Evaluation of Hydroxyl Radical-Scavenging Abilities of Two Dietary Antioxidant Supplements, Microhydrin® and MegaHydrin™ by Fe^{+2}-EDTA Induced 2-hydroxyterephthalate Fluorometric Analysis

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ABSTRACT

The hydroxyl radical scavenging capacity and efficacy of two dietary antioxidant supplements, Microhydrin® and MegaHydrin™ were quantified by a recently developed method which measures a direct relationship between the hydroxyl radical scavenging capability of the antioxidant compound and the linear decrease in signal from the fluorescent 2-hydroxyterephthalate product created by reacting an Fe^{+2}-EDTA complex under the presence of a potential radical scavenger. Fluorescence signal half-inhibition, IC_{50}, values were obtained for Microhydrin® and MegaHydrin™ of 4.4 μM and 1.4 μM, respectively. The validity of the analysis was verified by electron spin resonance spectroscopy, spectraphotometric analysis of NAD^+/NADH ratios, mitochondrial membrane potential measurements and reduction assays of both cytochrome *c*(Fe^{+3}) to cytochrome *c*(Fe^{+2}) and epinephrine to adenochrome.

INTRODUCTION

Free radicals and the resultant oxidative stress on an organism have been the subject of analysis for clinicians, nutritionists, biologists and chemists alike. Of all the reactive oxygen species (ROS), the hydroxyl reactive oxygen species is known to be one of the most reactive and physiologically harmful [1], suspected in such pathologies as atherosclerosis, oncogenesis, cataractulargenesis and DNA mutation [2-4].

The investigation into the involvement of free radicals in the fore mentioned pathologies has been ongoing for decades and has conveyed that the buildup of oxidized species occurs after the concentration of reaction products supercedes that which the cell's natural antioxidant system can effectively neutralize the radical or oxidized product [5].

The use of dietary antioxidant supplementation has been established as a potential protocol to decrease the detrimental effects of oxidative stress [6]. Some antioxidant compounds are known to be more effective than others, however, most commercially marketed antioxidant compounds have not been characterized with their *in vitro* and *in vivo* abilities to neutralize free radical species and there is no FDA regulation on their use and distribution. With the sheer number of dietary antioxidant supplements sold today, coupled with the level of grandeur of the biological and physiological ramifications of the direct and secondary products of intracellular reactions involving the hydroxyl radical call for an expressed need to quantify and characterize the reducing capacity of commercially available antioxidant nutritional supplements.

With the plethora of analytical techniques available, one might think that this would be an easy process, although the ability to effectively and accurately determine antioxidant capacity for hydroxyl free radicals has been a combative problem with scientists for some time.

The direct use of pH and reduction potential measurements (ORP), while giving an indication of the probability of a compound to act as an antioxidant, the technique does not define any specificity towards any particular ROS nor does it address any induction of cytotoxicity [7]. For example, the measurement of $LiAlH_4$, a violently reacting and toxic reducing agent would appear to be a great antioxidant by strictly using direct potential measurements with its large capacity to decrease ORP.

While electron spin resonance (ESR) spectroscopy is well known to measure the disappearance of ROS in a reaction, the methods used to analyze the radical species are not carried out under optimum biological conditions [8]. The Fenton reaction is commonly used to address the question of quantifying hydroxyl radical quenching efficacy, however, it is carried out at a pH that may hinder or bias the results [9].

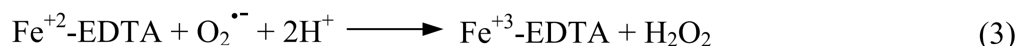
Photosensitizers, like malachite green, effectively introduce ROS into cells for *in vitro* analysis, however, have an inherent non-specificity to introduce both hydroxyl and superoxide radical species [10].

To address these problems with the study of hydroxyl radicals and antioxidant capacity, a new method was developed by Yang and Guo at Xiamen University utilizing the Fe^{+2}-EDTA hydroxylation of terephthalate to directly measure the radical-scavenging ability of antioxidant compounds at biological pH without the addition of H_2O_2 [11].

