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Humic Substances as Interferences in the Analysis of Nitrite in Water

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HUMIC SUBSTANCES AS INTERFERENCES IN THE ANALYSIS OF NITRITE IN WATER

by

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ABSTRACT

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Humic substances are of current interest because of their roles in environmental processes involving pollutants. It is also becoming recognized that humic substances may interfere in analysis of environmental samples though the possible adverse effects do not appear to be fully appreciated. The present effort focuses on determining whether humic materials interfere in the analysis of nitrite in water using the Griess reaction. This is a well-known reaction using nitrosation to give a diazonium salt followed by coupling with an appropriate reagent to form a dye. This colorimetric method continues to be applied in the laboratory and the field for nitrite. It was found that nitrite analyses at low ppm levels in water may be 50%-60% low in the presence of ppm amounts of specific humic acids. It was shown that the interference is due to molecular association of the Griess dye with the humic acid. The interference results in less color, and with some humic acids, a shift in the wavelength of maximum absorption.

This thesis is dedicated to my loving and wonderful
grandmother, Rita H. Joyner.

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CHAPTER I

INTRODUCTION

Objectives

The objective of this work is to determine whether humic materials interfere in the analysis of nitrite in water using the Griess reaction. This is significant since the Griess reaction continues to be applied in the laboratory and the field for nitrite. Secondary objectives are to evaluate any interference phenomena and to establish the mechanism for the interference. It is also intended through this work to provide guidelines on using the Griess reaction for analytical purposes in the presence of humic substances.

Background

The focus of this research is on humic substances as interferences in the analysis of nitrite in water using the Griess reaction. This section provides background on humic substances including examples of interferences and information on beneficial roles, i.e., remediation.

Successful implementation of field analytical methods involving water and soil requires knowledge of the potential effects of humic substances. The U.S. EPA has documented use of a variety of field analytical and site characterization technologies at contaminated sites.[1] A summary of the results indicates that several users experienced difficulty in extracting contaminants from soil and experienced other matrix

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The journal model for this thesis is the *Journal of Field Analytical Chemistry and Technology*.

interferences. Humic substances were not mentioned but could have been involved. The following sections serve to define humic substances [2]:

"Aquatic humic substances may be defined with an operational definition. They are colored, polyelectrolytic, organic acids isolated from water on XAD resins, weak-base ion-exchange resins, or a comparable procedure. They are nonvolatile and range in molecular weight from 500 to 5000; their elemental composition is approximately 50 percent carbon, 4 to 5 percent hydrogen, 35 to 40 percent oxygen, 1 to 2 percent nitrogen, and less than 1 percent for sulfur plus phosphorus. The major functional groups include: carboxylic acids, phenolic hydroxyls, carbonyl, and hydroxyl groups. Within aquatic humic substances there are two fractions, which are humic and fulvic acid. Humic acid is the fraction that precipitates at pH 2.0 or less, and fulvic acid is the fraction that remains in solution at pH 2.0 or less."

"Humic substances from soil are: organic substances extracted from soil by sodium hydroxide (typically 0.1 N), the fraction that precipitates in acid is humic acid (pH 1-2), and the fraction remaining in solution is fulvic acid. This definition is different from the operational definition of aquatic humic substances. Therefore, the best comparison is between the general chemical characteristics of humic substances from soil and water."

"Both are polyelectrolytic, colored, organic acids with comparable molecular weights, their elemental composition is similar, and so is their functional group analysis. This does not prove that humic substances from soil and water are the same. Merely, it demonstrates that their general chemical characteristics are similar, and the definition of aquatic humic substances seems consistent with the definition of humic substances from

soil."

Humic substances are heterogeneous, complex mixtures of organic compounds that are formed by chemical and biological degradation of plants.[3] They are also formed by degradative processes from microorganisms.[3] Humic substances actually contain three fractions: the humic and fulvic acid fractions as mentioned prior and a third fraction called humin. Humic acid (HA) is not soluble in water under acidic conditions (pH<2), but it is soluble at higher pH values. HA is the major extractable component of soil humic substances, and it is dark brown to black in color. Fulvic acid (FA) is soluble in water under all pH values. It is light yellow to yellow-brown in color. Humin is not soluble in water at any pH value, and it is black in color. The postulated relationships among the fractions are depicted in Table 1.

TABLE 1. Chemical properties of humic substances. [4]

Fulvic acid		Humic acid		Humin
Light yellow	Yellow brown	Dark brown	Grey-black	Black

----------increase in intensity of color---------->
----------increase in degree of polymerization---------->
2000----------increase in molecular weight----------->300,000
45%----------increase in carbon content----------->62%
48%----------decrease in oxygen content----------->30%
1400----------decrease in exchange acidity----------->500
----------decrease in degree of solubility---------->

The percentage of humus present in the three fractions of humic substances varies with different soil types. The humus of forest soils has a high FA content, and the humus of peat and grassland soils has a high HA content. The HA/FA ratio usually decreases

with increasing soil depth, but this is not always the case.

Humic substances have been known for over two centuries, and over this time they have been found to be a dichotomy in our environment, meaning that they play both beneficial as well as destructive roles. Humic substances are known to be beneficial in soil. They increase the percentage of total nitrogen in the soil, neutralize alkaline and acidic soils, act as a natural barrier (sorbent) to immobilize pollutants in soil [5], allow bioavailability of contaminants to degrading organisms [5], solubilize pollutants to allow washing of contaminated soil, and aid in cleaning the environment of heavy metals. An example of HA binding metal ions in an aqueous environment is as follows (eqs. 1-3) [6]:

$$HA \rightleftarrows A^- + H^+ \quad (1)$$

$$HA + Cu^{2+} \rightleftarrows CuA^+ + H^+ \quad (2)$$

$$2HA + Cu^{2+} \rightleftarrows CuA_2 + 2H^+ \quad (3)$$

Since humic substances are effective in dispersing, oxidizing, and reducing metal ions [7], they have a great influence on agricultural, geochemical, environmental, and pollutant treatment processes.[8-11] Association of contaminants with humic substances can decrease their toxicity toward organisms in the environment. However, this complexation can lead to either solubilization or immobilization of the contaminant. HAs and FAs can form soluble complexes that can migrate long distances. This can allow pollutants to enter aquifers and affect our drinking water supplies. Solubility of the complexes depends on the metallic ion, the cation charge, the degree of ionization of the organic molecule, the ionic strength of the media, and the degree of metal loading.[9] Another disadvantage of HAs toward the environment includes their potential utilization

as substrates for the production of carcinogens from the chlorination of drinking water.

Humic Substances as Interferences

Humic substances may interfere in field and laboratory analysis of environmental samples. They may produce a colored background in field and laboratory analysis, they may sorb target analytes, and they may react with analytical reagents.

Both colorimetric and immunoassay methods in the field sampling of explosives may be subject to positive interference from humic substances in soils, which results in yellow extracts.[12] When using colorimetric methods, the interference from humic substances may be significant for samples that contain less than 10 ppm of the target analyte. These interferences may be observable through visual checks for color background from humic substances before conducting the analysis. Many immunoassay methods use a reverse coloration process (less color, more analyte). The presence of humic substances may lead to lower estimates of analyte than actual.

Humic Substances in Remediation

In a March 1999 article from the EPA, the use of coal-derived HA material to remediate ground water contaminated with mining wastes was demonstrated by the U.S. Department of Energy (DOE) using waters from the Berkeley Pit in Butte, MT.[13] Pilot-scale demonstration results indicate that, in addition to removing heavy metals, application of an ion exchange/adsorbent polymer (HUMASORB-CS™) can produce a chelated micronutrient-enriched fertilizer product suitable for agricultural production.[13]

The Berkeley Pit is an open-pit mine that has been filling with acidic, heavy

metal waters since pumping operations stopped in 1982. A two stage process has been used on the Berkeley pit waters for removal of metals and organic contaminants. In the first stage, the water was treated with a liquid HUMASORB product. This product was used to remove iron as well as other agricultural micronutrients through formation of humates that precipitated as flocs. The flocs were separated in a solid/liquid separation unit, and the remaining metals were reduced using a cross-linked, immobilized solid HA product (HUMASORB-CS).

It is interesting to note that the chemistry of humic substances in remediation may also be important in how HAs interfere in analytical methods. HAs may sorb analyte and/or analytical reagents. Additional studies have been conducted to evaluate HUMASORB in the treatment of chlorinated organics. Early tests using HUMASORB-CS in the treatment of chlorinated organics revealed that the half-life was less than two hours for trichloroethene and tetrachloroethene in comparison to 15.3 and 6.3 hours using zero-valent iron technology. Additional tests performed at Temple University confirmed that the HA material did adsorb chlorinated organic contaminants as well as degraded contaminants through the process of reductive dehalogenation. HUMASORB-CS is also being evaluated for effectiveness in a simulated barrier system with barriers at depths of 10 feet and 100 feet. A simulated waste stream containing a mixture of metals, organics, and radionuclides was passed through the barriers at pressures of 10 pounds per square inch gauge (psig) and 100 psig for more than eight months, with no observed breakthrough.[13]

The following sections describe the chemistry of humic substances and the Griess reaction. The Griess reaction is a classical reaction used in the analysis of nitrite in

water. Possible effects of humic substances on the Griess reaction have not been examined previously.

CHAPTER II

CHEMISTRY OF HUMIC SUBSTANCES

Isolation

When studying aquatic humic substances, they must first be separated from the bulk of other organic and inorganic constituents. As cited by Thurman in Reference 2, in 1958, Jeffrey and Hood evaluated five methods of concentration of trace organic compounds from seawater. At that time, these researchers concluded that coprecipitation of organic compounds with ferric chloride was the most effective technique removing 95% of the dissolved organic matter. Other methods tested by these investigators included: electrodialysis, liquid extraction, carbon adsorption, and ion exchange. However, these methods had substantially lower recoveries in comparison to the precipitation technique, although it was concluded to be a slow and tedious method for large volumes of water. They noted that column adsorption chromatography was a simpler procedure for large volumes of water using charcoal as the adsorbent, but they found that humic substances couldn't be eluted efficiently from the charcoal.

Freeze concentration is another technique used to concentrate humic substances in water, and was first tried by Black and Christman (as cited in Reference 2) in 1963 and then by Fotiyev (as cited in Reference 2) in 1971. However, it was also slow and concentrated the inorganic solutes in the sample. Using ion exchange and desalting with gel filtration, such drawbacks have been overcome. Liquid extraction has been used with some success to isolate humic substances from water.[2] The following serve as examples [2]: In 1957, Shapiro (as cited in Reference 2) extracted color organic acids from pond

water with ethyl acetate and butanol, and in 1971, Martin and Pierce (as cited in Reference 2) isolated the HA fraction only using isoamyl alcohol and acetic acid. In 1968, Khaylov (as cited in Reference 2) isolated humic substances from seawater and fresh water using a chloroform emulsion method, but it was most successful for humic acid. In 1974, Eberle and Schweer (as cited in Reference 2) extracted humic substances as ion pairs using a tetrabutyl ammonium salt and chloroform. The drawback of these methods is that none of them are quantitative, and water samples can not be measured by carbon analysis to determine the amount of humic organic carbon that is removed using these methods. However, it should be noted that liquid extraction of ion pairs does remove color efficiently (greater than 90 percent).

