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# OxisResearch™

A Division of OXIS Health Products, Inc.

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## BIOXYTECH® HAE-586™

### Spectrophotometric Assay for Hydroxyalkenals

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number 21043

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## INTRODUCTION

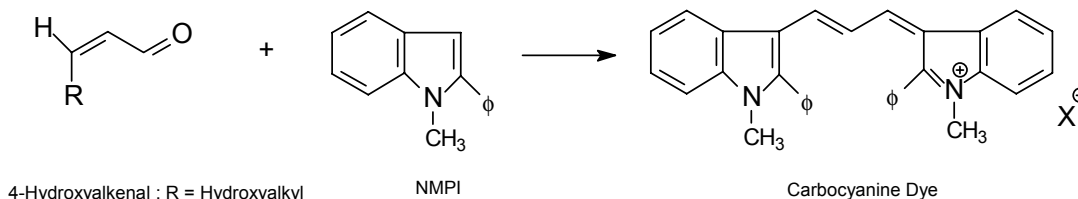
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### The Analyte

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds including malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). Therefore, measurement of HAE can be used as an indicator of lipid peroxidation (1). HAE react readily with nucleophilic groups, leading to protein and nucleic acid adducts. The most abundant 4-hydroxyalkenal formed in lipid peroxidation is 4-hydroxy-2-nonenal (HNE). HNE adducts are cytotoxic (2,3) and mutagenic (4,5). HNE can also function as a “second toxic messenger”, altering signal transduction and gene expression (4,6). HNE plays a significant role in neurodegenerative processes (6,7,8). HNE-protein adducts are found in oxidized LDL (9,10) and may contribute to foam cell formation in atherosclerosis (11). HAE is also produced in the thermal decomposition process of culinary oils (12). The HAE-586 method measures free 4-hydroxyalkenals in biological samples. An extraction step quantitatively separates HAE from MDA, allowing determination of HAE even in the presence of excess MDA.

### PRINCIPLES OF THE PROCEDURE

The HAE-586 method<sup>1</sup> (13) is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with 4-hydroxyalkenals at 45°C. One molecule of a 4-hydroxyalkenal reacts with 2 molecules of NMPI to yield a stable carbocyanine dye (14), as shown in **Figure 1**.



**Figure 1.** N-methyl-2-phenylindole (NMPI) reacts with a 4-hydroxyalkenal to form an intensely colored carbocyanine dye with a maximum absorption at 586 nm.

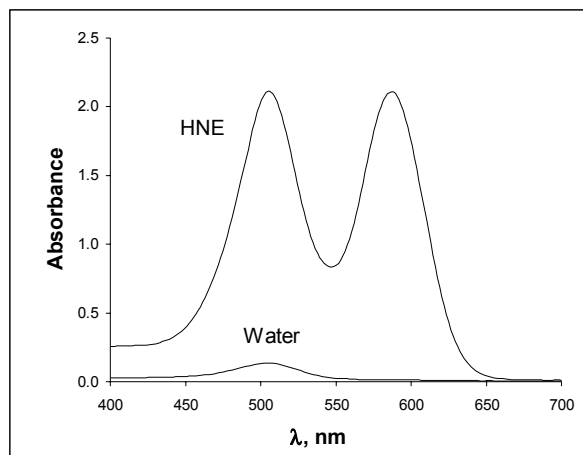
In the presence of methanesulfonic acid (R2, MSA), 4-hydroxyalkenals react quantitatively with NMPI to form the 586 nm chromophore, and all HAE forms the same 586 nm chromophore. Thus, the HAE-586 assay measures the total free HAE content of the sample (14). Saturated aldehydes, which are by-products of the reaction of HAE with NMPI, form a second product with maximum absorbance at 505 nm. As an example, the spectrum of the products formed from the reaction of 4-hydroxy-2-nonenal (HNE) with NMPI is shown in **Figure 2**. Both the 586 nm and 505 nm chromophores are shown.

HAEs are extracted from an aqueous biological sample into an organic solvent. Aliquots of the extract are dried under a stream of nitrogen or under vacuum (e.g., in a Centrivap™) and analyzed using the chemistry described above.

