Hydrogen Peroxide Measurement in Seawater by (p-Hydroxyphenyl) Acetic Acid Dimerization. Anal. Chem., 60, 2711-2715

ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 1988

Impact Factor: 5.64 · DOI: 10.1021/ac00175a014

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RECEIVED for review April 7, 1988. Accepted September 23, 1988. J.D.M. gratefully acknowledges the Inorganic Analytical Research Division of the National Institute of Standards and Technology (formerly the National Bureau of Standards) for a guest worker appointment during which this research was conducted in collaboration with the University of Maryland.

Hydrogen Peroxide Measurement in Seawater by (*p*-Hydroxyphenyl)acetic Acid Dimerization

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The dimerization of (*p*-hydroxyphenyl)acetic acid by hydrogen peroxide in the presence of peroxidase has been adapted to the determination of hydrogen peroxide in seawater. The fluorometric method presented requires only one mixed reagent for sample analysis and produces a stable product that does not require immediate measurement. Changes in reaction stoichiometry resulting from naturally occurring organic material have been addressed. The detection limit, defined as 3 times the standard deviation of the blank, is 4 and 5 nM for open ocean and coastal seawater, respectively.

Hydrogen peroxide has been reported in natural surface waters in concentrations well above those expected from thermodynamic predictions (*1-5*). These elevated levels may have measurable effects on the distribution and cycling of naturally occurring trace compounds such as dissolved and particulate organic material and redox sensitive metals such as manganese, copper, and iron (*6*). Various analytical methods have been used for measurement of H_2O_2 in the environment. These include luminol chemiluminescence (*7*), peroxyoxalate chemiluminescence (*8, 9*), iodometric titration (*10*), photometric analysis (*11, 12*), the scopoletin-peroxidase-phenol fluorescent method (*1-3, 10, 13-16*), and the fluorescent dimerization of (*p*-hydroxyphenyl)acetic acid (POHPAA) (*9, 17-22*). The scopoletin-peroxidase-phenol method has been used for most surface river water, seawater, and groundwater measurements while POHPAA dimerization has been used mostly for atmospheric H_2O_2 measurements (both aqueous and gaseous). H_2O_2 analysis of surface waters by the POHPAA technique has been reported only for freshwater samples. Application of the POHPAA method to natural estuarine and open ocean waters offers some advantages over alternate methods and could provide an analytically consistent method for a wide range of atmospheric and oceanic measurements of H_2O_2. This paper presents an investigation of the applications and limitations of this method to the measurement of H_2O_2 in marine surface waters.

The use of POHPAA for the determination of sugars, peroxidase, and other oxidative enzymes was first presented by Guilbault et al. (*23*). Of the 25 substituted phenols that were surveyed, POHPAA was the most useful indicator substrate due to its high fluorescent coefficient when dimerized, its stability to autooxidation, and its low cost. The mechanism of its reaction with H_2O_2 in the presence of peroxidase probably proceeds by the donation of an electron to the activated enzyme and the subsequent dimerization of two POHPAA free radical intermediates. Figure 1 outlines a mechanism compiled from the one proposed for the dimerization of homovanillic acid (*24*), a compound with similar structure to POHPAA, and mechanisms known for the catalytic oxidation of phenolic compounds by peroxidase (*25-28*). Steps in the reaction that involve the stepwise reduction of the activated forms of peroxidase, compound I and compound II, are the same for most reactions catalyzed by peroxidase, including the POHPAA and the scopoletin methods.

For atmospheric samples, the application of flow injection techniques to the POHPAA method makes it consistent, rapid, and easy to use (*17, 19, 29-32*). The fluorescent POHPAA dimer is a stable product and many of the interferences that plagued the early chemiluminescent methods do not occur with POHPAA. The presence of common inorganic substances (iron, manganese, copper, zinc, sodium, potassium, chloride, bromide, iodide, sulfate, nitrate, phosphate, and ammonium) shows no effect on either the formation of the fluorescent dimer or its subsequent stability (*17, 19*). Benezene, toluene, and various aliphatic organic compounds have also been shown to have no effect on the method (*17*). The technique exhibits a limited response to the presence of methyl hydroperoxide, *n*-propyl hydroperoxide, and peroxyacetic acid. The signal resulting from these compounds can be isolated by using a controlled reaction of samples with catalase (*17, 19*). This selectively eliminates H_2O_2 and allows the determination of its concentration by difference. In this investigation, the specific analytical methods of Kok et al. (*19*) for the POHPAA technique have been modified for the measurement of nanomolar concentrations of H_2O_2 in seawater using batch mode analysis.