The method established measures the decrease in fluorescence in a direct relationship to hydroxyl radical scavenging capability by the following reaction scheme (1-4):

$$Fe^{+2}\text{-EDTA} + O_2 \longrightarrow Fe^{+3}\text{-EDTA} + O_2^{\bullet -} \quad (1)$$

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$



$$Fe^{+2}\text{-EDTA} + O_2^{\bullet -} + 2H^+ \longrightarrow Fe^{+3}\text{-EDTA} + H_2O_2 \quad (3)$$

$$Fe^{+2}\text{-EDTA} + H_2O_2 \xrightarrow{k_o} Fe^{+3}\text{-EDTA} + OH^- + \bullet OH \quad (4)$$

The premise for the assay follows the measurement of the fluorescent 2-hydroxyterephthalate (HOTP) created by the reaction of a Fe^{+2}-EDTA complex with molecular oxygen to produce a superoxide radical species which dismutates to H_2O_2 and then decomposed to form the hydroxyl radical. The idea of the method follows that the greater the antioxidant capacity of the compound, the less hydroxyl radical present and resultantly the less fluorescent adduct 2-hydroxyterephthalate is produced. The stoichiometry of the reactions leads to a linear and direct relationship between the amount of antioxidant substrate and the measurement of the radical scavenging capacity by the following reactions (5-6):

$$\text{terephthalate} + \bullet OH \xrightarrow{k_1} \text{HOTP} \quad (5)$$

$$\text{scavenger} + \bullet OH \xrightarrow{k_2} \text{other products} \quad (6)$$

This methodology was employed to analyze to dietary antioxidant supplements: Microhydrin® and MegaHydrin™, trademarked by RBC-Globenet (Irving, TX) and Flantech Group (Cottonwood, AZ), respectively. Microhydrin® is silica-based colloidal compound and MegaHydrin™ is a multi-mineral colloidal compound, both with interstitially embedded hydride

(H^-) anions. The mechanism for the both of the compounds is that the colloid acts as a carrier for the hydride ions, which in turn react as a reducing agent in solution. The profound attribute of these compounds is that while they are hydride anion based compounds, they do not react violently with water or any other tested solution, however, have a measured reduction potential of up to –750 mV [12]. Publications on MegaHydrin™ and Microhydrin® have shown significant abilities as antioxidants and internal energy producers, as well as clinically effecting cardiovascular responses to exercise by reducing lactic acid production and buildup [13-15].

This paper utilizes the recently developed Fe^{+2}-EDTA fluorometric analysis to quantify and compare the antioxidant properties and capabilities of MegaHydrin™ and Microhydrin® brand dietary supplements.

MATERIALS AND METHODS

Apparatus

The fluorometric analysis and relative fluorescence intensity were measured with a Jasco FP-750 spectrafluorimeter in a 1cm quartz cuvette. Readings were acquired with 326 nm excitation and 432 nm emission wavelengths. The parameters of the spectrafluorimeter were set to +/- 5 nm bandpass for both the excitation and the emission wavelengths.

Reagents

A stock solution of 1.0 mM terephthalate was prepared by the addition of 16.7 mg of terephthalic acid (Sigma-Aldrich, analytical grade) in 100 mL of 10 mM NaOH solution. A 1.0 mM ferrous ion solution was prepared by the addition of ammonium ferrous sulfate (Sigma-Aldrich) in 5.0 mM H_2SO_4. Catalase, 1090 U mg^{-1}, was prepared by a 1:10 dillution of sotck catalase (Sigma-Aldrich, 10,900 U mg^{-1}) in 50 mM phosphate buffer solution at pH 7.4. A solution was also prepared in ddH_2O of 1.0 mM EDTA (Sigma-Alrich).

A analytical working solution of was prepared from the preceding stock solutions in a 10 mL graduated cylinder containing, as added in the following order: 1.0 mL of 0.1 mM terephthalate, 0.30 mL of 1.0 mM EDTA, 1.0 mL of [SCAVENGER], 0.30 mL of 1 mM Fe^{+2} and 2.0 mL of 0.50mM phosphate/ catalase solution.

Working solutions, [SCAVENGER], of MegaHydrin™ and Microhydrin® were prepared in ddH_2O 15 minutes before their addition to the Fe^{+2}-EDTA analytical solution in the following incremental concentrations (μg/mL): 2.5, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 175.0, 200.0, 225.0, 250.0, 275.0 and 300.0.

Upon the addition of the Fe^{+2} to the analytical solution, the mixture was allowed to sit at room temperature for 6 minutes at which time the relative fluorescence intensities were measured.

Controls to determine the maximum and minimum fluorescence signal were obtained by the direct measurement of the analytical working solution sans the scavenger. Additional spectra were obtained of the scavenger in the analytical working solution sans the Fe^{+2} solution to check that no fluorescence was being emitted by the scavenger in the analytical solution.

