[27] Simultaneous Detection of Tocopherols and Tocotrienols in Biological Samples Using HPLC-Coulometric Electrode Array

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Introduction

Vitamin E is a generic term for all tocopherols and tocotrienols derived from a chromanol structure having the biological activity of RRR-α-tocopherol.[1,2] In nature, eight substances have been found to have vitamin E activity: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. These compounds are closely related homologues and isomers depending, respectively, on the number and position of methyl groups on the aromatic ring. Tocotrienols, formerly known as ζ-, ε-, or η-tocopherols, are similar to tocopherols except that they have an isoprenoid tail with three unsaturation points instead of a saturated phytyl tail. While tocopherols are found predominantly in corn, soybean, and olive oils, tocotrienols are particularly rich in palm, rice bran, and barley oils.[1,2] The structural complexity and the wide variation in biological activity of these compounds require reliable and sensitive analytical techniques for the isolation, separation, differentiation, and quantification of individual components in mixtures derived from various sample matrices.[3] Because of their low concentrations in biological samples, sensitivity is particularly a critical issue for the detection methods of tocotrienols.

Because of their low oxidative potential, the various forms of vitamin E can be analyzed by reversed-phase HPLC-electrochemical detection. Of importance, electrochemical properties of the various forms of vitamin E are not identical, requiring different detector potentials for the optimal detection of each form. Simultaneous detection of tocopherol and tocotrienol using single or dual channel channel electrochemical (EC) detection alone or in combination with UV detection has been reported.[4,5] Single or dual channel electrochemical detectors are typically used at settings that are suitable only for few analytes. In this way, sensitivity of detection of the other forms of vitamin E is compromised. Furthermore, gradient elution chromatography required for such multicomponent analyses has poor compatibility with the amperometric methods. The method reported herein

[1] M. G. Traber and L. Packer, *Am. J. Clin. Nutr.* **62,** 1501S (1995).
[2] M. G. Traber and H. Sies, *Annu. Rev. Nutr.* **16,** 321 (1996).
[3] S. L. Abidi, *J. Chromatogr. A* **881,** 197 (2000).
[4] M. Podda, C. Weber, M. G. Traber, R. Milbradt, and L. Packer, *Methods Enzymol.* **299,** 330 (1999).
[5] M. Podda, C. Weber, M. G. Traber, and L. Packer, *J. Lipid Res.* **37,** 893 (1996).

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was developed to simultaneously analyze various isoforms of tocopherol and tocotrienol from biological samples using gradient elution chromatography and a coulometric electrode array detector. This detector is based on the use of multiple coulometric electrochemical sensors in series, maintained at different potentials. This allows for a combination of detector potentials, each optimal for a specific analyte.

Principles of HPLC-EC Coulometric Detection

Electrochemistry involves heterogeneous chemical reactions between a compound and an electrode in which an electron is transferred from the solution to the electrode, or vice versa, and a measurable current is formed as a result. For such oxidation–reduction reactions to occur, energy in the form of an electric potential is required. In a traditional electrochemical detector, the potential is held constant (DC mode) and current is measured as a function of time. When an electroactive species flows through the electrode, current is formed. The magnitude of this current is proportional to the concentration of the compound in solution on the electrode. Most electrochemical detectors for HPLC operate in the amperometric mode. In such a mode, the solution of the compound only passes over a flat bed of working electrode. Under such conditions only a small flat surface area of the electrode is available for interaction with the analyte. As a result, only 5–15% of the electroactive species is oxidized or reduced by the electrode. In contrast, coulometric detectors use flow-through or porous graphite electrodes. The surface area of such electrodes is large, allowing almost 100% of the analyte to react with it. Thus, the efficiency in coulometric detection is approximately 100% compared to conventional amperometric detection, which has only 5–15% detection efficiency.

Coulometric Electrode Array Detector

HPLC-based spectrophotometric detection techniques were revolutionized with the development of the photodiode array (PDA) detector, which is capable of monitoring hundreds of wavelengths simultaneously. Matson *et al.*[6] conceptualized a PDA equivalent of an electrochemical detector and, in 1984, developed an HPLC-based detector containing a serial array of up to 16 coulometric electrodes. Like PDA, this detector provided the ability to evaluate peak purity, assign identity with higher confidence, and resolve coeluting solutes. Coulometric electrode series array sensors provide a route to multiply the resolving power of conventional LC by factors of 10 to 50. Femtogram level separations can now be performed for multiple components in both isocratic and gradient modes.

[6] W. R. Matson, P. Langlais, L. Volicer, P. H. Gamache, E. Bird, and K. A. Mark, *Clin. Chem.* **30,** 1477 (1984).