Iron, manganese, aluminum, lead salt, and calcium carbonate precipitation techniques were investigated by Jeffrey and Hood (as cited in Reference 2) in 1958, Williams and Zirino (as cited in Reference 2) in 1964, and Weber and Wilson (as cited in Reference 2) in 1975, but these techniques were found to be slow, only partially effective, and gave large ash contents. In 1964, Williams and Zirino (as cited in Reference 2) tried inorganic packings using silica, alumina, calcium carbonate, and magnesium oxide. Of these packings, alumina was found to be the most efficient, but all of the adsorbents were found to have low capacities and irreversible adsorption.

Various researchers have tried concentrating aquatic humus using ultrafiltration. This particular method is effective, and it gives a range of molecular weights. The most serious drawback to this method is that it is slow, and it only works best when analyzing colored waters that have high concentrations of aquatic humus.

Anion exchange is another method that has been used to isolate humic

substances, and has been found to be an effective adsorbent. However, according to Jeffrey and Hood, Packham, and Weber and Wilson (as cited in Reference 2), the sorbed organic matter is difficult to recover. It was found by Abrams, Sirotkina, and Leenheer (as cited in Reference 2) that weak anion exchange resins allowed more efficient elution than did strong anion exchange resins while maintaining high efficiency of adsorption.

In the late 1960's, Rohm and Haas (as cited in Reference 2) developed nonionic XAD resin polymers which led to a breakthrough in the isolation of humic substances and other compounds found in water. Before the development of these polymers, anion exchange had been tried, however, these resins irreversibly adsorbed organic matter; recovery of humic substances was low. Since organic acids are adsorbed in the protonated form, the solutions had to be acidified to pH 2.0 using concentrated hydrochloric acid and then pumped onto the XAD resin. Dilute sodium hydroxide was introduced to desorb the humic substances. Table 2 summarizes the advantages and limitations of the various methods used to isolate humic substances.

HA can be separated from FA by precipitation at pH 1.[2] This occurs because HA is less soluble having fewer carboxylate anions. On the average, HA has 3.5 to 4.5 mM/g of carboxyl groups in comparison to FA which has 5.0 to 6.0 mM/g. This lower amount of carboxyl groups in turn lowers the aqueous solubility of HA at this pH, which is why most natural waters contain 5 to 25 times more FA than HA. HA molecules are two to ten times larger than FA which also lowers its solubility. Also, the ash content and the phenolic content of HA is greater than that of FA. All of these factors account for the ease in which HA can be separated/precipitated from FA.

TABLE 2. Advantages and limitations of various isolation procedures for humic substances. [2]

Method	Advantages	Limitations
Precipitation	None	Fractionates sample, not specific for humus, slow on large volumes
Freeze concentration	All DOC concentrated	Slow, tedious procedure, concentrates inorganics
Liquid extraction	Visual color removal	Not quantified by DOC, slow for large volumes
Ultrafiltration	Also separates by molecular weight	Slow
Strong anion exchange	Efficient sorption	Does not desorb completely
Charcoal	Efficient sorption	Does not desorb completely
Weak anion exchange	Adsorbs and desorbs efficiently	Resin bleeds DOC
XAD resin	Adsorbs and desorbs efficiently	Resin must be cleaned to keep DOC bleed low

Structure

HAs are postulated to be complex aromatic macromolecules containing amino acids, amino sugars, peptides, and aliphatic compounds with linkages between the aromatic groups. Figure 1 shows one hypothetical structure. Free and bound phenolic OH groups, quinones, nitrogen and oxygen bridges, and carboxylic acid functional groups appear at various points. Hydroxybenzoic acid units also appear at

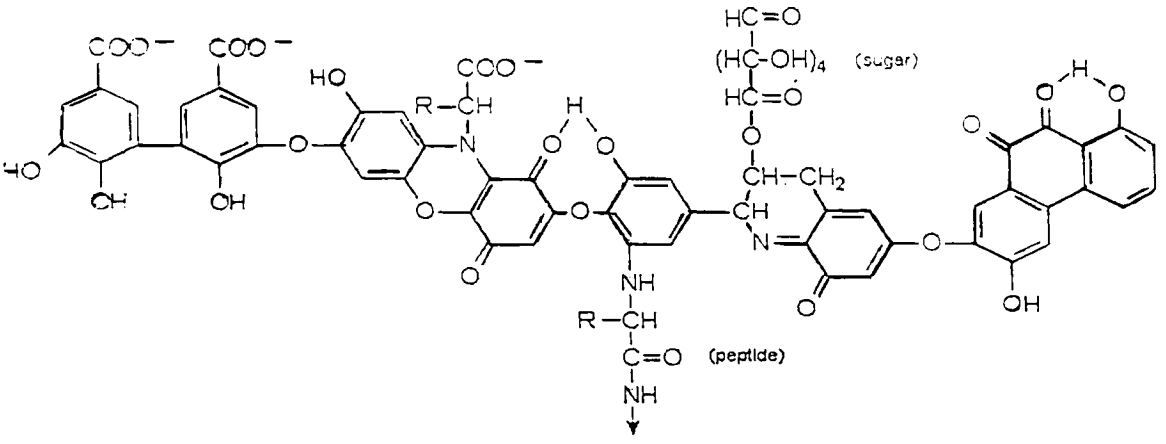

FIG. 1. Stevenson's hypothetical structure of humic acid [4] (modified to show carboxyl groups as anions).

various points in the structure. Hydroxybenzoic acids have been used in intermediary chemicals in a wide variety of industrial synthetic processes, and they may be regarded as a sort of primitive model compound for HA.[14] Since they are reasonable models for HA, several hydroxybenzoic acids were screened as interferences in the present research, including p-hydroxybenzoic acid and gallic acid monohydrate.

Two conformational models of a new building block of HA were recently proposed.[15] The modeling was based on the Steelink structure shown in Figure 2 and the TNB (Temple-Northeastern-Birmingham) structure shown in Figure 3. These structures differ somewhat from Stevenson's proposed structure (Figure 1) due to the presence of aniline and aminophenol functional groups. (Aminophenols were screened as interferences in the present research due to their presence in these building blocks.) It is important to point out that these structures are conformational (computer) models of postulated monomers (building blocks) of HA. They have not been synthesized.

Buffle [4] has proposed a structure of FA that contains aromatic and aliphatic functional groups that are extensively substituted with oxygen-containing functional groups. Buffle's hypothetical structure is shown in Figure 4.

Hatcher et al. [16] (1981c) analyzed a humic sample in which they reported "wet chemical" values for total acidity, carboxyl, phenolic, and carbonyl content in their sample to be 12.4, 9.1, 3.3, and 3.1 mmol/g, respectively. These functional groups are known to be present in HA, but a "true" or "actual" structure of HA has not been reported to our knowledge. Anyone working with HAs should be cautious since their structures have not yet been elucidated. The structures of HAs previously shown are postulated structures; they do not represent structures of HAs studied in the present

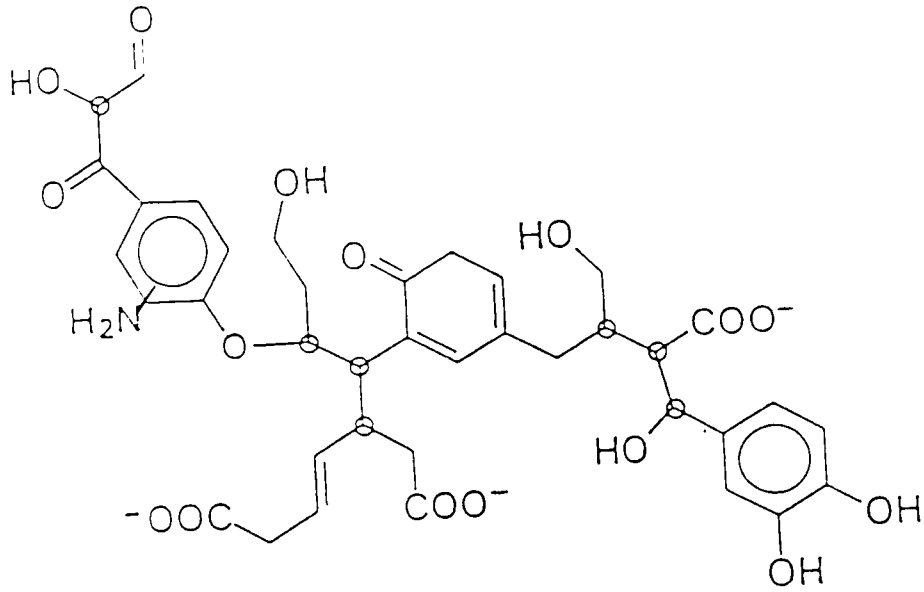

FIG. 2. Steelink structure of humic acid [15] (modified to show carboxyl groups as anions).

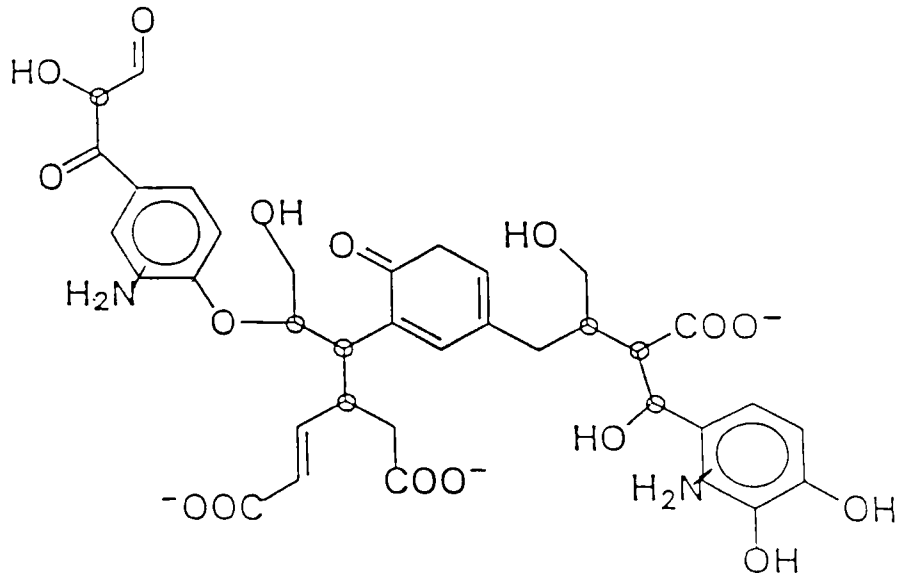

FIG. 3. TNB (Temple-Northeastern-Birmingham) structure of humic acid [15] (modified to show carboxyl groups as anions).