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<sup>1</sup> U.S. Patent 5726063

In the HAE-586 assay, a standard curve is prepared using HNE (provided). Since the extinction coefficient of the 586 nm product is the same for all HAE, the HAE content of an unknown sample can be calculated from the 586 nm absorbance of the sample and the HNE standard curve. For colored samples, a sample blank is run and the absorbance at 586 nm is subtracted from the assay sample to give the true absorbance of the carbocyanine dye.



**Figure 2.** Absorption spectra obtained from the reaction of NMPI with HNE (19  $\mu$ M) and a water blank in the presence of MSA.

## REAGENTS

### Materials Provided (for 100 tests)

Reagent R1	N-methyl-2-phenylindole in acetonitrile, 3 x 18 mL.
Reagent R2	Methanesulfonic acid, 1 x 16.5 mL.
HNE Standard	10 mM 4-hydroxynonenal diethylacetal in acetonitrile, 1 x 1 mL.
BHT	BHT (butylated hydroxytoluene) in acetonitrile, 1 x 2 mL.
Diluent	Ferric iron in methanol, 1 x 30 mL.
Dichloromethane	1 x 100 mL.

### Materials Required But Not Provided

- Spectrophotometer.
- Spectrophotometric cuvettes with a 1 cm optical path length (glass, quartz or polystyrene).
- Water bath or heating block set to control the temperature at  $45 \pm 1^\circ\text{C}$ .
- Disposable tubes with stoppers (glass and polypropylene).
- Microcentrifuge.
- Apparatus for drying samples at room temperature under nitrogen or vacuum (e.g., Labconco Centrivap<sup>TM</sup>).

### Warnings and Precautions

- **Acetonitrile** (R1, BHT, and Standard) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Causes irritation. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Methanesulfonic acid** (R2) is corrosive and may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Methanol** (Diluent) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Dichloromethane** is harmful if swallowed, inhaled or absorbed through the skin. Possible carcinogen/mutagen. Use with adequate ventilation. In case of contact with skin or eyes, flush immediately with plenty of water. Seek medical advice.
- **BHT** is harmful if swallowed, inhaled, or absorbed through the skin. Risk of serious eye injury. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

### Reagent Storage and Handling

- It is good practice to transfer the desired volumes of reagents for an experiment to clean glass test tubes or other containers and return the reagent bottles to 4°C storage.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 4°C.
- Unopened reagents are stable until the indicated expiration date.

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## **PROCEDURE**

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### **Reagent Preparation**

Dilution of the R1 solution for use in the assay. Add one volume (6 mL) of Diluent to three volumes (18 mL) reagent R1. Prepare this solution immediately before use. Do not leave the R1 reagent bottle uncapped (open to the atmosphere).

### **Preparing HNE Standard**

An HNE Standard is provided as the diethylacetal because free HNE is not stable. The acetal is hydrolyzed during the acid incubation step at 45°C, which will generate HNE. The Standard is provided as a 10 mM stock solution. Just prior to use, dilute the stock 1/500 (v/v) in water to give a 20 µM stock solution. Place at 0-4°C until use.

For the standard curve, pipet the the indicated volumes of the 20 µM Standard Solution and water to the reaction tube to give a total of 200 µL of the given standard. The concentrations given are for the final reaction mixture.

Recommended Addition Table for the HAE-586 Standard Curve

Volume of 20 µM Standard, µL	0	25	50	100	150	200
Volume of water, µL	200	175	150	100	50	0
Final concentration HNE, µM	0	0.5	1.0	2.0	3.0	4.0

### **Sample Preparation**

Note: Please read the appropriate **NOTES** sections before beginning the sample preparation procedure.

### **Extraction**

1. Place 1.00 mL of sample (or other known volume) in a glass tube.
2. Add 1.00 mL of dichloromethane (DCM) and stopper tightly.
3. Vortex at maximum speed for 3 x 30 seconds with a few seconds between cycles. Centrifuge (e.g., 3,000 x g for 15 minutes at 4°C) to separate phases.
4. Remove the DCM (lower) phase to a clean stoppered glass tube.
5. Place 200 µL aliquots (or other volume as needed) into clean glass tubes. Analysis in triplicate is recommended.
6. Dry aliquots at room temperature under a stream of nitrogen gas or under vacuum in a Centrivap™ or similar device.