The coulometric electrode array offers several advantages over conventional single-channel detectors beyond its high resolution. Using a progressively increasing oxidative array of 16 electrodes, compounds can be made to react at three consecutive sensors. The upstream electrode oxidizes a small portion of the analyte, the second dominant electrode oxidizes the bulk of the analyte, and the downstream electrode oxidizes the remainder. A particular standard eluting at a given retention time will always provide a predictable response across these three electrodes. The ratio of the response across these three electrodes remains constant and is referred to as ratio accuracy. The comparison of ratio accuracy of standard versus sample is powerful and an immediate indicator of peak purity. Compared to the standard, a lower ratio accuracy in the sample will indicate presence of a coelution.

HPLC Apparatus and Analytical Cells

In this method for the detection of various forms of tocopherols and tocotrienols, the HPLC system (ESA Inc., Chelmsford, MA) consists of the following components: (1) coularray detector (Model 5600 with 12 channels), PEEK tubing for an inert connection to the HPLC system; (2) UV detector (Model 520 UV/Vis HPLC detector) set up in-line with the coularray detector, with the eluent passing first through the UV/Vis detector; (3) pumps (two ESA Model 582 dual-piston pumps with gradient option), HPLC dynamic gradient mixer, and PEEK pulse damper; and (4) an autosampler (Model 542) with sample cooling and an integrated column oven. CoulArray for Windows-32 software is used for data acquisition and processing.

Column and Mobile Phases

MDA-150 (C_{18} column, 150 mm long $\times$ 4.6 mm i.d., 5-μm pore size; ESA Inc.) is used for the separation of tocopherols and tocotrienols.

Mobile Phase

A gradient is used consisting of a mixture of A (methanol : 0.2 *M* CH_3COONH_3, pH 4.4; 90 : 10, v/v) and B (methanol : 1-propanol : 1.0 *M* CH_3COONH_3, pH 4.4; 78 : 2 : 20, v/v/v). Solvents used to prepare the mobile phase are of HPLC grade. Mobile phases are filtered through 0.22-μm pore size nylon filters. The flow rate is maintained at 0.5 ml/min throughout the analysis. The following gradient program is used: the initial condition is 100% A and 0% B. The mobile phase is changed linearly over 10 min to 20% A and 80%, after which the mobile phase is changed linearly over the next 10 min to 100% B. The system is reverted back linearly over the next 5 min to the initial conditions, i.e., 100% A, where it is continued for 5 min to equilibrate.

Standards and Standard Curve

Authentic and high purity (>98%) tocopherol and tocotrienol compounds are from Sigma-Aldrich (St. Louis, MO), BASF (Germany), and Carotech Inc. (Malaysia). The stock solution of standards is prepared in HPLC-grade ethanol, and their concentrations are determined spectrophotometrically.[5] These compounds are highly unstable and oxidize readily at room temperature. To avoid oxidation, stock standard solutions are stored at $-80°$ or in liquid nitrogen. The concentrations of tocopherol and tocotrienol versus the corresponding peak area response have been plotted in Fig. 1. Injection of large amounts of the compounds may overwhelm the redox capacity of the electrode and may cause a deviation from the linear relationship between peak area and sample quantity. Repeated regression analysis of standards over a several-day period results in high correlation ($R = 0.99$), indicating low variability of analysis in different days. The concentration of vitamin E components used to prepare the standard curve is kept in a range that matches the concentration of these compounds in biological cell samples.