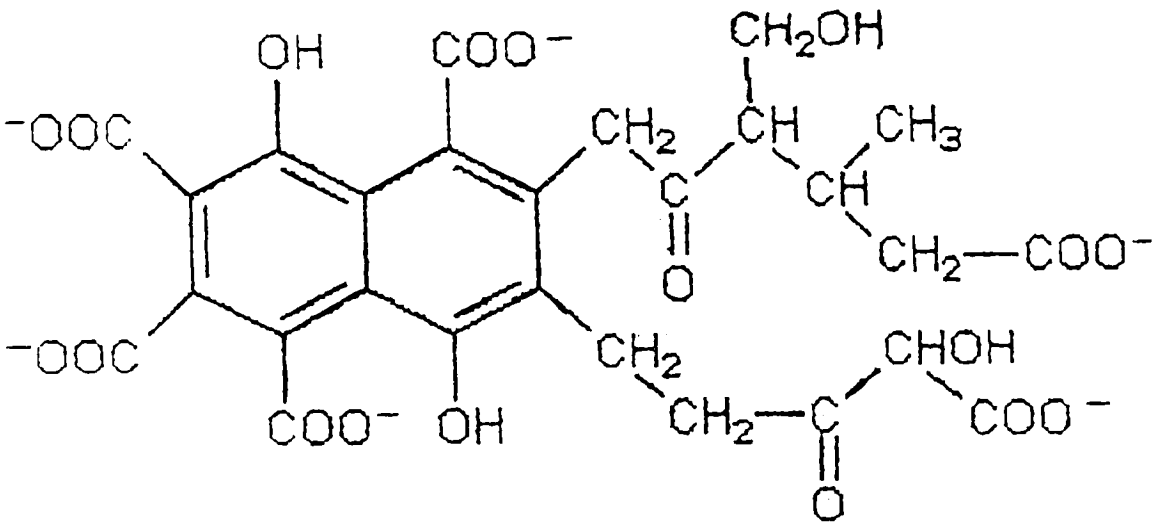

FIG. 4 Buffle's hypothetical structure of fulvic acid [4] (modified to show carboxyl groups as anions).

research (i.e., the structure for Fluka humic acid is not known). More recent structural information on a specific fulvic acid is given in the results and discussion section.

Formation

The major theories for the formation of humus in soil have been proposed by Stevenson in 1982 and is called "Humus Chemistry". The theories relate to: the lignin-degradation model, the polyphenol theory, and the sugar-amine condensation theory.

According to the lignin-degradation model, microorganisms partially metabolize lignin which then becomes soil humic substances. Carboxyl groups come from the oxidation of aliphatic side chains, methoxyl groups are lost, and phenols are produced through biochemical reactions.[2] The reactions proceed from HA, then through fragmentation and oxidation, to FA.[2]

According to the lignin-degradation and polymerization model, microorganisms degrade lignin and produce phenolic aldehydes and acids. These aldehydes and acids are enzymatically oxidized to quinones, and the quinones polymerize forming humic substances.

In the non-lignin-polyphenol-polymerization model, microorganisms decompose cellulose which produces polyphenols, and the polyphenols are enzymatically oxidized to quinones. The quinones then polymerize to form humic substances.

In the sugar-amine condensation model, the condensation of reducing sugars and amino acids forms polymeric humic substances. The sugars and the amino acids arise from the microbial decomposition of cellulose and polypeptides.

According to Stevenson [4], the lignin-degradation and polymerization model and

the non-lignin-polyphenol-polymerization model, are now the basis for the polyphenol theory, and the lignin-degradation model is considered to be incorrect. Also, the sugar-amine condensation model, is considered to be a viable theory for the formation of humic substances in seawater and in soil.

The theories cited above have merit, and all probably contribute to the formation of humus in soil. However, the origin of aquatic humic substances may be different. There may be other mechanisms of origin such as the degradation of aquatic organisms and bottom sediment.

It has been proposed that aquatic humic substances result from several processes, including: leaching of plant organic matter directly into the water, leaching of plant organic matter through the soil profile with subsequent alteration both chemical and biochemical within the soil, leaching of soil FA and HA into water, lysis of algal remains and bacterial action on phytoplankton, and ultraviolet oxidation of surface-active organic matter in the microlayer of streams, lakes, and seawater.[2] This is followed by polymerization reactions and reactions among phenolic, amine, and aldehyde functional groups from biological products in natural waters.[2]

Chemical Properties

Aquatic humic substances are the result of decomposition chemistry; they are not derived from ordered chemistry as is the case with biological products. This means that ordered biological molecules, i.e., carbohydrates, amino acids, cellulose, and lignin, are degraded and stripped of elements and molecules that microorganisms use for energy. It is believed that nitrogen, phosphorus, and carbohydrate are removed from these

molecules and that the number of carboxyl groups increase during the humification process. It is also thought that intramolecular interactions are important; however, intermolecular interactions are less important in dilute aqueous solutions such as in many natural waters. This results in crosslinking of carbon-carbon and carbon-oxygen bonds within the humic substance.

Humic substances perform various functions serving as surfactant flocculants, binding pesticides, and forming trihalomethanes (THMs). These processes may have adverse effects on water quality, taste, odor, color, and toxicity.

Humic substances have surfactant qualities due to their hydrophobic character. The hydrophilic end of a humic molecule points itself toward the aqueous phase, and the hydrophobic end of a humic molecule interacts with other humic molecules becoming surface active. An increase in surfactants from humic-like substances can be seen in small streams in spring during times of high water. Surfactants cause foaming and collect in backwater pools. The micelle that forms from this foam is able to dissolve oils and other hydrophobic constituents. For example, in 1980, Leenheer (as cited in Reference 2) reported that oil from small boats in the Rio Negro River in Brazil was quickly dispersed by humic substances, which contained 10 mg/L as FA. Also, several studies were done by Boehm and Quinn (as cited in Reference 2) in 1973 and by VanVleet and Quinn (as cited in Reference 2) in 1977 on the solubilization of hydrocarbons by surfactant-like organic matter in water.

It has been shown that humic substances can bind pesticides as well as other organic compounds. The majority of the research that has been done on this topic shows that the solubility of organic compounds is much greater when humic substances are

present versus when they are not. It has also been found that the solubilities of insoluble organic compounds increase two to three times when aquatic humic substances are present.

In 1983, Purdue (as cited in Reference 2) described a catalysis model that related the complexation of pesticides and other organic compounds to humic substances. He also discussed how the binding of pesticides by humic substances retarded the hydrolysis of pesticides. Prior to this in 1982, Carter and Suffet (as cited in Reference 2) made measurements using equilibrium dialysis between aquatic humic substances and DDT. These researchers found that the binding of DDT by humic substances varied with pH, ionic strength, and the presence of inorganic ions. They found that the log of the equilibrium constant, or log K of binding, varied from 4.83 to 5.74. This variation depended on the source of organic carbon in the sample. It appeared that more hydrophobic HA (Aldrich humic acid) had greater binding constants than the hydrophilic DOC (dissolved organic carbon) present in a New Jersey reservoir.[2] They also found that log K varied with the concentration of DOC, but they attributed this to possible leaking of DOC through the dialysis bag and to binding of DDT outside of the membrane.

In 1977, Rook (as cited in Reference 2) noted that humic substances in water were an important source of organic matter in the production of trihalomethanes (THMs) in chlorination of water and sewage. Both engineers and water treatment specialists are interested in the coagulation of humic substances to lower the production of THMs.[2] In 1965, Hall and Packham (as cited in Reference 2) found differences between the coagulation of clay suspensions and humic substances using aluminum sulfate or alum. They noted that turbidity was removed at pH's ranging from 6.5 to 7.5. This particular

pH range promotes precipitation of amorphous alum. They also found that clay increased the rate of precipitation of alum and that humic substances were removed at pH's that ranged from 5 to 6 which was slightly less than that for clay removal. As the amount of humic substances increased, the dose of alum had to be increased. However, when the amount of clay increased, the amount of coagulant could be slightly decreased. There was a direct relationship between the amount of coagulant and the color of the water.

In 1981, O'Melia and Dempsey (as cited in Reference 2) stated that Hall and Packham's work was limited to concentrations of humic substances of 25 ppm or greater and that differences existed for lower concentrations. They stated that the probable cause of coagulation is the adsorption of the negatively charged humic substances with the positively charged aluminum polymers which resulted in the formation of colloidal precipitates that flocculate if the overall charge of the Al-humic polymer is near zero.[2] At concentrations of humic substances less than 5 ppm, flocculation kinetics limit aggregation. They suggested two removal processes at low concentrations of humic substances [2]: First, aluminum polymers can interact with humic molecules in the pH range of 5 to 6. The resulting colloid will not aggregate to a size large enough for settling, because of flow flocculation kinetics, but is removed by direct filtration. Second, aluminum hydroxide that is precipitated at pH 6.0 to 7.5 may absorb humic substances from dilute solution and settle them.

In a 1986 article, Malcolm and MacCarthy [17] cautioned that commercial HAs may vary and that there was a need to include standard and reference samples in research studies.

CHAPTER III

BACKGROUND ON THE GRIESS REACTION

The formation of pigments from various nitrosatable compounds (principally aniline derivatives), nitrous acid, and various coupling reagents (principally naphthalene derivatives) was first described by Johann Peter Griess in 1864.[18] The products are of major importance to the dyestuff industry. The reaction is of equal utility as a measure of nitrite, as Griess first demonstrated in 1879.[19] This reaction has been used in the analysis of nitrite in foodstuffs and continues to be used today in various applications.

The Griess reaction steps include: nitrosation, diazonium ion formation, and coupling (Figure 5). The azo dye that is produced using the reagents shown is purple-pink in color and has a wavelength of maximum absorbance of 540 nm. A typical run using the Griess reagent system for nitrite is described in the experimental section. The nitrosation requires acid, the diazonium ion formation is an internal rearrangement, and the coupling proceeds at different rates dependent upon the pH. When dyestuffs are produced, the diazonium salt is prepared with excess nitrous acid. It is then crystallized and allowed to react with the coupling reagent at the optimal pH which is close to neutrality. As shown in Figure 5, we used sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride as the Griess reagent. For nitrite analysis, the reaction is carried out at a pH that is a compromise between the optimal pH values for the two pH-dependent reactions, with all three reaction steps continuing simultaneously at limiting nitrite concentrations.[20]

When analyzing nitrite in meat, sample preparation must take place before the

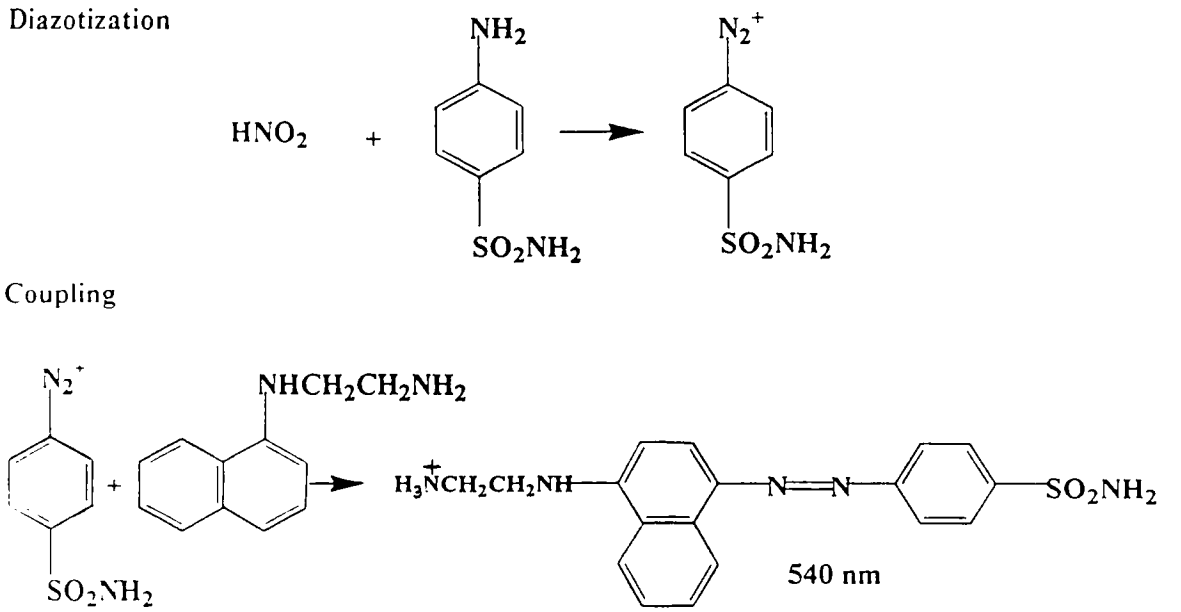

FIG. 5. Griess reaction for nitrite.[20]