EXPERIMENTAL SECTION

Reagents. Catalase (C-100, 44 200 units mg^{-1}), peroxidase (P-8375, type VI, 300 purpurogallin units mg^{-1}; or P-8250, type II, 200 purpurogallin units mg^{-1}), and hydrogen peroxide (H-1009, 30% (w/w)) were obtained from Sigma Chemical Co. and used as received. Crystalline (*p*-hydroxyphenyl)acetic acid (Sigma Chemical, H-4377) was further purified by recrystallization in

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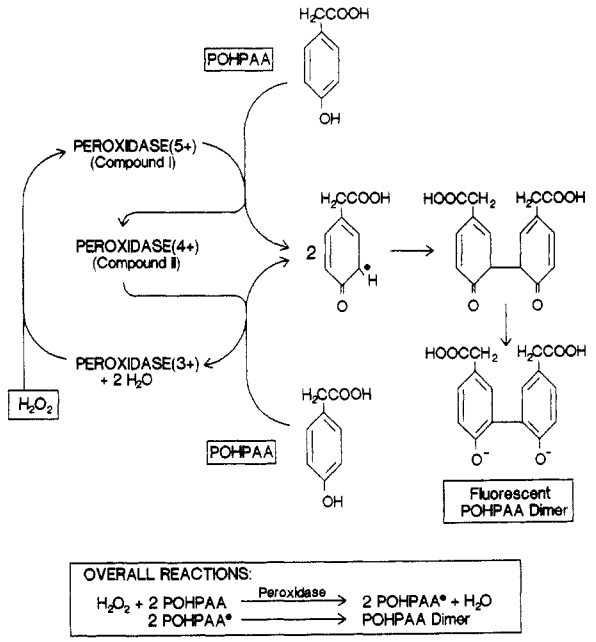

Figure 1. Proposed mechanism for the peroxidase-catalyzed dimerization of (*p*-hydroxyphenyl)acetic acid. Peroxidase(3+) is oxidized to peroxidase(5+) by H_2O_2. POHPAA reduces peroxidase(5+) to peroxidase(4+) resulting in the production of a POHPAA radical. Peroxidase(4+) is reduced to peroxidase(3+) by POHPAA also resulting in a POHPAA radical. Two POHPAA radicals dimerize to produce a fluorescent product. Overall stoichiometry is H_2O_2:dimer = 1:1. Numbers in parentheses are oxidation states. Dot indicates radical.

warm water. All other chemicals were reagent grade. A 0.25 M tris(hydroxymethyl)aminomethane (Tris) solution, adjusted to pH 8.8 with 0.5 M HCl, was used to buffer sample solutions at a pH that maximizes fluorescence without causing $Mg(OH)_2$ precipitation from seawater. All solutions were made with purified distilled Milli-Q water (Millipore Corp.) having a final resistance of approximately 18 MΩ cm^{-1} and passed through a 0.2-μm filter.

Equipment. All fluorometric measurements were made on a Perkin-Elmer Model 203 spectrofluorometer. Solutions were introduced into a 0.3-mL volume, 1 cm path length quartz glass flow cell using a sipper system consisting of Teflon tubing and a timed pump. This procedure gives reproducible sample delivery and rinse characteristics. The excitation and emission wavelengths that optimized POHPAA dimer fluorescence were 313 and 400 nm, respectively. The spectrofluorometer was zeroed with Milli-Q water and adjusted for fluorescence by using standard quinine sulfate solutions. This eliminates error from instrument drift and allowed direct quantitative comparison of all fluorometric analyses in this study. Hydrogen peroxide stock solutions were stored refrigerated in glass containers. FEP Teflonware was used for storage of all reagent solutions and seawater samples.