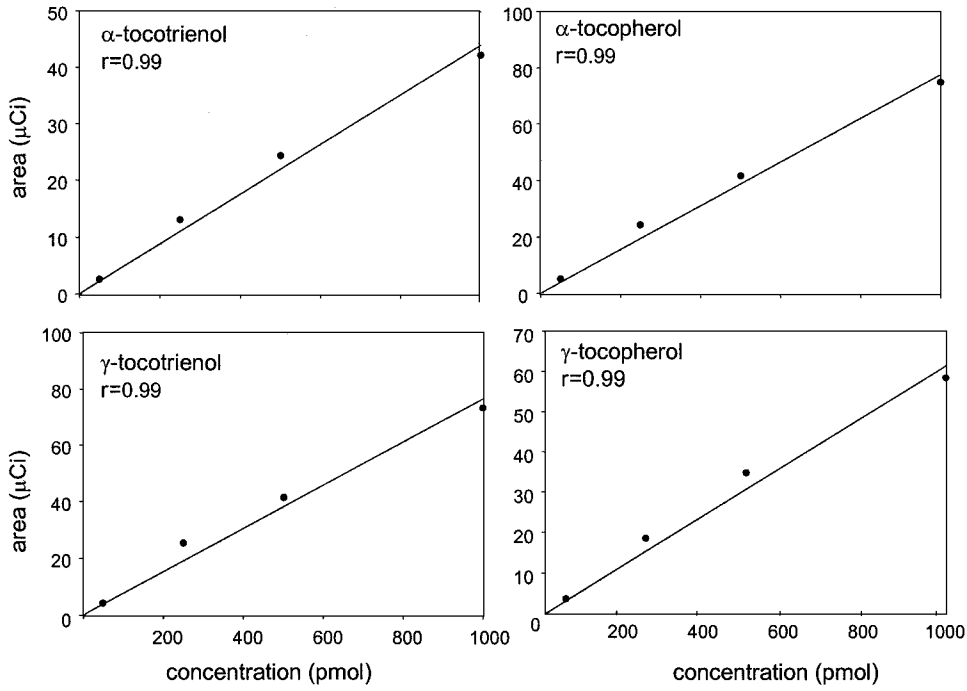

FIG. 1. Representative standard curves for α- and γ-tocopherols and tocotrienols. The conditions of chromatography are described in the text.

Current–Voltage Response Curve

The optimal use of an electrochemical detector for liquid chromatography requires knowledge of the appropriate potentials to drive the desired electrochemical reaction. This potential is dependent on a large number of factors, including the nature of the electrode surface, pH, composition of the mobile phase, and chemistry of the compound of interest. A plot of current generated (peak height) versus applied potential difference is commonly referred to as a hydrodynamic (HDV) voltammogram or a current–voltage (C-V) curve.

The optimum potential for the oxidation of α- and γ-tocopherol/tocotrienol is determined by injecting the compounds onto the column and by adjusting the potential difference across electrode 1–10 from 0 to 1000 mV. The optimum potential for the quantitative measurement of α-tocopherol/tocotrienol and γ-tocopherol/tocotrienol is 200 and 240 mV, respectively (Fig. 2). Using these settings of potential differences, the maximum peak area with minimal background in response to a given injection of vitamin E components is obtained. The C-V curve clearly indicates that the α forms (200 mV) of tocopherols/tocotrienols oxidize more readily than the corresponding γ forms (240 mV).

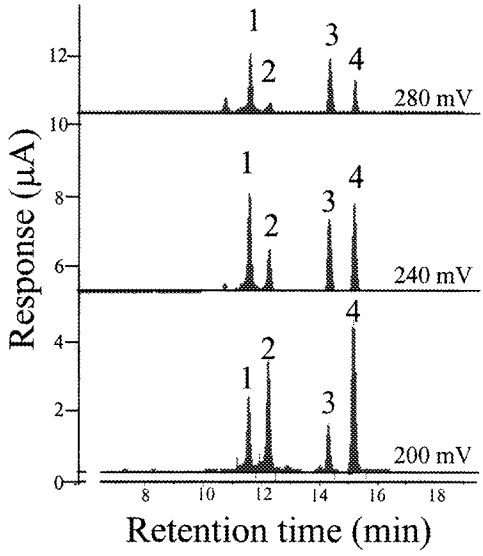

FIG. 2. Current–voltage response for (1) γ-tocotrienol, (2) α-tocotrienol, (3) γ-tocopherol, and (4) α-tocopherol. The responses of α-tocopherol and α-tocotrienols were maximal at 200 mV, whereas maximal responses of γ-tocopherol and γ-tocotrienols were observed at 240 mV.

Detection of Tocopherol and Tocotrienols in Biological Samples

To demonstrate that the method is applicable for biological samples, vitamin E components were analyzed from tissues of rats supplemented intragastrically with a tocotrienol-rich fraction (TRF) isolated from palm oil. TRF is provided in the form of Tocomin (78% tocotrienols and 22% tocopherols) by Carotech Inc. (Malaysia).

Supplementation and Tissue Collection

Eight-day pregnant rats are fed (intragastrically) daily with 1 g/kg body weight of TRF suspended in vitamin E-stripped corn oil for 9 days. The control group is fed intragastric daily vitamin E-stripped corn oil. Both groups of rats receive a standard laboratory diet (Harlan-Teklad, Indianapolis, IN). A third group of nonsupplemented rats is maintained on a vitamin E-deficient diet (Harlan-Teklad). On day 17 of pregnancy, rats are killed and tissues are collected from the mother. Tissues are rinsed in ice-cold phosphate buffered saline, pH 7.4 (PBS), and snap froze in liquid nitrogen. The samples are kept stored in liquid nitrogen until extraction and HPLC analysis. HPLC assays are done within 1 week of storage.