Griess reagents can be added. Fox [20] found that the amount of nitrite in cured meats depends on how much "free" or "bound" nitrite is originally present and on how the sample preparation procedure affects other compounds that interfere in the color reaction. Fox used the same colorimetric reagents so that direct comparisons could be made of the effect of the various preparation procedures. In 1963, Sawicki et al.[21] wrote an article on fifty-two different methods for the determination of nitrite, but they also varied their preparation procedure so the effectiveness of different reagents cannot be accurately evaluated from their study. To evaluate the effect of residual reactants on the Griess reaction, one must determine the critical reaction parameters by systematically studying an analogous series of reactants under varying conditions.[20]

Various optimal operating conditions for the Griess reaction have been established since the late 1800's depending on the application. Ilosvay [20] first recommended the use of acetic acid in the reaction. It is claimed that this gives a pH range (2.5-3.0) of maximal conversion for the sulfanilic acid/1-naphthylamine reactions. However, this has not been adopted universally. For maximal pigment production, the nitrosated species has to be in at least 100-fold excess over nitrite. If nitrite reacts with the coupling reagent, incomplete color formation could occur in analytical applications. It is interesting to note that nitrosation of a coupling reagent, 1-naphthylamine, is the basis for a method of nitrite determination.[22] There can be interfering reactions or side reactions of the reagents. The possibilities have been discussed by Fox [20] in detail. However, over the years improvements have been made in the choice of Griess reagents and the reaction conditions.

As a secondary result of their study, Fox et al.[20] developed some data as to the

sources of variability in the Griess analysis. The results of their study showed that the amount of diazo pigment that formed from the reaction of a variety of aniline and naphthylamine derivatives with nitrite was dependent upon the following factors: kind and concentration of reagents used including the position of the ring substituents, specific combinations and relative concentrations of the nitrosatable species and the coupling reagent used, reaction of nitrite with ring substituents other than the amino group, reaction of nitrite with the coupling reagent, formation of more than one pigment, oxidation of the diazonium intermediate, oxidation of the pigment, oxides of nitrogen in the air, reduction of the diazonium ion by residual reductants, formation of semistable nitroso-reductant intermediates, and pre-reaction of nitrosated species and nitrite.

In addition to pH and temperature factors, the factors mentioned above are important to some degree to all the aniline and naphthylamine derivatives studied by Fox.[20] It may be assumed that they are also important to any other compounds that have been studied or have been proposed to be studied for the purpose of determining nitrite.[20]

The Griess reaction has been studied extensively and the conditions for use in analytical applications have been optimized with time. A current U.S. Environmental Protection Agency method [23] was chosen for use in the present research. However, the background on the Griess reaction cited above and the possible interfering paths are important to consider when anomalies are encountered.

CHAPTER IV

EXPERIMENTAL

Reagents and Materials

The Griess reagent was prepared using sulfanilamide, 99 +% (Aldrich, Lot No. AU 06116ER), N-(1-naphthyl) ethylenediamine dihydrochloride, 98 % A.C.S. reagent grade (Aldrich, Lot No. CU 08517LS), granular sodium acetate, analytical grade (Mallinckrodt), 12 M hydrochloric acid, and distilled water free of nitrite or nitrate. A nitrite stock solution was prepared by dissolving 0.1493 g of anhydrous sodium nitrite, 99.99 +% Aldrich® Reagent Plus™ (Sigma-Aldrich, Co., Lot No. 04820KS), in distilled water and diluting to 1000 mL. The resulting solution had a concentration of 99.5 ppm nitrite and was preserved with 2 mL of chloroform, analytical grade (Mallinckrodt, Lot No. 4440KMLY). Ten mL of this stock solution was then diluted to 1000 mL to prepare the nitrite standard solution. The resulting standard solution had a concentration of 0.995 or 1 ppm nitrite. This standard solution was used in the preparation of all nitrite standards using the EPA Method 354.1.[23]

The following HAs were used in this research: Fluka Chemika humic acid (Ash~20%, Analysis Number: 38537/1 194, Fluka Chemika 53680), Aldrich humic acid (Aldrich, Catalog No. H1, 675-2, Lot No. 01902AR), sodium salt, IHSS (International Humic Substances Society) Leonardite humic acid standard 1S104H-5, IHSS peat humic acid reference 1R103H, and IHSS soil humic acid standard 1S102. Suwannee fulvic acid 1R101F was also tested.

Other chemicals included: 3-aminophenol, 98% (Aldrich, Catalog No. 10, 024-2,

Lot No. 01511LM), p-aminophenol HC1 (Eastman), 3-hydroxybenzoic acid, 99% (Aldrich, Catalog No. H2, 000-8), p-hydroxybenzoic acid (1520, Eastman Kodak, Co.), tannic acid, A.C.S. reagent grade (Aldrich, Catalog No. 40, 304-0, Lot No. 07818TR), and gallic acid monohydrate, 98 +% A.C.S. reagent grade (Aldrich, Catalog No. 39, 822-5, Lot No. 09312PS). Once the HA and FA solutions were prepared (specific preparation of these solutions are discussed below), they were filtered three times with Fisherbrand Filter Paper (Fisher Scientific, Catalog No. 09-795B, porosity: coarse, flow rate: fast, diameter: 7 cm).

Equipment

The instrument used in this research was the Hitachi Model U-2010 UV/VIS spectrophotometer, and the cuvettes used in the instrument were Fisherbrand® 1 cm glass cells (Catalog No. 14-385-912B). Also, pH measurements were taken using Fisher Scientific Accumet 910 pH Meter. The pH meter was calibrated using Buffer Solution pH 7.0 Certified (Fisher Scientific, ±0.01 @ 25 °C SB107-500, Lot No. 964974-24) and Buffer Solution pH 4.0 Certified (Fisher Scientific, ±0.01 @ 25 °C SB101-500, Lot No. 964936-24).

Specific Procedures

When performing a typical run using the Griess reagent system for nitrite, the spectrophotometer was first calibrated by placing an opaque, black cuvette in the sample compartment and zeroing the instrument and then doing the same procedure by placing the black cuvette in the reference compartment. A blank was run before any samples were analyzed by filling the Fisherbrand® 1 cm cells with deionized water (the cells were

handled by touching only the two translucent rougher sides of the cuvette), wiping any excess water on the two smooth transparent sides of the cuvettes with Kimwipes® X-L Kimberly-Clark®), and placing the cuvettes in both the sample and reference compartments. This gives a zero absorbance baseline before any samples were analyzed. The spectra produced gave absorbance on the y-axis (usually ranging from 0-1, depending on the sample being analyzed) and wavelength in nanometers on the x-axis (always starting at 300 nm and ending at 700 nm). Typically as a control, 0.2 ppm nitrite was analyzed (other concentrations of nitrite were studied, but the majority of studies analyzed 0.2 ppm nitrite).

To prepare a 0.2 ppm nitrite standard, 80 mL of deionized water was added to 20 mL of 1 ppm nitrite standard solution using Class A glass pipets (Kimex USA or Fisherbrand), 2 mL of the Griess reagent was next added, and the color medium was allowed to develop for 15-20 minutes in LDPE (low density polyethylene) Nalgene® 125 mL bottles. Typically, when analyzing the various humic substances, their initial concentrations were 60 ppm. To prepare solutions with 60 ppm HA/FA/humic substance present, 80 mL of 60 ppm HA was added to 20 mL of 1 ppm nitrite standard solution, 2 mL of the Griess reagent was next added, and the color medium was allowed to develop for 15-20 minutes. After all dilutions were made, the resulting humic substance concentration went from 60 ppm to approximately 47 ppm and the resulting nitrite concentration went from 1 to 0.2 ppm (in a real world application, the sample received for analysis would have 1 ppm nitrite and 60 ppm HA). To analyze the 0.2 ppm nitrite standard control, 3 mL of the control was placed in a cuvette which was placed in the sample compartment, and 3 mL of deionized water was placed in another cuvette which

was placed in the reference compartment. The wavelength of maximum absorbance for the Griess azo dye produced, regardless of concentration of nitrite present, was 540 nm; however, the absorbance increased with an increase in the concentration of nitrite. To analyze a sample with 60 ppm HA replacing the 80 mL of deionized water, 3 mL of the sample was placed in a cuvette which was placed in the sample compartment, and 3 mL of HA diluted with deionized water (diluted so that the concentration of the reference compartment matched exactly the concentration of HA in the sample compartment) and acidified with one drop of 12 M HCl (acidified so that the pH of the reference compartment matched exactly the pH of the sample compartment) was placed in another cuvette which was placed in the reference compartment. The wavelength of the sample was shifted from 540 nm to longer wavelengths and the absorbance of the sample usually decreased depending on the type of humic substance being analyzed.

When analyzing nitrite, a meaningful procedure must include a quality assurance program. As a QA/QC procedure, everyday that a new experiment was performed, the Griess reagent solution was tested by analyzing a fresh 0.2 ppm nitrite standard solution. If the absorbance of this standard solution varied by more than ±0.002 absorbance units, a new Griess solution was prepared before any samples were analyzed.

Humic Acid Solutions

When preparing the HA/FA/humic substance solutions, first a 200 ppm stock solution was prepared. Certain HAs and other chemicals, for example, Fluka humic acid and p-hydroxybenzoic acid, were difficult to dissolve in deionized water. Therefore, 3 mL of 0.1 M NaOH was added to the 200 ppm stock solutions. This significantly

improved their solubility, but in all cases, the humic solutions still required filtration (vacuum filtration and Fisherbrand Filter Paper). The Suwannee FA went very easily into solution and did not require the addition of NaOH and did not have to be filtered.

For all samples tested, dilutions were made from each 200 ppm stock solution to achieve sample concentrations of 10, 30, 60, and 99 ppm. A 10 ppm sample solution was prepared by taking 50 mL of a 200 ppm stock solution and diluting with deionized water up to 1000 mL, a 30 ppm sample solution was prepared by taking 150 mL of a 200 ppm stock solution and diluting with deionized water up to 1000 mL, a 60 ppm sample solution was prepared by taking 300 mL of a 200 ppm stock solution and diluting with deionized water up to 1000 mL, and a 99 ppm sample solution was prepared by taking 495 mL of a 200 ppm stock solution and diluting with deionized water up to 1000 mL.

CHAPTER V

RESULTS AND DISCUSSION

EPA Method 354.1 for the Analysis of Nitrite

The method chosen for nitrite analysis was the EPA Method 354.1, which utilizes the Griess reaction to detect nitrite in water.[23] As indicated above, the method involves diazotization of sulfanilamide by nitrite in water under acidic conditions followed by coupling with N-(1-naphthyl) ethylenediamine dihydrochloride to produce a pinkish-purple color which is read spectrophotometrically at 540 nm. It is indicated that there are very few known interferences at concentrations less than 1,000 times that of the nitrite. However, the presence of strong oxidants or reductants in samples will readily affect the nitrite concentrations. The EPA method is applicable to the analysis of nitrite in drinking, surface and saline waters, as well as domestic and industrial wastes. It is stated that the method is applicable in the range from 0.01 to 1 ppm nitrite.[23]

A nitrite calibration curve was prepared for the range 0.0 to 0.20 ppm. Griess reagent (2 mL) was added to 100 mL of each nitrite standard solution. The solutions were allowed to stand for 20 minutes as directed though it was observed that the color formation appeared "instantaneous". The EPA method stated that the pH of the reaction solution should be between 1.5 to 2.0. However, the pH of our solutions was consistently in the range of 2.2-2.4. The absorbance was taken three times for each concentration of nitrite using different solutions to establish the precision of the method. Though the blank contained no added nitrite, a slight absorbance (0.0034) appeared which was subtracted from the other readings. The absorbance at 540 nm for each standard was

then plotted versus concentration of nitrite in ppm. Table 3 shows the individual absorbance readings and Figure 6 shows the calibration curve for nitrite. A comparison to the EPA method could not be made since a calibration curve was not included.