### **Assay**

1. Prepare standards in the respective assay tubes. Add 200 µL of water to each dried DCM sample.
2. Add 650 µL of diluted R1 reagent to each tube.
3. Mix by briefly by vortexing each tube.
4. Add 150 µL R2 Reagent.
5. Stopper the tubes and mix well by vortexing each sample.
6. Incubate at 45°C for 60 minutes.
7. Any turbidity must be removed: transfer each sample to a polypropylene tube and centrifuge (e.g., 10,000 X g for 10 minutes at 4°C) to obtain a clear supernatant.

8. Transfer the clear supernatant to a cuvette.
9. Measure absorbance at 586 nm vs a water blank. The color is stable for at least two hours at room temperature or 4°C (14).

### Calculations

1. Using the standard data, perform a linear regression of  $A_{586}$  on [HNE] :

$$A_{586} = a[\text{HNE}] + b$$

2. Calculate the concentration of analyte in the sample

$$[\text{HAE}] = \frac{A_{586} - b}{a} \cdot \text{df}$$

Where [HAE] = Concentration of 4-hydroxyalkenals in the sample

$A_{586}$  = Absorbance at 586 nm of sample

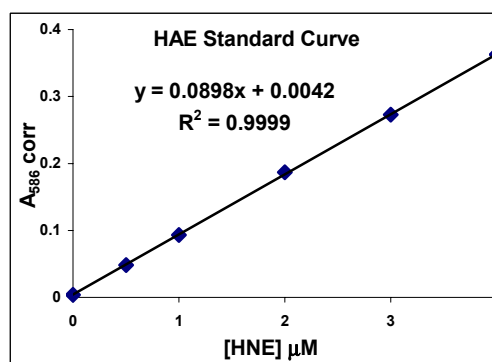
a = Regression coefficient (slope)

b = Intercept

df = Sample dilution factor

#### Example

To illustrate the calculations, consider the following experiment: A 0.500 mL biological sample was extracted with 1.00 mL of DCM. Three 300  $\mu\text{L}$  aliquots of the DCM phase were dried. The assay was carried out as described above on standards and unknowns in triplicate. The average  $A_{586}$  value for the zero concentration standard was subtracted from the average  $A_{586}$  values of the other standards and from the average sample  $A_{586}$  value to give corrected absorbances ( $A_{586}$  corr). A plot of  $A_{586}$  corr vs. [HNE] for the standards was constructed (**Figure 3**, see below).



**Figure 3.** Plot of  $A_{586}$  corr vs. [HNE] for the standards in the HAE-586 reaction

The equation for calculating [HAE] is thus:

$$[\text{HAE}] = \frac{(\text{Sample } A_{586} \text{ corr}) - 0.0042}{0.0898} \cdot \text{df}$$

The sample had an average  $A_{586}$  corr = 0.1729. Steps in the calculation of sample [HAE] are:

- 1) Use the equation to calculate [HAE] in the reaction mixture.

$$[\text{HAE}] = \frac{0.1729 - 0.0042}{0.0898} = 1.879 \mu\text{M}$$

- 2) Correct for the dilution of the sample. There are two dilutions to correct for:

- a) 300  $\mu\text{L}$  of the DCM phase was used and the final reaction volume was 1000  $\mu\text{L}$ :

$$\text{df}_a = 1000/300 = 3.333$$

- b) 500  $\mu\text{L}$  of biological sample was extracted with 1,000  $\mu\text{L}$  of DCM: thus the [HAE] in the biological sample is twice that in the DCM phase:  $\text{df}_b = 2.00$

Total dilution factor is thus:  $df = 3.33 \times 2.00 = 6.67$

[HAE] in biological sample =  $1.879 \mu\text{M} \times 6.67 = 12.5 \mu\text{M}$

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## PERFORMANCE CHARACTERISTICS

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### Linearity

The absorbance at 586 nm is a linear function of the HNE concentration over the range from 0.5  $\mu\text{M}$  to 20  $\mu\text{M}$ . Repeated testing of standard curves yielded an average slope of .09049 (standard deviation .0032), corresponding to an apparent extinction coefficient of 90,492. (**Figure 3**).