Hydrogen Peroxide Stock Solutions. A working stock solution of approximately 1 mM H_2O_2 was prepared by volumetric dilution of 30% (w/w) H_2O_2. Millimolar concentrations of H_2O_2 in distilled water can be determined by measuring UV absorbance at 240 nm (Abs_{240}) (*32*). A series of Milli-Q solutions containing varied additions of H_2O_2 were analyzed by titration with $KMnO_4$ solutions of known concentration. The Milli-Q solutions were then measured for Abs_{240} with a Shimadzu UV-260 recording spectrophotometer. A linear Beer's law plot with a correlation coefficient of greater than 0.9999 was obtained for H_2O_2 concentrations $\geq 10^{-4}$ M H_2O_2. A molar absorptivity for H_2O_2 absorbance at 240 nm was determined to be 38.1 ± 1.4 mol^{-1} cm^{-1}. UV spectroscopy was used daily to provide a rapid, straightforward check for H_2O_2 stock solution stability. A stock solution analyzed in this manner was used to make 10^{-6} and/or 10^{-5} M H_2O_2 working standard solutions just prior to their use in standard addition measurements.

RESULTS AND DISCUSSION

Blank Determination and Limits of Detection. The natural fluorescence of seawater (λ_{ex} = 313 nm, λ_{em} = 400 nm) varies both spatially and temporally. Samples taken throughout Narragansett Bay, RI, over a period of 3 days in March of 1987 showed natural fluorescence values that differed by a factor of 3 when using a sensitivity setting appropriate for H_2O_2 analysis of these waters. Because of such variations, it is important to determine reagent and H_2O_2-free blanks in the same seawater for which the analysis is to be done. Samples reacted for 5 min with catalase (*13*, *17*, *19*) have been used to obtain H_2O_2-free seawater blanks without significant loss of other hydroperoxides. Catalase was found to exhibit fluorescence at the wavelengths used for POHPAA dimer analysis. For catalase additions used in this study (Table II), this signal is small compared to the natural fluorescence of coastal seawater from Narragansett Bay but is larger than the natural fluorescence of open ocean seawater. For greatest accuracy in blank determinations, the measurement of fluorescence in three solutions other than the sample is required: the seawater alone (NAT), seawater with catalase added (CAT), and seawater that has been reacted with catalase for 5 min followed by the addition of a fluorometric reagent (FL) consisting of Tris buffer, POHPAA, and peroxidase. Blank determination is then accomplished by using the following formula:

$$\text{blank} = \text{NAT} + (\text{FL} - \text{CAT})$$

The blank determined in this manner is appropriate for both samples and standard additions and accounts for the natural fluorescence and added fluorescence due to the fluorometric reagent without including catalase fluorescence in the H_2O_2-free sample. The limit of detection, calculated as 3 times the standard deviation of five replicate blank determinations, was 4.7 nM for coastal seawater and 3.5 nM for open ocean seawater.

Reagent Concentrations. For atmospheric peroxide measurements, final POHPAA concentrations in the sample typically fall in the micromolar range (*17*, *19*). While these concentrations are appropriate for H_2O_2 in the range found in atmospheric samples (10^{-6}–10^{-4} M), they are too high for analysis of the nanomolar concentrations found in surface seawater (*2–5*). Experiments to determine an appropriate POHPAA concentration for nanomolar H_2O_2 analysis were undertaken in coastal seawater collected from Narragansett Bay, RI, and in open ocean seawater from the Sargasso Sea. Five standard additions resulting in added H_2O_2 from 2 to 100 nM were performed for POHPAA concentrations ranging from 10^{-7} to 10^{-5} M. Samples with less than 10^{-6} M POHPAA showed little or no increase in fluorescence after H_2O_2 additions. Samples containing POHPAA concentrations greater than 10^{-5} M showed unstable fluorescence and proved difficult to measure due to a slow, significant increase in fluorescence during the measurement. This may indicate new dimer production in the closed fluorometric cell due to a spontaneous conversion of the POHPAA molecule to the fluorescent dimer (*18*) or photochemical production due to prolonged exposure to the excitation light (313 nm). A stable fluorescence signal was observed for POHPAA concentrations between 10^{-6} and 10^{-5} M in both coastal and open ocean seawater. A final POHPAA concentration of 5×10^{-6} M was adopted for subsequent H_2O_2 measurements.