TABLE 3. Absorbance values (AV) for various concentrations of nitrite.

Concentration (ppm)	Absorbance Readings	Average Absorbance	Average Absorbance Minus Blank	Standard Deviation	%RSD
0	0.0047, 0.0026, 0.0028	0.0034	0	-------	-------
0.01	0.0074, 0.0068, 0.0071	0.0071	0.0037	0.0003	8.1
0.02	0.0149, 0.0156, 0.0153	0.0153	0.0119	0.0004	3.4
0.03	0.0238, 0.0233, 0.0236	0.0236	0.0202	0.0003	1.5
0.04	0.0354, 0.0346, 0.0351	0.0350	0.0316	0.0004	1.3
0.06	0.0484, 0.0484, 0.0482	0.0483	0.0449	0.0001	0.22
0.08	0.0639, 0.0647, 0.0636	0.0641	0.0607	0.0006	0.99
0.10	0.0865, 0.0869, 0.0858	0.0864	0.0830	0.0006	0.72
0.20	0.1672, 0.1656, 0.1668	0.1665	0.1631	0.0008	0.49

Effect of Humic Acid Concentration

The first HA examined was a Fluka product at 10, 30, 60, and 99 ppm (Figure 7 shows absorbance spectra of Fluka humic acid at these concentrations). The

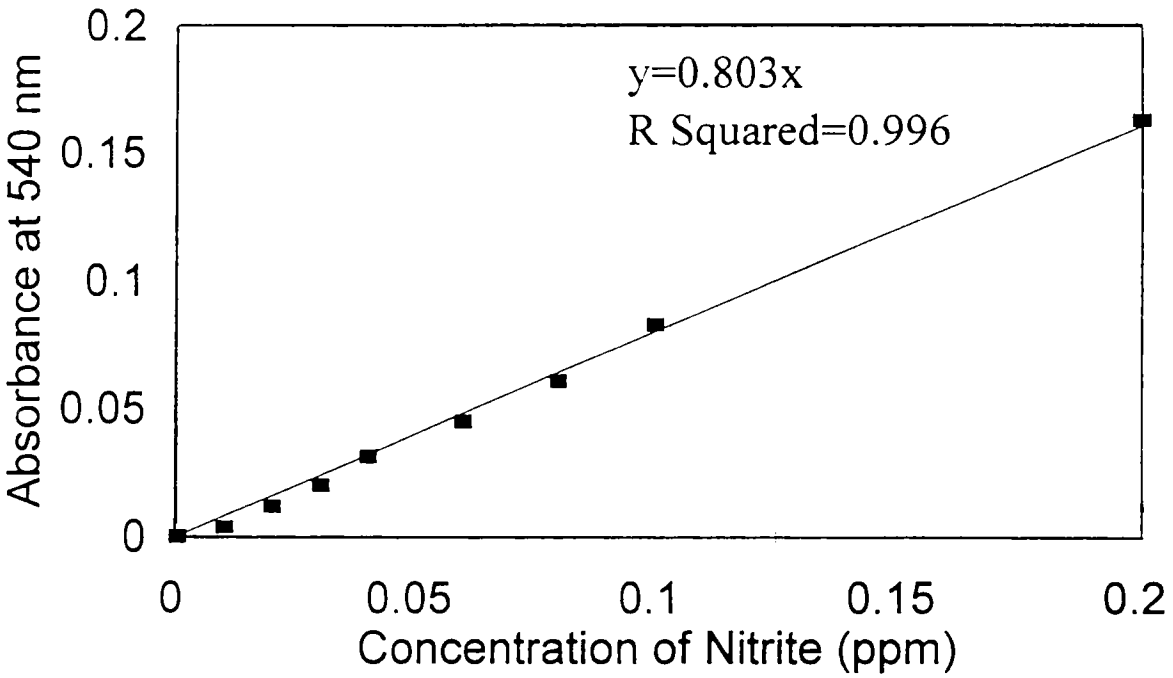

FIG. 6. EPA Method 354.1 nitrite calibration curve (error bars lie within the data symbols).

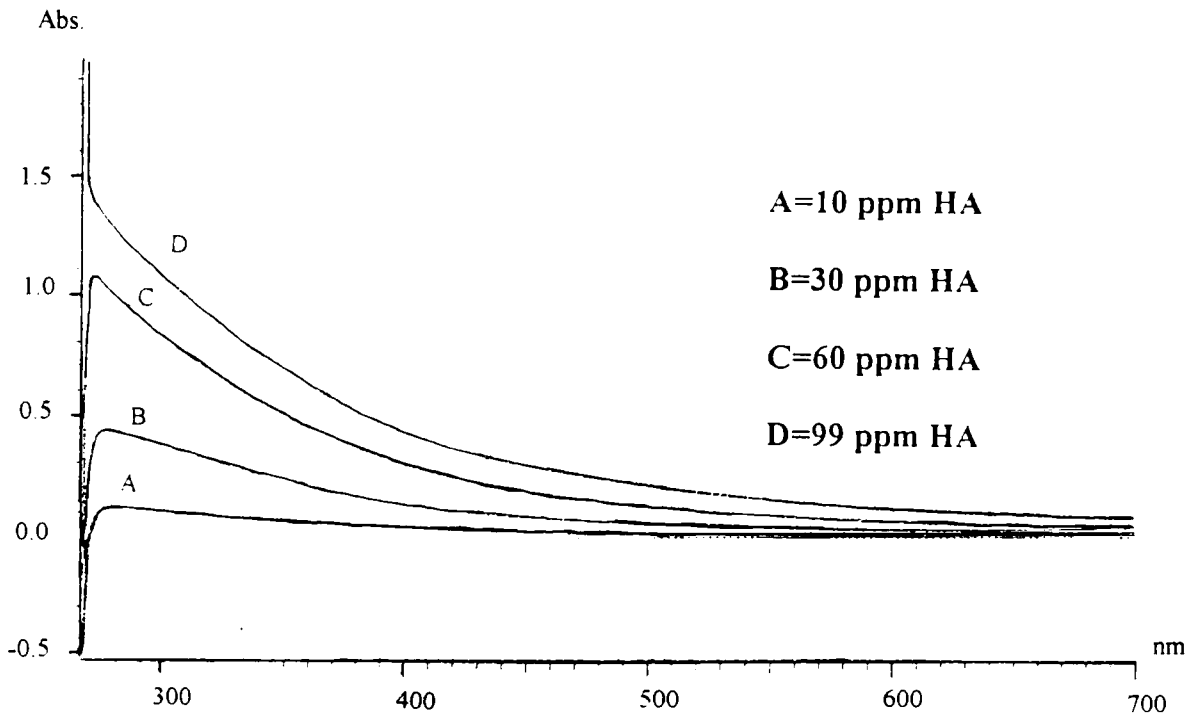

FIG. 7. Background absorbance produced by 10, 30, 60, and 99 ppm Fluka humic acid.

concentration of nitrite was kept constant at 0.2 ppm. First, the absorbance of a 0.2 ppm nitrite standard was measured (no HA added). The standard contained 80 mL of deionized water, 20 mL of 1 ppm nitrite standard solution, and 2 mL of Griess reagent. Next, in place of the deionized water, 80 mL of 10, 30, 60, and 99 ppm Fluka humic acid were added, respectively. The volume of nitrite was kept constant. The experiment was repeated three times to determine precision. Table 4 shows the data obtained from one of the performance runs and Figure 8 shows the spectra obtained.

TABLE 4. Effect of Fluka humic acid on the Griess dye absorption from 0.2 ppm nitrite.

Concentration of Fluka Humic Acid Added	0 ppm HA Added (Control)	10 ppm HA Added	30 ppm HA Added	60 ppm HA Added	99 ppm HA Added
Absorbance	0.182	0.180	0.163	0.136	0.113
Wavelength of Maximum Absorbance	540.0 nm	541.0 nm	541.5 nm	547.5 nm	557.5 nm

As can be seen, the presence of Fluka humic acid results in a decrease of the Griess dye absorption as well as a shift in the wavelength of maximum absorbance from 540 nm to longer wavelengths. Table 5 shows the data obtained from an average of three runs. These are plotted in Figure 9.

The absorbance at 540 nm for the same runs are shown in Table 6 and plotted in Figure 10 simulating what would be recorded following the directions of the method and without the benefit of knowing the wavelength of maximum absorbance had shifted. Little difference is noted between measuring at the wavelength of maximum absorption

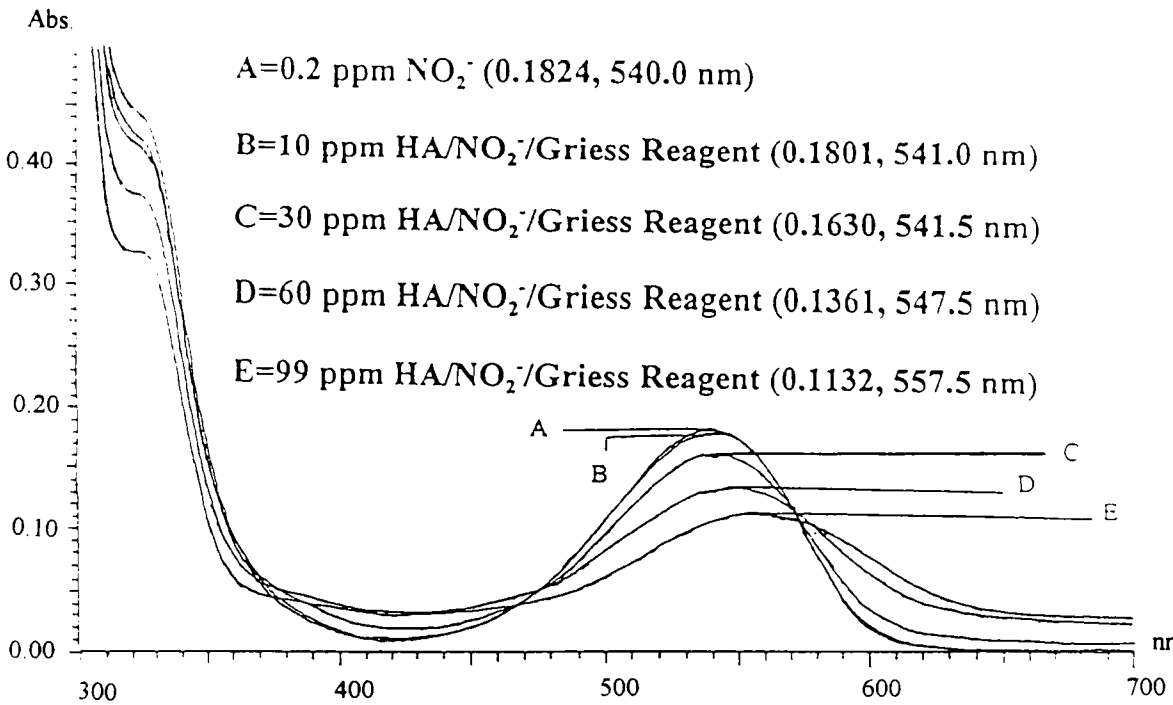

FIG. 8. Spectra showing the effect of Fluka humic acid on the Griess dye absorption from 0.2 ppm nitrite.