### Specificity

The specificity of the HAE-586 method was determined by measuring the absorbance at 586 nm of representative enaldehydes, aldehydes, and other compounds. Those values were compared to HNE at the same concentrations (14). The % values are the  $A_{586}$  value of the compound relative to HNE. For example, MDA gives the same color yield as HNE. However, in the HAE-586 assay, interference from MDA is completely eliminated by the extraction step. Other water-soluble compounds, such as acrolein and methylglyoxal will be largely eliminated by the extraction step as well.

#### HAE-586 Specificity.

Substance	%
MDA	100
HNE	100
Acrolein	10.3
Heptadiene	6.9
Methylglyoxal	1.5
Trans-2-nonenal	1.3
Trans-2-hexanal	0.2
Hexanal	0.5

### Precision

Precision of the HAE-586 method was estimated by measuring three levels of HNE, in buffer, each day for 10 days (15). Precision results are given in  $\mu\text{M}$ .

#### Precision.

Concentration	HNE		
	Low	Medium	High
Days	10	10	10
Mean	0.9896	1.7880	4.3008
SD Within Run	0.0504	0.0488	0.1256
CV Within Run (%)	5.1	2.7	2.9
SD Total	0.0584	0.0752	0.1424
CV Total (%)	5.9	4.2	3.3

## **Sensitivity**

The lower limit of detection is defined as 5.185 standard deviations (n=10) from the blank absorbance at 586 nm. The sensitivity of the HAE-586 method for HNE is shown below.

### Sensitivity

Average $A_{586}$	0.0030
Standard deviation, $A_{586}$	0.0015
Detection limit, $A_{586}$	0.0078
Detection limit, $\mu\text{M}$ (in reaction mixture)	0.0743

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## **NOTES**

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### **Interference**

- **Reducing Sugars.** Sucrose or fructose, at concentrations of 50 mM or greater in the sample, will cause a high bias in the assay (16).
- **Antioxidants.** Vitamin E (10  $\mu\text{M}$ ), probucol (10  $\mu\text{M}$ ) and ethoxyquine (100  $\mu\text{M}$ ) will cause a decrease in the values obtained for the 4-hydroxyalkenals of 5%, 5%, and 18%, respectively. Glutathione (200  $\mu\text{M}$ ), ascorbate (100  $\mu\text{M}$ ), and BHT do not interfere. (14).

### **Limitations**

- **Free HAE.** The HAE-586 assay measures only free HAE in samples. The conditions of the assay do not provide for liberation of HAE bound to proteins or other biological molecules (16). HAEs are sufficiently reactive that they rapidly combine with thiol groups and other nucleophiles on proteins in tissues, forming stable adducts that are not liberated by heating at high temperatures in acid; as a consequence, there is very little free HAE in tissue (1).
- **Normal Tissue.** Normal tissues have very low levels of free HAE, typically 10 pmol/mg protein (17, 18). Assay of a 1.0 mL sample containing 50 mg of protein derived from normal tissue will give absorbance values at 586 nm of 0.01 or less in the standard HAE-586 assay. Caution must be taken not to interpret very low absorbance values (near zero) as an accurate reflection of analyte concentrations in biological samples. If larger sized biological samples are available, extraction on a larger scale and drying of DCM aliquots of greater than 200  $\mu\text{L}$  can be used to enhance sensitivity.

### **Sample Preparation**

- **Sample Oxidation.** OXIS Health Products recommends that butylated hydroxytoluene (BHT) be added to a final concentration of 5 mM prior to homogenization of tissue or cells. BHT is supplied as a 100 X stock (500 mM) solution in acetonitrile. If no antioxidant is added, new lipid peroxidation can occur during homogenization resulting in biased values (17).
- **Tissue.** Sample homogenates should be made as concentrated as possible, (i.e., approximately 20% - 30% or 200-300 mg tissue per mL buffer). The concentration of protein in the homogenate should be determined and used to normalize the HAE value. Homogenates not assayed immediately must be stored at  $-70^{\circ}\text{C}$  or lower (19, 20).
- **Cell culture.** Cells should be washed to remove protein and other constituents from the media, then lysed by 2 or 3 freeze/thaw cycles or by sonication. It is recommended that a lysate from at least  $10^7$  cells be added to the HAE-586 reaction mixture (i.e., 1.0 mL of  $5 \times 10^7$  cells per mL extracted with 1.0 mL DCM and 200  $\mu\text{L}$  aliquots of the DCM phase used per assay). Lysates not assayed immediately must be stored at  $-70^{\circ}\text{C}$  or lower (19, 20).