By use of this concentration of POHPAA, a range of peroxidase concentrations from 0.0 purpurogallin units per liter of sample (U/L) to 1067 U/L were examined for reactivity and stability. Results are presented in Table I. All peroxidase concentrations above 10 U/L gave significant fluorescence after 15 min. Samples containing more than 500 U/L showed increasing fluorescence over a period of 20 h. A peroxidase concentration of 235 U/L was used in subsequent experiments to maximize sensitivity while ensuring stability of the derivatized product. Measurement procedures that provide con-

Table I. Stablity of Derivitized Product with Varied Peroxidase Concentrations: [POHPAA] = 5×10^{-5} M, 5 nM H_2O_2 Added

	fluorescence, arbitrary units		
peroxidase[a]	0.25 h	1 h	20.5 h
0	0	0	0
12	11	20	19
30	25	26	26
116	26	27	27
230	29	28	32
558	29	30	59
1067	32	35	83

[a] Purpurgallin units/liter; one unit will form 1 mg of purpurgallin in 20 s from pyrogallol at pH 6.0 and 20 °C.

Table II. Reagent Concentrations Used for Analysis of Seawater

	concentration			
	stock solution	fluorometric reagent	sample solution	sample blank
POHPAA	2.55×10^{-2} M	2.55×10^{-4} M	5×10^{-6} M	5×10^{-6} M
peroxidase[a]		1.2×10^{4}	235	235
catalase[b] (44 200)	1:20 dilution			$1:10^3$ dilution
Tris (pH 8.8)	0.25 M	0.25 M	4.9×10^{-3} M	4.9×10^{-3} M

[a] Purpurgallon units/liter; one unit will form 1 mg of purpurgallin in 20 s from pyrogallol at pH 6.0 and 20 °C. [b] units/milligram of protein; one unit will decompose 1.0 μmol of H_2O_2 per minute at pH 7.0 and 25 °C.

trolled reaction times may allow the use of higher peroxidase concentrations for increased reaction rates and sensitivity.

Procedures. Based on experiments with varied reagent concentrations, the values presented in Table II were chosen for use in measurements of H_2O_2 in surface seawater. A single mixed fluorometric reagent containing POHPAA and peroxidase together in Tris buffer was found to give the same response to H_2O_2 as when POHPAA and peroxidase, each in Tris buffer, were added to the sample separately. The addition of a mixed fluorometric reagent has proven convenient for determinations of H_2O_2 and was therefore used in subsequent analyses. EDTA and formaldehyde have been used in the measurement of rainwater samples to mask interferences by trace metals and bisulfite (*17–19*). Inclusion of EDTA and HCHO to our method caused no alteration in the response of this method to standard additions of H_2O_2 to Narragansett Bay water. Consequently, only those compounds listed in Table II were included.

The following procedures have proven useful for field measurements. The mixed fluorometric reagent was premeasured into FEP Teflon vials and refrigerated until a seawater sample was added. This reagent was prepared daily to prevent possible loss of enzyme activity over time. A mixing ratio of 50 parts of sample to 1 part of reagent was used. This provided a good signal with minimal blank fluorescence and sample dilution. For blank determinations, catalase was added to the sample 5 min prior to the fluorometric reagent to obtain H_2O_2-free seawater (*17*). The natural fluorescence of each seawater sample was also determined for use in blank determinations. If derivatized samples could not be measured within 30 min of the initial reaction, they were refrigerated for up to 12 h until fluorescence could be measured.

Standard additions using either 10^{-5} or 10^{-6} M H_2O_2 stock solutions were made to seawater samples already mixed with the fluorometric reagent. This order of addition was used to maximize the reaction of H_2O_2 with peroxidase prior to possible consumption by other seawater constituents. The H_2O_2 concentration added in the highest standard addition was at least equal to the concentration preexisting in the sample. For this study, standard additions ranging from 5 to 200 nM proved useful. Sample H_2O_2 concentrations were calculated by dividing the slope of the best linear fit to the standard addition data into the blank-corrected fluorescence. Representative H_2O_2 values (Table III) are similar to previously reported values for hydrogen peroxide in seawater (*1–5*). The sensitivity of the spectrofluorometer was set to a level that gave maximum fluorescence for the highest standard addition without going off scale. Preliminary analysis of samples in order to scale reagent concentrations or spectrofluorometer settings was unnecessary. H_2O_2 concentrations ranging from the detection limit to about 100 nM can usually be analyzed without a change in the sensitivity setting.