TABLE 5. Effect of Fluka humic acid on the Griess dye absorption from 0.2 ppm nitrite (data obtained from three separate trials).

Concentration of Fluka Humic Acid Added	0 ppm HA Added (Control)	10 ppm HA Added	30 ppm HA Added	60 ppm HA Added	99 ppm HA Added
Absorbance Readings	0.182, 0.181, 0.172	0.180, 0.175, 0.170	0.163, 0.166, 0.165	0.136, 0.140, 0.137	0.113, 0.118, 0.117
Average Absorbance	0.179	0.175	0.165	0.138	0.116
Standard Deviation	0.006	0.005	0.002	0.002	0.002
%RSD	3.1	2.8	0.91	1.4	2.0

TABLE 6. Effect of Fluka humic acid on the Griess dye absorption from 0.2 ppm nitrite at 540 nm.

Concentration of Fluka Humic Acid Added	0 ppm HA Added (Control)	10 ppm HA Added	30 ppm HA Added	60 ppm HA Added	99 ppm HA Added
Absorbance Readings	0.182, 0.181, 0.172	0.180, 0.175, 0.170	0.163, 0.166, 0.164	0.134, 0.138, 0.137	0.107, 0.112, 0.116
Average Absorbance	0.179	0.175	0.164	0.136	0.112
Standard Deviation	0.006	0.005	0.001	0.002	0.004
%RSD	3.1	2.8	0.79	1.7	3.9

or at 540 nm. This is a reflection of the broadness of the absorption band. The broadness does not appear to be impacted by the presence of HA. Whether one measures at 540 nm or the wavelength of maximum absorption it is clear that the presence of Fluka

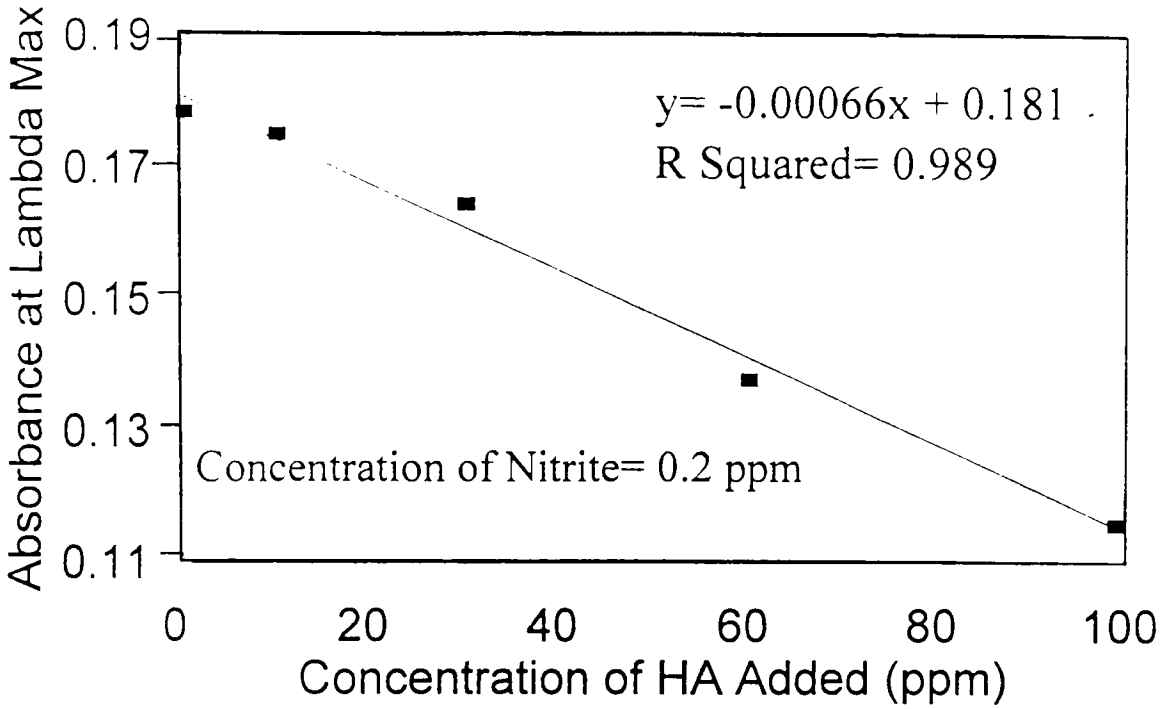

FIG. 9. Absorbance for nitrite at lambda max vs. amount of Fluka humic acid added (error bars lie within the data symbols).

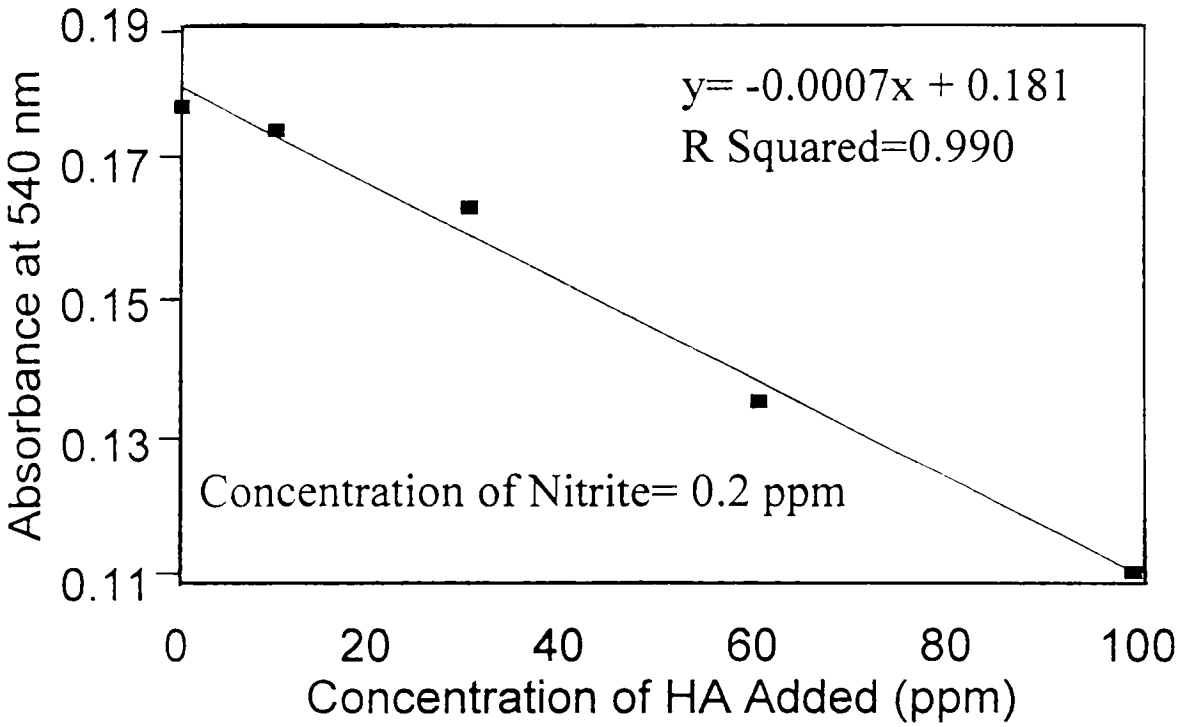

FIG. 10. Absorbance for nitrite at 540 nm vs. amount of Fluka humic acid added (error bars lie within the data symbols).

humic acid does interfere with the analysis of 0.2 ppm nitrite to give lower values than actual.

Effect of Nitrite Concentration

We chose to examine the effect of a set concentration of Fluka humic acid (addition of 60 ppm with a final concentration of 47 ppm) on the Griess determination of nitrite in the range of 0.2 ppm-0.9 ppm. Absorbance data in the absence and presence of HA using the Griess reaction procedure are given in Tables 7 and 8, respectively.

TABLE 7. Absorbance at 540 nm for 0.2 ppm-0.9 ppm nitrite using the Griess reaction.

Concentration of Nitrite (ppm)	Absorbance Readings	Average Absorbance	Standard Deviation	%RSD
0.2	0.172, 0.168, 0.169	0.170	0.002	1.2
0.5	0.437, 0.434, 0.435	0.436	0.001	0.32
0.7	0.639, 0.638, 0.638	0.638	0.001	0.13
0.8	0.736, 0.742, 0.733	0.737	0.005	0.62
0.9	0.809, 0.857, 0.809	0.825	0.028	3.4

Figures 11, and 12 show spectra for the 0.2 ppm nitrite standard and 0.9 ppm nitrite standard with and without the Fluka humic acid.

Table 8 gives the percent absorbance decrease for the various concentrations of nitrite as a result of the HA being present. The greatest absorbance decrease (53%) was

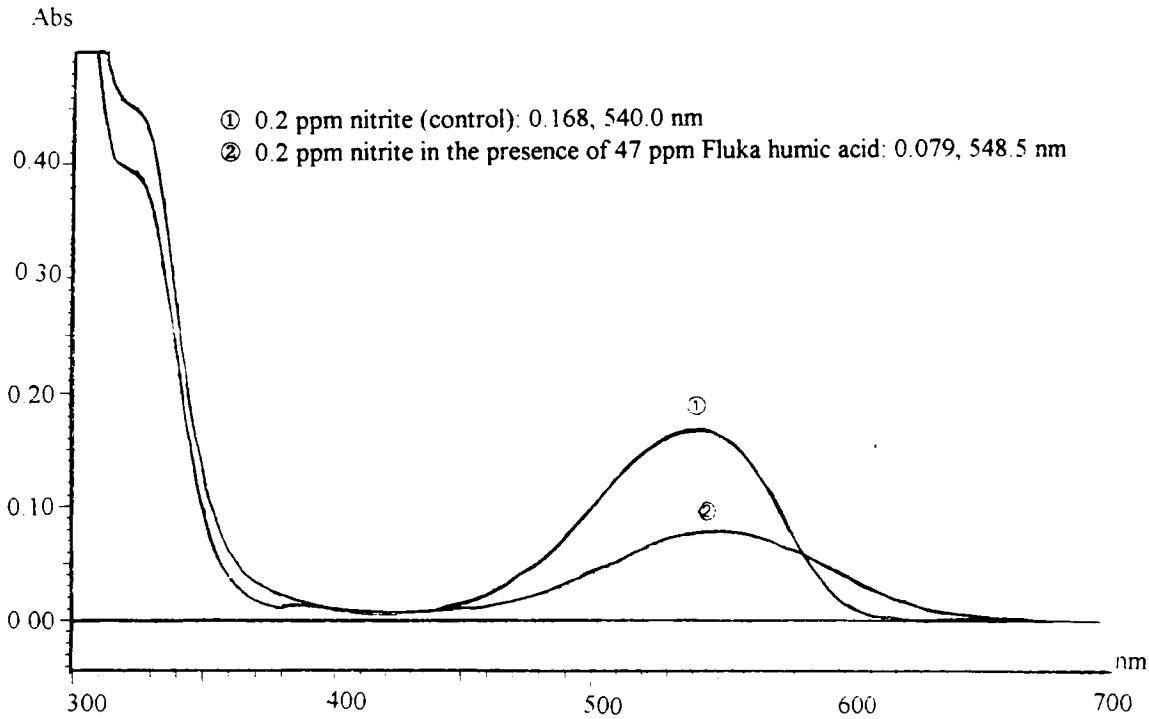

FIG. 11. Spectra showing the absorbance of 0.2 ppm nitrite with and without the presence of 47 ppm Fluka humic acid.