- **Extraction.** DCM causes protein in the sample to precipitate. After centrifugation of the extracted sample, both the aqueous (top) and DCM (bottom) phases should be clear with a narrow zone of precipitated protein floating on the DCM phase. If difficulty is experienced in clarifying the DCM phase, two alterations in the standard procedure can be attempted. (1) Centrifuge at higher speed after ascertaining that the glass tubes being employed can withstand the higher centrifugal field. (2) Double the volume of DCM relative to the aqueous sample (e.g., use 1.00 mL of DCM and 0.500 mL of sample). This often results in better precipitation of protein from samples having a higher protein concentration.

### **Assay Performance**

- **505 nm Product.** A 505 nm reaction product (pink color) can sometimes be observed in samples or blanks. This is due to the production of chromophores other than those that produce the 586 nm peak. Ordinarily the 505 nm product will not interfere with the absorbance at 586 nm (21).
- **Sample Oxidation.** The kinetics of color development on the sample should be followed in comparison with that of the HNE diethylacetal supplied with the reagent set. The  $A_{586}$  of the sample should reach a plateau and then remain stable. Continual increase in the  $A_{586}$  indicates a non-HAE reactivity (interference) or oxidation is occurring in the reaction mixture (21).
- **Turbidity.** A wavelength scan from 450 to 700 nm should be performed on the clarified sample reaction mixture and compared to that obtained with the HNE control. The lack of a peak at 586 nm or a continuous increase in the baseline would suggest interference or non-specific reactivity in the sample (21).
- **Sample Blank.** Turbid or hazy samples will give biased values in the assay resulting from a contribution of light scattering to the measured absorbance at 586 nm. If the reaction mixtures do not clarify upon centrifugation an individual sample blank should be prepared. The Sample Blank is made by substituting 650  $\mu$ L of 75% acetonitrile/25% Diluent (ie. 3 volumes of acetonitrile and 1 volume of Diluent) for the R1 reagent in the sample reaction mixture. Correct for any  $A_{586}$  contribution due to the sample by subtracting the Sample Blank from the sample (R1 reaction).

### **Third Derivative Spectroscopy**

- **Derivative Spectroscopy.** Specificity and sensitivity can be improved using 3<sup>rd</sup> derivative spectroscopy to analyze the HAE-586 data. Derivative spectroscopy helps to eliminate or reduce the effects of a large 505 nm peak, drifting baseline and absorptions from endogenous substances in biological samples. Most modern spectrophotometers include derivative spectroscopy in the application software. Please contact OXIS Technical Service to obtain a complete description of the improved methodology (22).

### **Reagent Preparation**

- **R1 Dilution.** Failure to dilute the R1 reagent with Diluent will result in the formation of a two phase reaction mixture.
- **Assay Buffers.** Buffers composed of nucleophilic species (e.g., Tris) may react with and deplete the sample of HAE. These species should be avoided. Similarly, use of thiols such as dithiothreitol or 2-mercaptoethanol should also be avoided. Potential interference from buffers can be assessed by measuring the concentration of HNE (hydrolyzed HNE diethylacetal, Standard) over time in the chosen buffer and comparing with an identical sample of HNE diluted into water.

To evaluate a buffer, proceed as follows.

1. Prepare a 0.1 M MSA solution by diluting 66  $\mu$ L of R2 with 934  $\mu$ L of water.
2. Mix 50  $\mu$ L of Standard with 50  $\mu$ L of 0.1 M MSA in a glass tube and vortex. Allow to stand at room temperature for two minutes (this gives a 5 mM solution of HNE).
3. Dilute 20  $\mu$ L of this HNE solution to 10 mL using the buffer being evaluated (10  $\mu$ M HNE).
4. Prepare an identical dilution of HNE into water.

5. Allow both solutions to stand at room temperature. Periodically remove 200  $\mu$ L aliquots from each sample and analyze in the HAE-586 assay. A significant loss of HNE in the buffer, relative to water, is an indication that the buffer should not be used in the sample preparation. Phosphate buffers do not interfere and are recommended for tissue extraction. The buffer pH in the extraction step should be 7 or higher.

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