Sample Stability. A study of the temporal stability of the POHPAA dimer (produced using the reagent concentrations listed in Table II) in seawater samples showed <5% increase in fluorescence over a period of 24 h when refrigerated at 4 °C. This compares to an increase in fluorescence of up to 200% when stored at 20–25 °C for the same period of time. Narragansett Bay seawater samples containing from 5 to 100 nM of added H_2O_2 showed a similar long-term trend with an equal increase in fluorescence for all samples regardless of the amount of H_2O_2 added. This is consistent with the observations of Kok et al. (*19*) regarding the spontaneous dimerization of POHPAA in rainwater samples, a process unrelated to H_2O_2 concentration. The increase occurs with no change in the standard addition slope. Consequently, if standards and samples are measured after an equivalent time, H_2O_2 analysis should be possible for samples which show relatively large long-term increases in fluorescence. To minimize possible errors due to spontaneous dimerization, however, both reagents and samples for which the timing of the measurements cannot be strictly controlled should be refrigerated. The use of this method with flow injection analysis or air-segmented flow analysis as has been described for atmospheric measurements of H_2O_2 (*17*, *19*, *29–31*) should minimize problems associated with time instability of the derivitized product.

Reaction Stoichiometry and Standard Addition Slopes. As shown in the proposed mechanism in Figure 1, the reaction of one H_2O_2 molecule with two POHPAA molecules in the presence of peroxidase is expected to result in the formation of one POHPAA dimer. Verification of this stoichiometry using fluorescence requires the use of a fluorescent coefficient (fluorescence/concentration). The fluorescent coefficient published for the POHPAA dimer (*23*) cannot be directly applied to this study since its fluorescence varies with the spectral bandwidth, intensity, and path length of the light used for excitation, along with spectrofluorometer settings such as excitation and emission wavelength, sensitivity, and scale adjustments. In order to determine a fluorescent coefficient appropriate to our experimental conditions, measurements made in this study were related to a consistent fluorescence scale established by daily reference to the fluorescence of Milli-Q water and quinine sulfate solutions. An attempt to determine a useful fluorescent coefficient was made by using Milli-Q samples containing reagent concentrations listed in Table II and 10^{-3} M added H_2O_2. This excess H_2O_2 should result in the dimerization of all POHPAA in solution with a resulting dimer concentration equal to half the original POHPAA concentration. The fluorescent coefficients obtained in this manner were variable and provided little stoichiometric information when applied to Milli-Q samples with nanomolar concentrations of H_2O_2. Uncertainty in the calculation of the resulting dimer concentrations may be due

Table III. H_2O_2 Standard Addition Comparison[a]

	Abs_{260}	concn factor[b]	slope[c]	rel stoichiometry[d]	$[H_2O_2]$
Milli-Q water	0.000		0.39 ± 0.02	1.00	9 ± 1
Sargasso Sea					
surface	0.066	1.0	0.39 ± 0.01	1.00	139 ± 6
100 m			0.39 ± 0.01	1.00	<3.5
Narragansett Bay					
RI Sound	0.170	2.6	0.34 ± 0.03	0.87	25 ± 1
mid-bay	0.342	5.2	0.18 ± 0.01	0.46	38 ± 1
upper-bay	0.480	7.3	0.11 ± 0.01	0.28	115 ± 3

[a] Stoichiometry arbitrarily set at 1:1 ($[H_2O_2]$:[dimer]) for Milli-Q samples. H_2O_2 concentrations are nanomolar (±1 standard deviation of triplicate samples). [b] Calculated from increase in UV absorbance at 260 nm. [c] Best fit slope for linear regression of H_2O_2 additions (nM) and fluorescence (arbitrary units, equivalent scale for all measurements). [d] Slope/slope for Sargasso Sea water.

to spontaneous dimerization (*19*), possible disproportionation and continued oxidation of the radical intermediate (*26*), and the unknown degree to which the reaction goes to completion. Consequently, the values for standard addition slopes given in the following discussion do not represent the absolute stoichiometry of the reaction.