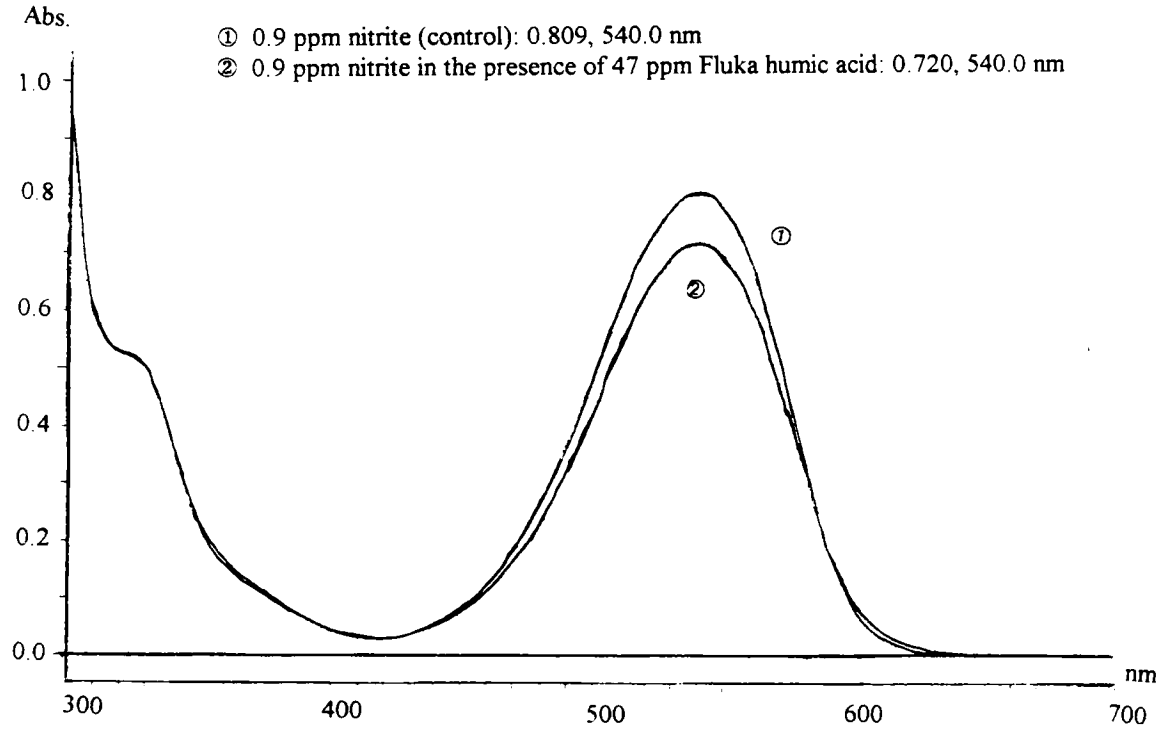

FIG. 12. Spectra showing the absorbance of 0.9 ppm nitrite with and without the presence of 47 ppm Fluka humic acid.

evident for the lowest concentration of nitrite (0.2 ppm). The absorbance decrease was

TABLE 8. Effect of 47 ppm Fluka humic acid on the analysis of 0.2 ppm-0.9 ppm nitrite using the Griess reaction.

Concentration of Nitrite (ppm)	Absorbance Readings	Average Absorbance	Standard Deviation	%RSD	Average Percentage Absorbance Decrease
0.2	0.081, 0.079, 0.080	0.080	0.001	1.6	53
0.5	0.253, 0.249, 0.251	0.251	0.002	0.72	43
0.7	0.498, 0.496, 0.490	0.495	0.004	0.89	23
0.8	0.645, 0.657, 0.640	0.647	0.009	1.4	12
0.9	0.720, 0.752, 0.721	0.731	0.018	2.5	11

only 11% for the 0.9 ppm nitrite sample. The presence of the HA led to shifts of the wavelength of maximum absorption to 548.5 nm and 546.0 nm with the 0.2 ppm and 0.5 ppm nitrite samples, respectively. The wavelength of maximum absorption for the higher nitrite concentrations stayed at 540 nm.

Mechanism of Humic Acid Interference

The mechanism of the HA interference in the Griess analysis of nitrite may be explained on the basis of one of the following: reaction of HA with nitrite, diazotization of HA, coupling of HA with the diazonium salt of sulfanilamide, and

reaction/association of HA with the azo dye from the Griess reaction. Each was examined as described next.

Reaction of HA with nitrite. An experiment was performed in which 25 mL of 30 ppm Fluka humic acid was mixed with excess sodium nitrite (0.200 g) giving 0.116 M sodium nitrite and its absorbance was monitored over a week period. The same was done for a control (25 mL of deionized water mixed with the same amount of excess sodium nitrite) and for 30 ppm Fluka humic acid alone.

Diazotization of HA. An experiment was performed in which Fluka humic acid, nitrite, and N-(1-naphthyl) ethylenediamine dihydrochloride were allowed to develop color adhering to the procedure of the EPA Method 354.1 except that the sulfanilamide was absent. This experiment was repeated several times using 30, 60, and 99 ppm Fluka humic acid solutions (80 mL), 1.0 ppm nitrite solution (20 mL), and 2 mL of the Griess reagent with only the N-(1-naphthyl) ethylenediamine dihydrochloride present. The HA took the place of the sulfanilamide. Color should appear if the HA diazotized and then coupled with the N-(1-naphthyl) ethylenediamine dihydrochloride. Absorbance spectra were measured of the various test solutions. No color formation was observed leading to the conclusion that the HA did not diazotize. If HA had diazotized, the diazonium salt did not couple with the naphthyl reagent to give color. If the latter was true, the decrease in absorbance in the Griess reaction for nitrite in the presence of HA might be explained.

Coupling of HA with the diazonium salt of sulfanilamide. An experiment was performed in which solutions of 10, 30, 60, and 99 ppm Fluka humic acid (80 mL), 1.0 ppm nitrite solution (20 mL), and the Griess reagent (2 mL) containing sulfanilamide

were added together and allowed to stand for 20 minutes. Absorbance spectra were obtained, but no changes were observed relative to controls. It appears that Fluka humic acid does not couple with the diazonium salt of sulfanilamide otherwise some absorption changes including the appearance of visible color would have been measured. An experiment was also performed in which solutions of 60 ppm Fluka humic acid (50 mL), 400 ppm nitrite (50 mL), and the Griess reagent with only the sulfanilamide component (2 mL) were mixed together and allowed to stand for 20 minutes. (The concentration of the nitrite was 400 ppm before dilution and 196 ppm after being mixed with the humic acid and the Griess reagent with only the sulfanilamide.) Though the nitrite concentration (and also the sulfanilamide diazonium salt concentration) were high, no changes were evident in the absorption spectrum.

Reaction/association of HA with the azo dye from the Griess reaction. The EPA Method 354.1 procedure was modified to determine whether the HA was reacting/associating with the azo dye formed in the Griess reaction with nitrite. The 1.0 ppm nitrite solution (20 mL) and the Griess reagent (2 mL) were mixed and allowed to stand for the usual 20 minute time period. The modification was that the mixture was more concentrated than normal. The rate of dye formation was very rapid irrespective of the concentration difference. Adding 80 mL of water and immediately checking the wavelength of maximum absorbance showed no difference with a control run in which 80 mL of water were added at the beginning of the 20 minute period.

However, when a solution of 60 ppm (80 mL) HA was added to the more concentrated Griess reaction solution (which had been standing for 20 minutes) and immediately measured, a lower absorbance was noted (0.085 vs. 0.161). The wavelength

of maximum absorbance had shifted to a longer wavelength (566.0 nm). Allowing the solution to stand for 20 minutes did not result in further changes.

These experiments provide clear evidence that the decrease in absorbance and wavelength shift first noted in analyzing nitrite in the presence of Fluka humic acid are due to the association of the azo dye product with the HA. Chemical reaction (in lieu of molecular association) is unlikely in view of the immediate change noted on adding HA in the modified procedure.

Binding of metal ions to humic substances is well known.[6,24,25] Humic substances can control metal ion concentration in soils and natural waters and can affect the mobility of metals through soils and aquifers. Benedetti et al.[24] describe metal binding to humic substances and summarize the different processes and HA properties that affect binding. It is not surprising that the emphasis on cation binding to humic substances has been on metal ions because of environmental importance. No references have been found on the binding of cationic dyes with humic substances. A reference was found on the fluorescence quenching of humic substances by cationic nitroxides due to enhanced attraction of the cations to the anionic surfaces.[26] It is believed that our work on humic substances in the Griess system represents the first report of cationic dye binding.

As described above, each of the humic substances tested in the Griess reaction with nitrite gave a lower than expected dye product absorbance and in some cases a shift of the absorption band to longer wavelengths. It was interesting to find that the UV absorbance at 228 nm of $Ru(NH_3)_6^+$ was reported to decrease as a function of increasing HA in solution.[27] This was used as confirmation of $Ru(NH_3)_6^+$ binding to HA.

Formulating a specific model for binding of the cationic Griess dye to humic

substances is difficult since structures for the humic materials tested are not known. As pointed out in the literature [24], binding of metal ions to HA is strongly influenced by the intrinsic chemical heterogeneity and polyelectrolyte behavior. The degree of ionization of HA functional groups such as phenols and carboxylic acids will be determined by the pH. (Phenols and carboxylic acids are of major importance in HA.) The pH will also influence the charge of the HA. The cation binding may be influenced by factors such as pH, ionic strength, concentration ratio of cation to humic ligand, competitive binding between cations, intermolecular and intramolecular heterogeneity of binding sites, and size and shape differences between humic substances. There are many studies reported on binding of metal ions but none could be found on cationic dyes. For example, Leenheer et al.[28] assessed Suwannee River fulvic acid (also examined in the present study with the Griess dye) for its ability to bind Ca^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} ions at pH 6 before and after extensive fractionation that was designed to reveal the nature of metal binding functional groups. A structural model of the hydrogen form of the metal binding fraction of the FA was portrayed. A corresponding structural model of a calcium complex of the metal binding fraction from Suwannee River fulvic acid was also shown as an inner complex. It is not known whether this particular fraction of the Suwannee River would also bind the Griess dye. It is speculated that the Griess dye forms an outer complex with the humic substances. Sufficient information is not available to formulate a specific structural model.

A very simple model of the molecular association of the Griess dye with humic substances is binding through electrostatic interaction between the cationic dye and the anionic carboxylates of the humic substances (HS):

$$\text{Griess dye}^{+} + \text{HS-COO}^{-} \rightleftarrows (\text{Griess dye}^{+})(\text{HS-COO}^{-}) \quad (4)$$

Questions arise as to the importance of carboxylates in binding of cationic Griess dye in view of the low pH (2.4) at which the Griess reaction is performed. One might expect that the pK_as of the functional groups are much higher. Leenheer et al.[29] also investigated the strong-acid, carboxyl group structures in FA from the Suwannee River. Carboxyl groups at pK_a 3.0 or less accounted for 33.4% of the total carboxyl content. Acid group structures included sulfur and nitrogen acids, oxalate half-esters, substituted malonic acids, keto acids, and aromatic and olefinic acids. It was calculated that 7.7% (0.46 mmol/g) of total carboxyl activity was from aromatic carboxyl groups whose pK_a is 3.0 or less. It is not unreasonable to expect carboxylate anions to be present from humic substances at pH 2.4 as in our studies.