Extraction of Vitamin E from Biological Samples

Weigh approximately 150–200 mg tissue quickly, grind under liquid nitrogen using a mortal and pestle, and homogenize with a Teflon pestle on ice in a Potter–Elvenhjem tube containing PBS (1 ml/100 mg tissue) and butylated hydroxytoluene (BHT, 10 mg/ml stock, 50 μl BHT stock/100 mg tissue). Transfer the homogenate to a screw-cap glass tube, add 0.1 *M* sodium dodecyl sulfate (SDS, 1 ml/100 mg tissue), and vortex vigorously for 30 sec. Take 100 μl of homogenate for protein analysis (described later). To the rest of the homogenate, add ethanol (2 ml/100 mg tissue), mix briefly and sonicate on ice water, and extract homogenate with hexane (2 ml/mg tissue). Dry an appropriate aliquot under nitrogen and resuspend in mobile phase B. Filter the samples using microfilterfuge tubes (Rainin, Woburn, MA) fitted with a 0.22-μm nylon filter before injecting to HPLC.

A representative chromatogram form spleen of rats demonstrates that these vitamin E components are well separated using the present HPLC method (Fig. 3). A small amount of α- and γ-tocotrienols was observed in the spleen of a rat that was supplemented with vitamin E-stripped corn oil and maintained on a standard laboratory diet, suggesting the presence of these vitamin E forms in a standard rat diet. This is consistent with an earlier report in which the presence of these compounds was demonstrated in a standard laboratory mouse diet.[5] TRF feeding increased α- and γ-tocotrienol content in the rat tissue several folds (Fig. 3). A significant decrease in α-tocopherol, as well as other vitamin E forms, was observed in the spleen of nonsupplemented rats that were maintained on a vitamin E-deficient diet.

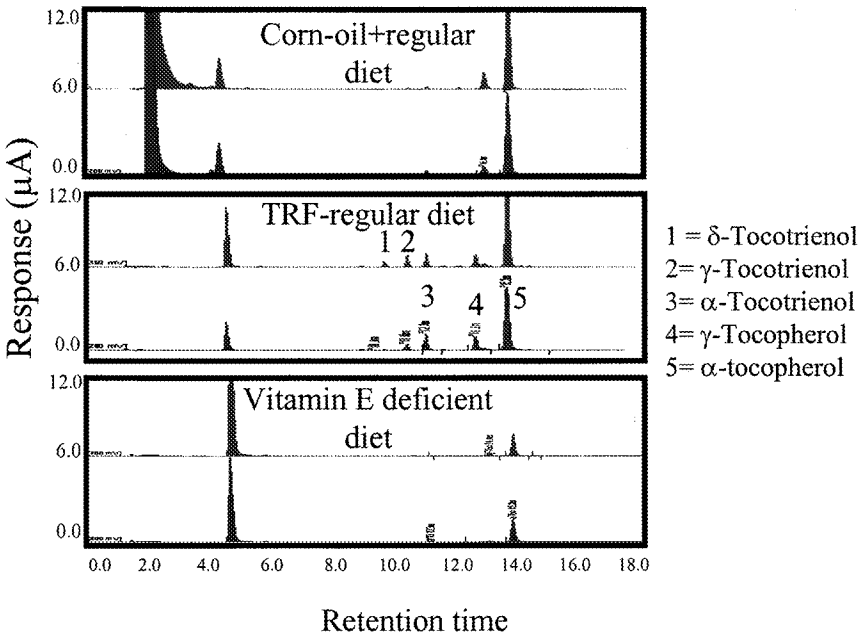

FIG. 3. Chromatograms of tocotrienols and tocopherols detected in spleen of rats supplemented (intragastrically) with (top) vitamin E-stripped corn oil and maintained on a standard rat diet; (middle) tocotrienol-rich fraction (TRF) derived from palm oil and maintained on a standard rat diet; and (bottom) nonsupplemented and maintained on a vitamin E-deficient diet. Individual peaks are labeled as 1–5.

In summary, the HPLC-EC coulometric electrode array detection method described here sensitively detects isoforms of tocopherols and tocotrienol from biological samples (Fig. 3). The detection limit for these compounds for this HPLC method is about 50 fmol. Using the extraction protocol, the recovery of tocopherol and tocotrienol from tissues was greater than 95%. Moreover, reproducibility of this HPLC method is excellent, with coefficient of variations ranging from 3 to 5%. Using this method, we showed that the optimum potentials for the detection of various isoforms of tocopherol/tocotrienols are different. Therefore, maintaining an electrochemical sensor in a series at various potentials, each optimal for a given vitamin E isoform, will provide enhanced sensitivity in the detection of these compounds in biological samples where the concentration of some of these vitamin E forms is very low.