An assumption can be made that standard additions acquired in Milli-Q water, without excess H_2O_2, will most closely approach the expected 1:1 stoichiometry. Support for this assumption comes from noting that throughout this study the largest values for standard addition slopes were in all cases found for Milli-Q samples. Comparison of the standard addition slope and relative stoichiometry for Milli-Q samples (Milli-Q arbitrarily set at 1:1, H_2O_2:dimer concentrations) with those obtained for open ocean seawater and Narragansett Bay seawater are shown in Table III.

Variations in the performance of the POHPAA method due to differing salinity do not explain the observed slope changes. Open ocean seawater (salinity = 35 ‰) has a slope very similar to Milli-Q while water from upper Narragansett Bay (salinity = 25 ‰) results in lower slope values. A nonstoichiometry similar to that suggested by our observed slope variations has been reported for H_2O_2 analysis of groundwater samples using the scopoletin–peroxidase method (*16*). Holm et al. (*16*) reviewed substances that may alter the stoichiometry of the oxidative quenching reaction of scopoletin by H_2O_2 in the presence of peroxidase. Suggested compounds include nitrite, ethanol, amino acids, thiols, diamines, iodide, phenols, chlorinated phenols, and phenolic functional groups found on aquatic humic substances. These suggestions seem reasonable considering the wide variety of phenols and aromatic amines known to be oxidizable by peroxidase compound I (*28*). Holm et al. (*16*) further suggest that the observed nonstoichiometry in the scopoletin method may be caused by oxidative coupling of naturally occurring dissolved organic matter (DOM) in the presence of H_2O_2 and peroxidase.

Using POHPAA for the analysis of H_2O_2 in river waters, Zepp et al. (*22*) have also seen variations in the standard addition slopes. The addition of either commercially available fulvic acid (Contech, ETC, Ottawa, Canada) or naturally occurring hydrophobic organic solutes isolated from the Suwannee River, GA, caused a decrease in the response of the method relative to that observed for distilled water. Zepp et al. (*22*) observed decreases in their standard addition slopes of up to 60% after additions of fulvic acid to distilled water. This magnitude is similar to the slope changes observed here for Narragansett Bay. Zepp et al. (*22*) conclude that for freshwaters, peroxidase-based H_2O_2 assays should be calibrated to each system that is studied. On the basis of our Narragansett Bay data, a similar calibration should be applied to estuarine waters. Slope variations of the magnitude observed within Narragansett Bay could lead to errors in H_2O_2 concentrations of a factor of 4 if it is assumed that a single stoichiometry applied to all sample sites. Clearly, the use of a standard addition procedure for estuarine waters is required for accurate H_2O_2 measurements using this technique.

Table IV. Addition of Hydrophobic Organic Material to Sargasso Sea Water

Abs_{260}	concn factor[a]	slope[b]	rel stoichiometry[c]
0.066	1.0	0.39 ± 0.01	1.00
0.188	2.8	0.24 ± 0.01	0.62
0.324	4.9	0.18 ± 0.01	0.46
0.486	7.4	0.15 ± 0.01	0.38
0.684	10.4	0.09 ± 0.01	0.23

[a] Calculated from increase in UV absorbance at 260 nm. [b] Best fit slope for linear regression of H_2O_2 additions (nM) and fluorescence (arbitrary units, equivalent scale for all measurements). [c] Slope/slope for Sargasso Sea water.

Effect of Marine Organics. The possible link between naturally occurring organic material and the performance of peroxidase-based H_2O_2 analyses (*16*, *22*) was further evaluated for marine samples. Three liters of freshly collected Narragansett Bay water was filtered (0.2 μm), acidified (pH 3.0), and passed through two C-18 Sep-Paks (Waters, Inc.) at approximately 15 mL/min. The hydrophobic material adsorbed onto the Sep–Pak was eluted in 10 mL of methanol and evaporated to 2.88 g in a class 100 clean bench. Microliter quantities of the isolated organic material were added to 100-mL samples of Sargasso Sea water.