Characteristics of the Griess Azo Dye and its Complexes with Humic Acid

Effect of order in adding HA. It was interesting in the experiments described in the previous section that the wavelength of maximum absorbance of the Griess azo dye in the presence of Fluka humic acid was 548.5 nm (0.079 AV) and 566.0 nm (0.085 AV) both for 0.2 ppm nitrite using the regular and modified methods, respectively. It was established that the 566.0 nm did not change after allowing the solution to stand for 20 minutes.

An experiment was performed in which the HA and Griess reagent were mixed first followed by addition of the nitrite solution. The regular procedure described in the experimental section is to mix the HA solution with the nitrite solution followed by the

Griess reagent solution. The regular procedure gives 548.5 nm as mentioned several times above. In the case of mixing the HA and Griess reagent first, the wavelength of maximum absorbance was found to be 563.0 nm.

Two procedures have now been found that give the absorption near 565.0 nm. In one case, HA was added to a solution in which the Griess azo dye had already been formed. In the other case, a solution of HA and Griess reagent were mixed with nitrite.

The nature of the results imply that the wavelength of maximum absorbance of the dye complex with HA in the constant acid pH of the Griess reaction is subject to changes in the polarity of the HA surface. Changes in the wavelength of maximum absorbance with changes in reagent order of addition may reflect changes in the HA polarity at the point of dye aggregation/association. These are interesting observations worthy of further study.

Effect of solvent polarity. The effect of solvent polarity was examined by comparing the visible absoprtion of the Griess azo dye in water and isopropanol. Allowing the nitrite solution (1.0 ppm, 20 mL) to react with the Griess reagent (2 mL) followed by addition of water gives 540.0 nm (0.168 AV). If 80 mL of isopropanol are added rather than water, the wavelength of maximum absorbance shifts to 517.5 nm (0.109 AV). This implies that the aggregation of the azo dye on the surface of the HA creates a more polar environment for the dye. It also brings out the possibility that aggregation of dyes on the surfaces of humic substances can lead to characterization and differentiation of humic substances.

Effect of pH. The Griess reaction for nitrite requires acid conditions. However, the wavelength of maximum absorbance for the azo dye product and its complex was

checked as the pH was allowed to increase. Drops of 6 M NaOH were added to Griess reaction solutions (from 0.2 ppm nitrite) in the presence and absence of Fluka humic acid (47 ppm). As the NaOH was added, the absorbance decreased and the wavelength of maximum absorbance shifted to lower ones. The control (no HA) shifted from 540.0 nm to 486.5 nm (pH 5.12). The solution absorption with Fluka humic acid went from 562.5 nm to 509.0 nm (pH 6.15).

Though these pH experiments are preliminary, they bring out that the Griess dye visible absorption depends on pH (this is expected in view of the amino groups in the dye). It also brings out that the complexes are formed between the dye and the HA at higher pHs as reflected by the wavelength of maximum absorbance in comparison to a control in the absence of HA.

These results imply that dye complexation with HA may be useful to characterize the HA as a function of pH.

Effect of Humic Substances

Several HA and one FA were examined for interference effects in the Griess method for nitrite. Table 9 contains dye absorbance data obtained from several humic substances (each at 47 ppm after dilution) and nitrite (0.2 ppm after dilution).

The modified procedure involved addition of the humic substance after nitrite has been allowed to react with the Griess reagents. It is clear that the interference effect is not limited to Fluka humic acid. The absorbance of the azo dye from the Griess reaction with nitrite is lower in all cases in comparison to the control without any humic substance. The greatest decrease (61%) was found with IHSS soil humic acid standard

TABLE 9. Effect of other humic acids at 47 ppm on the Griess dye absorption from 0.2 ppm nitrite.

Humic Substance (47 ppm)	Absorbance (Regular EPA Procedure)	Lambda Max (Regular EPA Procedure)	Absorbance (Modified EPA Procedure)	Lambda Max (Modified EPA Procedure)
Control (No HA or FA)	0.168	540.0 nm	0.161	540.0 nm
Fluka	0.079	548.5 nm	0.085	566.0 nm
Aldrich	0.093	558.0 nm	0.081	561.0 nm
Leonardite	0.124	543.0 nm	0.101	544.0 nm
IHSS Peat HA Reference	0.089	558.0 nm	0.085	559.5 nm
IHSS Soil HA Standard	0.066	556.0 nm	0.070	556.0 nm
IHSS Suwannee River FA Reference	0.126	542.0 nm	0.134	542.0 nm

sample. Also, each humic substance showed at least a slight shift in the absorption peak to longer wavelengths with the greatest shift (18 nm) being exhibited by the IHSS peat humic acid reference sample. Absorbance decreases were also noted using the modified EPA procedure though wavelength shifts were not as pronounced as with the Fluka humic acid.

Humic substances should be expected to give interferences generally in the Griess reaction for nitrite with the magnitude depending on the specific substance and the procedure. This puts a burden on the practitioner to either know the type of humic

substance present or to take steps to remove the humic substance during sample preparation. It is interesting to speculate whether the Griess reaction using a specified amount of nitrite could be used as a method for humic substances.

Effect of Chemicals Related to Humic Acids

Several hydroxybenzoic acids and aminophenols were checked as interferences in the Griess reaction for nitrite (Table 10) since hydroxybenzoic acid and aminophenol units appear in structures for HA (Figures 1 and 3). As shown in Table 10, tannic acid resulted in a small shift in wavelength relative to the control. Both tannic and gallic acids gave small absorbance decreases implying interaction with the dye. However, the absorbance decreases were smaller than those observed with humic substances (Table 9). Though many other HA related materials can be screened, the probability of finding effects as high as observed with humic substances appears low.

Advice for the Practitioner

HA concentrations in the literature are usually reported in terms of DOC (dissolved organic carbon) rather than ppm. Elemental analysis data was provided by the International Humic Substances Society (IHSS) for the IHSS soil humic acid standard, IHSS Leonardite humic acid standard, and the IHSS peat humic acid reference used in the present research. Therefore, we were able to convert our concentrations for these HA from ppm to DOC. Each had a concentration of 47 ppm in final dilution, and the following are their percentage carbon values and DOC values, respectively: IHSS soil humic acid standard (58.13% C, DOC (27mg/L)), IHSS Leonardite humic acid standard (63.81 % C, DOC (30 mg/L)), and IHSS peat humic acid reference (56.84 % C, DOC (27

TABLE 10. Effect of hydroxybenzoic acids and aminophenols at 47 ppm on the Griess dye absorption from 0.2 ppm nitrite.

Structural Component (47 ppm)	Absorbance (Regular EPA Procedure)	Lambda Max (Regular EPA Procedure)	Absorbance (Modified EPA Procedure)	Lambda Max (Modified EPA Procedure)
Control (No Benzoic Acid or Aminophenol)	0.168	540.0 nm	0.161	540.0 nm
p-hydroxybenzoic acid	0.175	540.0 nm	0.162	540.5 nm
3-hydroxybenzoic acid	0.176	540.0 nm	0.157	540.0 nm
p-aminophenol	0.166	540.0 nm	0.159	540.5 nm
3-aminophenol	0.165	540.0 nm	0.160	540.0 nm
Tannic acid	0.158	543.5 nm	0.149	543.5 nm
Gallic acid monohydrate	0.173	539.5 nm	0.159	540.0 nm

mg/L)). "Real world" water samples have DOC levels of less than 3 mg/L.[30] Therefore, HA probably will not cause much of an interference problem for the field analytical practitioner except in water with high organic matter levels such as those found in swamps. However, in these cases, the water will undoubtedly be colored (yellow) so the practitioner will be aware of their presence and can remove them before analyzing any water samples. Experiments were performed using the Griess reaction to analyze 0.2 ppm nitrite in the presence of 8 ppm Fluka humic acid (both in final dilution) and little interference was found (there was just a very slight absorbance decrease and no shift in

the wavelength of maximum absorbance). Since HA concentrations are usually lower in the "real world" as compared to the concentrations used in this research, little concern is needed as to interference effects. However, with high concentrations of humic substances the practitioner can divide results by an appropriate factor if the type and concentration of the humic substance is known. In the case of 47 ppm of either Fluka humic acid or IHSS humic acid standard, the factors would be 0.53 and 0.61, respectively. Alternatively, humic substances can be removed altogether. For example, in a recent comprehensive study, analytical restricted access media (RAM) columns were investigated to determine their performance in decreasing the HA interference encountered in the trace analysis of acidic herbicides in environmental water samples when employing reversed-phase liquid chromatography with UV detection (RPLC-UV).[30] The RPLC-UV trace analysis of acidic analytes in water samples is always severely hampered by coextracted HA substances causing a severe baseline deviation.[30] It was shown that the use of an analytical RAM column in reversed-phase LC/LC significantly improved the baseline, allowing quantification at the required low levels in sample extracts without the use of additional cleanup.[30]

The practitioner should be aware of the potential interference effects of HA, but at concentrations of 10 ppm or less the effect will be small in the Griess reaction for analysis of nitrite.

CHAPTER VI

CONCLUSIONS

It is concluded that ppm levels of humic substances interfere in the Griess reaction (EPA Method 354.1) for low ppm levels of nitrite in water. EPA Method 354.1 is based on diazotization of sulfanilamide with nitrous acid followed by coupling of the diazonium salt with N-(1-naphthyl) ethylenediamine dihydrochloride to give an azo dye with a wavelength of maximum absorbance in the visible region at 540 nm. The presence of humic substances leads to a decrease in the dye absorbance and a shift in the absorption peak to longer wavelengths. The interference effect is not limited to a particular humic substance but was noted for each of the five HAs and one FA tested.

Fluka humic acid and IHSS humic acid standard at 47 ppm each with Griess azo dye from 0.2 ppm nitrite in final dilution led to decreases in absorbance of 53% and 61%, respectively. The greater the HA concentration, the greater the interference effect. However, it is projected, based on experiments with Fluka humic acid, that humic substance concentrations of 10 ppm or less will have a measurable but small effect.

For the same concentration of HA but with increases in amount of nitrite present leads to less interference of the humic substance. For example, 47 ppm of Fluka humic acid in samples containing 0.2 and 0.9 ppm nitrite led to decreases of Griess dye absorbance of 53% and 11.4 %, respectively.

It is concluded that the mechanism of the interference involves molecular association of the humic substance with the Griess azo dye product rather than reaction with either nitrite, individual reagents, reaction intermediates, or the azo dye.

The shift of the Griess azo visible absorption peak to longer wavelengths reflects an increase in polarity of the humic substance surface in comparison to the reaction medium. Adding isopropanol to the Griess reaction solution in the absence of HA results in a shift of the absorption peak to lower wavelengths reflecting the sensitivity of the azo dye absorption to polarity changes.

The results of the research suggest several interesting areas worthy of additional study. One area is the possibility of using the molecular association of the dye with humic substances to characterize their surfaces. There also appears to be a high probability that the molecular association effects can be used as a basis for methods of analysis for humic substances.

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