RESULTS

The reaction scheme for the direct measurement of the hydroxyl radical scavenging capacity (7-8) is based on the principle that any molecule introduced into the analytical solution that is able to react with the •OH will compete with the terephthalate for •OH and therefore will reduce the concentration of the fluorescent 2-hydroxyterephthalate (HOTP) produced in the reaction, resultantly decreasing the fluorescence signal.

$$\frac{F_0}{F} = 1 + \frac{k_2[C_{scav}]}{k_1[TP]} \qquad (7)$$

$$\log\left(\frac{F_0}{F} - 1\right) = \log[C_{scav}] + \log\frac{k_2}{k_1[TP]} \qquad (8)$$

Where F_0 is the maximum fluorescence signal, F is the measured fluorescence signal for the scavenger introduced analytical sample, $[C_{scav}]$ is the concentration of the scavenger and [TP] is the terephthalate concentration. Kinetic constants, k_1 and k_2 refer to reaction rates for [TP] and $[C_{scav}]$, respectively. Since the [TP] is held constant, the equation reduces to $\log(F_0/F\text{-}1)=$ $\log[C_{scav}]$

Plotting the log-log graph of the relative fluorescence signal, $\log(F_0/F\text{-}1)$, as a function of the concentration of scavenger, $\log[C_{scav}]$ results in a linear trend. **Figure 1** displays the results obtained for MegaHydrin™ and Microhydrin®.

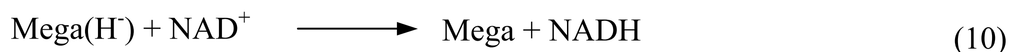
The concentration of MegaHydrin™ and Microhydrin® producing the half-inhibition of the maximum fluorescence intensity (IC_{50}) was calculated by the extrapolation of $\log(F_0/F\text{-}1)=0$ This is possible because when F= ½ F_0, $\log(F_0/F\text{-}1)=0$. The calculated IC_{50} values for MegaHydrin™ and Microhydrin® are 1.4 μM and 4.4 μM, respectively.

DISCUSSION

The analysis of both MegaHydrin™ and Microhydrin® by this newly developed technique distinctly convey a significant effectiveness as an antioxidant against •OH ROS at biological pH.

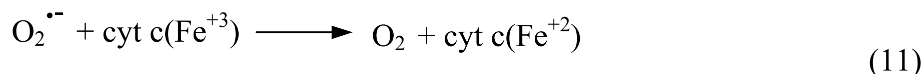
To confirm the results obtained by this quick, easy and cost effective method, additional tests were performed by outside sources to verify the in-house HOTP-fluorometric results. Three "traditional" analyses were performed by three major universities. Studies at the University of North Carolina at Chapel Hill (J.J. Lemasters, personal communications) showed spectraphotometrically, an increase in the $NADH/NAD^+$ ratio, in addition to an increase in mitochondrial membrane potential. The results indicate the stabilization of radical species by the electron donation of the hydride ion in solution by Microhydrin® and MegaHydrin™. MegaHydrin™ and Microhydrin® were shown by the Webb-Waring Institute for Cancer, Aging and Antioxidant Research at the University of Colorado Health Sciences Center (J.M. McCord, personal communications) to specifically reduce cytochrome c (9) and NAD^+ (10) by the following mechanisms:

$$\text{Mega}(H^-) + \text{cyt c}(Fe^{+3}) \longrightarrow \text{Mega} + \text{cyt c}(Fe^{+2}) + H^+ \quad (9)$$



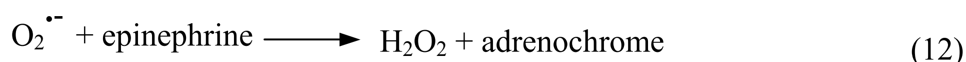
$$\text{Mega}(H^-) + NAD^+ \longrightarrow \text{Mega} + NADH \quad (10)$$

Additionally Microhydrin® and MegaHydrin™ had been shown to inhibit the reduction of cytochrome *c* by superoxide, (11)



$$O_2^{\bullet -} + \text{cyt c}(Fe^{+3}) \longrightarrow O_2 + \text{cyt c}(Fe^{+2}) \quad (11)$$

indicating that the superoxide radical was reduced. Because MegaHydrin™ reduced the cytochrome *c* in (6) and inhibited the cytochrome *c* reduction in (8), an additional assay was performed to clear up any confusion about Microhydrin® and MegaHydrin™'s role in radical reactions. The second assay observes the oxidation of epinephrine to adenochrome by superoxide (12):

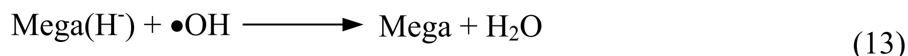


$$O_2^{\bullet -} + \text{epinephrine} \longrightarrow H_2O_2 + \text{adrenochrome} \qquad (12)$$

In (12), when Microhydrin® or MegaHydrin™ was added, the superoxide was scavenged, leaving epinephrine, illustrating the antioxidant activity of both compounds.

