are exposed to ROS, RNS or other Pro-oxidant species reported in literature.

DISCUSSION

The HPLC-CoulArray ECD method is both selective and sensitive (~5 pg on column). Free CSF levels of 3-nitrotyrosine were found to be 1.4 ± 0.7 nM (control patients). 3-Nitrotyrosine levels in hydrolyzed plasma proteins were $\sim 2/10^6$ modified residues/tyrosine residues in control patients.

PROTECTIVE MECHANISMS

Cells contain a number of protective mechanisms to prevent or limit the damage caused by pro-oxidant species. These include repair mechanisms (e.g., DNA), destruction of damaged species (e.g., proteins), and a suite of antioxidants including enzymes (e.g., superoxide dismutase; catalase), transition metal binding proteins (e.g., transferrin, ferritin), and low molecular weight species (e.g., ascorbic acid, glutathione, tocopherol, and ubiquinone).

MEASUREMENT

A number of analytical techniques exist for the measurement of individual low molecular weight antioxidants including colorimetric, spectrophotometric, and enzymatic approaches. Numerous HPLC-based approaches are reported in literature, with HPLC-ECD offering improved sensitivity and selectivity over HPLC-UV and FLD, without the need for derivatization. Gradient HPLC with coulometric array detection can be used to simultaneously measure numerous fat-soluble vitamins and antioxidants in biological tissues (e.g., human serum or plasma).

Conditions

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
Detection: CoulArray (Model 5600)
Potentials: 200, 400, 500, 700, 800, -1000, 200, 500 mv vs Pd

Sample Preparation

1. Serum or standard (0.2 mL) + (0.2 mL) diluent [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 1.0 mL of hexane
5. Vortex 10 min and centrifuge (4000x g, 10 min)
6. Withdraw 0.8 mL of supernatant and evaporate to dryness by vacuum centrifugation
7. Dissolve residue in 0.2 mL ethanol/BHA diluent by vortex-mixing, 5 min
8. Analyze 10 μL

RESULTS

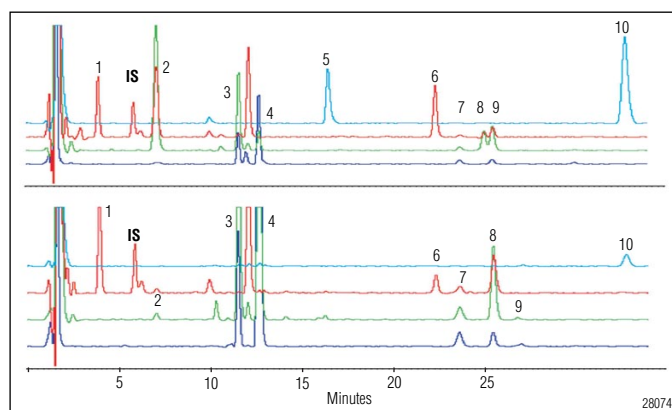


Figure 8. Extracted standards (Upper Figure) and extracted NIST Medium Level Serum (Lower Figure). 1) Retinol; 2) Lutein; 3) γ -tocopherol; 4) α -tocopherol; 5) vitamin K1; 6) retinyl palmitate (all trans); 7) lycopene; 8) α -carotene; 9) β -carotene; 10) coenzyme Q10; IS - Internal Standard-retinyl acetate

DISCUSSION

The CoulArray electrochemical detector is the only ECD that is fully gradient compatible. It offers sensitivity and selectivity, and also provides qualitative results based on an analyte's voltammetric profile. The assay had a limit of detection of <10 pg for most analytes, showed good linearity (typically $r^2 > 0.999$ for 0 to 1 $\mu\text{g/mL}$) and excellent intra-assay precision (% relative standard deviation (%RSD) using 18 replicates of a pooled serum extracted and analyzed using the NIST high control as the external standard) of typically <2% for the majority of analytes.

CONCLUSION

- HPLC with electrochemical detection is a very powerful analytical approach with great relevance to the field of free radical biology and medicine.
- HPLC with electrochemical detection can be used to study the production of pro-oxidant species, the damage they cause to biologically relevant macromolecules and the body's protective antioxidant defenses.
- The CoulArray detector is gradient compatible and is not only sensitive and selective but also produces qualitative data for analyte authentication.

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