The absorbance of light at 260 nm (Abs_{260}) over a 10-cm path length was used as an estimate of dissolved organic material in our samples (*33*). By use of Abs_{260}, concentration factors were calculated relative to Sargasso Sea water for both our natural samples and samples to which we added isolated organics. Organic additions were adjusted to cover the range of concentration factors observed for samples taken from Narragansett Bay. Samples with equal additions of methanol gave standard addition slopes that were identical with those obtained in Sargasso Sea water alone. The relation between Abs_{260} and the relative stoichiometry of the POHPAA method is shown in Tables III and IV. There is an inverse correlation between standard addition slope values and both Abs_{260} and added organic material. This suggests that interference by naturally occurring organic material can account for the nonstoichiometry of the POHPAA reaction observed in natural samples. These results for marine samples are very similar to previous observations made for peroxidase-based H_2O_2 analysis of freshwaters (*16*, *22*).

CONCLUSIONS

The dimerization of (*p*-hydroxyphenyl)acetic acid by H_2O_2 in the presence of peroxidase can be used to measure H_2O_2 in seawater. The method requires only one mixed reagent for sample analysis and produces a stable product that does not require immediate measurement. This is useful in H_2O_2 analysis which must be undertaken at remote sites and on shipboard. For manual batch mode analysis care must be taken to use appropriate concentrations of both POHPAA and peroxidase for analysis of nanomolar H_2O_2 concentrations. Refrigeration of reagents and reacted samples is suggested for best results when strict timing of fluorometric determinations cannot be achieved. Due to the possibility of changes in the reaction stoichiometry in samples containing various concentrations of dissolved organic material, the standard addition method should be used in calculating H_2O_2 concentrations. Analysis of waters exhibiting similar concentrations of dissolved organic material should not require repeated standard addition determinations.

Registry No. PHPAA, 156-38-7; H_2O, 7732-18-5; H_2O_2, 7722-84-1; peroxidase, 9003-99-0.

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RECEIVED for review April 12, 1988. Resubmitted September 6, 1988. Accepted September 15, 1988. This work was supported by Sea Grant College Program Contract Number NA85AA-D-SG094.

Direct Heated Interface Probe for Capillary Supercritical Fluid Chromatography/Double Focusing Mass Spectrometry

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A capillary supercritical fluid chromatograph was coupled to a double focusing mass spectrometer with a direct heated probe interface. The interface contained a fused silica transfer capillary containing a frit restrictor at the end which was heated to a temperature of 300–350 °C by resistance heating. The total column effluent was transferred into the mass spectrometer ion source. This interface not only preserved the chromatographic efficiency but also provided for mass spectral detection and identification of various high molecular weight, thermally labile, and polar compounds. Electron-impact and chemical ionization mass spectra of selected natural products and pharmaceuticals were obtained at the low nanogram level.

Applications of supercritical fluid chromatography (SFC) for the separation of compounds that are nonvolatile or thermally labile have been rapidly increasing. Supercritical CO_2 is the most frequently used SFC mobile phase, primarily because of its inert chemical properties, its ready availability in high purity, and its compatibility with the flame ionization detector. However, to fully take advantage of SFC, increased mobile phase polarity is essential. This can be achieved by doping CO_2 with polar modifiers (*1*, *2*) or by simply using more polar substances such as supercritical ammonia (*3*, *4*). However, problems with detector compatibility often arise when these mobile phases are used. Although various selective detectors such as UV absorbance (*5*), fluorescence (*6*), IR (*7–9*), and ion mobility spectrometry (*10*) have been demonstrated to be useful with SFC, the mass spectrometer is ideal, not only because of its mobile phase independence at low flow rates but also because of its inherent sensitivity and capability of providing structural information.

In spite of the fact that SFC was first introduced in the 1960s, only recently has supercritical fluid chromatography/mass spectrometry (SFC/MS) been studied intensively. Capillary SFC/MS was first demonstrated by Smith et al. (*11*). They reported a direct fluid injection (DFI) probe, which utilized a laser-drilled orifice or a crimped metal capillary tube for flow restriction. On the basis of this study, several modified versions (*12–15*) have been designed and used for improvement of SFC/MS performance. Recently, high flow rate SFC/MS interface designs (*16–18*) and SFC/Fourier transform MS (*19*, *20*) have been successfully demonstrated. In addition to these efforts, a vacuum nebulizing interface originally developed for the combination of liquid chromatography (LC) with MS (*21*), a direct liquid introduction (DLI) LC/MS interface (*22*), and a moving belt interface (*23*) were modified to couple packed column SFC with MS. Each of

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