The control assays performed at the University of California, Berkley (L. Packer, personal communications) used ESR to measure hydroxyl radical reduction. The conclusion of the assay was that MegaHydrin™ has antioxidant activity towards hydroxyl radicals.

In light of the presentation of all of the data, it is thereby postulated that the mechanism of Microhydrin® and MegaHydrin™ against the hydroxyl radical (13) is presumably:



$$\text{Mega}(H^-) + \bullet OH \longrightarrow \text{Mega} + H_2O \qquad (13)$$

Where the electron rich hydride anion reacts with the electron deficient •OH radical. These tests, in conjunction with the spectrafluorometric HOTP assay described in this paper concur with the results that MegaHydrin™ and Microhydrin® are indeed quenching the hydroxyl radical species reactions. The results of the reduction assays, ESR and the mitochondrial

membrane potential assays of NADH are consistent with the results presented by the technique used in this paper, validating its use as a method to quantify antioxidant activity. As previously described by Yang and Guo, this method provides an easy, effective and cost-efficient assay for antioxidant capacity at biological pH.

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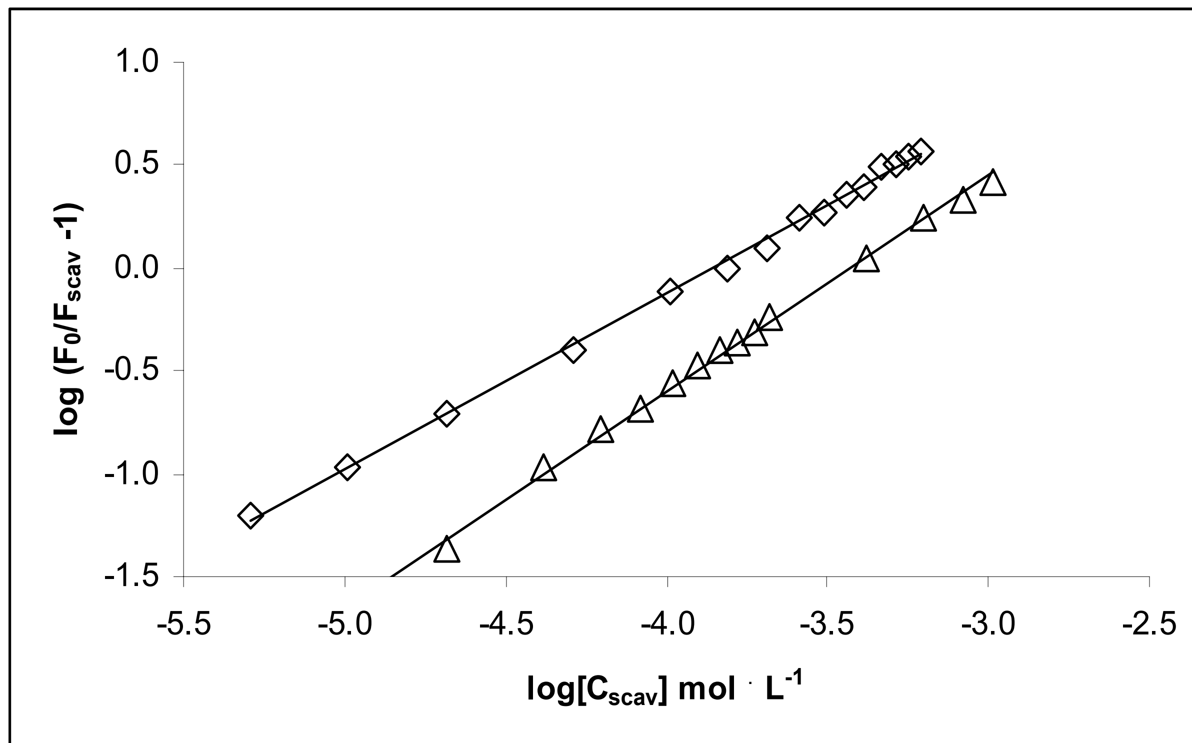
FIGURES

Figure 1:



LEGENDS

Figure 1: Chart of the relative fluorescence signal, log(F_0/F-1) as a function of the concentration of scavenger, log[C_{scav}], at 326 nm excitation and 432 nm emission wavelengths +/- 5 nm bandpass. Regression analysis of the averaged data for the three replicates resulted in a linear trend for both MegaHydrin™ (**-◊-**)and Microhydrin®(**-Δ-**), R^2=0.9979 and R^2=0.9970, respectively. The concentration of MegaHydrin™ and Microhydrin® producing the half-inhibition of the maximum fluorescence intensity (IC_{50}) was calculated by the extrapolation of log(F_0/F-1)=0. The IC_{50} values for MegaHydrin™ and Microhydrin® are 1.4 µM and 4.4 µM, respectively.